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Characterization, Antibiofilm, and Mechanism of Action of Novel PEG-Stabilized Lipid Nanoparticles Loaded with Terpinen-4-ol

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ABSTRACT: Terpinen-4-ol, an active component of tea tree oil, exhibits broad-spectrum antimicrobial activity. However, the high volatilization of terpinen-4-ol and its nonwettability property have limited its application. Our objective was to synthesize novel nanocarriers to deliver and protect terpinen-4-ol. The polyethylene glycol (PEG)-stabilized lipid nanoparticles were prepared and characterized by scanning electron microscope, Zetasizer, and differential scanning calorimetry. These nanoparticles had an average diameter of 397 nm and a Z-potential of 10 mV after being modified by glycine. Results showed that homogeneous particle size, high drug loading, stability, and targeting were obtained by the nanoparticles. Liquid chromatography/mass spectrometry showed a sustained release trend from nanoparticles for terpinen-4-ol. Minimum inhibitory concentration and minimum biofilm eradication concentration were tested against *Candida albicans* ATCC 11231. Studies on isolated mitochondria showed the blockage of biofilm respiration and inhibition of enzyme activity. The effects can be ascribed to localization of terpinen-4-ol on the membrane of mitochondria.

KEYWORDS: *terpinen-4-ol, Candida albicans, biofilm, lipid nanoparticle, succinate dehydrogenase*

INTRODUCTION

The essential oil derived by steam-distillation from the leaves of *Melaleuca alternifolia* is known as tea tree oil (TTO), a complex mixture of about 100 different compounds, mainly monoterpenes and their corresponding alcohols. Terpinen-4-ol is the major active component of tea tree oil. Terpinen-4-ol gained attention because of its antibacterial, antifungal, antiviral, and anti-inflammatory properties.[1−4] In recent years, it has especially gained popularity as a natural antimicrobial agent against microbial species (*Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*)[5−7] and drug-resistant bacteria (methicillin-resistant *Staphylococcus aureus* (MRSA)),[8,9] and fungi (*Saccharomyces cerevisiae*, *Candida albicans*).[10]

However, microorganisms in biofilms exhibit elevated resistance to both antibiotics and the host defense systems, which often results in persistent and difficult-to-treat infection.[11,12] *Candida albicans* (*C. albicans*) ATCC 11231 is able to form biofilms, which are complex structures consisting of surface-attached cells surrounded by a self-produced extracellular polymer matrix. *C. albicans* ATCC 11231 biofilm formation contributes to its pathogenesis in a number of conditions. Additionally, biofilms of *C. albicans* ATCC 11231 on surfaces pose a serious risk of food contamination in the food industry.[13] The nonwetting property of *C. albicans* ATCC 11231 biofilm limits the penetration of antimicrobial liquids into the biofilm and severely compromises their efficacy.

Because terpinen-4-ol is volatile and hydrophobic, its application is limited. One solution is to immobilize it in a pharmaceutical carrier, which would be important for its stability and antibiofilm activity. Lipid nanoparticles are alternative drug carriers. Compared to other particulate carriers, lipid nanoparticles have more advantages for drug loading, such as good tolerability and biodegradability. In recent years, the study of pharmaceutical carriers has markedly increased.[14] However, thus far, few reports have discussed terpinen-4-ol loaded polyethylene glycol (PEG)-stabilized lipid nanoparticles and their antibiofilm activities.

In this paper, terpinen-4-ol was immobilized in novel lipid nanoparticles by film sonication technology. Then we modified the lipid nanoparticles to improve their stability and targeting ability to *C. albicans* ATCC 11231 biofilm. The lipid nanoparticles were characterized by scanning electron microscopy (SEM), Zetasizer, and differential scanning calorimetry (DSC). The antibiofilm activity of the terpinen-4-ol loaded nanoparticle was evaluated by MTT assay. Meanwhile, to our knowledge, we are the first to report the mechanism of terpinen-4-ol-loaded nanoparticles against *C. albicans* ATCC 11231 biofilm.

MATERIALS AND METHODS

Materials. Glyceryl monostearate (GMS) and polyethylene glycol stearate (PEG-stearate) were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (Shanghai, China). 1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide (EDC) and glycine (Gly) were obtained from Shanghai Mychems Company. Albumin was obtained from Shanghai Pufei Biotechnology Co., Ltd. 2,6-Dichlorphenol indophenol (DCPIP) and phenazine methosulfate (PMS) were obtained from Shanghai Sangon Biotechnology Company. Terpinen-

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4-ol and rotenone were obtained from Sigma. Gly-PEG-stearate and the strain used in this study were from our laboratory. All the other reagents were made in China and of analytical grade or molecular biology grade.

Preparation of Lipid Nanoparticles. Drug-loaded lipid nanoparticles were prepared using the film sonication method. A number of preliminary experiments were conducted to select the most appropriate conditions for lipid nanoparticle formulation. In brief, 70 mg of GMS and 30 mg of Gly-PEG-stearate were dissolved in 3 mL of methylene chloride. The mixture was vortexed for 5 min and the solvent evaporated under reduced pressure. The resulting film was hydrated in 10 mL of PBS buffer (pH 7.0) containing 5 mg of terpinen-4-ol. Then, the solution was sonicated with an ultrasonic probe for 10 s at 15 W.

Physicochemical Characterization of Lipid Nanoparticles. *Particle Size Analysis.* The average diameter of nanoparticles was determined by scanning electron microscope (SEM). The aqueous nanoparticle dispersions were diluted with PBS buffer before analysis. Each value is the average of five measurements.

Zeta Potential. The particle charge was quantified as zeta potential (ZP) by using a Zetasizer (Malvern, U.K.) at 25 °C. Measurements were performed in bidistilled water adjusted with sodium chloride to a conductivity of 50 microsiemens (μS)/cm. The pH values of the samples were always 6.0 ± 0.5.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) was performed with a DSC Q100 (TA Instruments, USA). DSC scans were recorded at a heating rate of 2 °C/min. Samples were heated from 25 to 140 °C.

Liquid Chromatography/Mass Spectrometry (LC/MS). In previous reports,[15] terpinen-4-ol contents were measured by gas chromatography/mass spectrometry (GC/MS). In this study, we developed an LC/MS method to examine drug loading and release. Drug contents were measured by an API3000 LC/MS system (Applied Biosystems, USA). High performance liquid chromatography (HPLC) analytical separations were performed with an SCL-10A (Shimadzu, USA) pump and symmetry C_{18} column (4.6 × 75 mm) that was coupled directly to a mass spectrometer. Elution was carried out at room temperature by using water:methanol:formic acid (20:79.9:0.1, v/v/v) as a mobile phase at a flow rate 0.3 mL/min. Terpinen-4-ol had an m/z measured to 193.1 $[M + K]^+$ and 177.0 $[M + Na]^+$.

Encapsulation Efficiency and Drug Loading Content. To determine drug loading, the terpinen-4-ol loaded lipid nanoparticles dispersions were evaporated under reduced pressure to remove the free terpinen-4-ol in the solution. Then they were dissolved in methanol, and centrifuged at 10000 rpm at 4 °C for 10 min to remove the precipitate. The supernatants were determined using LC/MS. The concentrations of terpinen-4-ol were calculated by peak area. The encapsulation efficiency (EE) is defined as the ratio of the weight of encapsulation drug and total drug, and drug loading content (LC) is the ratio of the weight of encapsulation drug and total nanoparticles. EE and LC were calculated using the following equations:

$$\text{EE}\,\% = \frac{\text{To} - \text{Fo}}{\text{To}} \times 100\% \quad (1)$$

$$\text{LC}\,\% = \frac{\text{To} - \text{Fo}}{\text{Wp}} \times 100\% \quad (2)$$

where To and Fo are the weight of total and free terpinen-4-ol, respectively. Wp is the weight of lipid nanoparticles.

In Vitro Release Study. The dialysis technique was used to study the release of terpinen-4-ol from nanoparticles in aqueous solution containing 1% Tween 80. A 12 mL of terpinen-4-ol loaded lipid nanoparticle dispersion was poured into a dialysis bag (molecular weight cutoff 8000 Da) with the two ends fixed by clamps. The bags were placed in a beaker, and 200 mL of aqueous solution containing 1% Tween 80 was added. The beaker was placed into a magnetic stirrer (Dongxi, China) at room temperature at a rate of 100 times/min. At selected time intervals, 0.5 mL samples were withdrawn for analysis from the dialysis bag, evaporated under reduced pressure, diluted with methanol, and centrifuged at 10,000 rpm at 4 °C for 5 min. Samples were analyzed by LC/MS. All the operations were carried out in triplicate.

Stability of Lipid Nanoparticles. Terpinen-4-ol loaded nanoparticles were stored at room temperature for 4 weeks under a sealed glass bottle. The particle size, zeta potential, and drug encapsulation efficiency were determined to evaluate stability of the formulation.

Effects of Terpinen-4-ol Loaded Lipid Nanoparticles on Biofilms. *C. albicans* ATCC 11231 was grown by shaking in liquid medium (1% yeast extract, 2% peptone and 2% glucose, YPD) at 30 °C; cell density was assessed with a Varian Cary 50 Probe UV−visible spectrophotometer (Varian, USA) at 600 nm (OD_{600}). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were used to evaluate terpinen-4-ol antimicrobial activity. The methods were from our previous research and references.[16−18] Blank nanoparticles served as control.

A biofilm evaluation test was performed for *C. albicans* ATCC 11231. The antibiofilm activity of terpinen-4-ol-loaded nanoparticles was assayed against *C. albicans* ATCC 11231 cells by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. An initial inoculum of 1×10^3 cfu/mL was prepared for use in the biofilm evaluation test by diluting an actively growing culture in YPD. *C. albicans* ATCC 11231 at a density of 1×10^3 cfu/mL was seeded in a 96-well plate in the YPD. After culturing for 16 h, the media were exchanged with 200 μL of culture medium containing a specified concentration of terpinen-4-ol-loaded lipid nanoparticles or free drug or blank lipid nanoparticle dispersion. After 4 h of incubation, the culture medium from each well was removed, and the cells were washed twice with PBS (pH 7.0). Then, 200 μL of YPD culture medium and 20 μL of MTT solution (5 mg/mL in PBS) were added to each well. After 2 h of additional incubation, the media were removed, and formazan crystals were solubilized with 200 μL of DMSO for 15 min. The amount of formazan was then determined from the optical density at 560 nm by a BioTek ELX800 (BioTek, USA). The minimum biofilm eradication concentration (MBEC) was defined as the lowest terpinen-4-ol concentration resulting in a decrease in absorbance to <5% of the control. Control experiments were carried out using blank nanoparticle treatment. The results were expressed as percentages relative to the result obtained with control.

Preparation of C. albicans ATCC 11231 Mitochondria. Cells of a strain of *C. albicans* ATCC 11231 were incubated in a culture medium and then starved overnight. The cells were then centrifuged, washed twice with water, and weighed. One gram of wet cells was resuspended in 3 mL of preparation medium (0.6 mol/L mannitol, 0.2% albumin, 10 mmol/L imidazole, pH 6.8). Mitochondria from yeast cells were prepared as the reference.[19] To prepare mitochondria, the cell suspension described was treated in a French Press (Thermo, USA) at 200 MPa and 4 °C.

Succinate Dehydrogenase Activity. This activity was determined by measuring spectrophotometrically the reduction of 2, 6-dichloroindophenol in incubation medium containing 50 mmol/L potassium phosphate buffer (pH 7.6), 100 μmol/L KCN, 275 μmol/L phenazine methosulfate, 5 mmol/L sodium succinate, 5 μg of rotenone, plus variable concentrations of terpinen-4-ol and 2 mg of mitochondrial protein at room temperature. After 2 min, 2,6-dichloroindophenol was added. The absorbance changes were determined at 600 versus 590 nm.

RESULTS

PEG-Stabilized Lipid Nanoparticle Design. The PEGylated surfaces of the pharmaceutical carriers can improve the performance of these systems in biological environments.[20] Researchers have become interested in quantifying of the PEG density on the surface of nanoparticles and have developed mathematical models to describe the molecular arrangement of the polymer chains on the surface of the pharmaceutical carriers. According to these studies, increasing the density and length of PEG favored the stability of carriers. In the present study, we investigated the effect of the amount of PEG-stearate

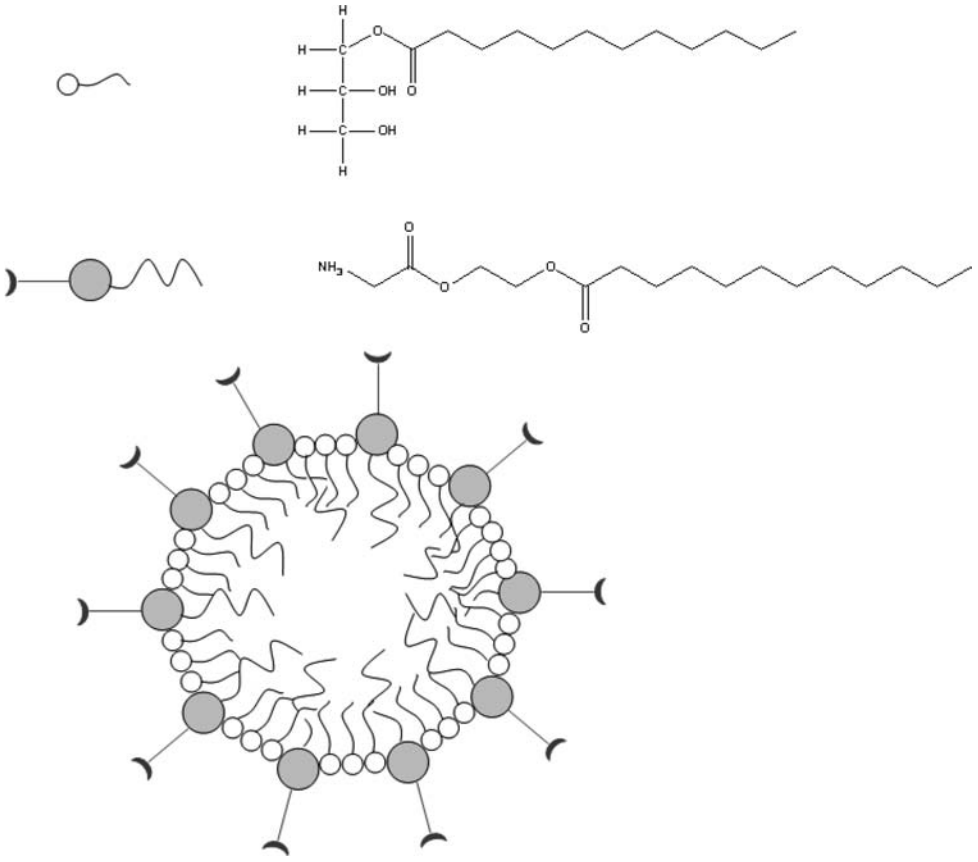

Figure 1. Schematic illustration of PEG-stabilized lipid nanoparticles after modification by glycine.

on the lipid nanoparticles. Our results indicated that the stability of lipid nanoparticles was high when a large amount of PEG-stearate was added to the nanoparticle formation medium; however, efficiency decreased with increases in the amount of PEG-stearate. We designed and synthesized novel glycine-modified lipid nanoparticles (Figure 1). In previous experiments, we optimized the formation conditions of nanoparticles based on the ensurance of the drug loading, stability, and target of nanoparticles. It is 30% of Gly-PEG-stearate in the nanoparticle formation medium.

Size Distribution and Zeta Potential Measurements. Using the film sonication method, we were able to produce physically stable lipid nanoparticles, both empty and terpinen-4-ol loaded. SEM analysis was performed to get more information about lipid nanoparticle size and shape. The shape of terpinen-4-ol-loaded lipid nanoparticles was almost spherical, and the size was about 397.0 nm (Figure 6 and Figure 2). The average diameter and size distribution of nanoparticles were determined by a Zetasizer at 25 °C. The values in Figure 2 indicated a fairly narrow size distribution of nanoparticles. The change in particle Z-potential was determined by measuring it before and after being modified by glycine. The initial Z-potential was less than 2.47 mV in lipid nanoparticle batches with PEG-stearate. We found higher Z-potential in surface-modified nanoparticle batches compared with unmodified nanoparticles. The Z-potential was 10.1 mV after being modified by glycine and was associated with the high dispersion stability.

Differential Scanning Calorimetry (DSC) Analysis. DSC is a useful method to evaluate the physical state of the lipid nanoparticle matrix, which affects the physicochemical properties and thermodynamic stability of lipid nanoparticle dispersions. To investigate the thermostability of lipid nanoparticles, DSC runs were performed by heating the bulk materials from 40 °C to120 °C. The heating run of bulk PEG-stearate showed the highest peak at 86.27 °C, and the highest peak was at 95.09 °C after modification by glycine. Regarding the effect of terpinen-4-ol on lipid nanoparticle thermostability, no significant change was noted after incorporating terpinen-4-

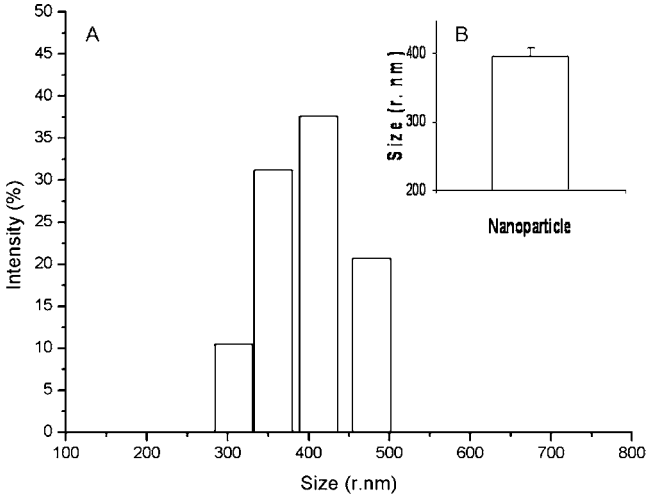

Figure 2. The diameter and size distribution of lipid nanoparticles after modification by glycine. Shown are the size distribution of the nanoparticles from a representative experiment (A) and average size of nanoparticles in aqueous solution from five independent experiments (B).

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ol into PEG-stabilized lipid nanoparticles (data not shown). The results from DSC indicated little change in the thermal properties of nanoparticles before or after modification by glycine (Figure 3). Meanwhile, the change of thermal properties also proved that glycine successfully bound to PEG (Figure 3).

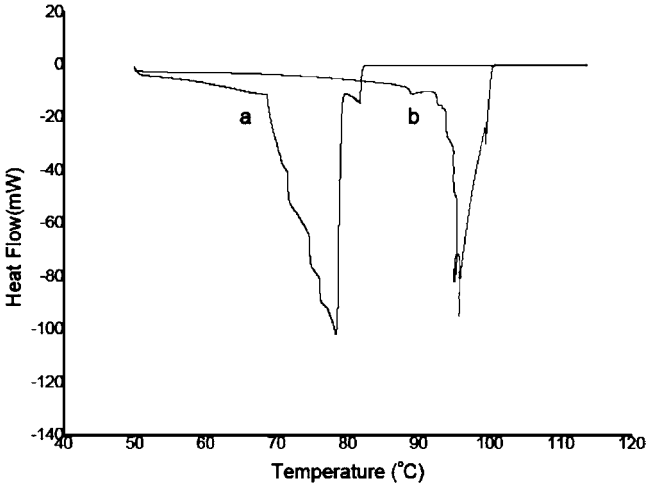

Figure 3. Differential scanning calorimetry (DSC) thermograms of PEG-stabilized lipid nanoparticles with (a) and without (b) modification by glycine.

LC/MS Analysis. To establish the optimum MS conditions, infusions of terpinen-4-ol standard solutions were performed. Declustering potential (DP) varied from 5 to 120 V, and collision energy (CE) varied from 5 to 45 V. Optimal DP and CE selected were DP = 80 and CE = 20 for terpinen-4-ol. All MS data were collected in positive ion mode. The MS spectrum showed that the m/z 193 ion corresponds to the $[M + K]^+$ of terpinen-4-ol, and the ion at m/z 177 is formed by adding Na^+ to m/z 154. The chromatograms of terpinen-4-ol obtained from the LC/MS analysis are shown in Figure 4. The peak at 4.9 min was due to terpinen-4-ol, as shown by the presence of the $[M + K]^+$ ion at m/z 193. In conclusion, our LC/MS analysis successfully determined the concentration of terpinen-4-ol in the lipid nanoparticles. The MS study also indicated that terpinen-4-ol easily received K^+ compared to Na^+ and H^+.

Drug Loading Analysis and Release Study. Terpinen-4-ol is volatile, which may make encapsulation difficult. In addition, terpinen-4-ol tends to adsorb onto surfaces such as glass and plastic, which can lead to distinct losses in the amount of terpinen-4-ol available for delivery. Low temperature condition of the dispersed aqueous phase was used in preparing lipid nanoparticles. In this study, drug loading content and encapsulation efficiency were calculated on a 5% theoretical loading using equations described in the experimental methods. Based on our previous experiments, the EE % and LD % were 82.4% and 4.1% in optimal conditions.

The studies have shown that incorporating volatile compounds into nanocarriers prevents their rapid evaporation.[21] To investigate the capability of lipid nanoparticles preventing the rapid evaporation of the incorporated terpinen-4-ol, the release was performed using the dialysis method. The cumulative release of terpinen-4-ol from lipid nanoparticles was 76.7% after 12 h (Figure 5). However, we did not detect any free terpinen-4-ol in the aqueous solution 20 h after incubation. Figure 5 clearly shows that the incorporation of terpinen-4-ol into lipid nanoparticles significantly decreased its evaporation.

Stability Study of Lipid Nanoparticles. After the lipid nanoparticle suspension was stored at room temperature for 4 weeks, the mean size of drug-loaded lipid nanoparticles was 405.7 nm, not significantly changed compared to the initial particle size (397.0 nm). After adding Gly-PEG-stearate to the lipid nanoparticle formulation, there was no visible aggregation in the system during storage (Figure 6). The results showed that modifying the lipid nanoparticles with glycine enhanced their stability.

Antibiofilm Study. The terpinen-4-ol-loaded nanoparticles showed better efficacy toward *C. albicans* ATCC 11231 with a longer duration effect than did terpinen-4-ol only. The

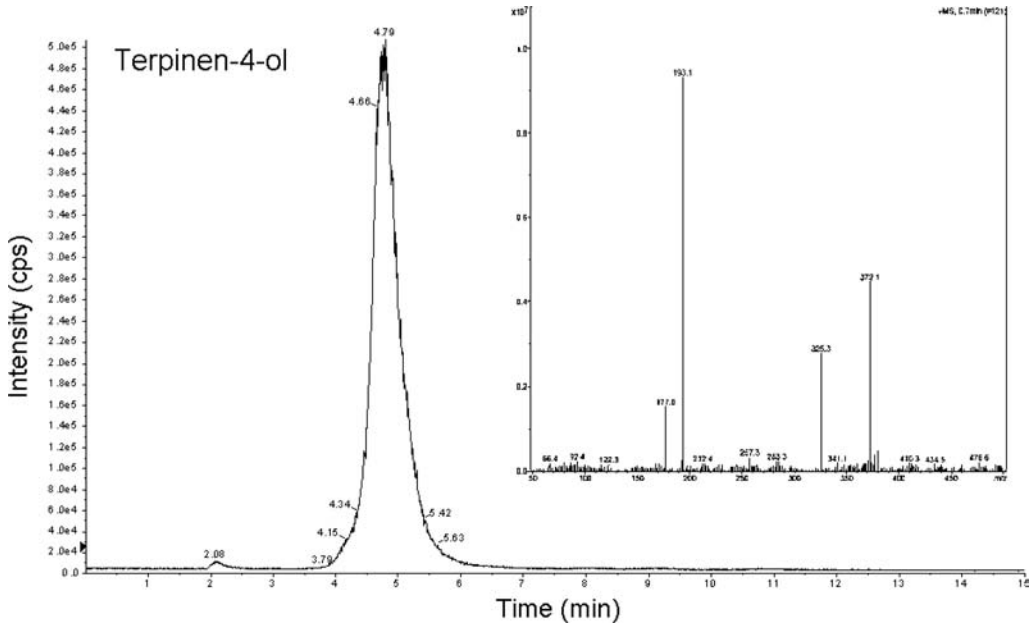

Figure 4. LC/MS and MS chromatogram of terpinen-4-ol. The major peaks are 193.1 $[M + K]^+$ and 177.0 $[M + Na]^+$ in the mass spectrum.

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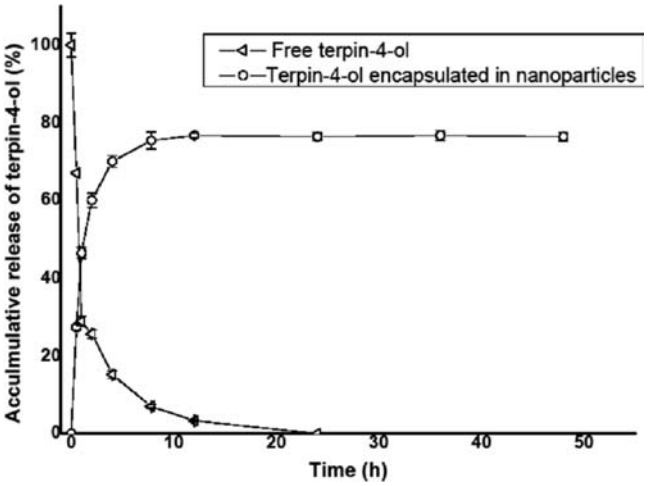

Figure 5. The cumulative release of free terpinen-4-ol and encapsulation terpinen-4-ol from PEG-stabilized lipid nanoparticles. The data represent mean ± SD from three independent experiments performed in multiple replicates.

antimicrobial activity of the samples against the tested strain was investigated with the MIC, MBC, and MBEC. The results showed that the mean MIC was 5 μg/mL; MBC and MBEC were 10 μg/mL. The morphological cellular changes can be seen clearly in the SEM images (Figure 7) where normal *C. albicans* ATCC 11231 cells grown in YPD medium exhibited a typical elliptical shape with a smooth surface (Figure 7a). The effects of terpinen-4-ol-loaded lipid nanoparticles on *C. albicans* ATCC 11231 biofilm viability measured by MTT staining are shown in Figure 8. The data showed that 10 μg/mL terpinen-4-ol effectively eradicated the biofilm. The decrease of *C. albicans* ATCC 11231 metabolic activity was observed after exposure to terpinen-4-ol-loaded lipid nanoparticles. The metabolic activity was 21.5% of control activity for biofilm exposed to lipid nanoparticles containing 10 μg/mL terpinen-4-ol, and only 9.7% of control activity for 20 μg/mL terpinen-4-ol exposure. Increasing terpinen-4-ol concentration up to 40 μg/mL or 80 μg/mL had no any additional effect on the kinetics of biofilm eradication.

Effect on *C. albicans* ATCC 11231 Respiration. Succinate dehydrogenase is an enzyme complex that is bound to the inner mitochondrial membrane of cells. It is the only enzyme that participates in both the citric acid cycle and the electron transport chain.[22] We found previously that terpinen-4-ol can inhibit the respiration of *C. albicans* ATCC 11231 mitochondria. We also found that respiration of *C. albicans* ATCC 11231 mitochondria was sensitive to rather low concentrations of terpinen-4-ol. Figure 9 shows that terpinen-4-ol can inhibit succinate dehydrogenase activity and block the mitochondria respiration chain. When terpinen-4-ol concentration was increased to 20 μg/mL, increasing it had no any additional effect on the kinetics of succinate dehydrogenase inhibition.

DISCUSSION

In most environments, *C. albicans* ATCC 11231 is able to grow as a biofilm, in which a phenotype different from their planktonic counterparts.[23] Biofilms consist of structured communities of cells adhering to a surface and have phenotypical diversity.[11] The previous results showed that *C. albicans* ATCC 11231 biofilms were extremely nonwetting, which limited the penetration of antimicrobial liquids into the biofilm and severely compromised their efficacy. The contact angle of a liquid on a solid surface is the angle formed at the droplet edge between the solid−air and liquid−air interfaces.[12] High surface-tension liquids are repelled by the surface of biofilms, so that the droplets meet the surface at a contact angle >90°. Persistent biofilm nonwettability represents a significant obstacles for many of the most commonly used biocides. In previous experiments, we found that *C. albicans* ATCC 11231 biofilm was able to resist wetting of terpinen-4-ol. Our findings further suggested that a novel drug-loaded carrier could resolve the problem. The preparation method and reagents used in the article have been widely reported in many peer-reviewed articles. The studies showed that the preparation and reagents are suitable for adaptation to the food industry.[24,25] Nanocarriers with a PEG shell reportedly tended to disperse in colloidal suspension, and the lipid nanoparticles showed more stability in the process of treatment.[26] The negative charges on the cell surface of *C. albicans* may attract nanoparticles with the positive charge after being modified with glycine and may result in an increase in bactericidal activity of terpinen-4-ol against the biofilm. Meanwhile, these drug-loaded nanoparticles were bound to the surface of biofilm using EDC as a coupling reagent. Terpinen-4-ol can be released gradually into the interior of cells by diffusion. As a result, the problem of terpinen-4-ol droplets being repelled by the surface of *C. albicans* ATCC 11231 biofilm was avoided.

Earlier studies showed that terpinen-4-ol caused membrane damage and provoked whole-cell lysis.[3,27−30] MTT staining is one of the methods used to evaluate the viability of microbial biofilms based on reduction of tetrazolium salts to formazan by viable metabolishing cells.[31] Using this method, we showed that terpinen-4-ol-loaded lipid nanoparticles efficiently killed *C.*

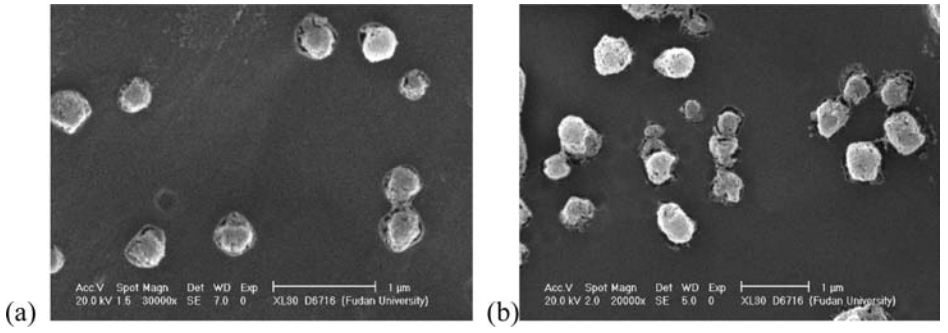

Figure 6. Scanning electron microscopy photographs of PEG-stabilized nanoparticles: (a) 0 h; (b) after 4 weeks.

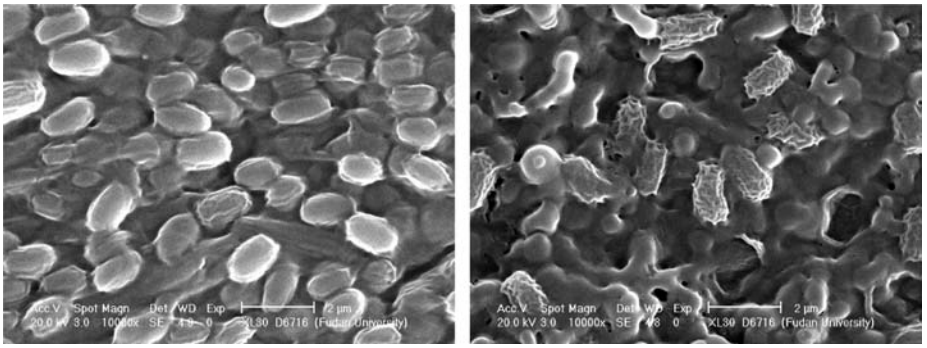

Figure 7. Scanning electron microscopy images of *C. albicans* ATCC 11231 biofilm: (a) control; (b) after 4 h exposure to PEG-stabilized lipid nanoparticles containing 10 μg/mL terpinen-4-ol.

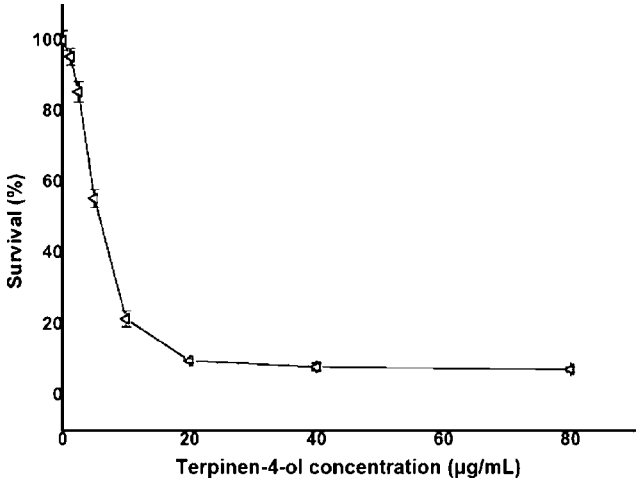

Figure 8. Survival curve for *C. albicans* ATCC 11231 biofilm after treatment with terpinen-4-ol-loaded lipid nanoparticles. Cell viability was estimated by MTT assay and expressed as a percentage of blank nanoparticle controls. All data represent the mean ± SD of triplicate experiments.

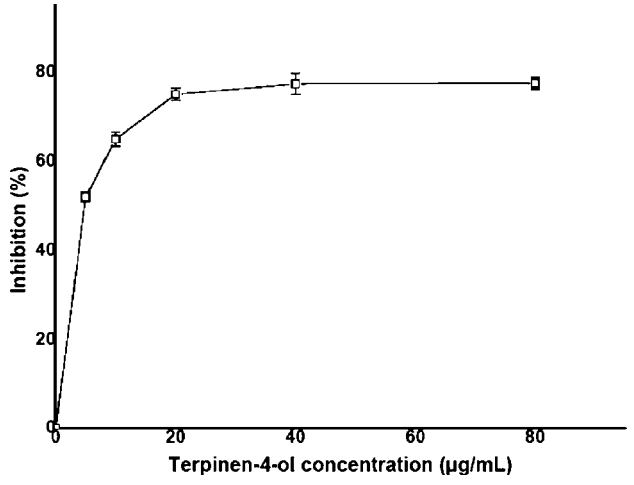

Figure 9. Effects of terpinen-4-ol-loaded lipid nanoparticles on succinate dehydrogenase activity. Blank nanoparticles served as control. Inhibition was determined by measuring spectrophotometrically the reduction of 2,6-dichloroindophenol in incubation medium after the treatment. Results are expressed as mean ± SD (n = 4).

albicans ATCC 11231 in both suspension and biofilm. In addition, to get isolated mitochondria of *C. albicans* ATCC 11231 cells, breaking cells by high-pressure homogenization was investigated. The effects of terpinen-4-ol on the *C. albicans* ATCC 11231 mitochondria were studied, and the results showed that inhibition localized in the mitochondria and that terpinen-4-ol blocked the respiration of *C. albicans* ATCC 11231 mitochondria. The inhibition of succinate dehydrogenase with isolated mitochondria was found at a low concentration of terpinen-4-ol by using the PMS method. In conclusion, we confirmed that the antibiofilm activity of terpinen-4-ol-loaded lipid nanoparticles results from its ability to disrupt the cell membrane structure and blocking of the respiration chain by the inhibition of succinate dehydrogenase, which is bound to the inner mitochondrial membrane of cells.

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Notes

The authors declare no competing financial interest.

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