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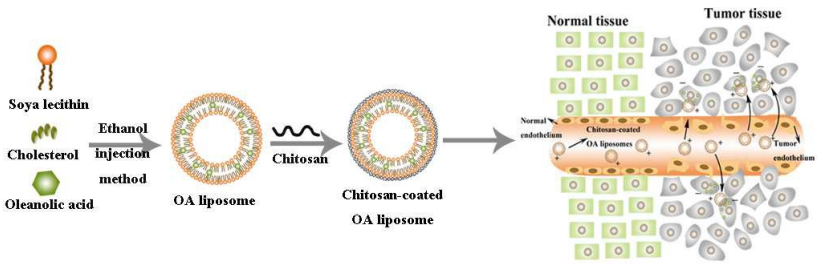

Chitosan modified OA liposomes can achieve the desired effect of tumor-targeting drug delivery and improve the anti-tumor efficacy.

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ARTICLE

Preparation and Study on Anti-tumor Effect of Chitosan-coated Oleanolic Acid Liposomes

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The purpose of this work was to prepare and study the anti-tumor effect of chitosan-coated oleanolic acid (OA) liposomes. Chitosan-coated OA liposomes had a marked positive charges (19.9 ± 0.814 mV), which were inclined to combine with the negative charges on surface of tumor cells, and then targeted and inhibited the growth of tumor cells. The average size of chitosan-coated OA liposomes was around 167.44 nm, and the dimension was more easily trapped into the tumor tissue. The chitosan-coated OA liposomes possessed the stronger rigidity and stability than those of ordinary liposomes, which can prevent the leakage of encapsulated drugs from liposomes. The fourier transform infrared spectroscopy (FTIR) result indicated that chitosan already anchored the liposomes successfully. The chitosan-coated OA liposomes exhibited a slow, controlled OA release at pH 7.4, and a rapid release at pH 5.5 in *vitro*, which was beneficial on controlling tumor-targeting drug release. Additionally, MTT experimental results proved that the chitosan-coated OA liposomes can achieve more ideal anti-tumor effects than OA solution and OA liposomes. The study showed that chitosan modified liposomes not only solve the poor water solubility of OA, but also improve the anti-tumor efficacy, hence, it is a most promising drug carrier.

Introduction

In recent years, the pharmaceutical industry has been successful in discovering many new drugs which are potential candidates for the treatment of cancer. However, there are still more than 8 million people are killed by the disease every year worldwide and the number is growing.[1] The clinical application of the most traditional chemotherapeutics is usually limited owing to concentrations insufficient of therapeutic drug accumulation in the tumor tissue or serious toxic effects on normal organs. The clinical chemotherapeutic agents, such as doxorubicin, cyclophosphamide or combination drugs exhibit undesirable toxicity and serious side-effects.[2] So there is an urgent need to develop a therapeutic agent, which has few or no side-effects on normal organs.[3, 4] In this respect, a large number of natural compounds have been studied.

Oleanolic acid (OA), a naturally pentacyclic triterpenoid compound which is widely distributed in many traditional Chinese medicines (e.g., *fructus forsythia*, *radix ginseng* and *fructus ligustri lucidi*).[5] OA has a variety of biological actions, such as antioxidant, anti-inflammatory, antidiabetic and antifungal properties.[6, 7] In recent years, it was found that OA had marked anti-tumor effects, and exhibited cytotoxic activity toward many cancer cell lines in culture.[8] However, being hydrophobic (logP=6.32, pKa=5.11),[5] OA has poor aqueous solubility and low dissolution rate in the gastrointestinal tract, which significantly limits its effective absorption and bioavailability in body. Therefore, it is essential to develop newly intelligent drug delivery overcoming this obstacle for improving OA therapeutic effect.

Up to now, liposomes have been widely investigated as drug carriers. They are frequently used to improve the treatment effect of various water soluble/insoluble drugs by improving

bioavailability, solubility and retention time.[9] Until now, a number of hydrophobic drugs have been encapsulated into liposomes, such as paclitaxel,[10, 11] ursolic acid,[12] vincristine,[13] nimodipine[14] and curcumin,[15] ect. The solubility and therapeutic effect will be improved by entrapped in liposomes. Liposomes are self-assembling structures of a lipid dispersion in water and a promising tool for drug delivery.[16] Many different approaches have been applied to assemble these lipid vesicles, including thin-film hydration, solvent injection, reverse-phase evaporation, sonication, and membrane extrusion.[17] Liposomes are of great application prospect of drug carries because of their increased therapeutic efficacy and decreased toxicity compared with the drug prototype. A large array of different drugs has been encapsulated in liposomes for the treatment of cancer.[18] According to research, the pore sizes in solid tumor vasculature endothelium vary from 100 to 200 nm,[19] which is much larger than those of normal tissue.[20, 21] Organs and tissues with discontinuous endothelium such as the kidney glomerulus have the pores ranging from 40 to 60 nm.[21, 22] Thus the liposomes are generally too large for glomerular filtration. A number of investigations have shown that liposomal nanoparticles tend to passively extravasate and accumulate in tumor through the leaky vasculature, which is the characteristic of solid tumor.[23, 24] However, the liposomes may be removed by macrophages of the liver and spleen in circulation, because the sinusoidal endotheliums of the liver and spleen have pores up to 150 nm.[21, 25] Therefore, the ideal size distribution is within 150-200 nm for drug delivery, which is beneficial to accumulate in tumor tissues. Meanwhile, the liposomes' clinical applications are affected by targeted release or poor availability of encapsulated drug and its stability in *vivo*. Therefore, various liposome formulations have been studied to solve these problems. Our group has successfully prepared the OA-loaded PEGylated liposomes and achieved significant anti-tumor effect through the enhanced permeability and retention (EPR) effect.[26, 27]

Furthermore, polysaccharide anchored liposomes have attracted increasing interest for its advantages in drug delivery systems: some polysaccharides can anchor their hydrophobic groups into the phospholipid bilayer of liposomes by hydrophobic interaction to form a hydrophilic shell on the liposomes surface. The hydrophilic polysaccharide shell can not only increase the physical stability of liposomes, but also provide steric protection for liposomes, which can escape adsorption of opsonins and phagocytosis of macrophages, so as to prolong their circulation time in blood.[28] Chitosan, a homopolymer of (1,4)- linked 2- amino- 2-deoxy- β -glucan, is made through the deacetylation of chitin, which is the second most prolific, renewable natural polysaccharide after cellulose.[29] Chitosan has been used in many biomedical applications because of select properties such as nontoxicity, biocompatibility, biodegradability and bioadhesivity.[30-32] In this study, chitosan was selected as the positively charged polysaccharide to be used as the anchoring material to coat on the liposomes surface. Chitosan has an amino group, which can be combined with protons, and make chitosan carrying the positive charges.[33] When the chitosan coats on the surface of the liposomes, the chitosan layer not only has the characteristics of the other polysaccharides shell, but also can make the surface of liposomes loading positive charges. The research indicated that tumors exhibit a lower extracellular pH than normal tissues, and the surface of the tumor cells has more negative charges than that of normal cells.[34, 35] Positively charged chitosan-coated OA liposomes can be combined with the negative charges on the surface of the tumor cells. In addition, chitosan may interact with the cell membrane and change the structure of the related tight junction proteins, eventually improve the osmosis of drugs.[36] The chitosan-coated OA liposomes tend to aggregate in the tumor cells, causing release of the drug at the tumor site, which also reduce the toxicity to the normal tissues. Thereby the chitosan-coated OA liposomes can target and inhibit the growth of tumor cells effectively.

In this study, an ethanol injection method was used to prepare OA liposomes, and then chitosan was modified on the surface of liposomes. Atomic force microscopy and transmission electron microscopy were used to observe the morphology of the liposomal vesicles. The stability, release rates and loaded charges of the OA formulations in *vitro* were investigated. The anti-tumor effects of OA formulations on HepG2 cells were detected by MTT assay.

Materials and methods

Materials

Oleanolic acid and Soya lecithin were purchased from Shenyang Tianfeng Biological Pharmaceutical Co., Ltd (Shenyang, China). Chitosan (degree of deacetylation: 92%) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cholesterol, surfactant Tween-80 and anhydrous ethanol were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Sephadex G-75 was obtained from Beijing BioDee Bio Tech Corporation Ltd (Beijing, China). Phosphate-buffered saline was sourced from Sigma Chemical Company (Henan, China). Acetic acid glacial was obtained from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd (Tianjin, China). Methanol and acetonitrilewere purchased from Tianjin Four Friends Fine Chemicals Co., Ltd and were of high-pressure liquid chromatography (HPLC) grade. All other chemical agents used were of analytical grade.

Preparation of OA liposomes and chitosan-modified OA liposomes

OA liposomes were prepared by an ethanol injection method which developed by Batzri and Korn.[37] In brief, the hydrophobic components soya lecithin, cholesterol and OA (in weight ratio of 50: 6: 5) were dissolved in 3 mL of anhydrous ethanol as the lipid phase, which was continuously stirred by electric heating magnetic stirrer until completely dissolved. Tween-80 (0.1%, v/v) was dissolved in 10 mL phosphate-

buffered saline (PBS, pH 6.5) at 43 °C as the aqueous phase. The lipid phase was injected drop wise into the aqueous phase under electric heating magnetic stirrer. The translucent blue opalescent OA liposomes were produced spontaneously after further evaporation of the residual ethanol.

For chitosan-coated OA liposomes, briefly, 0.1% chitosan was dissolved in 0.1 M acetic acid glacial solution (pH 3.5) to obtain a chitosan solution. Chitosan solution was added drop wise to the above liposomal suspension under the magnetic stirrer at room temperature,[38] and then the mixture was incubated at room temperature for 2 hours. The two kinds of liposomes were kept at 4 °C.

Characterization of chitosan-coated OA liposomes

PARTICLE SIZE AND ZETA POTENTIAL

Malvern Zetasizer ZS (Malvern Instruments, UK) was used to measure the sizes and surface zeta potentials of the OA liposomes and chitosan-coated OA liposomes. The mean liposome diameters and zeta potentials were determined by dynamic light scattering (DLS) and electrophoretic mobility measurement, respectively. All characterization measurements were repeated three times at 25 °C.

TRANSMISSION ELECTRON MICROSCOPY (TEM)

The two liposome formulations were stained and analyzed using a TEM (HT7700, Japan). The samples for TEM were prepared by a standard procedure. 3% tungstophosphoric acid was used as a negative staining agent. The negative staining agent and liposome suspension sample were mixed with 1:3 (v/v). Then the carbon film-coated copper grid was placed on the mixed samples for ten minutes, and the excess solution was removed with a filter paper. The samples were air-dried, and then observed under the TEM.

ATOMIC FORCE MICROSCOPY (AFM)

AFM was also used to probe into the surface morphology of the liposomal vesicles. Imaging and force measurement of the liposomes were conducted in tapping mode employing a Multimode AFM, which equipped with Scan Asyst, E-type scanner, Nanoscope V controller, (all from Veeco/broker, America) and a silicon tapping tip having the maximum scan area of 10 μm^2. 30 μL of suspension was dropped onto the surface of a new cleaved mica sheet (about 1 cm×1 cm), which was air-dried on a super clean workbench for the detection. Under the Scan Asyst mode, the images of the liposomes were obtained. In addition, the values of the height representative of liposomal vesicles were obtained by using the software of this apparatus. Separately, the particle sizes were measured using the DLS before the particles were adsorbed onto the mica sheet surface. These two parameters were introduced to describe the change of the height of particle against the particle diameter, which regards as the rigidity of the submicron-size particles (H/P) according the following equation.[39] The scanner was calibrated following the standard procedural offered by digital instruments.

$$H/P = \frac{\text{Mode of height of adsorbed particles (H)}}{\text{Particle size of particles (P)}}$$

FOURIER TRANSFORM INFRARED SPECTROSCOPY（FTIR）

The structure of chitosan-coated OA liposomes was determined via FTIR, which was exploited to demonstrate the chitosan coated liposomes successfully. The chitosan-coated OA liposomes with lyoprotectants were first freeze-dried to powder, using a vacuum freeze-drying machine. A small quantity of lyophilized sample was mixed with pre-dried KBr powder, and then the mixture was grind into fine powder by agate mortar. Finally the fine powder was pressed into a thin KBr pellet with a hydraulic press at 10,000 psi. The FTIR spectra of the samples were recorded on a Nicolet iS10 (USA) spectrometer. The scanning was done in the range 400-4000 cm^{-1} with speed of 2 mm/s at room temperature. A small amount of pure chitosan was also measured as a control.

ENCAPSULATION EFFICIENCY (EE)

The encapsulation efficiencies of OA liposomes and chitosan-coated OA liposomes were determined by dextran gel column chromatography, protein purification system combining with high-performance liquid chromatography (HPLC). The preprocessed Sephadex G-75 was filled with a gel chromatographic column (1.0 cm × 25 cm) to separate free OA from the liposomes entrapped drug. 1 mL sample of OA liposomes or chitosan-coated OA liposomes was injected into the column and eluted by double-distilled water at 215 nm and 2.0 mL / min. The free drug remained in the gel, then the liposomes outflowed from the column and were collected at 6.0 min for high performance liquid chromatography.

The original liposomes and the eluted liposomes were dissolved in the same amount methanol for demulsification, and then centrifuged at 10000 rpm for 30 min. Finally, the supernatant was filtered with an organic membrane (0.45 μm). The amounts of OA in the eluted liposomes and the original liposomes were detected by HPLC, respectively. An Agilent Zorba × 300 SB-C18 column (5 μm, 250 mm × 4.6 mm) was used. The mobile phase, consisting of acetonitrile and 0.1% acetic acid in water (88 : 12, v/v), was maintained at a flow rate of 0.8 mL/min. The ultraviolet detector wavelength was 215 nm and the injection volume was 20 μL. As well, OA standard solution was analyzed using above method. The peak area of response to the concentration of OA was linear in the appropriate range (r = 0.9999). Drug encapsulation efficiency (EE) was calculated from the following equation:

$$EE\ (\%) = \frac{\text{amount of OA in the eluted liposomes}}{\text{amount of OA in the original liposomes}} \times 100$$

Stability study

In the process of storage, liposomes may be mutual confluence leading to changes of their morphology and characteristics, furthermore, resulting in leakage of drug from the liposomes.[40] Hence, the physical stability is a crucial factor for liposomes. In

the study, the OA liposomes and chitosan-coated OA liposomes were stored in 4 °C and 25 °C for a period of 0, 5, 10, 20 and 30 days, respectively. At the different time point, the leakage rates for the liposomes were analyzed to evaluate their stability. All experiments were performed in triplicate. The leakage rates were calculated according to the following equation:

$$\text{Leakage rate (\%)} = \left(1 - \frac{\text{EE (\%) of liposomes after storage}}{\text{EE (\%) of the original liposomes}}\right) \times 100$$

Drug release in *vitro* study

In order to realize the release behavior in *vitro* and the pH sensitivity of the nanoparticles, drug release effects from OA liposomes and chitosan-coated OA liposomes were determined using a dynamic dialysis method at pH 5.5 and 7.4. In this study, buffered mediums (pH 5.5 and 7.4 phosphate buffers) were used to simulate the tumor environment and the normal body environment, respectively. The liposomal samples (1 mL) were placed into the dialysis bags (molecular weight cutoff 12-14 k Da), and the bags were pretreated in the usual manner. Then the dialysis bags containing liposomal samples were immersed in 200 mL of phosphate buffers (pH 5.5 or 7.4) at 37 °C using a rotational speed of 100 rpm. At the different time intervals, the OA contents of samples in the dialysis bags were determined. In the end, the residual OA contents in the liposomes were analyzed by HPLC. All experiments were performed in triplicate.

Anti-tumor effects assay

The anti-cancer effects of OA solution, OA liposomes, chitosan-coated liposomes without OA and chitosan-coated OA liposomes were evaluated using the MTT assay with HepG2 (liver carcinoma) cells. Briefly, HepG2 cancer cells were seeded in 96-well flat-bottomed plates at a seeding density of 2.0×10^4 cells / well and grown for 24 hours at 37 °C in a 5% CO_2 atmosphere. At that time, cells were attached and grew. The above samples were filtered through sterile 0.22 μm filter membrane, and then they were diluted with phosphate-buffered saline (pH 7.4) to obtaine various drug concentrations of 187.5, 93.75, 46.88, 23.44, 11.72 and 5.87 μg / mL. Each sample was done in triplicate (100 μL / well) into the appropriate well of culture dishes. Blank controls were processed with culture medium. The drug-treated cells were incubated for 24 hours under the conditions mentioned above, and the cells' viability was analyzed using the MTT agent to investigate the anti-cancer effects of all the formulations. After 24 hours, the medium was discarded and the cells were incubated with 200 μL fresh medium containing 0.5 mg / mL MTT for 4 hours. After removed the unreduced MTT, 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbances of the samples were measured at 490 nm using a microplate reader. The assays were performed for three times.

Results and discussion

Characterization of chitosan-coated OA liposomes

PARTICLE SIZE AND ZETA POTENTIAL

In this study, the zeta potentials were determined by electrophoretic mobility measurement. The original liposomes had a marked negative charges (-12.3 ± 1.9 mV), which were inverted to positive value (19 ± 0.814 mV) after the incubation with chitosan. Surface zeta potentials of two liposome samples can be seen from the Fig. 1. The inversion indicated that chitosan has conjugated on the OA liposome surface. The strong positive charges implyed the presence of cationic amine groups of chitosan on the particle surfaces. Positively charged chitosan-coated OA liposomes can be combined with the negative charges on the surface of the tumor cells. Hence, the chitosan-coated OA liposomes tend to aggregate in the tumor cells, and causing release of the drug at the tumor site,[36] which can inhibit effectively the growth of tumor cells.

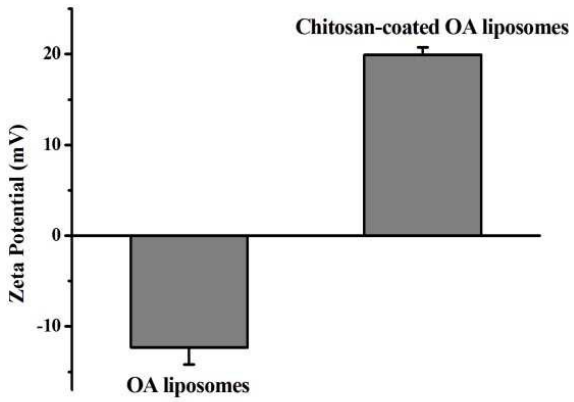

Fig. 1 Surface zeta potentials of OA liposomes and chitosan-coated OA liposomes. Data represent mean ± SD (n=3).

The particle sizes were detected by the DLS. The size distributions with the log-normal fitting curves of OA liposomes and chitosan-coated OA liposomes were shown in Fig. 2. The average diameter of OA liposomes was around 118.76 nm, however, the mean size of chitosan-coated OA liposomes was about 167.44 nm, which was evidently larger than that of the original liposomes. The increase on particle size of the chitosan-modified OA liposomes attributed to the adsorption of chitosan, which also indicated the formation of coating layer on the surface of the liposomes. The particles sizes of both samples were relatively uniform, which also indicated without contact and fusion phenomena on the liposomes. The sizes of liposomes may be a key factor for targeting to tumor tissues and release the antitumor drugs, because nanoparticles size of 150-200 nm is beneficial to accumulate in tumor tissue.[19, 21, 22, 25] Hence, the modified liposomes' diameter (167.44 nm) was more conducive to the tumor tissue.

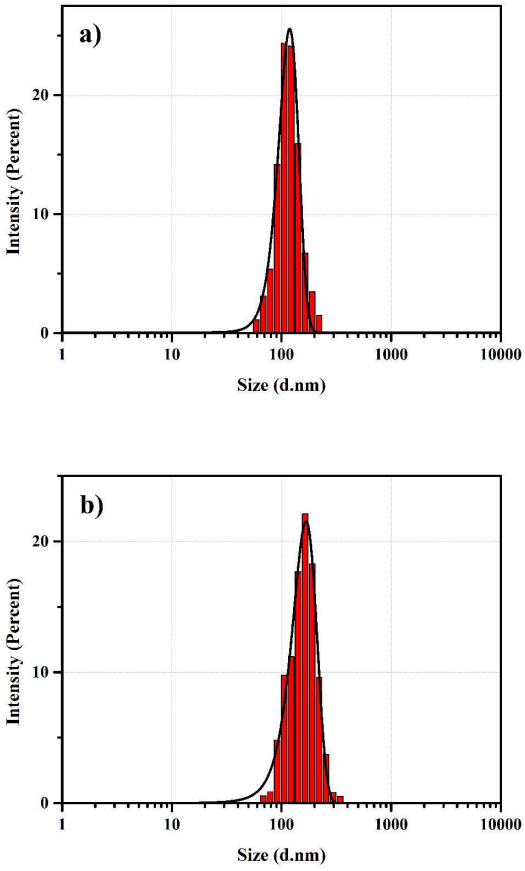

Fig. 2 Size distribution of the liposomal vesicles with the log-normal fitting curve: a) OA liposome; b) chitosan-coated OA liposomes.

Transmission Electron Microscopy

The morphologies of two liposome samples were visualized using TEM. The structures were shown in Fig. 3. It could be seen that both of the liposomes presented an almost spherical morphology, yet no significant morphological differences between them were observed. The action of chitosan on liposomes is due to a combination of adsorption coagulation and bridging between them.[41] The strong adsorption between the polymer and liposomal bilayer resulted in a smooth surface of liposomes, so it was difficult to distinguish the chitosan on the liposome surface. Other previous articles also reported the same situation.[42, 43]

Atomic force microscopy

AFM of liposomal vesicles were shown in the Fig. 4. The two vesicles were spherical and monodispersed. The heights of the OA liposomes and chitosan-coated OA liposomes were about 75.8 nm and 182.6 nm, respectively. Meanwhile, the middle sag was appeared on the OA liposomes. The ratio of the height to the particle diameter reflects the rigidity of liposomal vesicles, and strong rigidity liposomes presented a hemisphere without middle sag.[39] Thus, the rigidity of chitosan modified liposomes was stronger than that of the ordinary liposomes. Rigidity great influenced the stability of liposomes, which also connected with the drug release and residence time in the blood circulation. Therefore, the stability of the chitosan-coated OA liposomes may be enhanced.

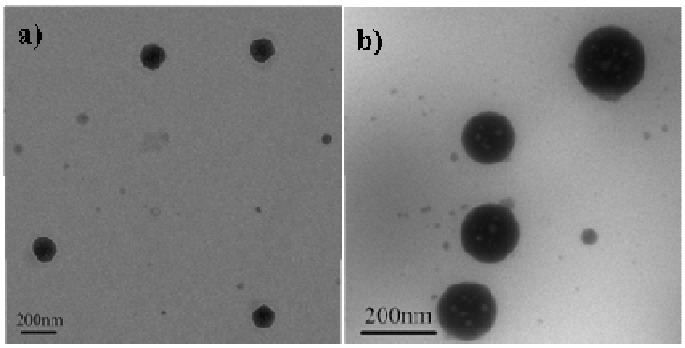

Fig. 3 TEM of liposomal vesicles. a) OA liposomes; b) Chitosan-coated OA liposomes.

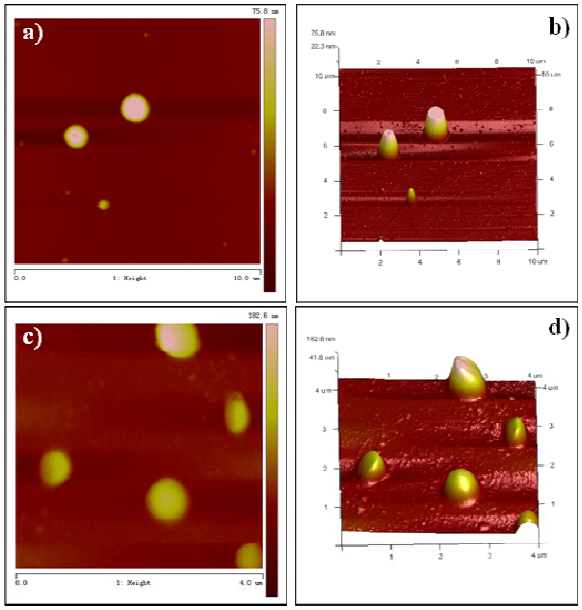

Fig. 4 AFM images of liposomal vesicles. a) and b) OA liposomes; c) and d) chitosan-coated OA liposomes; b) and d) were three-dimensional maps.

Fourier transform infrared spectroscopy

The FTIR spectra of pure chitosan and chitosan-coated OA liposomes were shown in Fig. 5. In the pure chitosan, the FTIR spectrum exhibited a strong peak at 3433 cm^{-1} which is attributed to axial stretching vibration of O-H superimposed to the N-H stretching band and the inter hydrogen bonds of the polysaccharide. The band at 2920 cm^{-1} was seen in the FTIR spectrum of the pure chitosan, which corresponds to the C-H stretching. The band at 1633 cm^{-1} indicates the presence of chitosan-NHAc (acetyl) units (with > C=O stretching).

Meanwhile, the stretching vibration of C-O was found at 1051 cm^{-1}. The similar result has been reported by Krishnapriya KR et al,[44] Hailong Peng et al.[45] and Xiaofei Liang et al.[46] Characteristic peaks of chitosan were also distincted in the chitosan-coated OA liposomes, indicating that the chitosan was successfully modified on the OA liposomes.

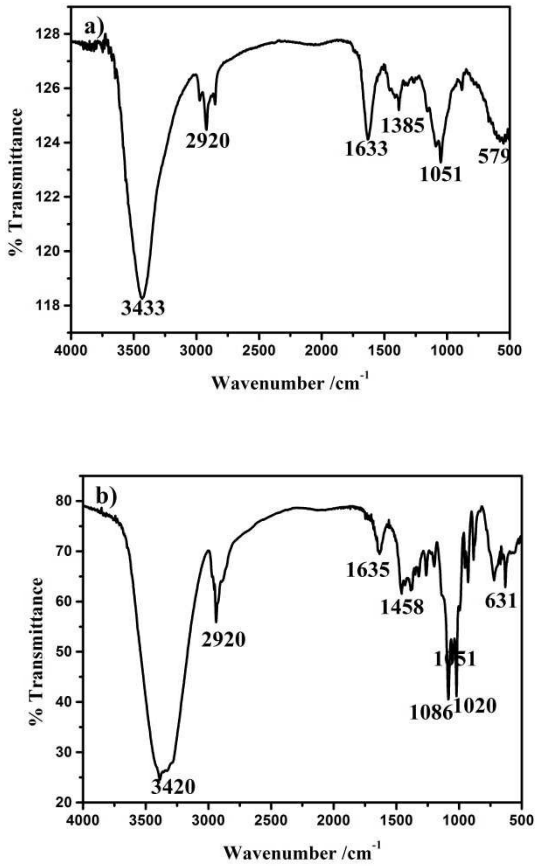

Fig. 5 FTIR spectra: a) pure chitosan; b) chitosan-coated OA liposomes.

ENCAPSULATION EFFICIENCY

The encapsulation efficiency was determined by gel chromatography separation and HPLC. The EE of OA was 94.3% for conventional liposomes, and chitosan-coated OA liposomes' EE was 94.7%. In consequence, almost all of the drugs were encapsulated into liposome vesicles. At the same time, it can be seen that the EE of both samples were very close, which only showing 0.4% difference.

Stability study

When the liposomes are stored for a long time, drug leakage usually occurs. In order to reduce drug loss, it is essential to study the stability of liposomes. In this study, OA liposomes and chitosan-coated OA liposomes were stored at 4 °C and 25 °C for a period of 0, 5, 10, 15, 20, 25 and 30 days, and then evaluated their leakage rates. Fig. 6 showed the effects on leakage rates of the liposomes. An insignificant difference was found in the leakage rates between the two liposomal formulations stored at 4 °C and 25 °C. Both the leakage rates of OA liposomes and chitosan-coated OA liposomes, were evidently reduced at 4 °C compared with 25 °C. For instance, the leakage rates of chitosan-coated OA liposomes were 28.92% ± 1.58% and 18.72% ± 1.18% stored at 25 °C and 4 °C after 30 days, respectively. The results indicated that temperature was an important factor in the leakage rate. The low temperature (4 °C) was more beneficial to preservation of liposomes integrity, the probable reason was that the high temperature expedited the hydrolysis and oxidation of liposomes.

It's worth noting that the leakage rates of chitosan-coated OA liposomes were lower than those of OA liposomes'. For example, the leakage rates were 18.72% ± 1.18% for the former and 29.11% ± 0.98% for the latter at 4 °C after 30 days storage. The main reason was that the chitosan shell increased the liposomal particles' charges, and prompted repulsion between nanoparticles, thereby improving the stability of the nano system.[47] In addition, chitosan contains hydroxyl groups along its backbone which can form hydrogen bonds between the polymer and water molecules.[48] Chitosan by modifying the surface of the liposomes, could form a layer of dense protective film on the lipid membrane surface, reducing the lipid membrane fluidity, and improving the hydrophilicity of liposomes. Hence, chitosan improved the stability of liposomes to prevent the encapsulated drugs in liposomes leakage outward. It could be concluded that chitosan-coated OA liposomes had better storage stability than OA liposomes.

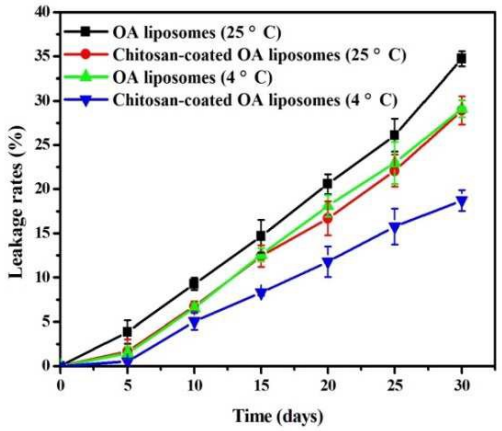

Fig. 6 Leakage rates of OA liposomes and chitosan-coated OA liposomes stored at 4 °C and 25 °C for 30 days. All values are expressed as mean ± standard deviation (n = 3).

Drug release in *vitro* study

In order to analysis the release behavior and investigate the pH sensitivity of the nanoparticles, drug release from OA liposomes and chitosan-coated OA liposomes in *vitro* were determined using a dynamic dialysis method at pH 5.5 and 7.4. Fig. 7 showed the release profiles of all delivery systems.

The release profiles of liposomal dispersions contained two phases, which included a relatively large burst effect in the initial stage, and thereafter showing a slower release phase. A similar phenomenon had been reported previously.[49] The burst release was mainly ascribed to drug detachment from the outer surface of the liposomes, while the later slow release part was due to the fact that the diffusion of the drug from the lipid bilayer across entered the release media.[50] Therefore, liposomes played a role to set back the internal phase drug released.

Under the normal physiological condition (pH 7.4), OA was released in the continuous way for 72 hours reaching 76.13% ± 2.56% and 66.61% ± 4.95% for OA liposomes and chitosan-coated OA liposomes, respectively. The encapsulation of OA into liposomes significantly slowed drug release, which indicatied the depot effect of liposomes, especially the chitosan-coated OA liposomal formulations. The sustained release behavior of OA from chitosan-coated OA liposomes was partly due to the increased stability of chitosan,which effectively prevented leakage of drug from liposomes; On the other hand, it may be because chitosan shell closely integrated with the liposome surface leading to slow drug release.[51]

Furthermore, the drug release of chitosan-coated OA liposomes was significantly enhanced under acidic conditions (pH 5.5). After 72 hours, 97.74% ± 4.45% of the encapsulated OA had been released at pH 5.5, whereas 66.61% ± 4.95% had been released at pH 7.4. Besides, the drug release of chitosan-coated OA liposomes under acidic conditions was also further enhanced than that of OA liposomes. The OA release rates from chitosan-coated OA liposomes were about 80.24% ± 2.72% and 96.45% ± 3.32% over 24 and 48 hours. Zhu et al. have reported that 40% and 46% DOX release from the pH-sensitive liposomes over 24 and 48 hours at pH 5.5.[35] Therefore, chitosan-coated OA liposomes exhibited a slow, controlled release of OA at pH 7.4, and then a rapid release with a decrease in the pH value. The chitosan-coated OA liposomes can achieve the desired effect of tumor-targeting and drug controlling release. This may be owing to the fact that positively charged chitosan-coated OA liposomes tend to aggregate, triggering release of OA in the acidic environment contrast with the neutral environment.

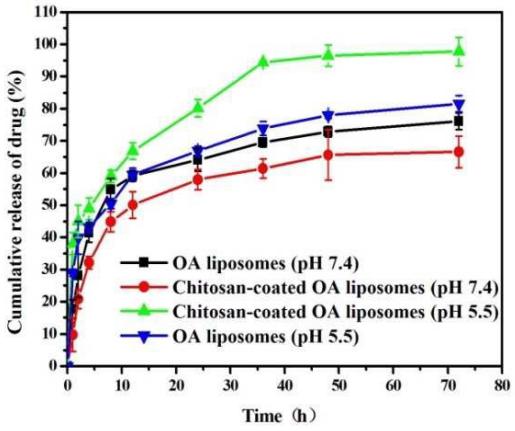

Fig. 7 Release profiles in *vitro* of OA liposomes and chitosan-coated OA liposomes. Data represent mean ± SD (n=3).

Anti-tumor effects assay

The anti-tumor effects of OA solution, OA liposomes, chitosan-coated liposomes without OA and chitosan-coated OA liposomes in *vitro* were investigated. The results were shown in Fig. 8 and Fig. 9. As shown in the Fig. 8, cell viability was measured quantitatively by a microplate reader. OA solution had the lowest anti-tumor effect, showing minimum inhibitory rate (26.07% ± 0.53% at 187.5 μg•mL^{-1}). The HepG2 cells treated with OA liposomes and chitosan-coated OA liposomes (187.5 μg•mL^{-1}) exhibited 65.0% ± 1.45% and 74.71% ± 0.76% cell inhibitory rates, respectively. As can be seen from the datum, the anti-cancer effect of OA was improved by entrapping in liposomes. Notably, chitosan-coated OA liposomes enhanced tumoricidal effect compare to the regular OA liposomes. The same effect was obtained when cells were treated using the low and middle OA concentration. The cell numbers were reduced in a concentration-dependent manner. Moreover, the chitosan-coated liposomes without OA can also inhibit the growth of cancer cells and the inhibitory rate was 15.72% ± 1.0%. This may be due to the action of chitosan, which also can inhibit the growth of tumor cells to some extent.[52-54]

In addition, anti-cancer effect was analyzed qualitatively by photographs. The cells were incubation with OA formulations after 24 hours, significantly better results were obtained for all OA formulations than the control group. More suspended dead cells were viewed in the OA formulations, which manifested that OA can inhibit the tumor cells growing, and the anti-tumor effect of chitosan-coated OA liposomes was most remarkable. After adding MTT, the amount of formazan crystallization can be clearly seen from the Fig. 9. The cells that were treated with chitosan-coated OA liposomes, can produce the minimum amount of formazan crystallization compared with other groups, which indicated that the anti-tumor effect was best among the formulations. The high inhibition rates of chitosan-coated OA liposomes probably due to the fact that

chitosan may interact with the cell membrane, and change the structure of the related tight junction proteins, eventually improve the osmosis of drugs.[36] Through a series of experimental results, chitosan-coated OA liposomes can achieve more ideal anti-cancer effects than OA solution and OA liposomes.

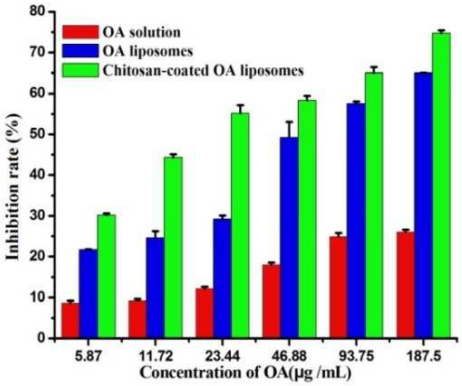

Fig. 8 Anti-cancer effects of OA solution, OA liposomes and chitosan-coated OA liposomes. All values are expressed as mean ± SD (n=3).

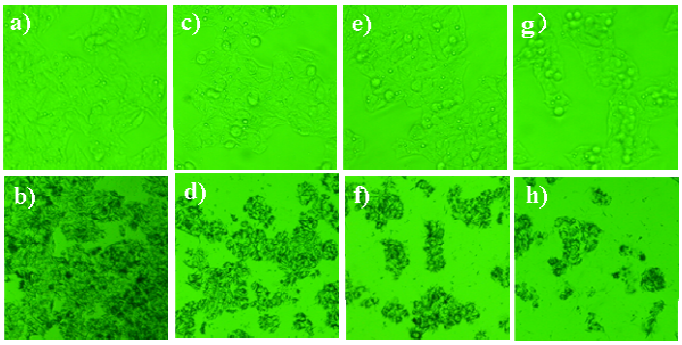

Fig. 9 Images of HepG2 cancer cells after 24 hours incubation with OA formulations. a) b) Control group; c) d) OA solution; e) f) OA liposomes; g) h) Chitosan-coated OA liposomes. Top row exhibits cells treated without MTT; Bottom row exhibits cells treated with MTT.

Conclusions

In this study, the chitosan modified OA liposomes were prepared and charactered, and their anti-tumor effects were studied. The average size of chitosan-coated OA liposomes was near 167.44nm, which was more conducive to targeting the tumor tissue. Meanwhile, the surface zeta potential of chitosan-coated OA liposomes was 19.9 ± 0.814 mV, whose high positive charges easily targeted the tumor tissues. Furthermore, the chitosan-modified OA liposomes were more rigid than those of the ordinary liposomes, so the stability of the chitosan-coated OA liposomes may be enhanced. The increasing stability of liposomes can delay drugs leakage. It was worth noting that chitosan-coated OA liposomes exhibited a slow, controlled release of OA at normal physiological conditions, and a rapid release in tumor tissues, so they can achieve tumor-targeting drug delivery and controlling release effects. The cells experiment results also indicated that chitosan-coated OA liposomes have more significant anti-cancer effects than OA solution and OA liposomes. Hence, chitosan-coated liposome is a promising drug carrier.

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