Enhancement of liposomal properties of thyme essential oil using lysozyme modification: Physicochemical, storage, and antibacterial properties

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ABSTRACT

Thyme essential oil (TEO) is a natural food antimicrobial agent derived of spice, but suffers from volatility and poor water solubility, which problem can be effectively solved by the encapsulation of liposomes. On this basis, a safe and common natural antibacterial protein, LYZ was used to modify the TEO liposomes (TEO-lips) for gaining better properties. 2.5 mg/mL TEO and 0.05 % LYZ/S100 mass ratio were the best formula for the preparation of LYZ-TEO-lips. After LYZ modification, the particle size and PDI increased, and the zeta potential decreased slightly. The modification of LYZ not only improves the thermal stability of TEO-Lips, weakens the influence of acid and salt ions on liposomes, but also improves the antibacterial properties of TEO-Lips. In brief, LYZ has the potential to improve the overall properties of liposomes and can provide a reference for the development of antimicrobial liposomes.

1. Introduction

Thyme essential oil (TEO) is derived from an ancient food spice, thyme. Thyme and carvacrol are its key active constituents, which are non-toxic, antiaging, antimicrobial, and have many immune-system-boosting benefits (Al-Moghazy, El-sayed, Salama, & Nada, 2021; Li et al., 2021; Liu & Liu, 2020; Rota, Herrera, Martínez, Sotomayor, & Jordán, 2008). TEO is not only a natural antimicrobial agent but can also be used as a food flavor additive for its unique flavor (Li et al., 2021). However, TEO has the disadvantages of poor water solubility, volatility, and sensitivity to light, heat, oxygen, and humidity (Benjemaa, Neves, Falleh, Isoda, Ksouri, & Nakajima, 2018), which may limit the direct application of TEO in the food industry. In this background, encapsulation techniques could improve TEO's performance, resulting in its widespread use in food products (Barros et al., 2022; Ozogul et al., 2020; Snoussi et al., 2022).

Liposomes are closed vesicles made of phospholipid bilayers that resemble biological membranes and have amphiphilic properties and efficient cellular uptake (Frenzel & Steffen-Heins, 2015; Tan, Wang, & Sun, 2021). It has been shown that terpenes in plant essential oils can promote the formation of stable essential oil liposome nanocarriers (Risaliti et al., 2019). In this context, TEO liposomes (TEO-Lips) have

been widely studied and applied (Al-Moghazy, El-sayed, Salama, & Nada, 2021; Cui, Ma, & Lin, 2016; Gil, Jerković, Marijanović, Manca, Caddeo, & Tuberoso, 2022; Lin, Zhu, Thangaraj, Abdel-Samie, & Cui, 2018). Nevertheless, liposomes are inherently unstable and prone to decomposition, which may lead to leakage of the contents (Tan, Wang, & Sun, 2021). Therefore, using polysaccharide or protein to modify liposomes is one of the ways to improve the application of essential oil liposomes (Semenova, Antipova, Martirosova, Zelikina, Palmina, & Chebotarev, 2021). The use of proteins to modify liposomes is relatively rare. The proteins commonly used for liposome modification are whey protein (Frenzel & Steffen-Heins, 2015), lactoferrin (Chen et al., 2020), and soy protein (Chen et al., 2022). In addition, Li was inspired by soybean oleosomes and achieved good results in modifying liposomes using soybean oleosomes-associated proteins (Li, Pu, Sun, Sun, & Tang, 2022).

Lysozyme (LYZ) has the advantage of being stable and inexpensive, which is considered to be generally regarded as safe (GRAS). In 1992, the FAO/WTO Association of Food Additives recognized that LYZ is safe for use in food. Several studies have explored the interaction between LYZ and liposomes (Tsunoda et al., 2001; Witoonsaridsilp, Panyarachun, Sarisuta, & Müller-Goymann, 2010). In some studies, it has been suggested that a possible binding mechanism of LYZ to liposomes. LYZ first undergoes electrostatic adsorption with the liposome, and it is

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Nomenclature

TEO	thyme essential oil
LYZ	lysozyme
Lip	liposome
S100	phospholipid S100
PBS	phosphate buffered saline
ABTS	2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
TBA	4,6-Dihydroxy-2-Mercaptopyrimidine
TCA	trichloroacetic acid
EE	encapsulation efficiency
RR	release rate
TEM	transmission electron microscopy
FT-IR	Fourier transform infrared spectroscopy
GC–MS	gas chromatography-mass spectrometry

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subsequently drawn into the core hydrophilic cavity by the liposome while generating a larger particle size (Wang et al., 2020). Meanwhile, the study by Dag confirmed that LYZ can be used as a cationic biopolymer to modify liposomes and may have a gaining effect on the properties of the latter (Dag, Guner, & Oztop, 2019). However, whether the lysozyme-modified liposomes have compound properties has not been further investigated, this is not conducive to further research on the mechanism of LYZ modification in liposomes.

According to the above, this study attempted to use LYZ to modify the TEO-Lips, a composite liposome with lysozyme, thyme essential oil and liposome co-existed was prepared. The effects of LYZ modification on the physical and chemical stability of TEO-Lips against acid and alkaline conditions and ionic concentrations, storage stability over 14 d, and antibacterial properties against *E. coli* and *S. aureus* were investigated. This study may provide a reference for the effect of LYZ as a modifier on TEO liposomes, and it may provide a new idea for developing liposomal systems of natural antimicrobial agents for food.

2. Materials and methods

2.1. Materials

TEO was purchased from Florihana (Caussols, France). LYZ (BR, CAS: 12650-88-3) from chicken egg white was purchased from Macklin (Shanghai, China). Phospholipid S100 (S100, 94 % purity) was purchased from Sigma-Aldrich (Milwaukee, USA). Cholesterol (95 % purity, CAS: 57-88-5) was purchased from Macklin (Shanghai, China). 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, 98 % purity, CAS: 30931-67-0) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH, 96 % purity, CAS: 1898-66-4) were purchased from Macklin (Shanghai, China). 4,6-Dihydroxy-2-Mercaptopyrimidine (TBA, ≥ 98 % purity, CAS: 504-17-6) and trichloroacetic acid (TCA, 99 % purity, CAS: 76-03-9) were purchased from Adamas (Shanghai, China). All reagents used in this study were analytical pure, and ultra-pure water was used throughout the experimental procedure.

2.2. Preparation of liposomes

Thin film hydration was used to create liposomes (Cui, Zhao, & Lin, 2015). The ratio of S100 to cholesterol was 8:1, and the final concentrations of S100 were fixed at 10 mg/mL (TEO were fixed at 2.5 mg/mL). Then, the materials were thoroughly dissolved by adding chloroform. Rotational evaporation was used to completely remove the chloroform from the combined solution between 40 and 45℃ until a homogeneous lipid thin film had formed on the flask wall. Water was added to the flask and shaken or stirred vigorously until the film was hydrated thoroughly.

The resulting liposome suspension was sonicated at 260 W for 5 min to obtain the blank liposomes (blank-Lips) or TEO liposomes (TEO-Lips) required for the experiment. The preparation method of LYZ-modified liposomes was slightly modified from the previous study (Dag, Guner, & Oztop, 2019). Briefly, LYZ was dissolved in a PBS solution at pH 6.5, and then, different LYZ-modified TEO-Lips solutions were configured according to different LYZ/S100 (w/w).

2.3. Morphological and physicochemical characterization of liposomes

2.3.1. Particle size, PDI and zeta potential

The morphological parameters of liposomes are determined using a Zetasizer Nanoseries (Malvern Instruments Zen 3600; Malvern, UK). Before measurement of the zeta potential, the liposomes need to be pre-diluted 10 times. All measurements were performed after 60 s of equilibration at 298 K and repeated thrice.

2.3.2. Encapsulation efficiency (EE)

The encapsulation effect of TEO in liposomes was measured by GC–MS (Agilent, California, America). The experimental method was slightly modified from the previous study (Lin, Gu, Sun, & Cui, 2019). The change of the peak area of TEO ethanol solution was measured in the range of 0.5 to 2.5 mg/mL and plotted the corresponding standard curve. The liposomes were centrifuged at $18,360 \times g$ for 90 min by high-speed centrifuge (Bioridge, Shanghai, China) to allow them to settle to the bottom as much as possible. Remove the supernatant and add anhydrous ethanol to break the emulsion—centrifuge again at high speed for 10 min. Finally, the supernatant was taken and measured by GC–MS. EE was calculated according to equation (1).

$$EE = W_t / W_T \times 100 \quad (1)$$

where W_t is the total TEO encapsulated in liposomes, and W_T is the total TEO added in liposomes.

2.3.3. Transmission electron microscopy (TEM)

TEM samples were prepared by referring to previous studies (Sebaaly, Jraij, Fessi, Charcosset, & Greige-Gerges, 2015). In brief, 10 µL of liposomes were dropped on a copper grid, air-dried for 60 s, then stained for 2 min at pH 6.5 using 10 µL of 1 % phosphotungstic acid solution, and finally observed by TEM (Hitachi, Tokyo, Japan) under a vacuum environment.

2.3.4. Fourier transforms infrared spectroscopy (FT-IR)

The samples were lyophilized in LC-12N-80C vacuum freeze dryer (Lichen, Shanghai, China) for 48 h. Subsequently, the solid liposomes were mixed with KBr using the compression method and made into thin slices for FT-IR spectrometer (Thermo Fisher Scientific, Sunnyvale, America) observation. During the test, each sample was scanned 32 times.

2.4. Determination of physicochemical and storage stability of liposomes

2.4.1. pH stability

PBS solution at different pH (2.0, 5.0, and 8.0) was mixed with liposomes in equal volumes and incubated at room temperature (25℃) for 2 h. The particle size changes were measured.

2.4.2. Ionic stability

NaCl at different concentrations (0, 100, 200, and 300 mM) was mixed with liposomes in equal volumes and incubated at room temperature (25℃) for 2 h. The particle size changes were measured.

2.4.3. Thermal stability

Determine the effect of high temperature on liposome stability by the degree of release. In brief, the samples were heated in a water bath at 80℃, and appropriate amounts of samples were taken into 100 kDa ultrafiltration tubes every 15 min. Then the tubes were centrifuged at

$1980 \times g$ for 10 min by medical centrifuge (Cence, Hunan, China). The absorbance of the filtrate from 0 to 1 h was measured by UV1901 Double Beam UV Spectrophotometer (AUCY, Shanghai, China), and the liposome retention rate was calculated according to the relevant equation (2).

Retention Rate % = $(W_t - W_r) / W_t \times 100$ (2)

where W_r is the content of TEO released, W_t is the total TEO encapsulated in liposomes.

2.4.4. Phospholipids oxidation

Lipids can be oxidized and generate reddish-brown trimethoprim with TBA under high temperatures or acidic conditions, and the maximum absorption wavelength of this substance is 532 nm. The degree of oxidation of phospholipids (MDA) can be examined based on this principle. Our method was slightly modified from those reported previously (Lin, Gu, Sun, & Cui, 2019). An equal amount of TBA-TCA-HCl solution was used as a blank without adding any sample. Furthermore, 0.5 mL of the liposomes to be tested was mixed with 2.5 mL of TBA-TCA-HCl solution, heated in a water bath for 30 min, and then the mixture was immediately cooled in ice water to prevent further reaction. Then, 2 mL of TBA-TCA-HCl solution was added to the solution and centrifuged at $1980 \times g$ for 10 min. The absorbance values of the supernatants of different samples were detected at 532 nm using a Biotek Synergy HTX. The MDA was calculated as shown in equations (3) and (4).

MDA (μg/mL) = $A_{532} \times 4.15$ (3)

MDA (ng/mg phospholipid) = MDA (μg/mL) / $V_p \times 100$ (4)

where A_{532} is the absorbance of the supernatants at 532 nm, V_p is the phospholipid content per volume in liposomes.

2.4.5. Storage stability

Variation in morphological parameters of liposomes was measured during 15 d of storage. The release rate (RR) of the liposomes was measured every 2 d for 14 d by using GC–MS. The RR was calculated as shown in equations (5).

RR % = $W_r / W_t \times 100$ (5)

where W_r is the content of TEO released, W_t is the total TEO encapsulated in liposomes.

2.5. Determination of antimicrobial property of liposomes

2.5.1. Preparation of the bacterial stock solution

Prepare E. coli or S. aureus as follows: first, a small amount of the bacteria was dipped using an inoculation loop, transferred to an LB liquid medium, and shaken at a constant temperature of 37℃ from 14 to 16 h, until the bacteria had grown to logarithmic growth stage. Then, the bacterial solution was plate scribed on NA solid medium and repeatedly purified and incubated 2 to 3 times. Eventually, the purified bacteria were inoculated in a liquid medium and cultured again to the logarithmic growth stage, which was the bacterial stock solution required for the experiment. The bacterial stock solution needs to be transferred and cultured every 3 or 4 d to prevent it from being contaminated.

2.5.2. Bacterial growth curves reflected by OD600

In general, the concentration of bacteria is linearly related to OD600, so that OD600 can reflect bacterial growth to some extent. LB liquid medium containing different liposomes or free TEO was prepared respectively and inoculated with 1 % of the medium volume of E. coli and S. aureus stock solutions. The changes of bacterial OD600 were measured over 24 h, and the growth curves of different bacteria were plotted under each sample separately.

2.5.3. Antibacterial effect

The bacterial stock solution was diluted using 0.9 % saline to obtain a bacterial suspension with an initial concentration of 10^5 to 10^6 CFU/mL, and different samples were added. Incubate at 37℃ for 24 h at a constant temperature. Subsequently, the plate counting method determined

the number of residual colonies in different samples. When counting bacteria, the countable number of colonies on a plate should be between 30 and 300.

2.5.4. Storage antibacterial property

The bacterial solution was diluted using an LB liquid medium to 10^4 to 10^5 CFU/mL. Different samples were added to the culture medium and incubated at 37℃. The plate counting method determined the number of residual colonies in different samples from 0 to 3 d.

2.6. Statistic analysis

Three replicate groups were set up for each experiment. The processed data were plotted in the experiment using Origin 2021 software (Origin Lab, Northampton, USA). One-way ANOVA was performed using SPSS with a significance level of 5 %.

3. Results and discussion

3.1. Particle Size, PDI, zeta potential, and EE

3.1.1. Determination of the optimum TEO concentration

Table 1 displays the variations of morphological parameters and the encapsulated efficiency of TEO-Lips with different TEO adding concentrations. In the experiment results, the average particle size of blank liposomes (Blank-Lips) without encapsulated material was 76.4 ± 1.8 nm. The particle size of TEO liposomes (TEO-Lips) gradually increased with the TEO concentration from 1.5 mg/mL to 3.5 mg/mL, increasing from 96 nm to 122.2 ± 0.7 nm. The PDI of Blank-Lips was 0.287 ± 0.018. The PDI of liposomes is slightly larger than 0.3 when the TEO concentration is lower than 2.0 mg/mL. When the TEO concentration was 2.5 mg/mL, the PDI of liposomes plunged to 0.154 ± 0.012. A PDI value lower than 0.3 indicates that the liposomes possess a more uniformly distributed particle size (Lin, Zhu, Thangaraj, Abdel-Samie, & Cui, 2018). Zeta potential is one of the factors for assessing the stability of liposomes. The average zeta potential of the Blank-Lips was determined to be −29.6 ± 0.4 mV. When TEO was added at a concentration of 2.5 mg/mL, the zeta potential of TEO-Lips was relatively lowest among TEO-Lips at −25.3 ± 0.7 mV. The absolute value of zeta potential below 10 mV indicates that the liposomes are unstable, 10 to 20 mV indicates low stability, 20 to 30 indicates moderate stability, and 30 to 40 indicates high stability (Esposto, Jauregi, Tapia-Blácido, & Martelli-Tosi, 2020). Therefore, the liposomes prepared in this experiment have moderately good stability. Meanwhile, the highest EE of 39.97 ± 0.26 % was obtained when TEO was added at a concentration of 2.5 mg/mL. That means the liposomes at this TEO concentration reflected the best

Table 1
Average TEO liposome Morphological parameters and EE with different TEO concentrations. Dissimilar superscript letters represent diverse significant differences between data ($P < 0.05$).

Parameters	different concentrations of TEO (mg/mL)					
	0 (Blank)	1.5	2.0	**2.5**	3.0	3.5
Particle size (nm)	76.4 ± 1.8[a]	96.7 ± 2.2[b]	96.4 ± 1.6[b]	**117.9 ± 1.0[d]**	115.1 ± 0.1[c]	122.2 ± 0.7[e]
PDI	0.287 ± 0.018[b]	0.305 ± 0.012[b]	0.302 ± 0.030[b]	**0.154 ± 0.012[a]**	0.161 ± 0.009[a]	0.165 ± 0.005[a]
Zeta potential (mV)	−29.6 ± 0.4[a]	−24.3 ± 1.7[c]	−23.7 ± 1.4[d]	**−25.3 ± 0.7[b]**	−23.5 ± 1.8[d]	−25.2 ± 2.6[b]
EE (%)	—	26.96 ± 0.34[a]	32.25 ± 0.19[b]	**39.97 ± 0.26[e]**	38.53 ± 0.34[d]	36.83 ± 0.14[c]

Different letters indicate significant differences ($P < 0.05$).
± Represents the standard error.

encapsulation effect.

The absolute value of the zeta potential of the liposomes decreased slightly after encapsulation with TEO, probably because the addition of TEO caused a change in the fluidity of the lipid membrane and thus affected the stability of liposomes. A study of flaxseed oil liposomes found that the phospholipid ratio to oil (flaxseed oil) may influence the encapsulation effect. That study suggests that moderate addition of flaxseed oil could fill the gaps caused by imperfect phospholipid alignment and thus improve the integrity of the liposomal membrane (Song, Tian, Yang, & Sun, 2022). Nevertheless, excessive incorporation of flaxseed oil could cause membrane asymmetry and distortion, resulting in changes in the lipid membrane bilayer alignment. This result can be used as a reference for this study. In addition, when the amount of TEO added is too little, TEO may be associated with phospholipids due to the principle of similar solubility. In this case, most of the TEO stayed on the surface of the lipid membrane, and only a few TEO was encapsulated into the liposomal core. This inference could also explain the phenomenon that the encapsulation efficiency of liposomes increases and then decreases as the TEO concentration changes. In conclusion, a 2.5 mg/mL TEO addition concentration is most likely the optimum concentration for liposome encapsulation.

3.1.2. Determination of the optimum LYZ addition

Table 2 demonstrates the changes in morphological parameters and encapsulation efficiency of LYZ modified TEO-Lips at different mass percentages (w/w) of LYZ and S100. According to the results, the morphological parameters of TEO-Lips gradually increased with increased LYZ additions. In this process, the particle size and PDI of TEO-Lips increased relatively slowly when the LYZ addition was 0.01 % to 0.07 %, and the particle size of liposomes increased from 117.9 ± 1.0 nm to 128.5 ± 2.4 nm, and the PDI increased from 0.154 ± 0.012 to 0.178 ± 0.017. When the LYZ addition was 0.13 %, liposome particle size and PDI were 140.0 ± 4.2 nm. When LYZ was added at 0.15 %, liposome particle size and PDI increased more significantly, reaching 173.6 ± 3.6 nm and 0.249 ± 0.024, respectively. In terms of zeta potential, adding a small amount of LYZ (0.01 %) caused a rapid increase in TEO liposome zeta potential from -25.3 ± 0.7 mV to -18.7 ± 0.6 mV. However, the change in zeta potential caused by LYZ was relatively small until the addition of 0.05 % LYZ, which only increased to -16.8 ± 0.5 mV. When LYZ addition was more significant than 0.05 %, the LYZ-TEO liposome potential rapidly increased until it increased to $-8.3 \pm$

0.2 mV at 0.15 %, indicating that the liposomes were unstable. The effect of LYZ on the EE of TEO-Lips also showed a similar trend. With the LYZ addition increased from none to 0.05 %, the corresponding EE of liposomes decreased from 39.97 ± 0.26 % to 36.25 ± 1.12 %. And then, the EE rapidly decreased to 30.45 ± 2.17 % when the addition of LYZ was 0.05 % to 0.07 %. Until the addition of LYZ increased to 0.15 %, the EE of LYZ-TEO-Lips decreased to 24.51 ± 1.03 %.

Interactions between LYZ and liposomes were likely to be responsible for these results. Related studies have shown that the binding mechanism between LYZ and liposomes is mainly electrostatic, followed by a small number of hydrophobic interactions (Wang, et al., 2020). The zeta potential shows that the TEO-Lips have a negative surface charge, while the positive surface charge of LYZ may allow LYZ to neutralize the charge with the liposomes. Therefore, the zeta potential of TEO-Lips gradually increased with increasing amounts of LYZ addition, eventually reaching the potential range representing the instability of the liposomes. In addition, the binding of LYZ to liposomes may be permeable (Dag, Guner, & Oztop, 2019). That may be the reason for the increase in liposome particle size. Therefore, a LYZ addition concentration of 0.05 % may be the threshold that leads to a decrease in liposome stability. Based on this, a concentration of 0.05 % LYZ/S100 mass ratio (w/w) was used to prepare the subsequent desired LYZ-TEO-Lips.

3.2. TEM

Theoretically, liposomes should generally exhibit a spherical shape with smooth edges (Song, Tian, Yang, & Sun, 2022). As shown in Fig. 1A, the Blank-Lips have a smooth and round appearance, indicating a relative stability arrangement of phospholipid molecules in a stack. Fig. 1B displayed that the TEO-Lips have an approximately spherical shape, but their edges were rougher than the Blank-Lips. That was presumably due to a slight distortion of the liposomes caused by the addition of TEO, which resulted in a change in the fluidity of the lipid membrane. The TEO-Lips with the addition of LYZ were shown in Fig. 1C. It showed that after adding LYZ, a slight deformation of the TEO-Lips, and their edges became rough. At the same time, the uniformity of the liposome size distribution was reduced, which may have led to an increase in PDI. TEM showed that the absolute particle size of Blank-Lips, TEO-Lips and LYZ-TEO-Lips was consistent with the morphological parameters.

3.3. FT-IR

FT-IR can be used to identify the functional groups of a substance through characteristic peak analysis and determine whether there are interactions between substances through changes in peak intensity. Fig. 1D displayed the FT-IR spectrum of TEO, LYZ, and different liposomes.

3086 cm^{-1}, 2968 cm^{-1}, and 2925 cm^{-1} are the three characteristic peaks of TEO (Lin, Zhu, Thangaraj, Abdel-Samie, & Cui, 2018). They almost disappeared in the spectrum of TEO-Lips, representing that TEO was successfully encapsulated. The main peak of the TEO-Lips was similar to those of the Blank-Lips, indicating that the addition of TEO hardly affected the liposomes. After encapsulation of TEO, a slight blue shift in liposome from 3425 cm^{-1} to 3444 cm^{-1} was observed (O—H stretching in the hydrogen bonding), suggesting that the addition of essential oil may have slightly increased the mobility of the liposomal membrane, resulting in a slight decrease in stability. These results are in agreement with the particle size measurements. After modification of LYZ, this peak in LYZ-TEO-Lips hardly moved. It indicated no or weak hydrogen bonding between LYZ and TEO-Lips.

The peak at 2965 cm^{-1} of LYZ represents the asymmetric and symmetric stretching vibration of CH$_2$, which can reflect the alteration of the hydrophobic interaction force in the system. A red shift from 2965 cm^{-1} to 2926 cm^{-1} was observed after adding LYZ, indicating that the hydrophobic interaction force between LYZ and TEO is enhanced, which is consistent with the finding reported in the literature (Wang, et al.,

Table 2

Average LYZ-TEO liposome morphological parameters and EE were prepared by different ratios of LYZ/S100 (w/w). Dissimilar superscript letters represent diverse significant differences between data ($P < 0.05$).

Different ratios of LYZ/S100 (w/w)	Particle Size	PDI	Zeta Potential	EE%
0.00	117.9 ± 1.0^a	0.154 ± 0.012^a	-25.3 ± 0.7^a	39.97 ± 0.26^g
0.01	126.1 ± 2.2^b	0.163 ± 0.006^a	-18.7 ± 0.6^b	35.22 ± 0.85^e
0.03	125.6 ± 1.2^b	0.153 ± 0.014^{ab}	-16.4 ± 0.7^c	34.95 ± 1.34^e
0.05	$\mathbf{128.3 \pm 1.6^c}$	$\mathbf{0.173 \pm 0.019^{ab}}$	$\mathbf{-16.8 \pm 0.5^c}$	$\mathbf{36.25 \pm 1.12^f}$
0.07	128.5 ± 2.4^c	0.178 ± 0.017^{bc}	-13.1 ± 0.1^d	30.45 ± 2.17^d
0.09	132.4 ± 1.6^d	0.195 ± 0.021^{cd}	-14.0 ± 0.6^d	28.96 ± 1.83^c
0.11	135.4 ± 2.6^e	0.204 ± 0.010^{de}	-12.9 ± 0.5^e	30.22 ± 1.48^d
0.13	140.0 ± 4.2^f	0.219 ± 0.007^e	-11.8 ± 0.5^f	27.44 ± 1.10^b
0.15	173.6 ± 3.6^g	0.249 ± 0.024^f	-8.3 ± 0.2^g	24.51 ± 1.03^a

Different letters indicate significant differences ($P < 0.05$).
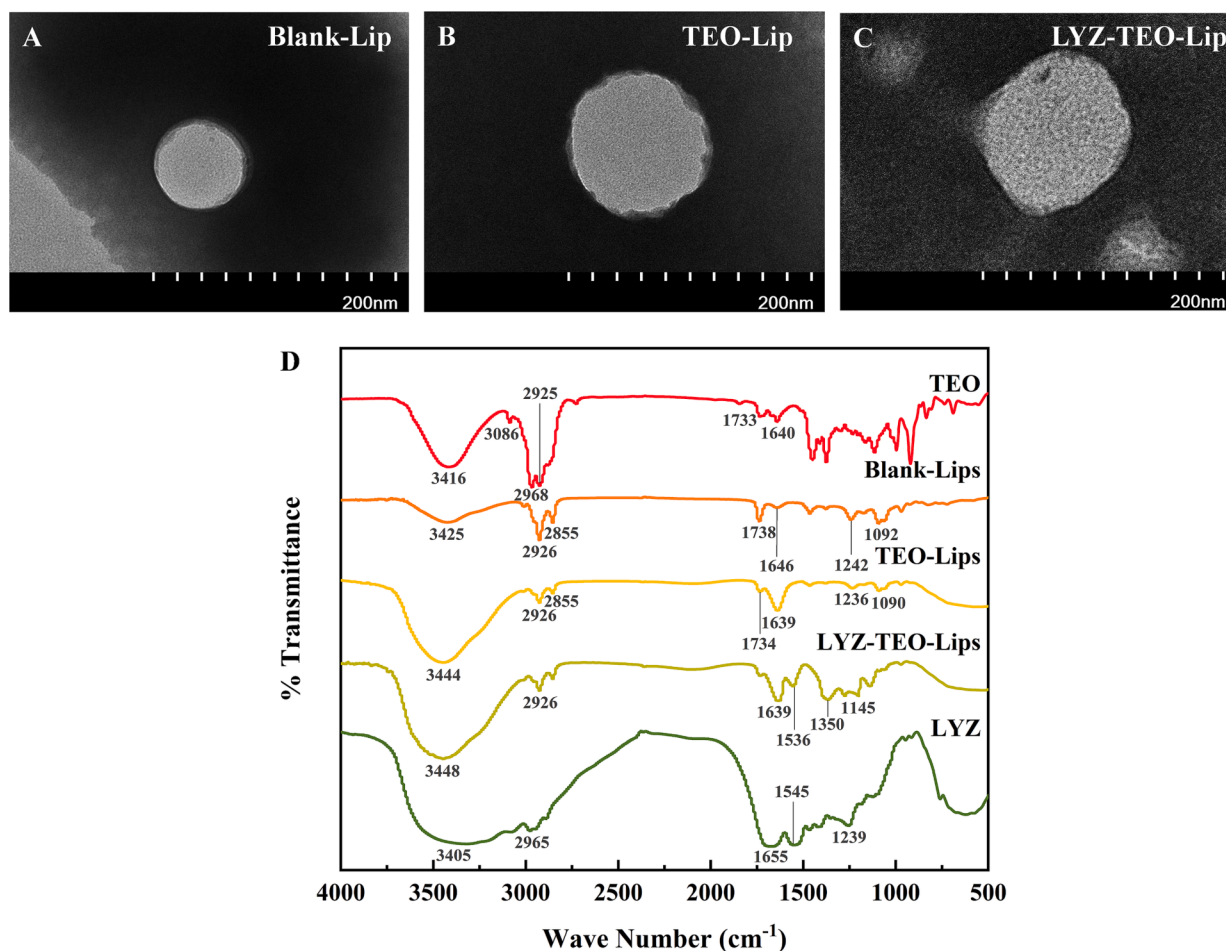$\pm$ Represents the standard error.

Fig. 1. TEM images of (A) Blank-Lip, (B) TEO-Lip, and (C) LYZ-TEO-Lip. (D) FT-IR spectrum of TEO, Blank-Lips, TEO-Lips, LYZ-TEO-Lips, and LYZ.

2020).

Three peaks at 1655 cm^{-1}, 1545 cm^{-1}, and 1239 cm^{-1} in the LYZ spectrum represent amide I, II, and III, respectively (Ro et al., 2017). Interestingly, the LYZ-TEO-Lips spectrum also showed the characteristic peaks for LYZ in these band ranges to varying degrees, further suggesting that there may be some interaction between LYZ and TEO-Lips.

The characteristic peak of the LYZ's spectrum at 1545 cm^{-1} is the stretching vibration of the secondary N—H bond in NH^{3+}, which slightly redshifted to 1536 cm-1 in the LYZ-TEO-Lips spectrum. Furthermore, characteristic peaks of Blank-Lips and TEO-Lips at 1092 cm^{-1} and 1090 cm^{-1} represent the PO^{2-} stretching band. After adding LYZ, the kurtosis verged to flatten. We speculated that the above redshift and alteration of kurtosis were caused by the PO^{2-} (negative charge) in the phospholipids of S100 interacting with LYZ (positive charge) through the electrostatic force. This speculation is in keeping with the decrease in liposomes' zeta potential due to electrostatic binding. In this interaction, LYZ contributed to the increase of the liposomal phospholipid bilayer's polarity, and the PO^{2-} group at the polar head tended to expose.

Overall, the FT-IR analysis demonstrated that LYZ mainly bound electrostatically to the liposomes, accompanied by hydrophobic interaction. TEO has a good encapsulation in liposomes and hardly reacts with liposomes, except for a slight effect on the fluidity of the lipid membrane. The liposomes were profitable material for encapsulating essential oil, and the LYZ's modification can promote its property.

3.4. Physicochemical stability of liposomes

3.4.1. pH stability

As food is likely to be in an acidic or neutral acidity range, it is necessary to study the effect of pH on liposome applications (Frenzel & Steffen-Heins, 2015). We observed that all liposomes increased in particle size at pH 2.0 (Fig. 2A), probably due to the higher swelling capacity of liposomes in acidic media (Shishir, Karim, Xu, Xie, & Chen, 2021). Besides, the PDI of TEO-Lips and LYZ-TEO-Lips (all below 0.2) varied relatively little at different pH (Fig. 2B), indicating that TEO-Lips solutions had a uniform distribution in different acidic and alkaline environments. Meanwhile, at pH 2.0, LYZ-TEO-Lips showed a minimum positive charge of 2.62 ± 0.30 mV (Fig. 2C), which was much smaller than Blank-Lips (10.50 ± 0.29 mV) and TEO-Lips (14.78 ± 0.26 mV). After adding LYZ, the TEO-Lips showed relatively minimal changes in different acidic and alkaline environments, most likely because LYZ improved the stability of TEO-Lips.

The isoelectric point of soy lecithin S100 was approximately around pH 6.7. When the pH is near the isoelectric point of phospholipids, the transition from the rigid to the ordered/gel phase of the lipid bilayer occurs, and its fluidity decreases significantly, which leads to a decrease in the stability of the liposome formed (Zimmermann, Küttner, Renner, Kaufmann, & Werner, 2012). Therefore, the change in surface charge of the liposomes was weak at pH 5.0 or pH 8.0 (proximity to the isoelectric point of the lipid membrane), which was not as pronounced as at pH 2.0. Moreover, the PDI of the Blank-Lips was relatively high in the environment of pH 2.0, whereas, after adding TEO, the PDI of TEO-Lips and LYZ-TEO-Lips was low (distributed more homogeneous), which indicates the TEO can attenuate the effects of the acid environment on liposomes.

While pH alters, the amount of protein surface charge changes (Güzey & McClements, 2006). When the acidity of a protein reaches near the isoelectric point (IEP), the protein can be deposited
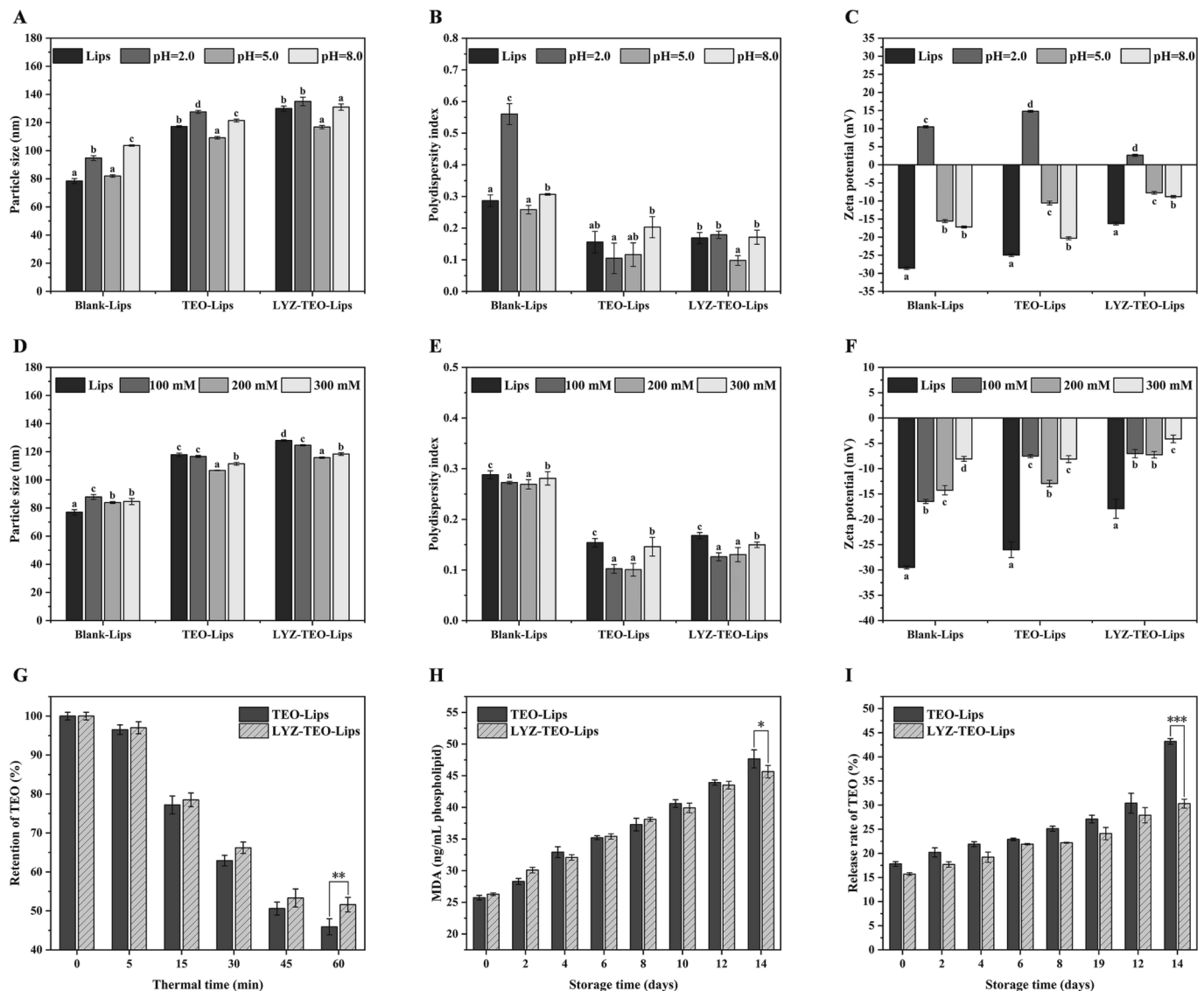
Fig. 2. (A) Particle size (B) PDI and (C) Zeta potential of Blank-Lips, TEO-Lips, and LYZ-TEO-Lips, at different pH environments. (D) Particle size (E) PDI and (F) Zeta potential of Blank-Lips, TEO-Lips, and LYZ-TEO-Lips, at different NaCl concentrations. (G) Retention of TEO of TEO-Lips and LYZ-TEO-Lips, at 80 °C. (H) Phospholipids oxidation and (I) Release rate (%) of TEO of TEO-Lips and LYZ-TEO-Lips in 14 d storage. Different letters at the top represent statistically significant results of the one-way ANOVA ($P < 0.05$), and different number of * represent the degree of significance of the difference.

electrostatically on the liposome surface due to the binding of the protein's positively charged amino group and the negatively charged liposomes (Tan, Wang, & Sun, 2021). For this reason, it is feasible to use protein modifications to improve liposome stability. At the same time, it is known from the properties of LYZ that LYZ is a highly alkaline protein (IEP = 11.16) and shows a positive charge in the pH range of 7.4 to 10.6 (Witoonsaridsilp, Panyarachun, Sarisuta, & Müller-Goymann, 2010). Compared with the other liposomes, the LYZ-TEO-Lips showed less significant changes in particle size and potential at different pH, which may be because electrostatic deposition attenuates the mobility of the liposome membrane.

In conclusion, the particle size, PDI, and potential of LYZ-TEO-Lips at various pH conditions changed to smaller than other liposomes. We supposed that the modification of LYZ in liposomes was easier to cope with acid-base changes.

3.4.2. Ionic stability

When applying natural antimicrobial agents to food, salt in food may impact liposome stability. Therefore, it is necessary to explore salt's

effect on liposome stability. The particle size (Fig. 2D) of Blank-Lips was 77.00 ± 1.87 nm in the absence of NaCl addition, which increased to 87.85 ± 1.73 nm at 100 mmol/L NaCl addition and decreased significantly to 84.66 ± 2.20 nm at 300 mmol/L NaCl addition. The particle size of TEO-Lips showed a decreasing trend. The particle size of TEO-Lips showed a decreasing trend. The particle size of TEO-Lips decreased significantly from 117.90 ± 1.15 nm to 111.49 ± 0.97 nm, and that of LYZ-TEO-Lips decreased significantly from 127.99 ± 0.55 nm to 118.41 ± 0.97 nm with the increase of NaCl concentration. On the other hand, the PDI (Fig. 2E) of the three liposomes showed a consistent trend with increasing NaCl concentrations (from 0 to 300 mmol/L). The PDI of the three liposomes decreased significantly when the concentration of NaCl was from 0 to 200 mmol/L and then rebounded somewhat when the concentration of NaCl reached 300 mmol/L. The PDI with a significant decrease followed by an increase, and an overall decreasing trend, which was also reported by Shishir et al (Shishir, Suo et al., 2021). Overall, the increase in salt concentration led to a decrease in the PDI of liposomes. The zeta potentials (Fig. 2F) of Blank-Lips, TEO-Lips and LYZ-TEO-Lips, along with the increase of salt concentration,

showed a decreasing trend from −29.50 ± 0.29 mV, −26.01 ± 1.55 mV and −117.90 ± 1.89 mV at NaCl concentration of 100 mmol/L, respectively, when the NaCl concentration was At 300 mmol/L, the zeta potential reached −8.06 ± 0.49 mV, −8.11 ± 0.69 mV, and −4.13 ± 0.75 mV, respectively.

Particle aggregation brought on by lessened electrostatic repulsion of mineral ions, which was also noted in Block liposomes created by Zidovska (Zidovska, Ewert, Quispe, Carragher, Potter, & Safinya, 2009), may be the source of the shift in the particle size of Blank-Lips. On the other hand, the smaller particle size of TEO-Lips and LYZ-TEO-Lips may be caused by water leaking from liposomes, which causes the liposomes to shrink (Cheng et al., 2019). The zeta potential of negatively charged Blank-Lips rapidly decreases with increasing salt content due to the influence of ionic strength on the ionization constant of the charge inserted into the lipid-like bilayer (Witoonsaridsilp, Panyarachun, Sarisuta, & Müller-Goymann, 2010). Reversely charged metal ions can attach to the liposome surface and lower the electrostatic charge on the surface of the lipid membrane, which may make the lipid vesicles more compact and stiffer (Shishir et al., 2021). In conclusion, the LYZ-TEO-Lips showed relatively little variation in stability in salt ions.

3.4.3. Thermal stability

Investigating the stability of liposomes at high temperatures may provide a reference for their application in food thermal processing. The results are shown in Fig. 2G. In the present study, the retention in TEO-Lips and LYZ-TEO-Lips decreased from 100 % to 45.9 ± 2.06 % and 51.6 ± 1.87 %, respectively, after 1 h of high-temperature heating. The result suggested that there probably existed some effects between LYZ and TEO-Lips. The mechanism for this effect may be that the modification of LYZ made the structure of the liposome membrane more rigid, and the TEO-Lips transformed more slowly at high temperatures accordingly (Frenzel & Steffen-Heins, 2015). Besides, since the mechanism of action of LYZ with liposomes may be through membrane permeation to the liposome interior, it may lead to a tighter arrangement of the liposome membranes in a two-dimensional array (Foteini, Pippa, Naziris, & Demetzos, 2019). Thus, whether LYZ is attached to the liposome surface, fills the liposomes' bilayer gap, or is absorbed and embedded in the hydrophilic cavity of the liposomes, it may make the structure of individual liposomes hard and stable. Beyond that, the diversity in permeability of the different TEO-Lips in the present results may also be related to their phase-transition temperature (Tm). Because the Tm of phospholipids can be altered accompanied by changes in interactions with other substances, adding LYZ may have increased the Tm of the liposomes and consequently enhanced the thermal stability (Frenzel & Steffen-Heins, 2015). That led to differences in the exudation of different liposomes in this study. This result was further evidence that the modification of LYZ may enhance liposome stability.

3.5. Determination of storage stability of liposomes

3.5.1. Phospholipids oxidation

The unsaturated bonds in soy lecithin are susceptible to oxidative degradation, so liposome lipid oxidation products (MDA) were assessed to determine the oxidative stability of liposomes (Min, Nam, & Ahn, 2010). The initial amounts of MDA at 0 d for TEO-Lips and LYZ-TEO-Lips were 25.73 ± 0.37 ng/mL and 26.28 ± 0.21 ng/mL, respectively (Fig. 2H). After 14 d of storage, the final amounts of MDA for both were 47.66 ± 1.42 ng/mL and 45.65 ± 0.98 ng/mL, respectively. After 14 d of storage, there was a significant difference in the final MDA content produced by LYZ-TEO-Lips and TEO-Lips. This result indicated that LYZ slightly retarded the oxidation of TEO-Lips.

3.5.2. Release rate (RR)

This study explored the RR of TEO from liposomes over 14 d. In the result, TEO-Lips with or without LYZ-modified respectively displayed a RR of 17.8 ± 0.48 % and 15.7 ± 0.27 % at 0 d. After 14 d, the release

rates reached 43.2 ± 0.59 % and 30.3 ± 0.94 %, separately (Fig. 2I). It can be shown that the addition of LYZ retarded the controlled release of TEO from the liposomes.

Typically, lipid membranes were considered inert and passive scaffolds for membrane proteins, and the dynamic exchange of proteins in the membrane interface environment affects the function of biological membranes. Generally, there were two binding modes between biofilms and proteins, the binding of proteins to the surface of the biofilm or their penetration into its interior (Heider, Reimhult, & Metzner, 2018). The difference with other proteins used for liposome modification (e.g. whey protein, lactoferrin) was that literature have shown that the binding mode of LYZ to liposomes was an osmotic process (Tsunoda, et al., 2001). In conjunction with our previous speculation, LYZ could alter the state of the arrangement of the lipid membrane structure. This alteration may result in a tighter and stiffer membrane structure with lower mobility, thus ensuring the controlled release of the encapsulated substance from the liposomes. The measurable junction of release rates is consistent with this inference. Hence, it can be concluded that LYZ enhances liposome stability to a certain extent and prolongs the controlled release effect of TEO.

3.5.3. Morphological changes during storage

We studied the alteration of liposome morphological parameters of different liposomes at low (4 °C) and room temperature (25 °C) in 15 d, which can provide some basis for applying liposomes in food refrigeration or ambient storage.

In low temperature storage conditions, the particle size of the Blank-Lips, TEO-Lips, and LYZ-TEO-Lips did not change much during the storage time, respectively increasing by 2.14 nm, 2.87 nm, and 9.88 nm (Fig. 3A). The increase of PDI for liposomes tended to be flat, which indicates that the three liposomes had uniform distribution during the 15 days storage period (Fig. 3B). The zeta potential of the Blank-Lips, TEO-Lips, and LYZ-TEO-Lips increased from −29.30 ± 1.24 mV to −16.53 ± 0.75 mV, −24.97 ± 0.38 mV to −17.52 ± 0.61 mV, and −17.04 ± 0.48 mV to −12.25 ± 0.89 mV, respectively (Fig. 3C).

While storing liposomes at room temperature, the particle size of the Blank-Lips increased from an initial 75.92 ± 0.58 nm to 140.4 ± 1.73 nm (Fig. 3D), and the final size was almost twice the initial size. On the other hand, the TEO-Lips and LYZ-TEO-Lips showed a lower increase than Blank-Lips, and the increasing degrees were respectively by 30.00 nm and 37.27 nm. Liposomes show a more significant increase in PDI at room temperature than at low temperature conditions, the final PDI for the Blank-Lips, TEO-Lips, and LYZ-TEO-Lips were 0.369 ± 0.032、 0.207 ± 0.026 and 0.243 ± 0.014, respectively (Fig. 3E). The decreases in potential were dramatic for the Blank-Lips and TEO-Lips but relatively moderate for the LYZ-TEO-Lips (Fig. 3F).

The low zeta potential of LYZ-TEO-Lips is caused by the electrostatic interaction forces between LYZ with liposomes so that the surface charge of the liposomes is neutralized. It represents particle-to-particle instability in the solution system and is more likely to result in phenomena such as aggregation and sedimentation. However, the results show a relatively smooth change in liposome particle size over 15 d. There was no significant increase or decrease in the particle size of the three liposomes during this period, indicating that the system neither increased in size due to aggregation nor decreased in size due to liposome rupture. Overall, the modification of LYZ makes for the minor fluctuation in morphological parameters of LYZ-TEO-Lips, meaning it is the most stable.

In cryogenic and ambient storage, TEO-Lips or LYZ-TEO-Lips all showed good storage stability compared to liposomes not encapsulated with any substance. Therefore, we consider the liposome system to be stable after adding LYZ. As indicated by the changes in morphological parameters, LYZ may have had a stabilizing effect on the liposome system. It is consistent with our previous assumptions.
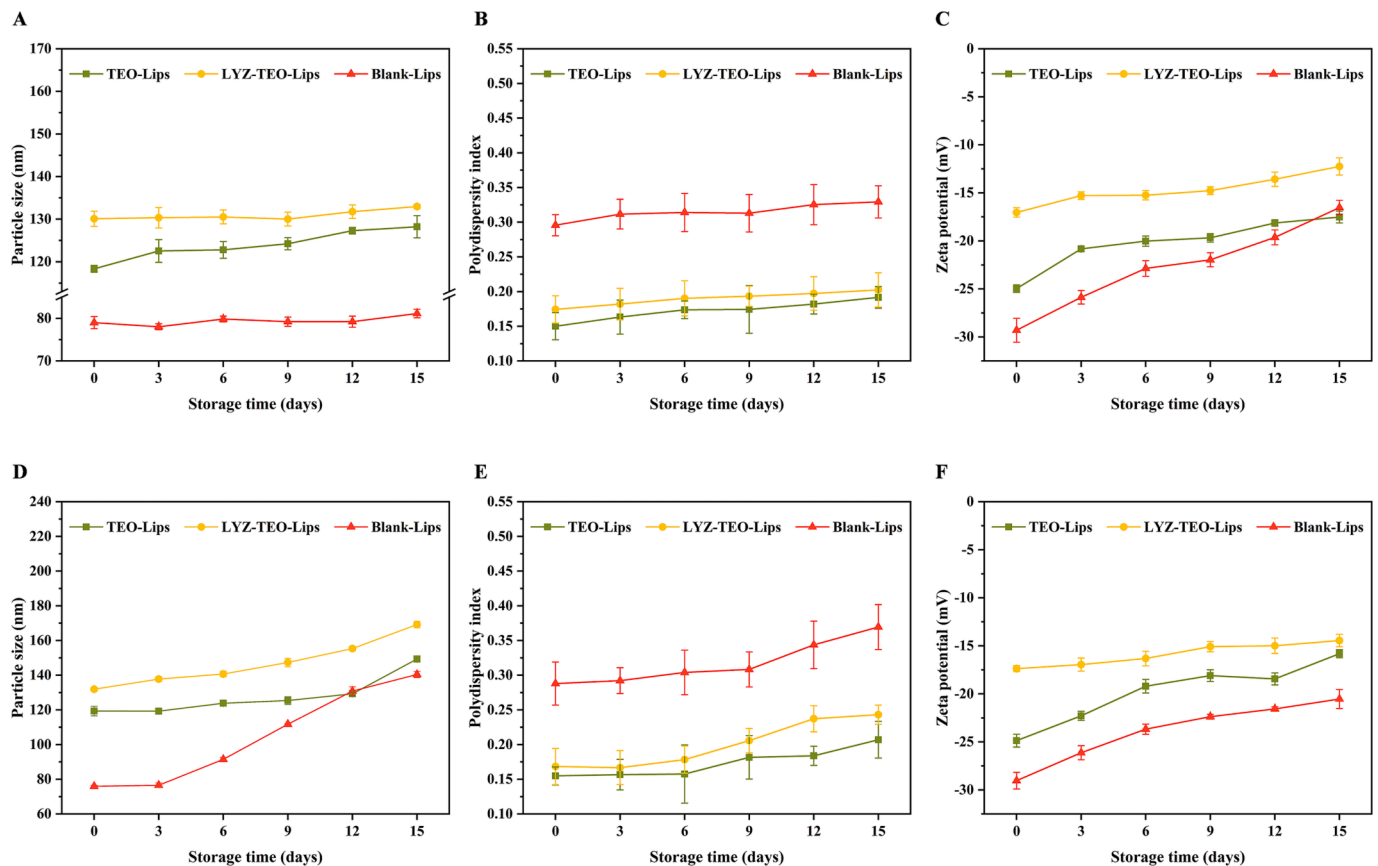
Fig. 3. (A) Particle size (B) PDI and (C) Zeta potential of TEO-Lips, and LYZ-TEO-Lips, and Blank-Lips in 15 d storage at 4 °C; (D) Particle size (E) PDI and (F) Zeta potential of TEO-Lips, and LYZ-TEO-Lips, and Blank-Lips in 15 d storage at 25 °C.

3.6. Determination of antimicrobial property of liposomes

3.6.1. Bacterial growth curves reflected by OD600

OD600 (turbidimetric method)/plate count can reflect the number of bacteria growing in the sample indirectly or directly. In general, the OD600 measurement was less precise than the plate count method because its value contains both live and dead bacteria, and the plate count method only reflects the bacterial growth status of live (Duedu & French, 2017). Nevertheless, OD600 is still a simple and rapid method for determining bacterial growth curves.

The growth curves of *E. coli* and *S. aureus* are shown in Fig. 4A and Fig. 4B. Normally, the growth curves of bacteria show logarithmic growth with an S-shape. In the results, the growth curves measured in this study were consistent with this trend. The growth curve of *E. coli* and *S. aureus* shows that after 24 h of incubation, the bacterial growth was Blank control > TEO > TEO-Lips > LYZ-TEO-Lips. Interestingly, the OD600 of LYZ-TEO-Lips was slightly higher than that of TEO-Lips in the growth curves of both bacteria during the first 6 or 8 h. It implied that the LYZ-TEO-Lips were less effective at inhibiting bacterial growth than TEO-Lips at this time. However, after 6 or 8 h, the antibacterial effect of LYZ-TEO-Lips was essentially stronger than that of TEO-Lips.

The previous results show that LYZ-TEO-Lips has a greater ability to control the release of TEO and therefore starts with a slower release rate. It may explain why it was observed that the antimicrobial ability of LYZ-TEO-Lips was slightly lower than TEO-Lips in the early stage but more substantial in the later stage, which was due to more TEO release in the later stage. In addition, TEO alone possessed a weaker antimicrobial ability, probably since TEO, as a hydrophobic substance, led to separation from the water phase in the medium, reducing its possibility of contact with bacteria (S. Li, et al., 2021).

3.6.2. Antibacterial effect

Fig. 4C and Fig. 4D show the colony counts of the two bacteria after incubation, respectively. The trends are generally in line with those presented by the growth curves. Bacteria treated with TEO, TEO-Lips, and LYZ-TEO-Lips decreased by 1.80 Log CFU/mL, 3.00 Log CFU/mL, and 3.02 Log CFU/mL in *E. coli*, and 0.99 Log CFU/mL, 1.88 Log CFU/mL, and 2.0 Log CFU/mL in *S. aureus*.

The different nature of *E. coli* and *S. aureus* was the main reason for the difference in inhibition in the results. In this study, TEO and its liposomes were more potent against Gram-negative bacteria. The possible explanation is that the main target of action of the active antimicrobial substances contained in TEO lies in the cell membrane of the microorganism (Ozogul, et al., 2020). These active substances interact with the proteins on the bacterial biofilm, thereby inducing the loss of bacterial cell contents and death (Milagres de Almeida et al., 2023). Besides, liposomes have a high degree of similarity to cell membranes, giving them an excellent cellular uptake capacity (Frenzel & Steffen-Heins, 2015). Therefore, due to good membrane compatibility, TEO-Lips will likely be released upon contact with bacterial biofilms. Since the thicker peptidoglycan layer in Gram-positive bacteria's cell walls, the cell wall makes it more difficult for TEO to reach the microbial membranes, thus presenting the results above.

3.6.3. Storage antimicrobial property

The antibacterial properties of the different samples against *E. coli* and *S. aureus* during 3 d of storage are shown in Fig. 4E and Fig. 4F. The average initial colony counts of *E. coli* and *S. aureus* in different samples were around 4.50 Log CFU/mL and 4.7 Log CFU/mL, respectively.

In the *E. coli* antimicrobial assay experiment, after 1 d of incubation, the number of bacteria in the Blank control and TEO experimental groups reached the logarithmic growth period. It stabilized around 9.67
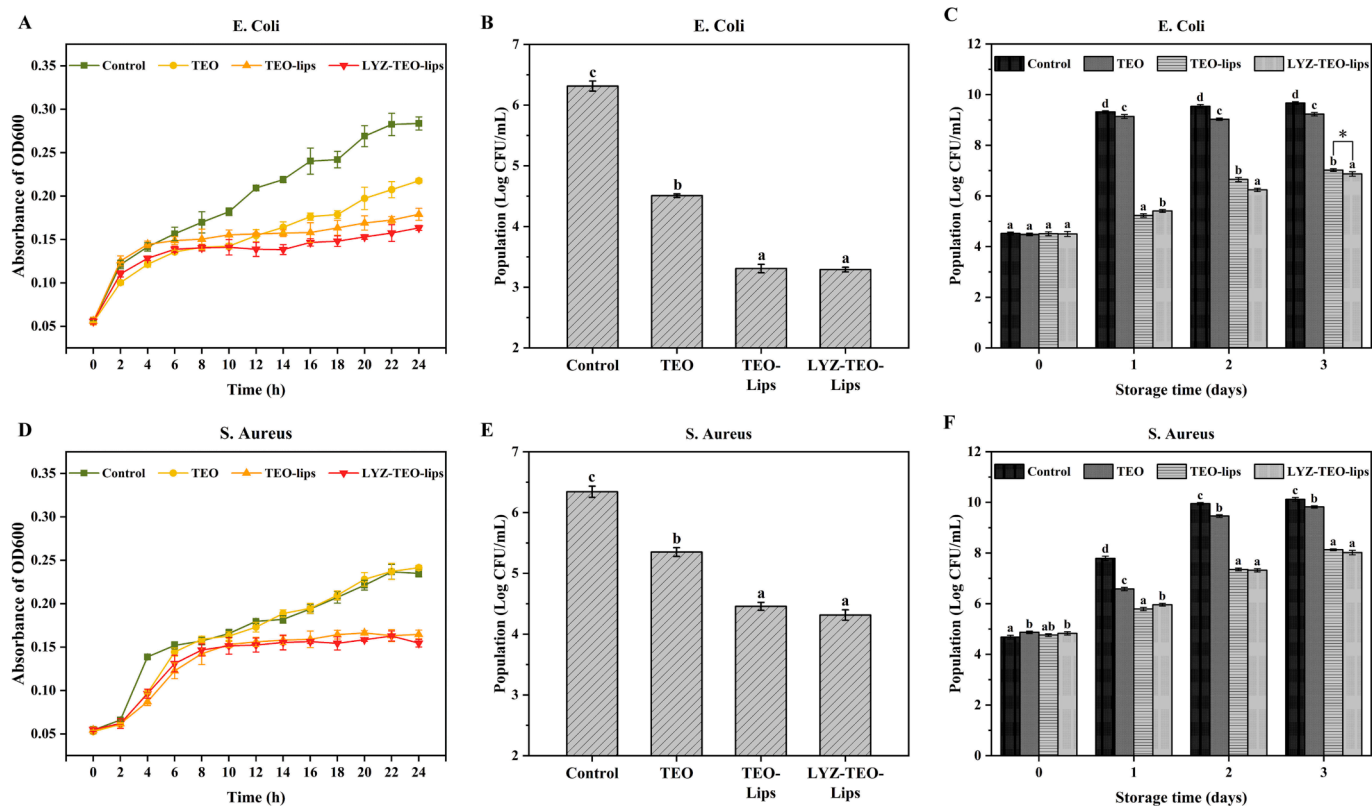
Fig. 4. Growth curves in OD600 of (A) *E. coli* (B) *S. aureus*, and final population counts of (C) *E. coli* (D) *S. aureus*, which were obtained after 24 h of treatment with blank control, TEO, TEO-Lips and LYZ-TEO-Lips. Storage antimicrobial properties of (E) *E. coli* (F) *S. aureus* of different liposomes. Different letters at the top represent statistically significant results of the one-way ANOVA ($P < 0.05$), and different number of * represent the degree of significance of the difference.

$\pm$ 0.05 Log CFU/mL and 9.23 $\pm$ 0.07 Log CFU/mL for 2 d. After 3 d of incubation, the final colony counts of TEO-Lips and LYZ-TEO-Lips groups were at 7.02 $\pm$ 0.06 Log CFU/mL and 6.87 $\pm$ 0.09 Log CFU/mL.

In the antibacterial experiment of *S. aureus*, the Blank control group and TEO experimental group also basically reached the logarithmic growth period after 2 d of incubation. After 3 d of incubation, the final colony counts of TEO-Lips and LYZ-TEO-Lips groups were at 8.13 Log CFU/mL and 8.02 Log CFU/mL.

In conclusion, in terms of antibacterial trends, TEO-Lips and LYZ-TEO-Lips greatly retarded the growth of both bacteria, probably due to the improved solubility of TEO by liposome encapsulation, which enhanced the possibility of TEO contact with bacterial biofilms. This finding is consistent with the results of previous experiments. Furthermore, the modification of LYZ enhanced the inhibitory effect of TEO-Lips on bacteria. Since both TEO and LYZ are natural antimicrobial agents for food, it is interesting to investigate the effect of LYZ on the antimicrobial properties of TEO-Lips. This experiment can provide ideas for the development of natural antimicrobial agent liposomes.

4. Conclusion

Previous reports have suggested that LYZ can improve liposome stability, but this effect has yet to be explored in depth. In our study, adding LYZ increased the particle size of TEO-Lips. FT-IR confirmed that the binding mechanism of LYZ to liposomes is mainly electrostatic. The modification of LYZ enhanced the antioxidant and antibacterial properties of the liposomes, shows well tolerance to acid and salt ions, and their stability at high temperatures, and has good stability during storage. In addition to that, the modification of LYZ provides better controlled release capability of TEO, maybe due to the LYZ reduced the fluidity of the liposome membrane, making TEO less likely to exude.

This finding could provide a reference for the effect of LYZ on the properties of antimicrobial liposomes. In future studies, protein-modified essential oil liposomes may be considered for use in the development of edible films or preservation of meat products.

CRediT authorship contribution statement

Dian Zhou: Data curation, Investigation, Writing – original draft. **Mohan Li:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Qinhong Li:** Conceptualization, Software. **Fang Geng:** Methodology, Resources, Visualization. **Shugang Li:** Project administration, Supervision. **Di Wu:** Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.fochx.2023.101057.

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