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Interaction mechanism of three egg protein derived ACE inhibitory tri-peptides and DPPC membrane using FS, FTIR, and DSC studies

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ABSTRACT

Understanding the interaction of food derived angiotensin converting enzyme (ACE) inhibitory peptides and intestinal epithelial cell membrane may help to improve their absorption. This research aimed to study the molecular interaction of ACE inhibitory tri-peptides ADF, FGR, and MIR with DPPC membrane during absorption process. The DPPC liposome was prepared and characterized, then used as a model membrane. The permeability of tri-peptides across the membrane was investigated using Fluorescence spectroscopy. The effect of tri-peptides on the structure and dynamics of DPPC bilayers was determined using Fourier transform infrared spectroscopy. The effect of tri-peptides on the phase transition temperature in the DPPC membrane was also analyzed using Differential scanning calorimetry. The results showed that ACE inhibitory tri-peptides ADF, FGR, and MIR can penetrate into both the membrane-water interface and hydrophobic region of DPPC bilayer, and the tri-peptide FGR have higher permeability across the membrane.

1. Introduction

Hypertension is a major risk factor of human health, and has become the main reason for about 19% of all deaths worldwide (Huang, Jia, Zhao, Hou, & Hu, 2021). Angiotensin converting enzyme (ACE) is considered as a key target for the prevention and management of high blood pressure. Many synthetic ACE inhibitors show strong anti-hypertensive activities, such as captopril, enalapril, and lisinopril (Liu et al., 2013). These inhibitors are associated with several side effects including coughing, skin rash, and angioedema (Kumagai et al., 2021). Various ACE inhibitory peptides have been identified from food proteins due to their safety advantages. In our previous studies, ACE inhibitory tri-peptides Ala-Asp-Phe (ADF), Phe-Gly-Arg (FGR), and Met-Ile-Arg (MIR) have been identified from hen eggs proteins (ovo-transferrin, myosin-10, ovomucoid, lysozyme, ovomacroglobulin, avidin, phosvitin, and ovalbumin), with half maximal inhibitory concentrations (IC_{50}) values of 27.75 ± 0.90 mM, 66.98 ± 1.40 μM, and 24.97 ± 0.80 mM, respectively (Zhao, Zhang, Yu, Ding, & Liu, 2020). Previous study demonstrated that dietary bioactive peptides have to penetrate the intestinal barrier, enter the blood circulation, and reach targeted tissues and organs in their active forms for exerting physiological functions (Shen & Matsui, 2018). The poor membrane permeability in the intestinal epithelial lead to low bioavailability of food derived bio-peptides, which limited their application (Yamamoto, Ukai, Morishita & Katsumi, 2020). Understanding the interaction of ACE inhibitory peptides and intestinal epithelial cell membrane may help to improve their intestinal absorption during peptides cross membrane process, and further exert functions (Ji, Zhao, Yu, & Wu, 2022).

Membrane is mainly composed of a phospholipid bilayer in which the polar heads of phospholipids arranged outside and the hydrophobic tails was inside (Mezzenga et al., 2019). Many unexpected or unknown influence factors make it complex to study natural membrane directly, thus, artificial model membrane has been developed to offer an alternative (Yuan, Piao, & Dong, 2021). In addition, the results obtained from model membrane systems are validated, and similar to that obtained from natural system (Knobloch, Suhendro, Zieleniecki, Shapter, & Koeper, 2015). Liposomes are particle vesicles formed by self-assembly of natural amphiphiles (*i.e.,* phospholipid, cholesterol) or synthetic amphiphiles in an aqueous environment (Pentak & Danuta, 2014b). The structure, composition, and physicochemical property of unilamellar lipid vesicle (liposome)-based artificial cellular membrane are similar to natural membrane (Yuan, Piao, & Dong, 2021). Therefore, liposomes have attracted great attention, and are widely used to simulate the membrane structure and study molecule-membrane

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interactions. In model systems, 1,2-dipalmitoyl-*sn-glycero*-3-phosphatidylcholine (DPPC) liposomes are often used to represent mammalian membrane. Ennaas et al. studied the interaction dynamics of antibacterial peptide collagencin and membrane using DPPC liposome (Ennaas et al., 2016). Investigating the interaction between other antimicrobial peptides or antigenic peptides with membrane based on DPPC liposome has also reported in previous studies (Dayane, Maria, João & Natalia, 2016; Sospedra, Mestres, Haro, Muñoz, & Busquets, 2002). However, few studies focused on the interactions between ACE inhibitory tri-peptides and membrane based on DPPC membrane.

The purpose of current study was to investigate the interaction of ACE inhibitory tri-peptides with DPPC membrane at molecular level. The DPPC liposome was prepared and characterized by the determination of particle size using dynamic light scattering. The location of tri-peptides in membrane was measured by Fluorescence spectroscopy (FS) techniques. The Fourier Transform Infrared spectroscopy (FTIR) method was used to study the effect of tri-peptides on lipid order, dynamics, and hydration state of head group by determining the variances of wavenumber of V_sCH_2, and $V_{as}PO_2^-$ of DPPC membrane. The phase transition property was obtained by determining the phase transition temperature of DPPC membrane in the presence of tri-peptides using Differential scanning calorimetry (DSC).

2. Materials and methods

2.1. Chemicals

ACE inhibitory tri-peptides ADF, FGR, and MIR were provided by Shanghai Science Peptide Biological Technology Co., Ltd. (Shanghai, China). 1,2-dipalmitoyl-*sn-glycero*-3-phosphatidylcholine (DPPC) was obtained from Ponsure Biological (Shanghai, China). 1,6-Diphenyl-1,3,5-hexatriene (DPH), [N,N,N,-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenylammonium(p-toluenesulfonate) (TMA-DPH), 1,2-dipalmitoyl-*sn-glycero*-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS) (pH 7.4) was obtained from Shanghai Future Industry Co., Ltd. (Shanghai, China). Tris-(hydroxymethyl) aminomethane (≥99%) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). All other chemicals were purchased from commercial sources and of analytical grade.

2.2. Preparation of DPPC liposome

The preparation of DPPC liposome was performed by binding thin layer film and ultrasonic methods (Zhu et al., 2016). To prepare pure DPPC liposome, 2 mg of DPPC was dissolved in 10 mL chloroform/methanol mixture (7/3, v/v) in a round-bottomed flask. DPH, TMA-DPH, and NBD-PE labeled liposomes (fluorescent liposomes) were prepared to determine the location of tri-peptides in membrane. Three different liposomes were prepared follow by the addition of 4 μL DPH (0.3 μM), 8 μL TMA- DPH (0.9 μM), and 50 μL NBD-PE (0.12 μM), respectively. The organic solvent was removed by evaporation at 50 °C (above the gel to liquid crystalline phase transition temperature) under vacuum with a rotary evaporator to obtain dried DPPC lipid thin film. The residual solvent was removed by storing the samples in fume hood overnight. The lipid film was hydrated for 1 h with 5 mL of PBS buffer (pH 7.4) and vortexing at 50 °C, and then the solution was subjected to sonication for 21 min.

2.3. Determination of DPPC liposome size

The average sizes of DPPC liposome were determined by dynamic light scattering (Nano-ZS 90, Malvern instruments, Worcestershire, UK) at room temperature. Each sample was equilibrated at 25 °C for 2 min, and was measured three times.

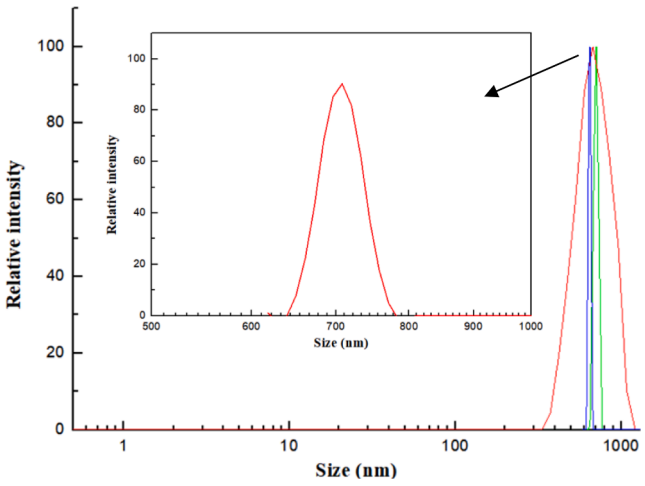

Fig. 1. The particle size distribution of DPPC liposome determined by dynamic light scattering.

2.4. Fluorescence spectroscopy measurement

Fluorescence spectroscopy was measured according to the method of Zhu et al with some modified (Zhu et al., 2016). Steady-state fluorescence spectroscopy measurements were performed using a Fluorescence Spectrophotometer (F-7000, HITACHI, Tokyo, Japan). Different concentrations of tri-peptides (2 μL) were added into 2 mL fluorescent liposomes to reach final concentration of 10, 20, 30, 40, 60 μg/mL, respectively. The 2 μL distilled water added into fluorescent liposomes was used as blank control. Then, the samples were allowed to equilibrate for 1, 5, 10, 15, 30, 60 min under water bath at 37 °C before measured, respectively. The excitation wavelength was set to 360 nm, and emission spectra were collected from 380 to 500 nm for DPH/TMA-DPH labeled liposomes. The excitation wavelength of 468 nm and emission wavelength between 500 and 600 nm were detecting for NBD-PE labeled liposomes. The fluorescence quenching results of tri-peptides on fluorescent liposomes were analyzed using following equation: quenching rate % = $(F_{bank}\text{-}F_{sample})/F_{bank} \times 100\%$, where F_{sample} refers to the fluorescence intensity of tri-peptides containing fluorescent liposomes, F_{bank} refers to the fluorescence intensity of distilled water containing fluorescent liposomes.

2.5. Fourier-Transform infrared (FTIR) spectroscopy analysis

The samples used for FTIR spectroscopy analysis were prepared as follows: Tri-peptide and DPPC liposome (total volume was 200 μL) at different molar ratio (0%, 10%, 20%, 40%, and 60%) were dissolved in 10 mL mixture of chloroform and methanol (7/3, v/v). The organic solvent was removed by the same preparation procedure of pure DPPC liposomes thin film. Then, the dried tri-peptide-lipid film was hydrated with 5 mL Tris-HCl, and the powder was obtained by a freeze-drying. The FTIR spectra of the tri-peptide-lipid was measured based on Scimitar 2000 instrument (Agilent, California, USA). The tri-peptide-lipid powder was mixed with 1% potassium bromide. Measurement parameters: spectral range, 400–4000 cm^{-1}; scan times, 32; resolution, 4 cm^{-1}.

2.6. Differential scanning calorimetry measurement

The tri-peptide-lipid sample for the DSC measurements were prepared follow the same procedure as that of the infrared study. DSC measurement was performed using a differential scanning calorimeter TA Q2000 (TA Instruments, USA). An empty aluminum crucible was used to calibrate the instrument, and tri-peptide-lipid powder (about 3–5 mg) was weighted into crucible for determine. The DSC

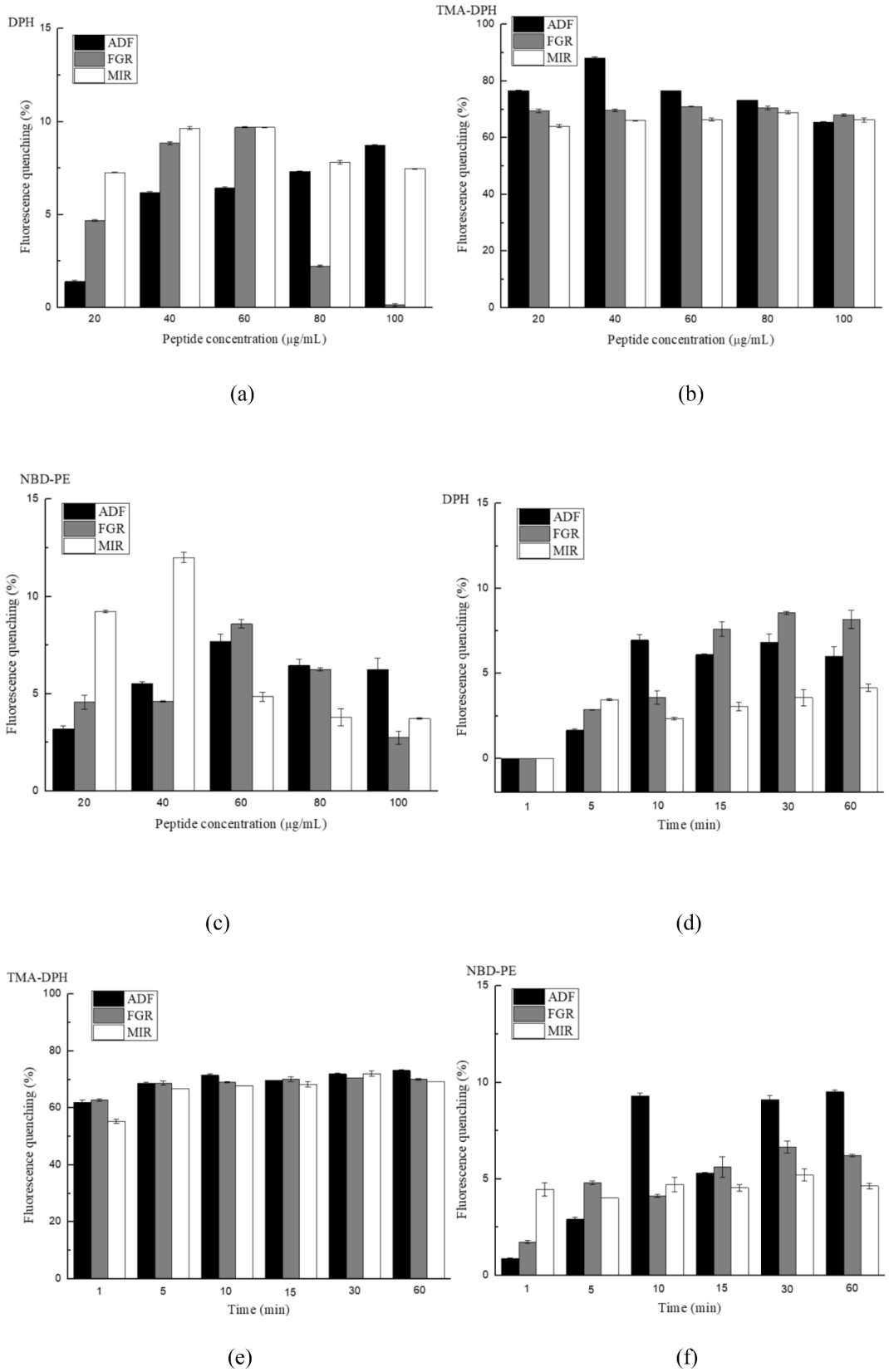

Fig. 2. The fluorescence quenching percentage of peptides ADF, FGR, and MIR on fluorescent probes DPH, TMA-DPH, and NBD-PE at different concentration and time. The effect of peptides concentration in quenching fluorescent probes DPH (a), TMA-DPH (b), and NBD-PE (c) of DPPC liposomes. The effect of reaction time between peptide (at the concentration of 60 μg/mL) and liposome in quenching fluorescent probes DPH (d), TMA-DPH (e), and NBD-PE (f) of DPPC liposomes.

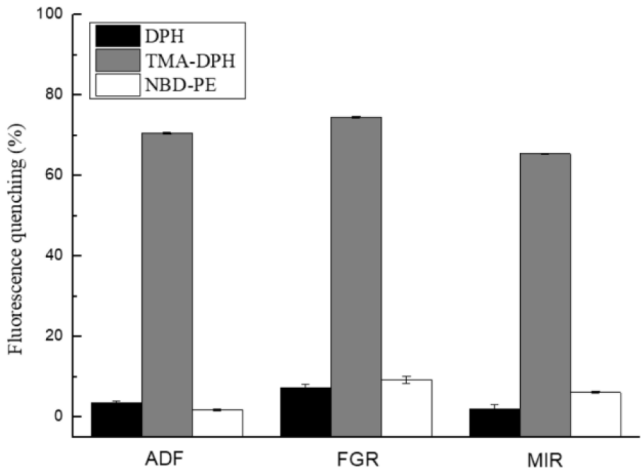

Fig. 3. The quenching percentage of peptides ADF, FGR, and MIR on DPH, TMA-DPH, and NBD-PE labeled fluorescent liposomes at the peptide concentration of 60 μg/mL and reaction time 30 min.

measurement was performed at a heating rate of 0.5 °C min^{-1} from 20 °C to 50 °C with a nitrogen flow. The phase transition temperature of DPPC lipid was obtained using the built-in software (TA Universal Analysis) of the equipment.

3. Results and discussion

3.1. Particle size distribution of DPPC liposome

The particle size distribution was determined to confirm and characterize the DPPC liposome, and the results are shown in Fig. 1. The size distribution results showed that the mean diameter of the DPPC liposome was 745.41 nm, and consistent with the results measured by Nystedt et al (Nystedt, Grnlien, & Tønnesen, 2021). The size of liposomes was according with the Large unilamellar vesicles (LUVs), which indicating the DPPC liposome was successfully prepared.

3.2. The effect of Tri-peptides on the quenching intensity of fluorescent liposomes

DPH, TMA-DPH, and NBD-PE are three fluorescent probes that behaved strong fluorescence upon binding to lipid, thus widely used for studies involved in membrane (Kaiser & London, 1998). Hydrophobic DPH distances 7.8 Å from the bilayer center, and TMA-DPH distances more shallowly than DPH in membrane (about 10.9 Å from the bilayer center) because of the cationic TMA group (Rodrigues, Gameiro, Prieto, & Castro, 2003). Fluorescent probe NBD-PE is located near the membrane surface. The fluorescence quenching percentage can indicate the depth of the peptides in the DPPC membrane. Therefore, the quenching of tri-peptides on fluorescent liposomes was used to determine the location of tri-peptides in DPPC bilayer in current study.

Different concentration of peptides and reaction time between tri-peptides and liposomes were used to determine the best concentration and time of tri-peptides in quenching fluorescent probes. Three tri-peptides have strong quenching intensity on DPH fluorescent probes at the concentration of 60 μg/mL as shown in Fig. 2a. And the different tri-peptides ADF, FGR, and MIR concentrations have almost not too much effect on fluorescence quenching of TMA-DPH fluorescent probes (Fig. 2b). Tri-peptides ADF and FGR have strongest quenching intensity on NBD-PE at the concentration of 60 μg/mL, and tri-peptide MIR at the concentration of 80 μg/mL (Fig. 2c). The results showed that the fluorescence quenching of tri-peptides ADF, FGR, and MIR on DPH fluorescent probes increased with reaction time within 30 min, as presented in Fig. 2d. For TMA-DPH fluorescent probe, tri-peptides ADF, FGR, and MIR have almost consistent quenching intensity at different reaction time as presented in Fig. 2e. And tri-peptides with strongest fluorescence quenching on NBD-PE fluorescent probe was observed at 30 min (Fig. 2f). Therefore, the tri-peptides concentration of 60 μg/mL and reaction time 30 min were used to determine the location of tri-peptides ADF, FGR, and MIR in the membrane.

All peptides have different fluorescence quenching percentage on fluorescent liposome, indicating that tri-peptides ADF, FGR, and MIR were not only located on the membrane-water interface regions but also inside the DPPC bilayer (Fig. 3). The fluorescence quenching percentage of tri-peptides on TMA-DPH were higher than that of DPH and NBD-PE, indicating that all tri-peptides able to enter into the midplane bilayer of the membrane. In addition, the tri-peptide FGR have higher fluorescence quenching percentage on the fluorescent liposome than that of tri-peptides ADF and MIR, may indicate that tri-peptide FGR penetrate membrane more rapidly. Tri-peptide FGR was consisted of residues Phe, Gly, and Arg. According to previous study, hydrophobic residues were more easily absorbed to the membrane surface (Alghamdi, Campbell, & Euston, 2019; Ulrih, Maricic, Ota, Sentjurc, & Abram, 2015). In addition, aromatic residues Phe also could strongly binding to membrane (Qian et al., 2018). Velasco et al. reported that aromatic residues initiate the process of antimicrobial peptides derived from scorpion venom penetrate into membrane (Velasco, Corzo, & Garduño, 2018). Positively charged Arg was reported that contributed to the interaction of peptides with negatively charged membrane head group by electrostatic attraction interaction (Qian et al., 2018). Therefore, the differences in tri-peptide sequence may lead to FGR with higher penetrate percentage across membrane.

3.3. The effect of tri-peptides on lipid acyl chain and headgroup of DPPC membrane

FTIR is an efficient spectroscopic method for getting molecular information of phospholipid membrane (Ezer, Sahin, & Kazanci, 2017). The FTIR was used to determine the effects of different concentrations of tri-peptides ADF, FGR, and MIR (from 0 mol% to 60 mol%) on the lipid acyl chain conformation and phospholipid head groups of DPPC membrane in current study (shown in Fig. 4 and Table 1). The wavenumber values of antisymmetric of phospholipid groups ($V_{as}PO_2^-$) of DPPC membrane was observed at 1209.3 cm^{-1}, and the wavenumber values of symmetric of methylene groups (V_sCH_2) of DPPC membrane was observed at 2848.8 cm^{-1}, which were consistence with the previous reported results (Arias, Cobos, Tuttolomondo, Altabef, & Díaz, 2020; Strugaa et al., 2021). As seen from Fig. 4a and Table 1, tri-peptide ADF decrease the wavenumber values of $V_{as}PO_2^-$ (from 1209.3 to 1207.4 cm^{-1}) at the concentration of 40 and 60 mol%. The wavenumber values of $V_{as}PO_2^-$ shift to lower value 1207.4 cm^{-1} in the presence of tri-peptide FGR (when the concentration over 20 mol%) (Fig. 4b). The wavenumber value of $V_{as}PO_2^-$ gradually shift to lower value as the increase of tri-peptide MIR concentration observed in Fig. 4c. The frequency shift of antisymmetric phospholipid groups ($V_{as}PO_2^-$) reflects the hydration state of phosphate headgroup (Schmid et al., 2018). The decrease of $V_{as}PO_2^-$ wavenumber indicated an increase of the hydrogen bond interactions between PO_2^- of DPPC membrane and peptides or water molecules (Ezer, Sahin, & Kazanci, 2017; Severcan, Sahin, & Kazanc, 2005). The results also suggested that the hydration degree of DPPC phosphate head region increased with the addition of tri-peptides ADF, FGR, and MIR (Schmid et al., 2018). The appearance of tri-peptide ADF and MIR shifts the V_sCH_2 wavenumber to a lower value from 2848.8 to 2846.9 cm^{-1} (Fig. 4d, 4f) at the concentration of 40 and 60 mol%, respectively. There was no variation for the V_sCH_2 wavenumber observed in the presence of different concentration tri-peptide FGR (Fig. 4e) may because it mostly binding with membrane head group. The wavenumber changes of symmetric stretching modes of methylene groups reflects the alkyl chain conformation change (Zhao & Feng, 2006). The addition of tri-peptides ADF and MIR leads to a decrease in wavenumber values, which indicates

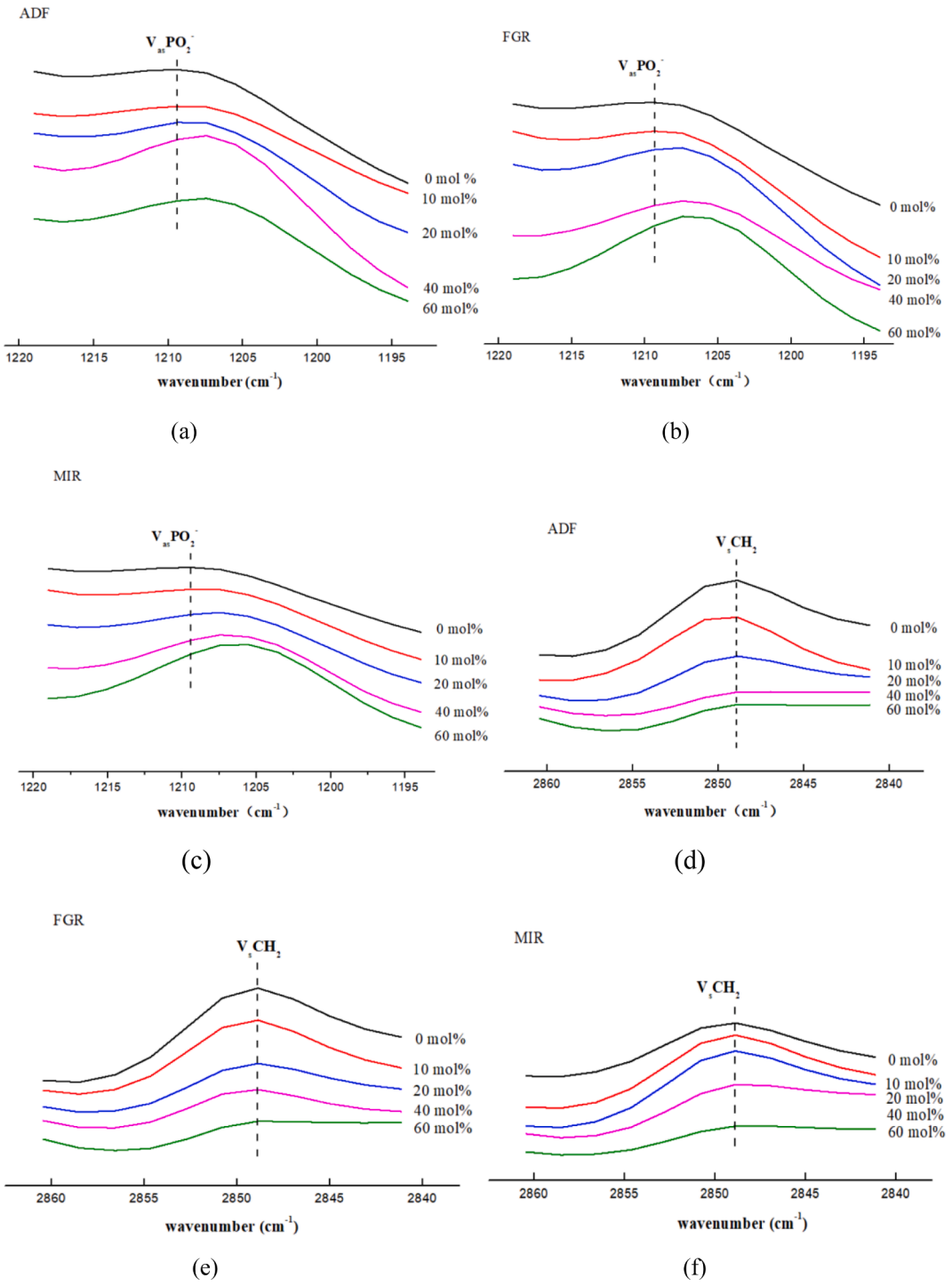

Fig. 4. FTIR spectra bands of DPPC membrane in the presence of different concentrations of peptides. The asymmetric phosphate vibration band in the presence of peptides ADF (a), FGR (b), and MIR (c), respectively. The symmetric CH2 stretching in the presence of peptides ADF (d), FGR (e), and MIR (f), respectively.

the increase of the number of trans conformers, thus implies an increase in the order of the DPPC bilayer and a tighter alkyl chain packing (Schmid et al., 2018; Severcan, Sahin, & Kazanc, 2005; Toyran & Severcan, 2007). In a previous study, the addition of cholesterol decreased the number of gauche conformer induced by drugs (paclitaxel) penetration, which makes the bilayer more ordered, consistent with the results of this study (Zhao & Feng, 2006). Therefore, food derived ACE inhibitory tri-peptides ADF and MIR may have the same membrane protection function as that of the cholesterol. Overall, the FTIR spectroscopy results indicated that ACE inhibitory tri-peptides ADF, FGR, and MIR interacted with both polar head group and tri-peptides ADF and MIR also penetrate into the hydrophobic regions of the bilayer.

3.4. The effect of tri-peptides on thermotropic properties of DPPC membrane

The DSC was used to characterize the effect of tri-peptides on thermotropic phase behavior of DPPC membrane. The DSC curves for pure DPPC membrane and that of in the presence of tri-peptides ADF, FGR, and MIR are shown in Fig. 5. The pure DPPC membrane shows two phase

Table 1
The wavenumber value of the PO_2^- asymmetric stretching and CH_2 symmetric stretching model of DPPC membrane in the presence of different concentration of peptides ADF, FGR, and MIR.

Molar concentration percentage (mol %)	Wavenumber (cm^{-1})					
	ADF		FGR		MIR	
	$V_{as}PO_2$-	V_sCH_2	$V_{as}PO_2$-	V_sCH_2	$V_{as}PO_2$-	V_sCH_2
0	1209.3	2848.8	1209.3	2848.8	1209.3	2848.8
10	1209.3	2848.8	1209.3	2848.8	1209.3	2848.8
20	1209.3	2848.8	1207.4	2848.8	1207.4	2848.8
40	1207.4	2846.9	1207.4	2848.8	1207.4	2848.8
60	1207.4	2848.8	1207.4	2848.8	1205.5	2846.9

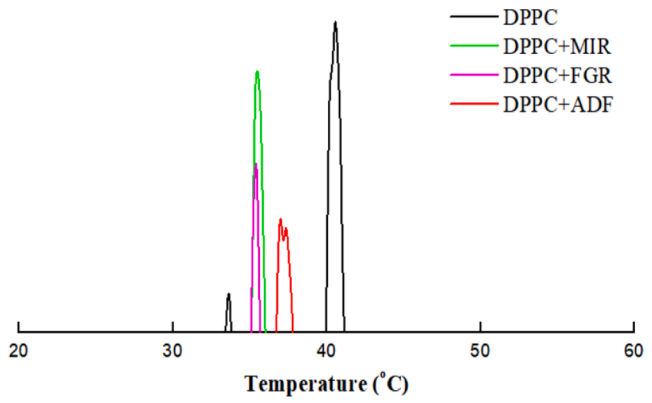

Fig. 5. The DSC curves for DPPC membrane in the absence and presence of peptides ADF, FGR, and MIR.

transition peaks, a low temperature peak around 34 °C which is the pretransition temperature (T_p), and the higher temperature peak around 41 °C which is the main phase temperature (T_m). The phase transition temperature values of DPPC membrane are in agreement with previous reported results (Pentak & Danuta, 2014). The T_p represents the bilayer structure transition from tilted gel to ripple gel conformation, and T_m corresponds to transition of the highly ordered gel state to disordered liquid crystalline phase (Pentak & Danuta, 2014). The presence of tri-peptides ADF, FGR, and MIR eliminated the T_p, and decreased the T_m values of DPPC membrane by 3, 5, 5 °C, respectively. Sospedra et al. observed the same effect when studying the interaction of peptides and membrane by DSC method (Sospedra, Mestres, Haro, Muñoz, & Busquets, 2002). T_p is reported to be highly sensitive to the presence of molecules in the phospholipid head groups (Ezer, Sahin, & Kazanci, 2017). The disappearance of T_p indicated that tri-peptides penetrated the membrane and interacted with the head groups (Pentak & Danuta, 2014). In addition, the decrease of T_m value indicated that the tri-peptides ADF, FGR, and MIR also penetrated into the hydrophobic regions of DPPC bilayer and increased the fluidity of DPPC bilayer. The variation of phase transition temperature also means the ACE inhibitory tri-peptides may change the physical properties of DPPC membrane (Alesksndrany & Sahin, 2020). The DSC results indicated that tri-peptides were able to across the membrane and well complemented the results obtained by fluorimetric and FTIR methods.

4. Conclusion

The interaction of ACE inhibitory tri-peptides ADF, FGR, and MIR with artificial membrane (DPPC liposome) was investigated using a combination of FS, FTIR, and DSC methods in the current study. All tri-peptides ADF, FGR, and MIR decreased the fluorescence intensity of fluorescent liposome, suggesting that they penetrated into the head and hydrophobic regions of DPPC bilayer. The wavenumber values of $V_{as}PO_2^-$ shift to lower values from 1209.3 to 1207.4 cm^{-1} and V_sCH_2 shift from 2848.8 to 2846.9 cm^{-1} with the addition of tri-peptides indicating that tri-peptides increased the hydration state of polar head groups and the order degree of DPPC membrane. Furthermore, the thermotropic phase properties of DPPC membrane results showed that tri-peptides changed the physical properties of DPPC membrane by diminishing the pretransition temperature and decreasing the main phase transition temperature about 3–5 °C of DPPC bilayer. And the DSC results also indicated tri-pepetides interacted with the head groups and penetrated into hydrophobic regions of membrane. In conclusion, ACE inhibitory tri-peptides ADF, FGR, and MIR have the capacity to permeate through the DPPC bilayer, they might have the membrane protection function, and change the physical properties of DPPC membrane. This study clarified the interaction mechanism of ACE inhibitory tri-peptides ADF, FGR, and MIR with DPPC membrane at molecular level, and will give insights in improving the absorption of ACE inhibitory peptides.

CRediT authorship contribution statement

Huizhuo Ji: Data curation, Formal analysis, Writing – original draft. **Wenzhu Zhao:** Visualization, Supervision, Investigation. **Zhipeng Yu:** Conceptualization, Methodology, Writing – review & editing, Supervision.

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.

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