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Modulation effect of core-wall ratio on the stability and antibacterial activity of cinnamaldehyde liposomes

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ABSTRACT

Cinnamaldehyde is an active component of some plant essential oils, which has broad antibacterial activity. However, the strong volatility and instability of cinnamaldehyde limits its application. Cinnamaldehyde was encapsulated by liposomes, and the effects of core-wall ratio on the stability and antibacterial activity during storage were investigated. The particle size during storage showed that cinnamaldehyde liposomes with high core-wall ratios aggregated more easily, and the retention ratio of it can maintained at around 60%. The increase of cinnamaldehyde loading could reduce the fluidity of the liposome membrane. The antibacterial activity of cinnamaldehyde liposomes against *Staphylococcus aureus* during storage was investigated by fluorescence labeling and the killing log value. It was found that liposome-encapsulated cinnamaldehyde might still inhibit bacteria by destroying cell membrane integrity after storage and the persistence was more efficient than that of pure cinnamaldehyde. Therefore, liposomes could improve the stability and long-term antibacterial activity of cinnamaldehyde.

1. Introduction

Staphylococcus aureus(*S. aureus*) is a well-recognized human pathogen that is implicated in a wide array of superficial, invasive and toxigenic infections, and remains a versatile and dangerous pathogen in humans(Lowy, 1998).With the improvement of living standards and application of natural product, the use of essential oils instead of chemical preservatives to inhibit *S. aureus* is one of the most promising ways to satisfy consumer demands (Ballester-Costa et al., 2013). Cinnamaldehyde is an oily liquid, transparent light-yellow color, with a strong cinnamon fragrance. It is the main ingredient and antibacterial component of cinnamon essential oil (Wang et al., 2018). Considering the strong inhibitory effects on a variety of bacteria and pathogenic fungi, cinnamaldehyde has been listed and approved by regulatory agency as a medical additive. However, the strong volatility, instability, and poor solubility of cinnamaldehyde limits its application in a variety of fields (Tian et al., 2016).

The microencapsulation of cinnamaldehyde can effectively improve the stability, control the release ability of its antibacterial activity in practical applications (Maronpot and Collins, 1993). The persistence of antibacterial activity of cinnamon oil microcapsules prepared by complex coacervation was higher than that of pure essential oils (Cui et al., 2016). With the development of microencapsulation technology, nanocapsules also have long-term inhibitory activities and slow-release effects (Kong et al., 2010).

Liposomes are bimolecular vesicles formed by lipid molecule in aqueous solution with a double molecular layer structure, which can simultaneously encapsulate oil-soluble substances and water-soluble substances (Lu et al., 2014). Because of the unique molecular structure and physicochemical properties of liposomes, its basic characteristics are mainly manifested in the following aspects (Aljamal and Kostarelos, 2011): good dispersibility in water, good biocompatibility, low diffusion rate of core material, slow release, and long residual activity. Currently, research on liposomes embedding essential oil is attracting more attention(Prakash et al., 2018). It was found that liposomes effectively protected eugenol which was the main component of clove oil (Sebaaly et al., 2015). Clove oil liposomes were applied to soybean products, showing its excellent antibacterial activity properties on *S. aureus* (Cui et al., 2015). Cinnamon essential oil and cinnamaldehyde were encapsulated for application in edible film, which effectively delayed the release rate of essential oils and improved the persistence of antibacterial properties (Makwana et al., 2014; Wu et al., 2015). Therefore, application of liposomes to encapsulate cinnamaldehyde might retain the functional activity of cinnamaldehyde and improve its stability during processing and storage (Cui et al., 2016).

There are many reports on the improvement of antibacterial activity

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after liposome embedded of cinnamaldehyde(Cui et al., 2016; Makwana et al., 2014), but there are few studies on whether liposomes play a prolonging antibacterial activity during storage. The long-acting sustained-release properties of liposomes during storage are the key to the application in medical industry. High entrapment efficiency is usually used as the evaluation index to optimize the process conditions of liposomes formulation (Chen et al., 2010; Duangjit et al., 2010). Therefore, we suggested that the combination of high encapsulation efficiency of cinnamaldehyde and good antibacterial stability in liposome formulation could be ideal for our system. Moreover, the method and mechanism of the core material released from liposomes are related to the core-wall ratios. Therefore, to present a clear view on the equilibrium point of entrapment efficiency, stability, and antibacterial activity during the storage, ethanol injection method was used to prepare cinnamaldehyde liposomes with three core-wall ratios.

The size distribution and microstructure of cinnamaldehyde-loaded liposomes were compared by using atomic force microscopy and dynamic light scattering technique. To get further insights on the influence of liposome encapsulation on the stability and bacteriostatic of cinnamaldehyde, the dependence of retention rate and killing log value on the storage time were compared. The effect of liposome encapsulation on the cell membrane of *S. aureus* were also investigated by fluorescence labeling.

2. Materials and methods

2.1. Materials

Egg yolk lecithin (EYPC) was obtained from Chemical Reagent Plant of East China Normal University (Shanghai, China). Chemical grade Tween 80 and analytical grade ethanol, petroleum ether, NaCl were from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Tryptone, beef extract were also from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Cinnamaldehyde (80% purity) was purchased from Guoguang Perfume Factory (Jiangxi, China). Standard cinnamaldehyde (98% purity) and propidium iodide (PI, 94% purity) were obtained from Aladdin Biochemical Polytron Technologies Inc (Shanghai, China). *S. aureus* was purchased from Zhongkehuchuang Biological Technology Co., Ltd (Beijing, China). 1, 6-diphenyl-1, 3, 5-hexatriene (DPH, 98% purity) was obtained from Sigma-Aldrich Co., Ltd (Shanghai, China).

2.2. Preparation of cinnamaldehyde liposomes

Cinnamaldehyde liposomes were prepared by ethanol injection method. Egg yolk lecithin (0.8 g), Tween 80 (0.2 g), and cinnamaldehyde were dissolved in 5 mL ethanol (40 °C). The weight of cinnamaldehyde was determined according to the core-wall ratios of 10%, 20%, and 30%. The ethanol solution was quickly injected into 40 mL of preheated hydration media (40 °C, 0.01 mol/L PBS, pH 6.5) with magnetic stirring. After 30 min of stirring, the ethanol was removed by rotary evaporation (control vacuum degree and temperature was 40 °C) to form an aqueous dispersion of liposomes. Then the suspension was cooled quickly and treated with ultrasonic sound. The ultrasonic power output was 200 W and the ultrasonic time was 3 min (1 s for the sonication and 2 s for the rest) using ultrasonic cell breaker (JY98, Nanjing Feiqi Industry and Trade Co., Ltd).

2.3. Encapsulation efficiency and retention rate of cinnamaldehyde liposomes

2.3.1. Determination of the total cinnamaldehyde content

The Tween 80 solubilization method was used to determine the total cinnamaldehyde content (Xia et al., 2006). A 0.5 mL cinnamaldehyde liposomes were moved into 10 mL volumetric flasks and then 1 mL Tween 80-ethanol solution (10%, w/v) was added and left to oscillate for 30 s. Each flask was filled with 10 mL of deionized water. The cinnamaldehyde concentration was detected spectrophotometrically by the change in absorbance at 285 nm. The blank liposomes were detected with the same method as the negative control. The cinnamaldehyde concentration was calculated according to the standard curve. The linear regression equation of the standard curve was y = 0.1583x-0.0009 (R^2 = 0.9998), where x was cinnamaldehyde concentration (μg/mL), and y was the absorption value. The linear concentration ranged from 1.112 μg/mL to 5.004 μg/mL.

2.3.2. Determination of the free cinnamaldehyde content

The organic solvent extraction method was used to determine the free cinnamaldehyde content (Tan et al., 2014). A 0.5 mL of cinnamaldehyde liposomes were moved into 15 mL centrifuge tubes and then 3 mL petroleum ether was added and left to oscillate for 2 min. After centrifugation (3000 r/min) for 5 min, the organic phase was collected and the above steps were repeated twice. The total supernatant was dried with nitrogen and then filled to 10 mL with ethanol. The cinnamaldehyde concentration was detected spectrophotometrically by the change in absorbance at 285 nm. The blank liposomes were detected with the same method as the negative control. The cinnamaldehyde concentration was calculated according to the standard curve. The linear regression equation of the standard curve was y = 0.1868x-0.0048 (R^2 = 0.9995), where x was the cinnamaldehyde concentration (μg/mL), and y was the absorption value. The linear concentration ranged from 0.996 μg/mL to 4.482 μg/mL.

The calculation formulas were as follows:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{total cinnamaldehyde} - \text{free cinnamaldehyde}}{\text{total cinnamaldehyde}} \times 100$$

$$\text{Loading capacity (\%)} = \frac{\text{total cinnamaldehyde} - \text{free cinnamaldehyde}}{\text{lipid content}} \times 100$$

$$\text{Retention rate (\%)} = \frac{\text{total cinnamaldehyde after storage}}{\text{total cinnamaldehyde initially prepared}} \times 100$$

2.4. Determination of particle size distribution

The liposomes were diluted to the appropriate gradient and loaded into the sample pool. The particle size distribution was measured by a multi-angle particle size analyzer (Nano Brook Omni, Brookhaven Instruments Co., United States) with a laser wavelength of 640 nm. The temperature was 25 °C, the scattering angle was 90°, and the measurements were conducted three times. The average particle size and the distribution of particle sizes (Polydispersity index, PDI) were recorded.

2.5. Analysis of fluorescence anisotropy

As a fluorescent probe, DPH was dissolved in tetrahydrofuran to obtain the concentration of 2×10^{-3} mol/L. The solution was diluted to 2×10^{-5} mol/L with 0.01 mol/L phosphate buffer (pH 6.5) upon use. The cinnamaldehyde liposomes were mixed with a DPH probe to make the final concentration of the DPH probe 1×10^{-5} mol/L. The mixed samples were placed at 37 °C for 60 min and then measured at room temperature (20 °C) by fluorescence spectrophotometer (F-7000, Hitachi, Japan). The excitation wavelength was Ex = 358 nm and the emission wavelength was Em = 425 nm.

The width of the slit was 5 nm when the fluorescence polarization was measured. When the vertical polarized light was first placed in the 0° position to excite the samples, the vertical polarization component $I_{0,0}$ and the horizontal polarization component $I_{0,90}$ were detected. When the horizontal polarized light was placed in the 90° position to

excite the samples, the vertical polarization component $I_{90,0}$ and the horizontal polarization component $I_{90,90}$ were detected(Engelke et al., 2001).

$$r = \frac{I_{0,0} - GI_{0,90}}{I_{0,0} + 2GI_{0,90}}$$

$$G = \frac{I_{90,90}}{I_{90,0}}$$

In the equation, r was fluorescence anisotropy, G was grating correction coefficient of the equipment.

2.6. Atomic force microscope observation (AFM)

The liposomes were diluted 5000 times with ultrapure water, and a few drops were dissipated at the mica film. The samples were placed at room temperature for 24 h to ensure that the samples were completely adsorbed on the mica film. Subsequently, the samples were placed under the scanning probe (Si probe) of atomic force microscope (Dimension ICON, Brook Technology Co., Ltd. Germany) and observed in ScanAsyst mode at room temperature and atmospheric pressure. The setting parameters were as follows: scanning rate = 0.977 Hz, resolution ratio = 512 × 512, scanning size = 5 μm × 5 μm. Image processing was used by NanoScopeTM software (Digital Instruments, version V614r1).

2.7. Evaluation of the antibacterial activity of cinnamaldehyde liposomes in vitro

S. aureus during the logarithmic growth period was centrifuged to remove the supernatant and then diluted to a concentration of 10^6 CFU/mL by normal saline water (Schmelcher et al., 2012). Compared with pure cinnamaldehyde, the cinnamaldehyde liposomes with different core-wall ratios were added to the suspension of *S. aureus* with a certain proportion, ensuring that the equivalent of cinnamaldehyde was 0.39 μg/mL. Then the suspension was oscillated at 37 ℃, with the speed of 150 r/min. A 1 mL solution of bacteria was suspended at set intervals to be diluted to the appropriate gradient, and then the suspension was evenly plated on the nutrient agar. After incubating at 37 ℃ for 24 h or 6 h, the colonies were counted. Three parallel experiments were performed and the average result was obtained. The killing log value (KLV) was calculated as follows (Tang and Ge, 2017):

$$KLV = \log_{10}N_0 - \log_{10}N_t$$

Where, N_0 was the living bacteria concentration of samples at initial time (CFU/mL), and N_t was the living bacteria concentration of samples after incubation (CFU/mL).

Pure and liposome-encapsulated cinnamaldehyde were stored at 37 ℃ for different times, and the above experiments were repeated to study the effect of storage time on antibacterial activity.

2.8. Cell membrane integrity of bacteria by fluorescence labeling

The pure and liposome-encapsulated cinnamaldehyde were added to the *S. aureus* suspension (the concentration of it was 10^8 to 10^9 CFU/mL) and oscillated for 24 h. After centrifugation at 6000 r/min for 15 min, the supernatant was removed and then the precipitate was washed by PBS three times followed by resuspending with the same volume of PBS. Propidium iodide (PI) was added to the samples (final concentration of it was 50 μg/mL) (Stiefel et al., 2015), and the samples were stored at 37 ℃ for 30 min in a dark place. After centrifugation, bacteria was washed by PBS until there was red color disappeared in the supernatant. The bacteria was resuspended with 50 μL PBS and then observed under a bright field and 488 nm excitation light with an inverted fluorescence microscope (Axio Vert A1, Carl Zeiss, Germany).

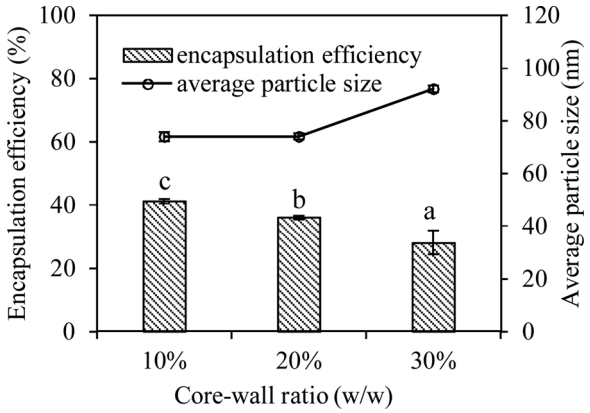

Fig. 1. Encapsulation efficiency and average particle size of cinnamaldehyde liposomes with different core-wall ratios.

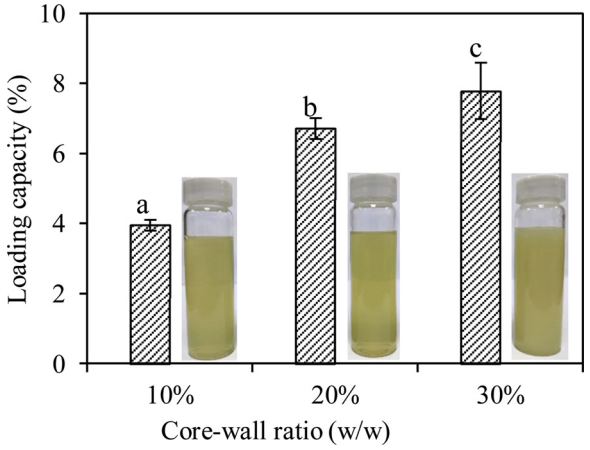

Fig. 2. Loading capacity and digital image of cinnamaldehyde liposomes with different core-wall ratios.

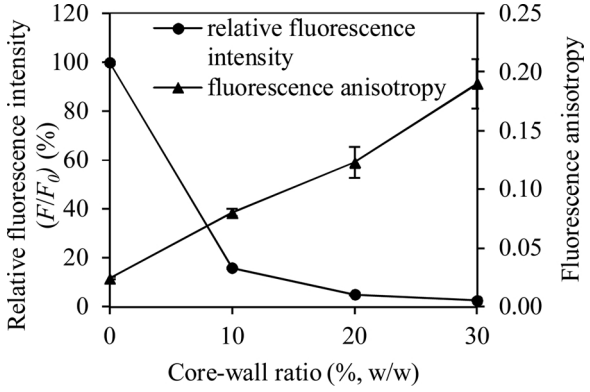

Fig. 3. Effect of core-wall ratio on the fluidity of cinnamaldehyde liposomes bilayer. F is the fluorescence intensity of cinnamaldehyde liposomes with different core-wall ratios, and F_0 indicated fluorescence intensity of pure liposomes.

2.9. Statistical analysis

All the experiments were performed on at least three individual samples. Results were expressed as mean value ± standard deviation (SD) of three or more determinations. All statistical significance ($p < 0.05$) were performed using one-way analysis of variance (ANOVA) with SPSS 19.0 (SPSS Inc., USA).

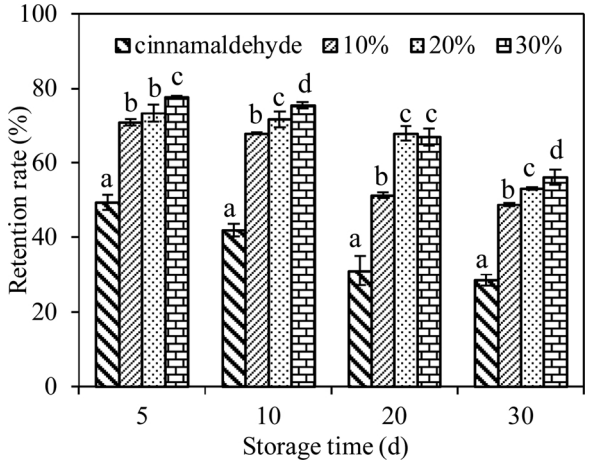

Fig. 4. Effect of core-wall ratio on the retention rate of cinnamaldehyde liposomes during storage at 37°C.

3. Results and discussion

3.1. Effects of core-wall ratio on encapsulation efficiency, loading capacity and membrane fluidity of cinnamaldehyde liposomes

Encapsulation efficiency is an important index to evaluate the encapsulating ability of liposomes. For some systems, adjusting the core-wall ratio is required to obtain the maximum entrapment efficiency. Therefore, three core-wall ratios were selected to prepare cinnamaldehyde liposomes, and the effect of the core-wall ratio on encapsulation effect and storage stability were investigated. As shown in Fig. 1, the encapsulation efficiency of cinnamaldehyde liposomes decreased with an increase of the core-wall ratio. This was mainly because cinnamaldehyde, a non-polar molecule, was largely arranged in the hydrophobic region of the lipid bilayer. The hydrophobic space was limited, resulting in the saturation of the lipid membrane when more cinnamaldehyde loading was increased. In addition, as shown in Fig. 2, the loading capacity of cinnamaldehyde liposomes increased with an increase of the core-wall ratio, ranging from 3% to 8%, which consistent with other published papers(Hosseini et al., 2013; Yoksan et al., 2010). It also seems that the transparency of liposome with a core wall ratio of 30% was lower than that of other two samples, which may be related to its encapsulation efficiency (lower than 20% and 10%). The more free cinnamaldehyde dispersed in water, the more turbid the solution may be.

As shown in Fig. 1, the average particle size of cinnamaldehyde was about 75 nm when the core-wall ratio ranged from 10% to 20%. However, once increasing the core-wall ratio to 30%, the average particle size of cinnamaldehyde liposomes was reached to 92.14 nm. It was suggested that the presence of more cinnamaldehyde increased the amount of the mechanical strength of liposomes, so the size of the liposomes could not be reduced to the same degree by the same ultrasonic condition. The polydispersity index (PDI) of cinnamaldehyde liposomes with different core-wall ratios was less than 0.3, indicating that the particle dispersion was uniform.

The mobility of lipid bilayer membranes is closely related to the properties and stability of liposomes. The fluidity of liposome membranes can be illustrated by the fluorescence polarization technique (Fraňová et al., 2010). DPH is a rigid rodlike small molecule with very strong hydrophobicity used as a fluorescent probe. Undissolved DPH has no fluorescence, but when it is located in the hydrophobic part of the liposome, there is energy transfer between DPH and the membrane groups, causing an increase of fluorescence (Pandey and Mishra, 1999). This kind of probe can achieve a strong fluorescence effect with a very small amount and without disturbing the structure of the bilayer

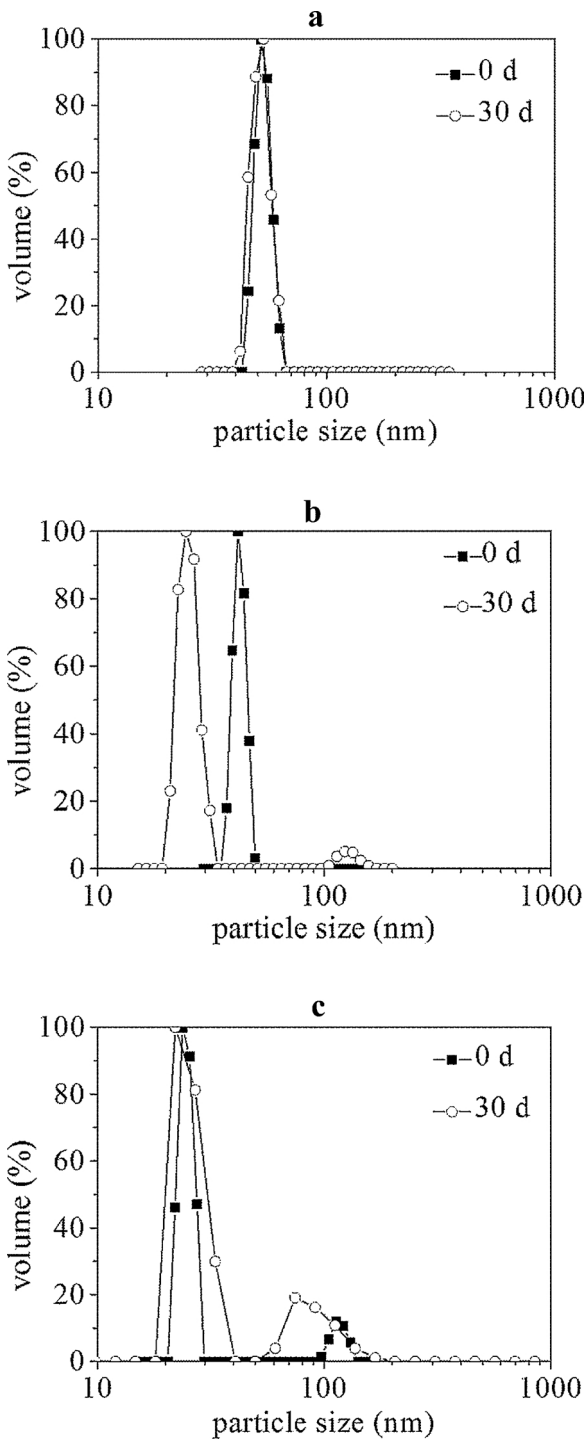

Fig. 5. Effect of core-wall ratio on the particle size distribution of cinnamaldehyde liposomes during storage at 37°C. (a) The core-wall ratio is 10%, (b) The core-wall ratio is 20%, (c) The core-wall ratio is 30%.

(Jemiola-Rzeminska et al., 1996). As shown in Fig. 3, the blank liposomes showed a very strong fluorescence, which indicated that DPH was mainly located in the hydrophobic region of the liposomes bilayer (Gradinaru et al., 2010). At a core-wall ratio of 10%, the fluorescence intensity of the DPH probe decreased rapidly, indicating that the DPH had produced fluorescence quenching when the core-wall ratio was low. Loading hydrophobic core in liposomes, the lateral diffusion of the head group in the liposomes moved the DPH chromophore near the aqueous phase, which resulted in a significant decrease in the fluorescence intensity of DPH. If the core-wall ratio was further increased, the

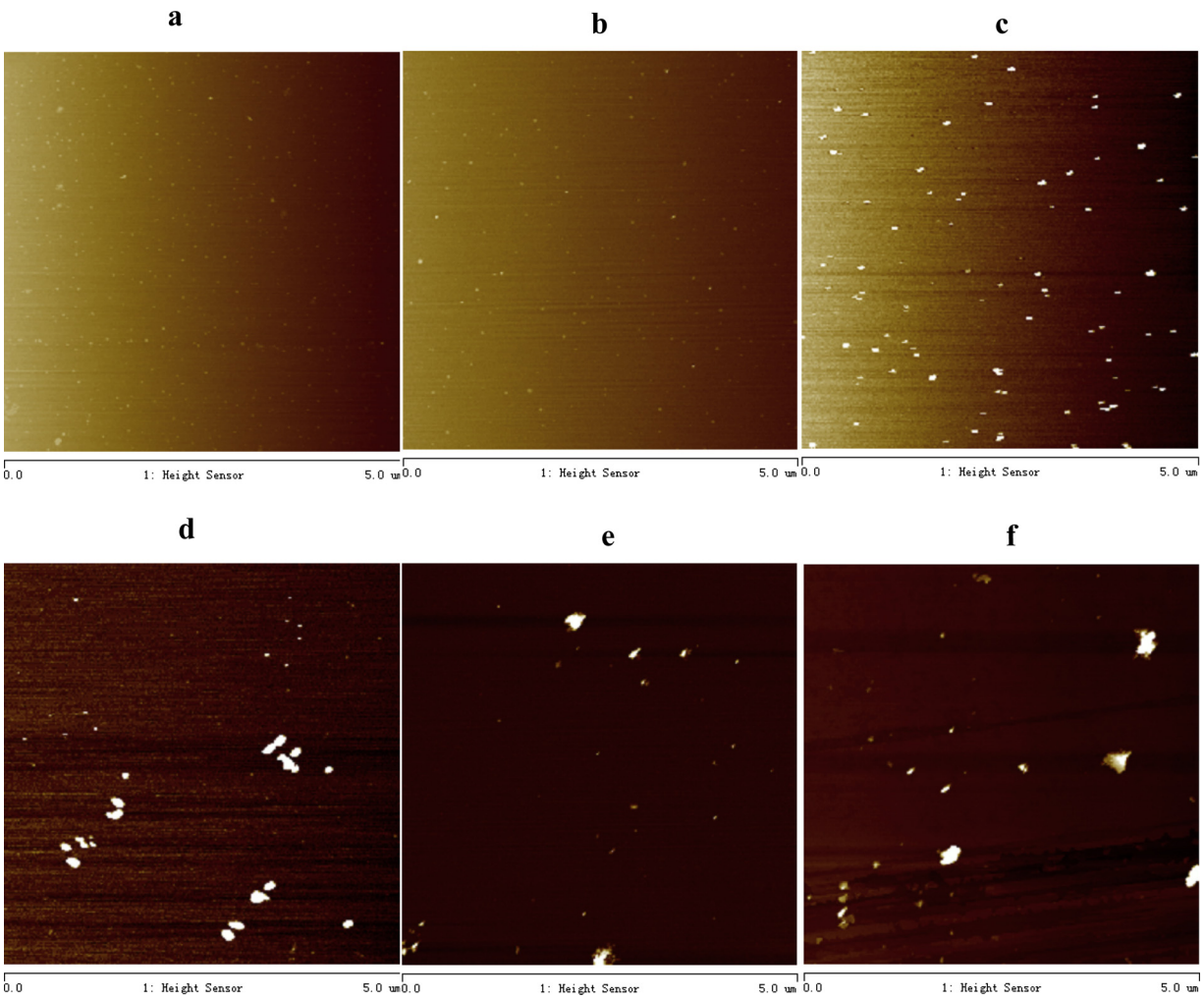

Fig. 6. Atomic force microscope images of liposomes during storage. (a), (b), (c) The core-wall ratio of cinnamaldehyde liposomes samples is respectively 10%, 20%, and 30% before storage. (d), (e), (f) The core-wall ratio of cinnamaldehyde liposomes samples is respectively 10%, 20%, and 30% after storage at 37°C for 30 days.

decreasing degree of DPH fluorescence intensity tended to be stable, which indicated that the distribution of cinnamaldehyde in the hydrophobic region was almost saturated. These results were in accordance with the decrease of encapsulation efficiency of the cinnamaldehyde liposomes in Fig. 1.

The structural properties of liposome membranes are characterized, which is based on the combination of fluorescent substances and membranes, causing changes of fluorescence parameters (Gradinaru et al., 2010). The increase of fluorescence anisotropy value indicates that the molecular arrangement becomes more regular, the membrane fluidity decreases and the protection of liposomes on the core material increases. As shown in Fig. 3, the fluorescence anisotropy of the hydrophobic region of the liposomes increased with the increase of the core-wall ratio, consistent with other published paper (Barker and Kennedy, 2017). Cinnamaldehyde as a nonpolar molecule was more easily embedded in the hydrophobic region of the liposomes bilayer and tightly coupled at its hydrophobic ends by hydrophobic interaction. As a result, the hydrophobic groups of lipid molecules were not easy to reverse, and the fluorescence anisotropy was increased. The increase of fluorescence anisotropy indicated that an increase of core-wall ratio could reduce the fluidity of the hydrophobic region of the bilayer. These results are in accordance with the tendency obtained in Fig. 1. The increase of core-wall ratio leads to an increase in average particle size. Because lipid membrane having a small curvature was less fluid (Shimanouchi et al., 2011), therefore, an increase of core-wall ratio can prevent core material from leaking to some extent.

3.2. Effects of core-wall ratio on storage stability of cinnamaldehyde liposomes

3.2.1. Changes of retention rate of cinnamaldehyde liposomes during storage

Liposomes are thermodynamically unstable systems, which can easily aggregate, flocculate, and precipitate in the process of preparation, storage, transportation, and application, leading to the leakage of core material (Takeuchi et al., 2001). The retention rate can directly reflect the change of cinnamaldehyde content in liposomes during storage. The cinnamaldehyde liposomes were placed at 37 °C for several days, and then the retention rate of cinnamaldehyde in liposomes was determined to compare with that of pure cinnamaldehyde. As shown in Fig. 4, the retention rate of pure cinnamaldehyde decreased to 49% in 5 days, while the retention of liposome-encapsulated cinnamaldehyde was maintained at around 70%. This indicated that the liposomes had a protective effect on cinnamaldehyde and delayed the loss of cinnamaldehyde. The retention rate of cinnamaldehyde liposomes with a core-wall ratio of 30% was the highest after 30 days, which meant that the protection ability of liposomes increased as the core-wall ratio increased. This tendency was consistent with the previous report of Hua et al. (2003), and agreed with the tendency obtained in Fig. 4. The increase of core-wall ratio helped the hydrocarbon chain of phospholipid molecules to tightly arrange, reducing the fluidity of the membrane and protecting the core material.

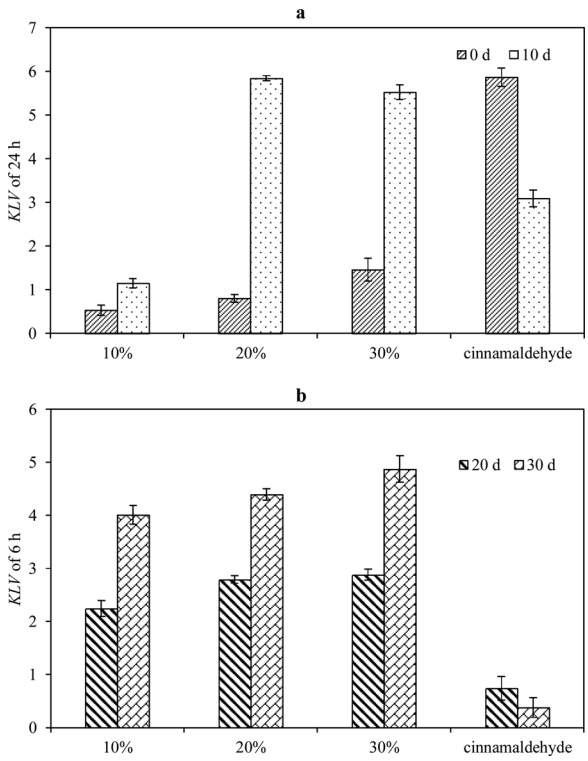

Fig. 7. The killing log value (*KLV*) of pure cinnamaldehyde and cinnamaldehyde-loaded liposomes with different core-wall ratio during initial (a) and final (b) storage at 37℃.

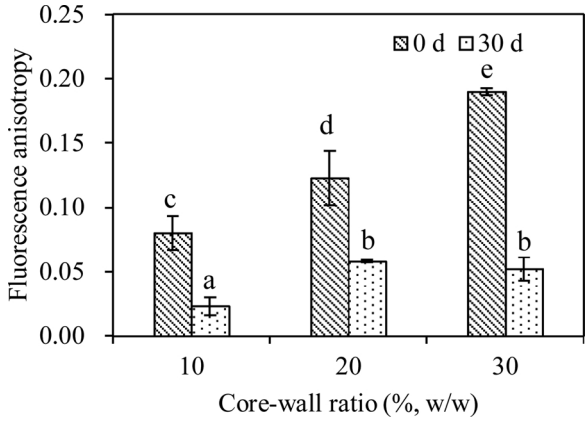

Fig. 8. Effect of core-wall ratio on the fluorescence anisotropy of the hydrophobic region of cinnamaldehyde liposomes during storage.

3.2.2. Changes of particle size distribution of cinnamaldehyde liposomes during storage

The size and distribution uniformity of liposome particles are related to their stability and directly affect the release and application of core materials. The cinnamaldehyde liposomes were stored at 37 ℃, and then the particle size distribution was measured by dynamic light scattering on different days. The results are shown in Fig. 5. The peaks of cinnamaldehyde liposomes with a core-wall ratio of 10% and 20% were unimodal with and relatively narrow, while the cinnamaldehyde liposomes with a core-wall ratio of 30% appeared bimodal at initial storage. After 30 days, the size of cinnamaldehyde liposomes with different core-wall ratios increased to varying degrees. This was because the curvature of liposomes with smaller particle sizes were bigger, leading more acyl chains of phosphatidylcholine to be exposed. At this time, the amphiphilic molecules in the system, such as Tween 80, could reduce the energy barrier required by the aggregation and fusion of liposomes through the hydrophobic interaction between the head group $(CH_2{-}CH_2{-}O)_n$ and induced fusion, resulting in the increase of liposomes size (Tasi et al., 2003). From the perspective of particle size distribution, cinnamaldehyde liposomes with a relatively low core-wall ratio had a smaller particle size and higher uniformity during storage.

Atomic force microscopy (AFM) is a powerful tool to study nanomaterials and bio-membranes. When the probe scans on the surface of the adsorption layer of liposomes, the micromorphology of the liposomes can be obtained directly by the interaction between the probe and the surface of the samples (Habib et al., 2014; Pentak, 2016). AFM has the advantage of nanometer resolution, which is prepared without any treatment (Ruozi et al., 2005). The ScanAsyst model can be used to scan the sample repeatedly. Some spherical or ellipsoidal particle images can be observed when selecting a certain range of scanning, as shown in Fig. 6. In the initial period of the preparation of cinnamaldehyde liposomes, liposomes with the core-wall ratio of 10% and 20% were more evenly distributed with singular peaks and no obvious aggregation or fusion. The size of the liposomes with the core-wall ratio of 30% was relatively larger than that of the other samples, and contained few aggregates. This was consistent with the change of particle size measured by dynamic light scattering. However, after 30 days of storage at 37 ℃, the obvious aggregation of vesicles could be found. The particle size increased and the dispersion was not uniform. The liposomes with a core-wall ratio of 20% and 30% showed irregular shape and the stability of liposomes decreased.

3.3. Effects of core-wall ratio on antibacterial properties of cinnamaldehyde liposomes

Cinnamaldehyde is a natural aromatic aldehyde, which has strong antibacterial activity against bacteria. In this paper, *S. aureus* was selected as the representative strain. The effect of liposomes encapsulation on the antibacterial activity of cinnamaldehyde was investigated, as well as the effect of accelerated storage time on the bacteriostatic effect was also studied.

In Fig. 7A, it could be seen that the killing log value (KLV) during 24 h of pure cinnamaldehyde treatment was significantly higher than that of cinnamaldehyde liposomes before accelerated storage at 37 ℃. This indicated that unencapsulated cinnamaldehyde could be fully exposed to *S. aureus*, demonstrating the greatest initial bacteriostatic effect. The bacteriostatic rate of cinnamaldehyde liposomes with higher core-wall ratios were faster than those of lower core-wall ratios. This was attributed that the encapsulation efficiency of liposomes reduced with the increase of the core-wall ratio, and more free cinnamaldehyde was exposed to microorganisms, thus presenting more efficient bacteriostatic action. After 10 days of accelerated storage, the killing log value during 24 h of pure cinnamaldehyde decreased significantly due to the volatilization and oxidative degradation of cinnamaldehyde. In contrast, the killing log value during 24 h of cinnamaldehyde liposomes with a core-wall ratio of 20% and 30% significantly increased, comparing with corresponding liposomes in their initial period.

The inhibitory effect during 24 h of cinnamaldehyde liposomes after prolonged storage was analyzed. The concentration of living bacteria incubated for 24 h in post-storage period (20days and 30days) was basically zero(*S. aureus* were almost all killed), indicating that the incubation time was too long to reflect the difference in antibacterial activity between liposomes with different core-wall ratios, since the increased rapidity of antibacterial activity of cinnamaldehyde liposomes. This situation may due to the fact that *S. aureus* was sensitive to antibacterial substances. It seems that only by adjusting the incubation time dynamically, can we get meaningful KLV experimental data. Therefore, in accelerated storage, the killing log value during 24 h was not suitable to reflect the difference between the liposomes, and the system incubation time was shortened to 6 h, then the killing log value was calculated. As shown in Fig. 7B, the antibacterial ability of pure

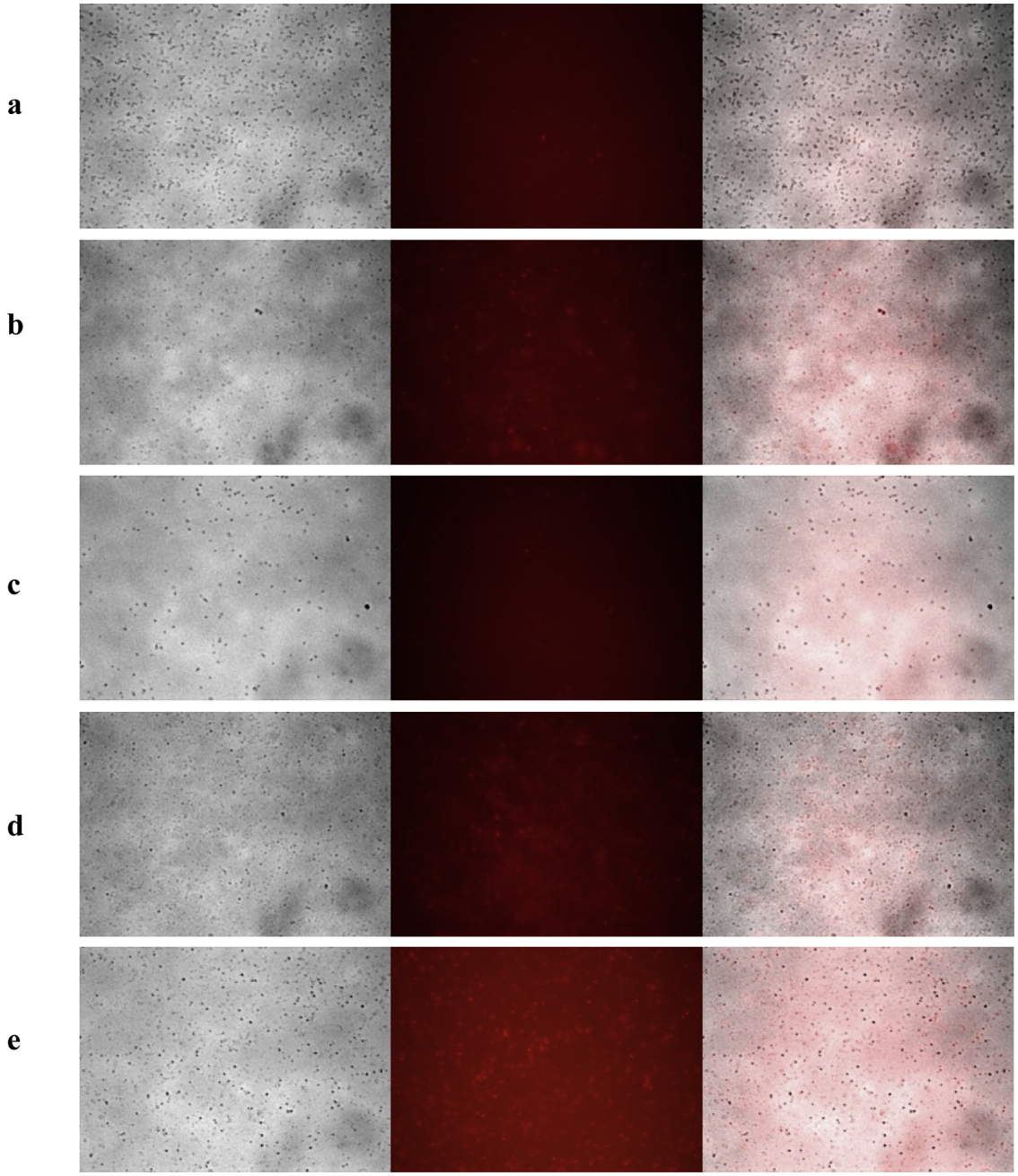

Fig. 9. Effect of pure cinnamaldehyde and cinnamaldehyde-loaded liposomes on propidium iodide fluorescence staining of *Staphylococcus aureus* before and after storage. (a) Effect of Negative control, (b) Effect of Cinnamaldehyde before storage, (c) Effect of Cinnamaldehyde storage at 37℃ for 30 d, (d) Effect of Cinnamaldehyde liposomes before storage, (e) Effect of Cinnamaldehyde liposomes storage at 37℃ for 30 d.

cinnamaldehyde decreased, while the antibacterial ability of cinnamaldehyde liposomes increased with the prolonging of storage time. In addition, the killing log value increased with the rising of core-wall ratio of liposomes, indicating that the stability of cinnamaldehyde was improved and the release of cinnamaldehyde might be controlled due to encapsulation of cinnamaldehyde in liposomes. Therefore, long-term bacteriostatic action of cinnamaldehyde could be achieved by liposomes.

In order to further demonstrate the relationship between the bacteriostatic effect and the fluidity of cinnamaldehyde liposome membranes, the fluorescence anisotropy of the hydrophobic region of cinnamaldehyde liposomes which were kept at 37 ℃ for 30 days was analyzed by the fluorescence spectrometer. The fluorescence intensity of the hydrophobic region (Fig. 8) showed that the micro viscosity of cinnamaldehyde liposomes with different core-wall ratios decreased after 30 days of storage, and the higher the core-wall ratio was, the greater the fluorescence anisotropy decreased. Our results indicated that the fluidity of the bilayer membrane increased with core-wall ratio increase, therefore the core material was more easily released from the liposomes, which facilitated the contact between cinnamaldehyde and *S. aureus*, and accelerate the death of the bacteria.

3.4. Changes of cell membrane integrity of bacteria

Fluorescent dye propidium iodide (PI) is a nuclear staining reagent that can combine with double strand DNA to form a complex, which will release red fluorescence. However, PI cannot penetrate the membrane of living cells, therefore, using fluorescence labeling techniques

to detect the PI staining of bacteria can quickly reflect the integrity of cell membrane and cell viability (Stiefel et al., 2015). The *S. aureus* was treated by pure and liposome-encapsulated cinnamaldehyde at 37 ℃ for 0 day and 30 days, and then the bacteria staining was characterized by inverted fluorescence microscope.

Fig. 9 is the microphotograph of *S. aureus* treated with different samples by PI fluorescence staining. In the case of the same initial concentration and same incubation time of experiment conditions, the PI staining rate of untreated *S. aureus* cells was low, and only a few bacteria were colored, close to the theoretical value of 0%. The reason why some bacteria were still colored maybe because those bacteria have already entered the decline period. Compared with the negative control, the PI staining rate of the bacteria treated by pure and liposome-encapsulated cinnamaldehyde increased significantly, and the fluorescence intensity increased. Our findings indicated that more *S. aureus* cell membranes were destroyed, so that PI entered the cell membrane combining with the DNA double chain. After 30 days of storage at 37 ℃, the PI staining rate of the pure cinnamaldehyde decreased significantly, which was almost like the negative control. However, the staining rate of cinnamaldehyde liposomes was not lower than that of the cinnamaldehyde liposomes before storage. These results showed that with the increase of storage time, the inhibition ability of cinnamaldehyde decreased due to its volatility and oxidation; however, the encapsulation of liposomes could enhance the stability of cinnamaldehyde and maintain its durability of bacteriostatic effects.

4. Conclusion

The encapsulation efficiency of cinnamaldehyde liposomes decreased with the increase of the core-wall ratio. The protective effect of the cinnamaldehyde liposomes with higher core-wall ratios were greater than that of liposomes with lower core-wall ratios during storage, while the former were more easily aggregated causing the increase of liposomal particle size. In the initial period of storage, the antibacterial ability of pure cinnamaldehyde against *S. aureus* was significantly better than that of cinnamaldehyde liposomes. However, the antibacterial ability of pure cinnamaldehyde weakened after prolonged time storage, and the cinnamaldehyde liposomes could still maintain high bacteriostatic activity. It indicated that the persistence of bacteriostatic effects of the cinnamaldehyde liposomes were better than that of pure cinnamaldehyde. With the increase of core-wall ratios, the bacteriostatic ability of the cinnamaldehyde liposomes increased. PI fluorescence staining confirmed that cinnamaldehyde liposomes could destroy the integrity of cell membrane leading to the death of *S. aureus*. Therefore, liposomes could endow cinnamaldehyde the improved stability and long-term antibacterial activity. The study was conducted to provide guidance for the rational design and fabrication of embedded systems for active components of natural plant essential oils.

Conflict of interests

The authors declare no conflict of interests regarding the publication of this article.

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