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Group 3 LEA protein model peptides protect liposomes during desiccation

Takao Furuki, Minoru Sakurai *

Center for Biological Resources and Informatics, Tokyo Institute of Technology, 4259-B-62, Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

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ABSTRACT

We investigated whether a model peptide for group 3 LEA (G3LEA) proteins we developed in previous studies can protect liposomes from desiccation damage. Four different peptides were compared: 1) PvLEA-22, which consists of two tandem repeats of the 11-mer motif characteristic of LEA proteins from the African sleeping chironomid; 2) a peptide with amino acid composition identical to that of PvLEA-22, but with its sequence scrambled; 3) poly-L-glutamic acid; and 4) poly-L-lysine. Peptides 1) and 2) protected liposomes composed of 1-palmitoyl 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) against fusion caused by desiccation, as revealed by particle size distribution measurements with dynamic light scattering. Indeed, liposomes maintain their pre-stress size distribution when these peptides are added at a peptide/POPC molar ratio of more than 0.5. Interestingly, peptide 1) achieved the comparable or higher retention of a fluorescent probe inside liposomes than did several native LEA proteins published previously. In contrast, the other peptides exhibited less protective effects. These results demonstrate that the synthetic peptide derived from the G3LEA protein sequence can suppress desiccation-induced liposome fusion. Fourier transform infrared (FT-IR) spectroscopic measurements were performed for the dried mixture of each peptide and liposome. Based on results for the gel-to-liquid crystalline phase transition temperature of the liposome and the secondary structure of the peptide backbone, we discuss possible underlying mechanisms for the protection effect of the synthetic peptide on dried liposomes.

1. Introduction

The traditional structure–function paradigm for proteins is that a uniquely rigid three dimensional (3D) structure is a prerequisite for functionality. In the past decade, however, the existence of proteins that are functional despite being entirely or partially unstructured has received great attention, and it is estimated that nearly one-third of eukaryotic proteins are of this type [1–13]. Such proteins are referred to as intrinsically disordered proteins (IDPs) [1,7,9–11,13], intrinsically unstructured proteins [3,5], or natively unfolded proteins [2,4], and are defined as proteins that lack well-defined 3D structures, instead adopting random-coil-like conformations in solution. Many IDPs are known to undergo a disorder-to-order transition as a requirement for biological function, often after interaction with a binding partner [1–13].

The late embryogenesis abundant (LEA) proteins are IDPs initially identified in cotton seeds at late stages of embryo development [14,15]. In many plants, LEA protein expression is closely linked to acquisition of tolerance against drought, freezing and salinity stresses [16–27]. LEA proteins are also found in animals [28], including nematodes [29–33], bdelloid rotifers [34,35], the African sleeping chironomid, *Polypedilum vanderplanki*[36], crustaceans [37], tardigrades [38,39], and springtails [40]. While LEA proteins can be classified into several groups according to gene expression pattern or amino acid sequence [16–23,25–27], almost all examples discovered in animals are group 3 LEA (G3LEA) proteins, although embryos of the brine shrimp *Artemia franciscana*[28] and tardigrades [38] express some group 1 LEA proteins.

The primary structure of G3LEA proteins is characterized by several tandem repeats of a loosely conserved 11-mer motif [28]. The 11-mer motifs are rich in polar residues, Lys, Glu, and Asp, rendering G3LEA proteins that are very hydrophilic [25,26,28], which probably account for their intrinsically disordered nature in solution [22,25,28]. However, G3LEA proteins can undergo structural transformation, predominantly to α-helical structures when they are dried, as revealed by in vitro FT-IR spectroscopic studies on various G3LEA proteins from a nematode (denoted as AavLEA1) [41], pea mitochondria (LEAM) [42], bullrush pollen (D-7 LEA) [43], a bdelloid rotifer (ArLEA1A) [35], and larvae of the African sleeping chironomid (PvLEA2 and PvLEA4) [44,45].

G3LEA proteins are able to function as protectants for proteins and membranes subjected to drought stress [25,28,46,47]. AavLEA1 reduces desiccation-induced aggregation or the concomitant loss of enzyme

Abbreviations: G3LEA, group 3 late embryogenesis abundant; PvLEA-22, 22-mer peptides constructed as the model peptides of the G3LEA proteins originating from an African sleeping chironomid (*Polypedilum vanderplanki*); Poly-L-Glu, poly-L-glutamic acid; Poly-L-Lys, poly-L-lysine; DLS, dynamic light scattering

* Corresponding author. Tel./fax: +81 45 924 5795.
E-mail address: msakurai@bio.titech.ac.jp (M. Sakurai).

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activities of lactate dehydrogenase (LDH) [48], citrate synthase (CS) [48], and water-soluble proteomes [49]. ArLEA1A also has anti-aggregation effects on CS during dehydration [35]. Recently, similar results have been reported for PvLEA4, which maintains the enzyme activity of dried LDH and protects α-casein against desiccation-induced aggregation [45]. FT-IR spectroscopy indicates the occurrence of an interaction between LEAM and the membrane surface in the dry state [50], which is relevant to the protection of liposomes during desiccation [42]. The addition of either ArLEA1B (a close homologue of ArLEA1A) [35] or LEA7 (G3LEA protein from the higher plant *Arabidopsis thaliana*) [51] reduces the gel-to-liquid crystalline phase transition temperature of liposomes in the dry state. On the other hand, no apparent membrane interactions were observed for AavLEA1 [35], ArLEA1A [35], or GmPM30 (a G3LEA protein from soybean) [52]. Currently, it remains unclear whether the 11-mer motif repeat regions in G3LEA proteins can contribute directly in protecting membranes against desiccation stress, since G3LEA proteins also include amino acid sequences other than the 11-mer motif [22,25,28].

Previously, we investigated the structural and functional properties of 22-mer and 44-mer chemically synthesized peptides that have two or four tandem repeats, respectively, of the 11-mer consensus motif from several anhydrobiotic organisms (chironomid, nematode, and plants) to unveil the physicochemical properties of the 11-mer motifs in G3LEA proteins [53]. Such G3LEA model peptides faithfully reproduce the conformational features of the parent G3LEA proteins in both the aqueous and dry states [44,53]. Furthermore, the model peptides for G3LEA proteins exhibit protective activity against protein aggregation induced by desiccation, highlighting a potential application as biological stabilizers [54].

In the present paper, we investigate whether a G3LEA model peptide derived from PvLEAs, possesses the ability to suppress desiccation-induced damage, such as fusion, of liposomes. Positive results could pave the way for use of this peptide in desiccation storage of a wide variety of biomaterials, including proteins and cells. In addition, we discuss the functional mechanism of the peptide through comparison with the following three kinds of peptides: a peptide with amino acid composition identical to that of a G3LEA model peptide, but with its sequence scrambled; poly-L-glutamic acid; and poly-L-lysine.

2. Materials and methods

2.1. Peptides

Four different peptides were examined: 1) PvLEA-22, which consists of two tandem repeats of the 11-mer motif characteristic of G3LEA proteins from the African sleeping chironomid, *P. vanderplanki*: AKDGTKEKAGE; 2) a peptide with identical amino acid composition to PvLEA-22, but with its sequence scrambled: AKEKEGTDKAGGAKDT GEKEKA. This peptide was called the control peptide previously [44,53, 54], although denoted as the scrambled peptide herein to avoid confusion with the following peptides: 3) poly-L-glutamic acid (poly-L-Glu); and 4) poly-L-lysine (poly-L-Lys). 3) and 4) are homopolypeptides of a charged amino acid contained in PvLEA-22. Chemically synthesized versions of 1) and 2) were purchased from Bex Co. (Tokyo, Japan). Homopolypeptides 3) and 4) were available from Sigma Chemical Co.: they are polydisperse mixtures of the corresponding peptides with chain lengths ranging from ca. 10-mer to 30-mer. When the concentration of these homopolypeptides was calculated, their chain length was assumed to be 20-mer.

2.2. Preparation of liposomes for turbidity and particle size distribution measurements

The phospholipid 1-palmitoyl 2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) was purchased from Sigma Chemical Co. Liposomes were prepared by extruding aqueous suspensions of POPC at 20 mg/mL, without buffer or with buffer of 50 mM Mops (3-Morpholinopropanesulfonic acid, pH 7), through a polycarbonate filter with a pore size of 100 nm diameter using a commercially available device (Mini-extruder; Avanti Polar Lipids, Inc., Alabama, USA). To the resulting liposome solution, the selected peptide was added at the desired concentration and then stirred. A droplet of 10–20 μL was dried on a Teflon plate in a vacuum desiccator at ambient temperature.

2.3. Aggregation assay

Initially, a simple aggregation assay was performed by measuring apparent absorbance, namely turbidity, due to light scattering at 400 nm (denoted as A_{400}), according to Ref. [55], with UV spectrophotometer U-2900 (Hitachi instruments, Hitachi, Japan). This was followed by a more detailed analysis of particle size distribution in suspension using a dynamic light scattering (DLS) analyzer (Zetasizer nano ZS; Malvern Instruments Ltd, Worcestershire, UK). For both of these measurements, the suspensions obtained after rehydration were diluted to avoid saturation in absorbance and dynamic light scattering.

2.4. Leakage experiments

Aqueous suspensions of POPC at 20 mg/mL were prepared with 50 mM Mops buffer (pH 7) plus 70 mM calcein as a fluorescent probe. After extrusion, the obtained liposome solution was passed through a NAP-5 column (Sephadex G-25, GE Healthcare, UK) equilibrated in 50 mM Mops buffer with 50 mM NaCl, to exclude the calcein not entrapped by the liposomes. The POPC concentration of the eluted solution was determined with the phospholipid assay kit (Wako Chemical Co., Japan) and then diluted to 10 mg/mL with 50 mM Mops buffer. The selected peptide was added to the liposome solution at the desired concentration. A droplet of 10 μL was dried on a Teflon plate in a vacuum desiccator at ambient temperature in the dark. Calcein fluorescence was measured with the fluorometer FP-6500 (JASCO, Tokyo, Japan) at an excitation wavelength of 490 nm and emission wavelength of 510 nm. Fluorescence of calcein is strongly quenched at the high concentration inside the liposomes, while it increases when the trapped calcein is released into the surrounding buffer. The fluorescence, F_a, was measured at first for the freshly prepared liposome solution. Then, the liposomes were lysed with 20% TritonX-100 and the fluorescence, F_b, was measured again. The fraction of calcein trapped in freshly prepared liposomes was calculated by $(F_b - F_a) / F_b$. The retention of calcein after the dehydration–rehydration treatment was calculated as follows: $[(F_b' - F_a') / F_b'] / [(F_b - F_a) / F_b]$, where F_a' represents the fluorescence after the dehydration–rehydration treatment without lysing and F_b' the fluorescence after the dehydration–rehydration treatment with lysing.

2.5. Fourier-transform infrared (FT-IR) spectroscopic measurements

FT-IR spectra were obtained using a Fourier transform infrared spectrometer (JASCO FT-6100 and IMV 4000, Tokyo, Japan) and measured with 128 scans at a resolution of 2 cm^{-1}. Dried samples were pressed between two KBr plates, and the temperature of the samples was controlled with a temperature controller, LK-600 (Linkam Scientific Instruments, Surry, UK), mounted on the stage of the above spectrometer. Aqueous samples were measured as a droplet by the method of attenuated total reflection, using an ATR PRO 450-S instrument (JASCO, Tokyo, Japan) at room temperature. To investigate peptide secondary structures, amide I absorption bands were analyzed. Spectral manipulations for Fourier-self-deconvolution and curve fittings were performed using the JASCO spectra manager version 2 software. When the amide I absorption band was analyzed for the aqueous samples, the following corrections were carried out to remove the contribution from water: (1) initially, the FT-IR spectra of pure water and aqueous pure liposomes were subtracted from the FT-IR spectra of the aqueous peptide alone

and its aqueous mixture with liposome, respectively; (2) subsequently, from these corrected FT-IR spectra, the potential contribution from the absorbance of water vapor in the atmosphere was removed by subtracting an FT-IR spectrum of the air in the laboratory where the experiments were carried out. FT-IR measurements were restricted to the samples without buffer for allowing easy analysis of the obtained spectra.

3. Results

3.1. Protective ability of G3LEA model peptides

The turbidity of liposome solutions without buffer greatly increased after dehydration–rehydration treatment (Fig. 1), suggesting that the desiccation stress caused damage to liposomes, such as fusion and collapse. Interestingly, this turbidity increase was appreciably suppressed when PvLEA-22 was added to the liposome solution before dehydration. The molar ratio of the added peptide relative to POPC molecules constructing the liposomes was varied over the range of 0 to 0.7. Hereafter, the mixture with a molar ratio of 0.1, for example, is denoted PvLEA-22 [0.1]/liposome. With increasing molar ratio, the turbidity after the dehydration–rehydration treatment was increasingly suppressed, and the post-treatment turbidity for PvLEA-22 [0.7]/liposome was as low as the value before dehydration (Fig. 1(a)). A very similar result was obtained for the mixture of the scrambled peptide and liposomes (Fig. 1(b)). Both poly-L-Glu and poly-L-Lys suppressed the increase in turbidity to a lesser extent than the LEA peptides, as shown in Fig. 1(c) and (d), respectively.

Fig. 2 shows the results of turbidity measurements for the added peptide [0.7]/liposomes when 50 mM Mops buffer solution at pH 7 was used. Overall, the relative effectiveness among the four different peptides of interest is consistent with the results of Fig. 1, being in the order of PvLEA-22, scramble > poly-L-Lys > poly-L-Glu.

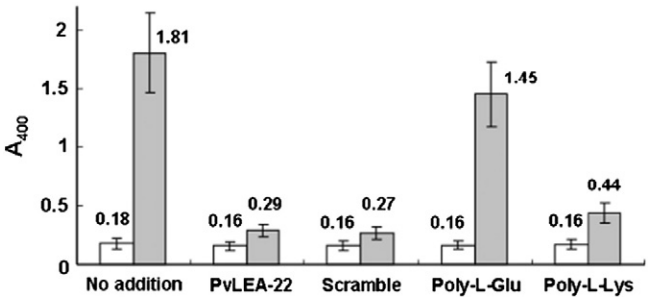

Fig. 2. Turbidity (apparent absorbance at 400 nm, A_{400}) of 50 mM Mops buffer solutions of peptide/liposome mixtures with the molar ratio of 0.7 for the added peptides relative to POPC, where the concentration of POPC was fixed at 20 mg/mL. White and gray bars correspond to the sample before dehydration and the rehydrated sample, respectively. The data represent the means from four separate independent tests, together with standard error.

To examine this effect on turbidity in more detail, we measured the particle size distribution of liposomes before and after the dehydration–rehydration treatment, without buffer, using DLS (Fig. 3). Before treatment, a single peak was obtained centering around 120 nm with a broad distribution: this feature was common to all four kinds of peptide/liposome mixture studied. When the peptide-free liposome solution was subjected to dehydration–rehydration, the peak position shifted to about 700 nm. The marked increase in diameter suggests that desiccation stress causes serious damage to liposome structure, such as aggregation and/or fusion. However, addition of PvLEA-22 to the liposome solution before dehydration reduced such effects. In fact, the peak position gradually decreased as the molar ratio of the peptide increased. At a molar ratio of 0.5, the resulting particle size distribution was very similar to that before treatment, although a broad shoulder remained on the right side of the main peak (Fig. 3(a)). At a molar ratio of 0.7, the original distribution was almost completely reproduced.

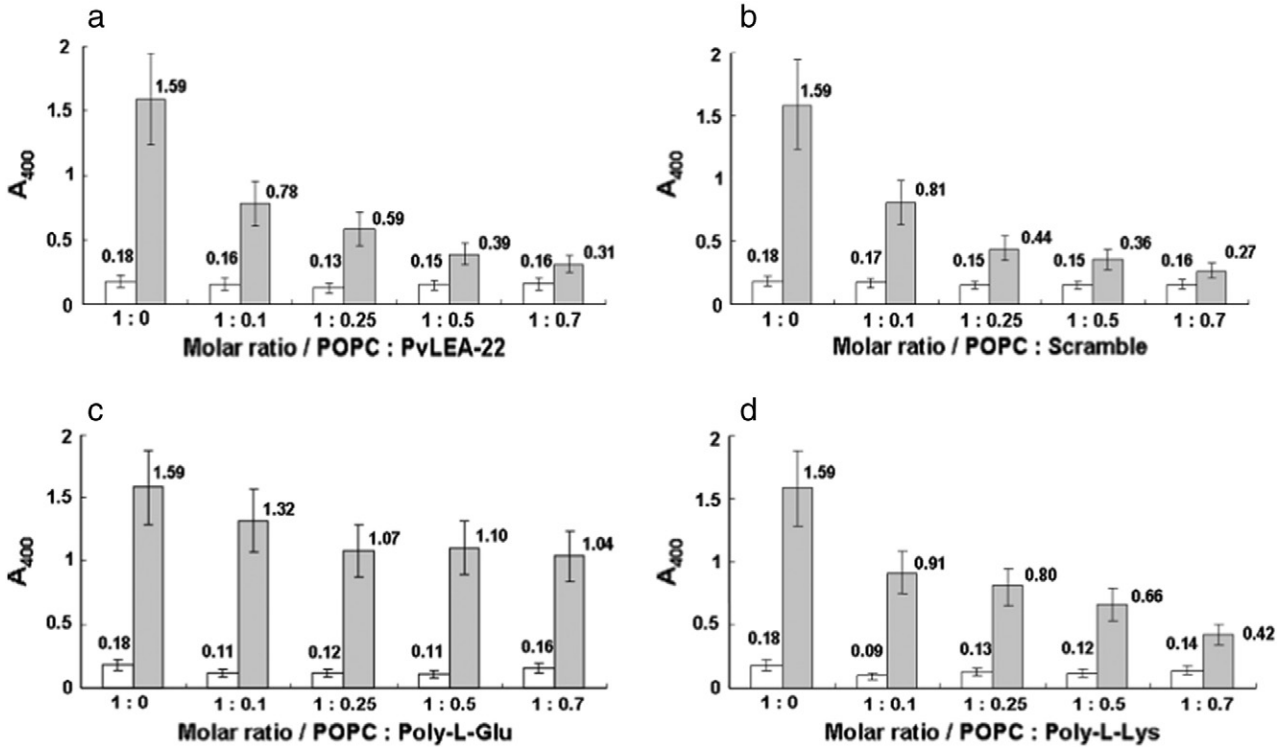

Fig. 1. Turbidity (apparent absorbance at 400 nm, A_{400}) of aqueous solutions of peptide/liposome mixtures with different molar ratios of peptides relative to POPC, where the concentration of POPC was fixed at 20 mg/mL. White and gray bars correspond to the sample before dehydration and the rehydrated sample, respectively. The data represent the means from four separate independent tests, together with standard error.

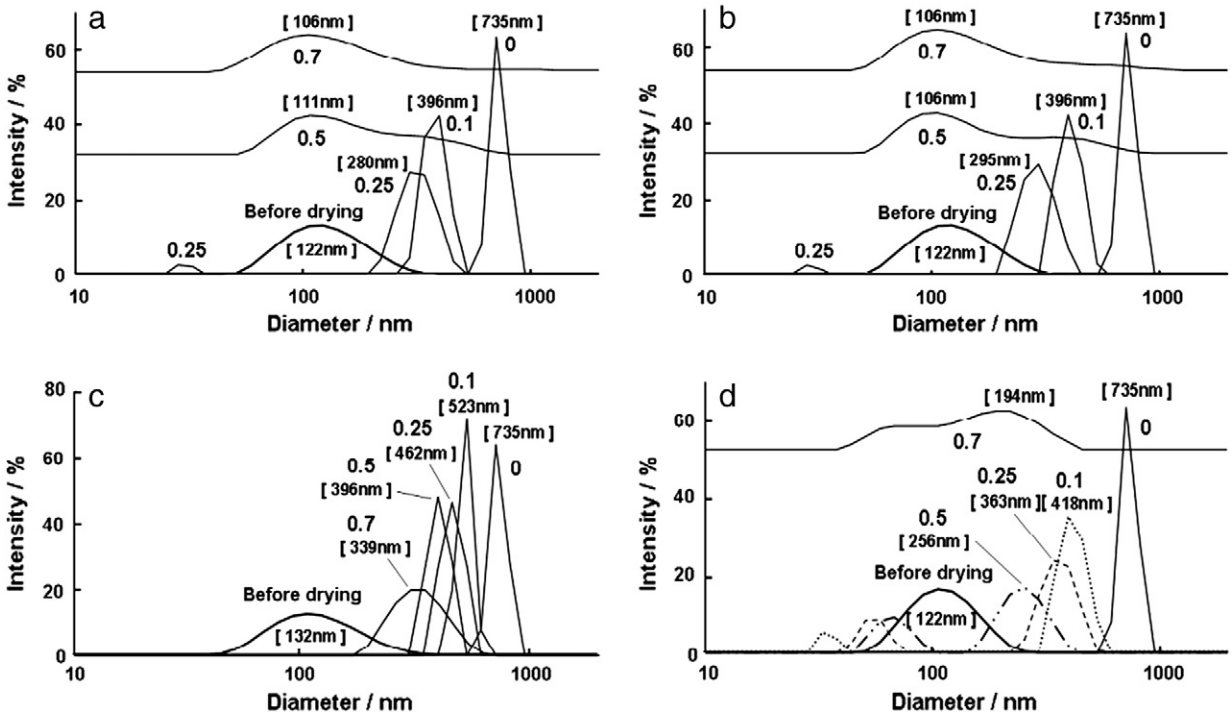

Fig. 3. Particle size distributions of aqueous solutions of the peptide/liposome mixtures after dehydration–rehydration treatment. (a) PvLEA-22, (b) the scrambled peptide, (c) poly-L-Glu, and (d) poly-L-Lys. The particle size distribution before dehydration, which was almost independent of the concentration of added peptide, is given by a bold line. The numbers, 0, 0.25, 0.5, and 0.7 indicate the molar ratios of added peptide to POPC. The diameter at the top of the intensity peak is given in a bracket.

Similar results were also obtained with the scrambled peptide/liposome mixtures (Fig. 3(b)). In the case of the poly-L-Glu/liposome mixture, the particle size distribution shifted towards the smaller end of the size range with increasing amount of peptide, but the original distribution was never reproduced, even at the highest molar ratio (Fig. 3(c)). With poly-L-Lys, more unfavorable results were obtained: the particle size distribution was split into two peaks (Fig. 3(d)), suggesting the disruption of liposomes.

Fig. 4 shows the particle size distribution of liposomes in 50 mM Mops buffer solution before and after the dehydration–rehydration treatments at the molar ratio of 0.7 for the added peptide relative to POPC. Similar to the case of buffer-free solution, the original size distribution of liposomes was almost kept in the presence of either PvLEA-22 or scramble, whereas such favorable effects were not observed for poly-L-Glu. With poly-L-Lys, the particle size distribution after the desiccation stress was more or less similar to the original distribution, although a broad shoulder still appeared on the right side of the main peak. In combination with the results of turbidity measurements, as a whole, the observed protective effects of the peptides tested are almost independent of whether the peptide/liposome mixture was initially prepared in water or buffered solution.

A decrease in liposome diameter should lead to a decrease in light scattering at 400 nm, that is, the observed turbidity. In fact, a good correlation was found between turbidity and the DLS peak position (see Fig. S1). As can be seen for the PvLEA-22/liposome and scrambled peptide/liposome mixtures, at molar ratios of 0.1 and 0.25 the DLS peak shifted into the smaller size region compared to peptide-free liposomes (Fig. 3(a) and (b)), and accordingly the turbidity is markedly decreased (gray bars in Fig. 1(a) and (b)). However, the particle size distributions at these molar ratios showed little overlap with the original distribution. Therefore, these smaller amounts of peptide are insufficient for maintaining the original liposome structure throughout the dehydration–rehydration treatment, instead requiring a molar ratio of

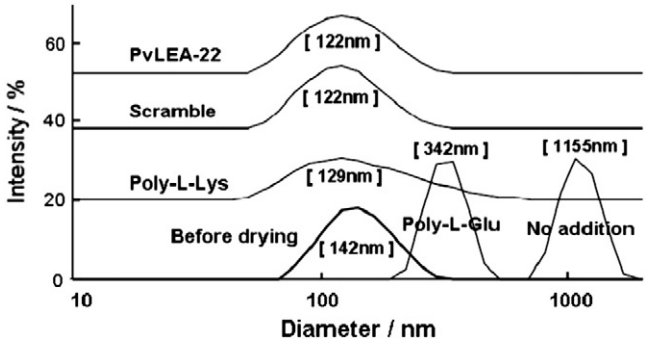

Fig. 4. Particle size distributions of 50 mM Mops buffer solutions of the peptide/liposome mixtures after the dehydration–rehydration treatment. The molar ratio of the added peptides relative to POPC was 0.7, where the concentration of POPC was fixed at 20 mg/mL. The particle size distribution before dehydration (bold line) was exemplified by the peptide-free case because it was found to be almost identical irrespective of the added peptides. The diameter at the top of the intensity peak is given in a bracket.

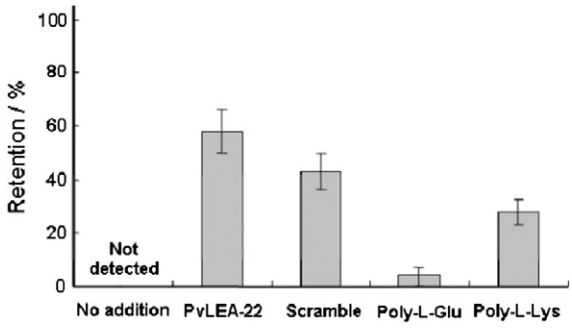

Fig. 5. Retention of calcein trapped in liposomes after the dehydration–rehydration treatment. The molar ratio of the added peptides relative to POPC was 0.7. The data represent the means from four separate independent experiments, together with standard error.

at least 0.5 relative to POPC molecules. In contrast, poly-L-Glu or poly-L-Lys was not fully protective even at the highest molar ratios used (Fig. 3(c) and (d)).

To get a deeper insight into the protective effects of the peptides, the leakage experiment of the fluorescent probe, calcein, trapped in liposomes was carried out. As shown in Fig. 5, the calcein inside the liposomes was virtually released when the liposomes without any of the peptides were subjected to the dehydration–rehydration treatment. However, the PvLEA-22[0.7]/liposome mixture retained as high as 60% of the trapped calcein after the desiccation stress. The scramble[0.7]/liposome mixture exhibited the comparable effects, although its fraction of the retained calcein was lower compared with the case of the PvLEA-22[0.7]/liposome mixture. The poly-L-Lys[0.7]/liposome mixture achieved only about 30% retention. These results of the leakage test are clear evidence of the relative effectiveness among the different peptides.

Taken together, the above results confirm the relative protective ability against dehydration stress in the order of PvLEA22 peptide > its scrambled equivalent > poly-L-Lys > poly-L-Glu. Thus, the protective activity strongly depends not only on the amino acid composition of the peptides used, but also on their amino acid sequence.

3.2. The gel-to-liquid crystalline phase transition behavior of dried peptide/liposome systems

The gel-to-liquid crystalline phase transition of liposomes can be monitored by the temperature-dependent shifts in the peak position of the CH_2 symmetric stretch vibration (hereafter denoted by $\nu[CH_2]$) in FT-IR spectra [56–58]. The gel-to-liquid crystalline phase transition temperature, T_m, is often determined as the transition midpoint in the sigmoidal curve of $\nu[CH_2]$ vs temperature [56–58]. From Fig. 6(a), T_m for the peptide-free dried liposome was estimated to be 58 °C, the temperature corresponding to $\nu[CH_2]$ at 2852.5 cm^{-1}. The data for the dried PvLEA-22/liposome systems are also given in Fig. 6(a), which shows that T_m is reduced with increasing molar ratio of PvLEA-22 to POPC. Similarly, the addition of the scrambled peptide to liposomes also led to the lowering of T_m (Fig. 6(b)). When poly-L-Glu was added to the liposome (Fig. 6(c)), the shift in T_m was smaller compared to PvLEA-22 and the scrambled peptide. In contrast, in the presence of poly-L-Lys (Fig. 6(d)), the phase transition behavior (sigmoidal shape) became obscure and the curve shifted largely to the lower temperature region. A reduction in T_m suggests that the lipid molecules making up the liposomes have a lowered packing density and concomitant activation of the dynamics of their hydrocarbon chains [56–58]. The degree of change in T_m among the four different peptides very likely reflects the difference in the mode and/or strength of interaction between the lipid bilayer and the added peptide. In particular, the abnormally large shifts and reduced cooperativity observed for the poly-L-Lys/liposome system may be attributed to the disruption of liposomes, consistent with the data on particle size distribution (Fig. 3(d)). This will be again discussed later in conjunction with the protection effect of poly-L-Lys.

3.3. Interaction between peptides and phospholipid head groups

The FT-IR spectroscopy peak assigned to the asymmetric stretch vibration ν[P═O] of the P═O group of phospholipids is known to appear around 1210–1280 cm^{-1} [56–58]. The peak position of ν[P═O] in the dried PvLEA-22 [0.1]/liposome mixture shifted to a lower wavenumber by more than 10 cm^{-1} relative to that (1261 cm^{-1}) of peptide-free dried liposomes (Fig. 7). Such lower wavenumber shifts of ν[P═O] have been also observed when liposomes were dried in the presence of native LEA proteins, indicating the hydrogen bond formation between the polar headgroup of phospholipid and native LEA proteins in the dried state [35,50,51]. Interestingly, the magnitude of the ν[P═O] shift became larger in the same order as did the T_m shifts: spectrum B (poly-L-Glu) < spectrum C (PvLEA-22) < spectrum D (poly-L-Lys) (Fig. 7, where the spectrum of the scrambled peptide is not shown, since it was almost identical to that of PvLEA-22).

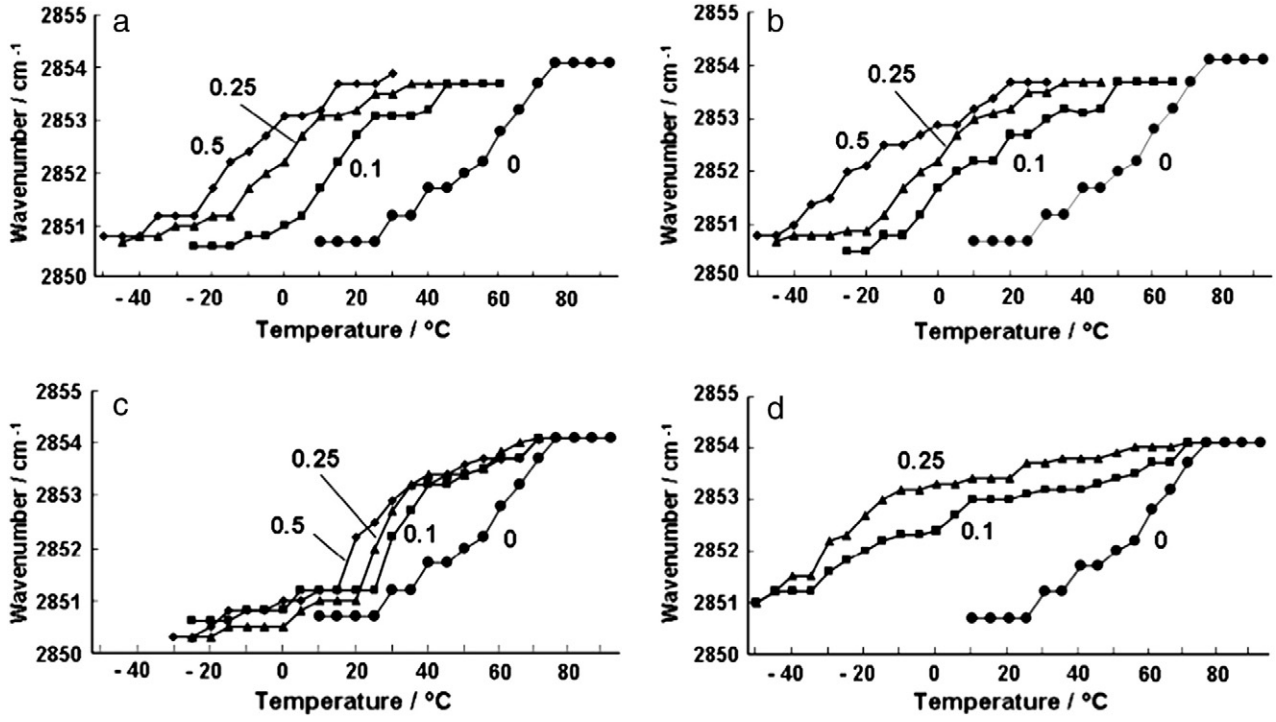

Fig. 6. Gel-to-liquid crystalline phase transition curves of dried liposomes, measured as a temperature-dependent shift of FT-IR peak position of $\nu[CH_2]$. (a) PvLEA-22, (b) the scrambled peptide, (c) poly-L-Glu, and (d) poly-L-Lys. The numbers inside the figure indicate the molar ratios of added peptide to POPC.

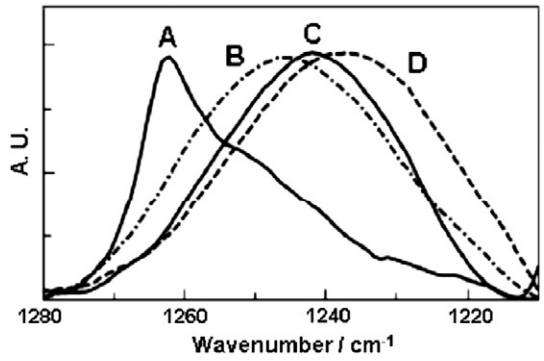

Fig. 7. Representative FT-IR spectra in the region including the asymmetric stretching vibration of the phosphate group, ν[P═O], in POPC. A: dried peptide-free liposome; B: dried poly-L-Glu [0.1]/liposome; C: dried PvLEA-22 [0.1]/liposome; D: dried poly-L-Lys [0.1]/liposome. The dried scrambled peptide [0.1]/liposome mixture gave an almost identical spectrum to that of C.

3.4. Secondary structure of the peptide in the dry peptide/liposome systems

In a previous study [44], the secondary structures of both the PvLEA-22 and scrambled peptides (alone) in the dry state were examined by FT-IR spectroscopy, while the structures in aqueous solution were examined by circular dichroism. For direct comparison between the hydrated and dehydrated states, FT-IR spectra were determined for both aqueous and dried samples. In solution, pure PvLEA-22 provided an amide I spectrum consisting of two major components around 1643 cm^{-1} and 1676 cm^{-1} (Fig. 8(a)), assignable to random coils and β-turn structures, respectively [59–62]. The aqueous PvLEA-22/liposome mixture exhibited very similar spectral features in the amide I region to pure PvLEA-22 in solution, regardless of the concentration of PvLEA-22 tested (data not shown). The amide I spectrum of dried PvLEA-22 had a predominant component at 1653 cm^{-1} (Fig. 8(b)), indicating a preponderance of α-helical secondary structure, as reported previously [44]. However, the PvLEA-22/liposome mixture in the dry state exhibited a quite different amide I spectrum from that of dried pure PvLEA-22 (Fig. 8(c)). When the molar ratio of PvLEA-22:POPC is 0.1 (PvLEA-22 [0.1]/liposome), for example, a large peak with a relative area up to 40% of the amide I band was observed around 1623 cm^{-1} (Fig. 8(d)), simultaneously with a minor component at 1685 cm^{-1}, suggesting preferential formation of β-sheet (β-strand) structures [59–62]. The relative strength of the component at 1623 cm^{-1} decreased with increasing molar ratio of PvLEA-22 relative to POPC (Fig. 8(c)). Overall, however, the content of β-sheet structure in dried PvLEA-22/liposome mixtures was still larger than other secondary structures. These results are summarized in Fig. 9(a).

Similarly to PvLEA-22, the amide I band for aqueous solutions of the scrambled peptide had two major components at 1643 cm^{-1} (random coil) and 1677 cm^{-1} (β-turn), as shown in Fig. S2(a). In the dry state, this peptide retained these major components (Fig. S2(b)), although the relative content of β-turn to random coil was increased. However, the dried scrambled peptide [0.1]/liposome gave a very different amide I spectrum to that of the dried pure scrambled peptide alone: a large peak, assigned to β-sheet structure, appeared at 1625 cm^{-1} (Fig.S2(c) and (d)). Again, this resembled the dried PvLEA-22 [0.1]/liposome mixture and, similarly, its peak intensity became weak with increasing molar ratio of the scrambled peptide relative to POPC (Fig. S2(c)), although β-sheet structure was still dominant (samples 3, 4 and 5, Fig. 9(b)).

The FT-IR spectrum of aqueous poly-L-Glu had two major peaks at 1642 cm^{-1} and 1668 cm^{-1}, indicating a predominant population of random coil conformations (Fig. S3(a)). Almost identical spectra were observed for the poly-L-Glu/liposome mixture in aqueous solution (data not shown). Pure poly-L-Glu in the dry state exhibited a somewhat different amide I spectrum (Fig. S3(b)), however, although this

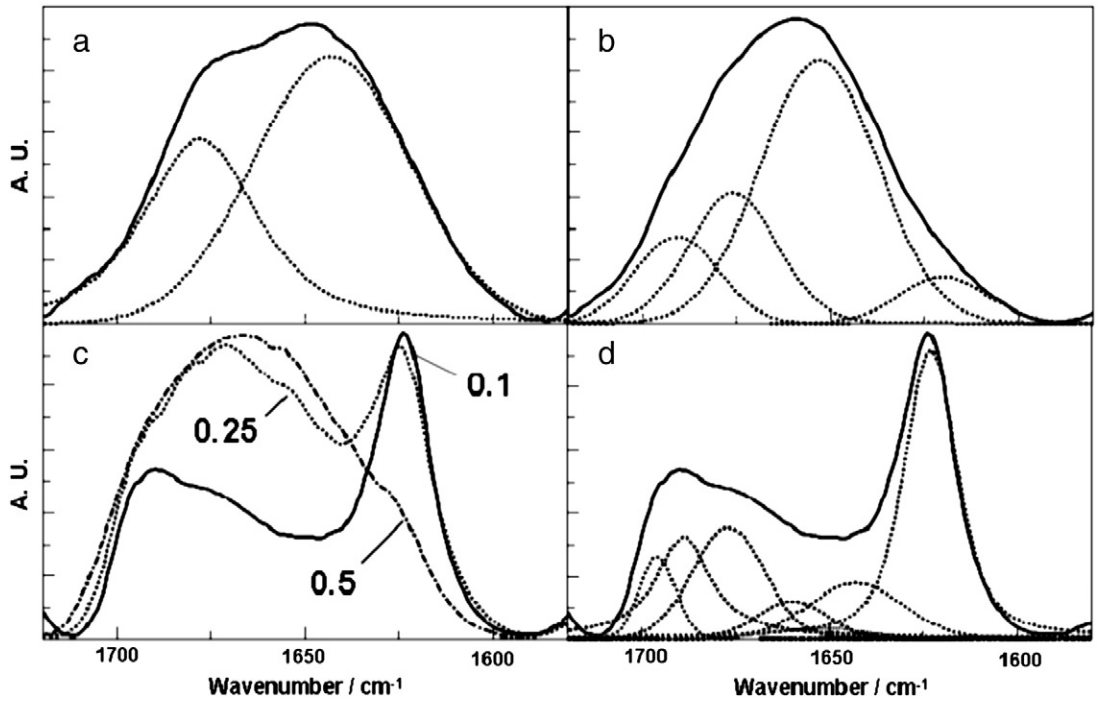

Fig. 8. FT-IR spectra in the amide I region of the following samples: (a) aqueous pure PvLEA-22, (b) dried pure PvLEA-22, (c) dried PvLEA-22/liposome (the numbers inside the figure indicate the molar ratios of PvLEA-22 relative to POPC), (d) dried PvLEA-22 [0.1]/liposome, whose original absorbance curve is the same as given in (c). In panels (a), (b), and (d), the original absorbance curve measured is shown as a solid line. Its constitutive spectral components are shown as broken lines, as evaluated by Fourier self-deconvolution followed by curve fitting. Panel (c) shows only the original absorbance curves. The data of (b) are the same as those of the corresponding sample in our previous study [44].

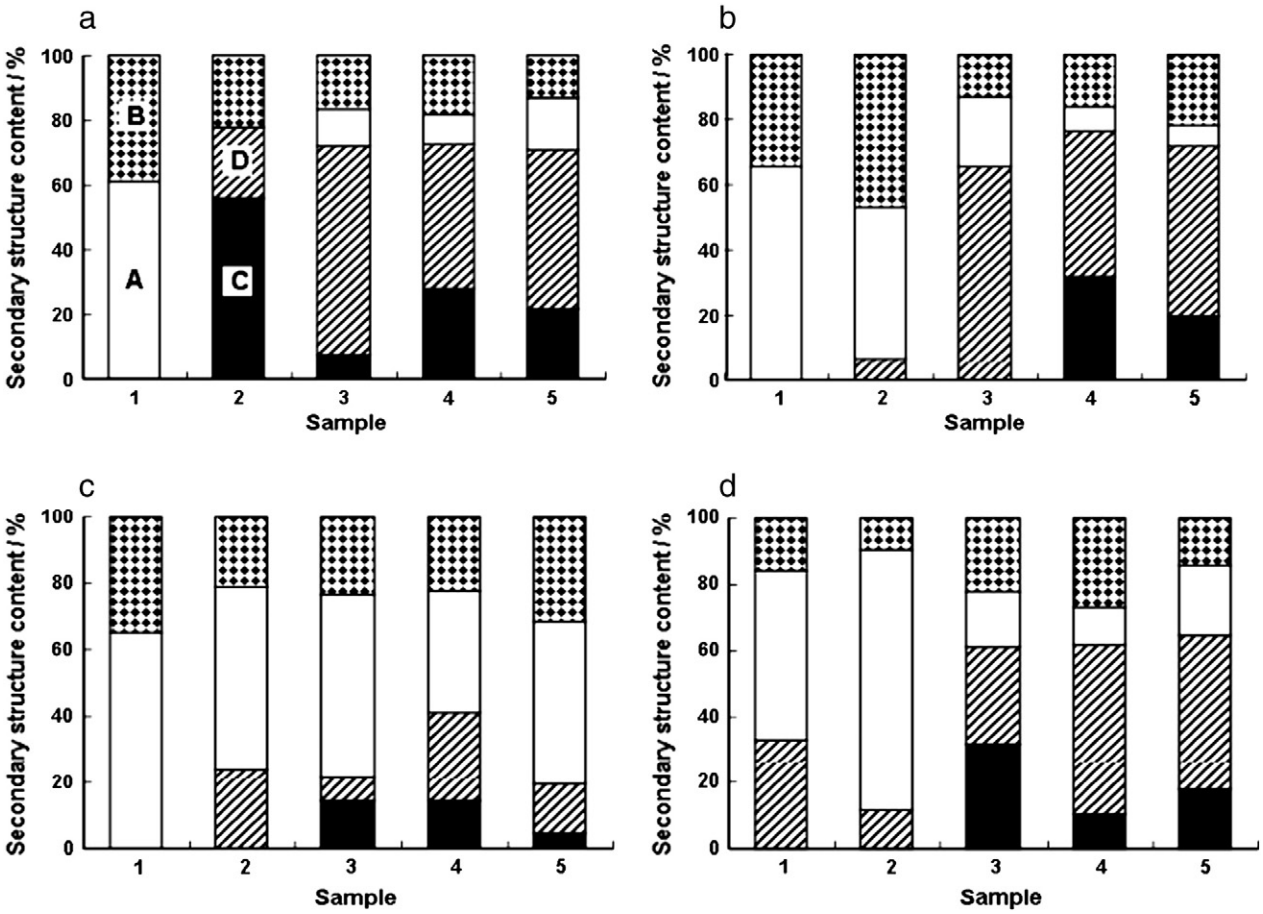

Fig. 9. Proportions of secondary structure elements for each of the samples studied. (a) PvLEA-22, (b) the scrambled peptide, (c) poly-L-Glu, and (d) poly-L-Lys. A: random coil, B: β-turn, C: α-helix, D: β-sheet. Sample 1: aqueous solution of pure peptide; sample 2: pure peptide in the dry state; sample 3: the mixture of peptide [0.1]/liposome in the dry state; sample 4: the mixture of peptide [0.25]/liposome in the dry state; and sample 5: the mixture of peptide [0.5]/liposome in the dry state.

dried polypeptide may still have a considerable proportion of random coil, as can be seen from the component at 1646 cm^{-1}. In the dried poly-L-Glu/liposome mixture, there were several components in the amide I band, located at 1649 cm^{-1} (random coil), 1660 cm^{-1} (α-helix), 1674 cm^{-1} (β-turn), and 1685 cm^{-1} (β-sheet), as shown in Fig. S3(d), although the most predominant component is random coil at a proportion of 50% or higher (samples 3, 4 and 5, Fig. 9(c)). This contrasts with the results for PvLEA-22 and the scrambled peptide.

The FT-IR spectrum of poly-L-Lys in aqueous solution had a major component at 1644 cm^{-1}, assigned to random coil, with a relative area of 51% of the amide I band (Fig. S4(a)). When this peptide was dried, the relative content of random coil increased, as shown by the large contribution of the component at 1646 cm^{-1} with a relative area of 79% (Fig. S4(b)). These results are consistent with those reported by Wolkers et al. [63], who stated that poly-L-Lys adopted the random coil in solution and that such featureless conformation was observed again when the small droplet of the aqueous poly-L-Lys was subjected to fast air-drying. However, the peptide in the dried poly-L-Lys [0.1]/liposome mixture had nearly equal proportions of the different secondary structures identified here (Fig. S4(d)): there are several spectral components at 1604 cm^{-1} (β-sheet), 1626 cm^{-1} (β-sheet), 1639 cm^{-1} (random coil), 1653 cm^{-1} (α-helix), 1668 cm^{-1} (β-turn), and 1684 cm^{-1} (β-sheet). However, with increasing peptide concentration, β-sheet structure became more predominant (samples 3, 4 and 5 in Fig. 9(d)), which is similar to the observations for PvLEA-22 and the scrambled peptide.

4. Discussion

Several G3LEA proteins suppress desiccation-induced damage such as fusion that can occur during the dehydration of liposomes [35,42,50,51]. In the present study, we showed that a 22-mer peptide derived from the repeating 11-mer motif in PvLEAs, denoted as PvLEA-22, has a significant protective ability for dried POPC liposomes when present at a molar ratio of more than 0.5 relative to liposome phospholipids. The results for the size distribution measurements were almost identical between PvLEA-22 and the scrambled peptide, which has the same amino acid composition as PvLEA-22, but with a scrambled sequence. However, the scrambled peptide showed a tendency to be inferior to PvLEA-22 in terms of preserving calcein trapped in the liposomes. We speculate that these peptides protect against desiccation damage by binding to the surface of liposomes and forming a physical barrier that inhibits liposome aggregation and fusion. A similar molecular shield function has been proposed as the mechanism by which G3LEA proteins and related peptides suppress protein aggregation [45–49,54]. The present study suggests that the characteristic amino acid sequence of G3LEA proteins, that is, the 11-mer motif, could more effectively work to shield the partner proteins and liposomes before receiving desiccation damage.

The mixture of PvLEA-22[0.7]/liposome achieved as high as 60% retention of the fluorescent marker, calcein, inside POPC liposomes after desiccation–rehydration treatment. This protective effect is comparable, or superior in some cases, to that of native LEA proteins. For example, the LEA protein from pea seed mitochondria, LEAM, retained only 20%

of a fluorescent marker, carboxyfluorescein, trapped in POPC liposomes after desiccation stress, even at the tested highest LEAM:lipid mass ratio of 1:3 (equivalent to a molar ratio of around 0.01:1) [50]. Interestingly, however, the retention level with LEAM was improved up to 40–50% when the liposome was prepared with the mixed lipids mimicking the lipid composition of the mitochondrial membrane.

According to a simple geometrical consideration, the size (longitudinal section) of one PvLEA-22 molecule (or one scrambled peptide) is 4.32 nm^2 when it adopts an extended β-sheet-like (β-strand) conformation. In comparison, the average area per lipid in a POPC liposome is 0.64 nm^2. Thus, one PvLEA-22 molecule could cover the cross-section (membrane surface) of a bundle of approximately 7 POPC molecules. Assuming that the inner and outer liposome surfaces are composed of the same number of lipid molecules, the minimum molar ratio of the peptide PvLEA-22 to the total number of POPC lipid molecules required to cover the whole membrane surface is 0.07. Therefore, a molar ratio of 0.5, the experimentally-determined quantity of peptide required to sufficiently protect the dried liposome, represents a considerable excess. There may be at least two reasons for this numerical discrepancy. First, the affinity (association constant) between the peptide and the membrane surface may be weak, such that an excess of peptide is needed to shift the equilibrium in the direction of complex formation. An alternative interpretation, which is not necessarily exclusive of the first, will be described later.

In this study, we obtained data that support a molecular shield mechanism. They are summarized as follows: on addition of the peptide, 1) the T_m of the liposomes was considerably lowered (Fig. 6); 2) the peak of the P═O stretching vibration, ν[P═O], shifted to a lower wavenumber (Fig. 7); and 3) the asymmetric stretching vibration of the C – N bond in the choline group, $\nu[N^+(CH_3)_3]$, shifted to a higher wavenumber (data not shown). Observations 2) and 3) suggest that the added peptide is interacting with the phospholipid head group. In particular, it is inferred from observation 2) that a hydrogen bond is formed between the P═O group and the peptide. We surmise that the binding of the peptide to the membrane surface weakens interactions between adjacent lipids, leading to the lowering of T_m (observation 1).

Recently, we performed a molecular dynamics (MD) simulation for model systems composed of a lipid bilayer of POPC and one or several PvLEA-22 molecules in aqueous solution [64]. This preliminary study suggests that the Lys residue in the peptide PvLEA-22 interacts with the membrane surface, such that its positively charged side chain, $-(CH_2)_4NH_3^+$, deeply intrudes into the head group region of the lipid bilayer and directly interacts with the negatively charged phosphate group of the lipid. This MD result fits very well with the observations in Fig. 7, and the role of Lys may be particularly relevant to the dramatic shift of ν[P═O] and T_m when poly-L-Lys was added to liposomes. Multiple electrostatic interactions are expected to occur between the consecutive amino groups of poly-L-Lys and juxtaposed lipid phosphate groups. However, poly-L-Lys actually was less effective in the protection function for dried liposomes than PvLEA-22 and the scramble peptide (Fig. 5). This may be interpreted as follows:

One of the most important features to be noted for poly-L-Lys is that the cooperativity of T_m transition is extremely low (Fig. 6(d)). This indicates that the self-assembled force between the lipid molecules is extremely lowered, which means that the liposome interacting with poly-L-Lys would have the larger risk of partial disruption. Therefore, even though poly-L-Lys/liposomes might be dried without receiving severe damage, the unstabilized membrane structure would be considerably disrupted in the subsequent rehydration process, leading to the large leakage of contents and the split of the particle size distribution.

PvLEA-22 and its scrambled equivalent both have six negatively-charged residues (Glu × 2, Asp × 4) and six positively charged residues (Lys × 6) in each molecule and hence are charged neutral. This charge balance likely allows the peptides to interact with the membrane surface neither too strongly nor too weakly, which may contribute to the optimal arrangement for its membrane-protective function.

In aqueous solution, both of PvLEA-22 and scrambled peptide were in the random coil state (Fig. 8(a), Fig. S2(a), and Fig. 9(a) sample 1 and Fig. 9(b) sample 1). When these peptides were dried, the former was preferentially transformed into α-helices (Fig. 8(b), 9(a) sample 2), whereas the latter was kept unstructured (Fig. S2(b), Fig. 9(b) sample 2). Namely, these peptides have intrinsically different conformational properties. However, they exhibited similar conformational changes when mixed with POPC liposomes in the dry state (Fig. 9), which implies that there are physicochemical interactions, like hydrogen bonding, between these peptides and the liposome as discussed above. Therefore, it is of great interest to discuss the relationship between the conformation of the peptides and its protective ability.

Dried pure PvLEA-22 adopts an α-helix-rich conformation (Fig. 9(a), sample 2). However, when this peptide is mixed with liposomes at a molar ratio of 0.1 (PvLEA-22 [0.1]/liposome), β-sheet becomes dominant in the dry state (Fig. 9(a), sample 3). As shown in Fig. 1 and 3, even at a molar ratio of 0.1 the peptide has some protective activity and the various FT-IR spectroscopy data all indicate an interaction between the peptide and liposomes (Fig. 6 and 7). According to our recent MD simulation for model systems composed of a lipid bilayer of POPC and several PvLEA-22 molecules in aqueous solution [64], PvLEA-22 molecules are preferentially bound to the surface of a POPC lipid bilayer in solution and the peptide–peptide association was not observed. Combining these observations, we infer that peptide molecules bind to the membrane surface with an extended, β-sheet-like conformation in the dry state, but at low molar ratios they only partially shield the surface, resulting in limited suppression of liposome damage. With further addition of the peptide (molar ratio ≥ 0.25), the protective effect improves (Fig. 1 and 3). Interestingly, the proportion of α-helix increases markedly with increasing molar ratio, although β-sheet still represents the largest fraction (Fig. 9(a), samples 4 and 5). There are two possible explanations for such appearance of α-helical structure. One is the crowding effects driving α-helix formation on the liposome surface. Something similar was supposed to happen with native G3LEA proteins such as LEAM [42,50]. The other is the presence of excess peptide, which does not directly interact with the membrane surface, because PvLEA-22 itself has a strong propensity to form α-helix in the dry state, as described previously [44].

An almost identical relationship between conformation and protective ability was observed for the scrambled peptide. In particular, it is noteworthy that α-helix was detected when this peptide was in excess (molar ratio ≥ 0.25) over liposome phospholipids. This is an unexpected result because, in contrast to PvLEA-22, the scrambled peptide alone does not gain structure on dehydration [44]. However, in some cases this peptide is known to form α-helix: for example, it undergoes a degree of structural transformation to α-helix on dehydration in the presence of cations, i.e. Na^+ or K^+ [44]. It is likely that the scrambled peptide crowding on the liposome surface and/or the excess of this peptide not directly binding the liposome surface experiences an environment that drives a conformational transition to α-helix.

Both the PvLEA-22 and scrambled peptides are known to vitrify at ambient temperature in the dry state: their glass transition temperatures are as high as 100 °C and 84 °C, respectively [53]. When mixed with liposomes, any excess peptide of either type would be expected to vitrify in the dry state, and might form inter-liposomal matrix in the space previously occupied by water, thereby helping to maintain the mechanical strength of intact liposomes in the dry state. This model corresponds to the so-called cytoskeleton model, which has been proposed as a functional mechanism for full-size LEA proteins [22,25,28]. According to Fig. 1 and 3, the liposomes are not protected until the molar ratio of peptide/POPC is more than 0.5, implying that glassy inter-liposomal matrix is indispensable for preserving liposomes in the dry state. The highly dense matrix can lead to its higher glass transition temperature, which is likely to help achieve higher retention of the solutes trapped in liposomes in the dry state. The current superiority

of PvLEA-22 to the scramble peptide may reflect such physicochemical nature.

5. Conclusion

In this study, the synthetic peptide PvLEA-22 has the ability to preserve liposomes in the dry state and, at a sufficient molar ratio, can maintain the pre-stress liposome size distribution. It is noteworthy that PvLEA-22 achieved the comparable or higher retention of a fluorescent probe inside liposomes than did several native LEA proteins published previously [50]. In contrast, the other peptides tested now exhibited less protective effects. These results demonstrate that the synthetic peptide derived from the group3 LEA (G3LEA) protein sequence can suppress liposome fusion under desiccation stress. We showed previously that G3LEA model peptides protect proteins from aggregation induced by desiccation [54] and the present findings enhance the potential of the model peptides as biostabilizers. When these peptides shield the surface of liposomes in the dry state, they preferentially adopt a β-sheet structure, while they are disordered in aqueous solution. In other words, they can change conformation in response to an interaction partner, similarly to IDPs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.07.009.

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