The role of membrane tension in the action of antimicrobial peptides and cell-penetrating peptides in biomembranes

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Abstract

For antimicrobial peptides (AMPs) with antimicrobial and bactericidal activities and cell-penetrating peptides (CPPs) with activity to permeate through plasma membrane, their interactions with lipid bilayer region in plasma membrane play important roles in these functions. However, the elementary processes and mechanisms of their functions have not been clear. The single giant unilamellar vesicle (GUV) method has revealed the details of elementary processes of interaction of some AMPs and CPPs with lipid vesicles. In this review, we summarize the mode of action of AMPs such as magainin 2 (Mag) and CPPs such as transportan 10 (TP10), revealed by the single GUV methods, and especially we focus on the role of membrane tension in actions of Mag and TP10 and the mechanisms of their actions. First, we explain the characteristics of the single GUV method briefly. Next, we summarize the recent view on the effect of tension on physical properties of lipid bilayers and describe the role of tension in actions of Mag and TP10. Some experimental results indicate that Mag-induced pore is a stretch-activated pore. The effect of packing of transbilayer asymmetric lipid on Mag-induced pore formation is described. On the other hand, entry of fluorescent dye, carboxyfluorescein (CF)-labeled TP10 (i.e., CF-TP10), into single GUVs without pore formation is affected by tension and high concentration of cholesterol. Pre-pore model for translocation of CF-TP10 across lipid bilayer is described. The experimental methods and their analysis described here are useful for investigation of functions of the other types of AMPs, CPPs, and proteins.

Keywords Single vesicles · Giant unilamellar vesicles · Pore formation · Membrane permeation · Pre-pores · Asymmetric lipid packing

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Introduction

Many peptides/proteins interact with integral and peripheral membrane proteins to trigger their functions. However, some peptides and proteins preferentially interact with the lipid bilayer region instead. Examples of this latter class include the well-known examples of antimicrobial peptides/proteins (AMPs) and cell-penetrating peptides/proteins (CPPs). AMPs produced by various organisms (e.g., animals (including humans), plants, and insects) act to either kill or suppress the growth of bacteria and fungi. One mechanism by which AMPs exert their antimicrobial activity is by damaging the plasma membranes of bacterial cells. This damage induces leakage of the internal contents of the bacteria and reduces their membrane potential. Therefore, AMPs destroy membranes by vastly increasing their permeability, thereby removing the controlled barrier that is one of the most important functions of the plasma membrane (Zasloff 2002; Melo et al. 2009; Hwang and Vogel 1998; Prophet et al. 2017). Unlike

ion channels (Hille 1992), AMPs do not necessarily form stable pores and holes whose sizes and selectivities are rigorously controlled for long periods of time.

CPPs also target the lipid bilayer region of the plasma membrane. In contrast to most AMPs, CPPs can enter the cytosol of eukaryotic cells without damaging the plasma membrane, and thus, CPPs have been used as tools for the cellular delivery of drugs and biomolecules (Bechara and Sagan 2013; Madani et al. 2011; Magzoub and Gräslund 2004; Stanzl et al. 2013; Zorko and Langel 2005). CPPs likely enter the cytosol via endocytic pathways such as clathrin-mediated endocytosis and by permeating the plasma membrane directly. Since endosome membrane is produced from the plasma membrane, their compositions are similar. Hence, in any pathways, CPPs must translocate across lipid bilayer region in the plasma membrane.

AMPs and CPPs are both highly positively charged peptides, but their functions differ greatly. The interactions of AMPs and CPPs with the lipid bilayer region of the plasma membrane are commonly studied using a suspension of large unilamellar vesicles (LUVs) (diameters 50 nm–1 μ m) composed of various lipid bilayers (i.e., the LUV suspension method). However, the experiments using the LUV suspension method provide ensemble average values of the physical characteristics of all the LUVs at various stages in their interaction with the peptides in a suspension, regardless of the biophysical technique being used to probe the system, resulting in the loss of important single vesicle/single molecule level information (Yamazaki 2008). For example, a common experimental approach using the LUV suspension method is the so-called “leakage (membrane permeation) experiment,” which has been extensively used to study the effects of AMPs, antibacterial compounds, CPPs, and various toxins (Matsuzaki et al. 1995, 1998; Aguilera et al. 1999; Jing et al. 2006; Bárány-Wallje et al. 2007; Yandek et al. 2007, 2008; Gregory et al. 2009; Qian et al. 2016). Such measurements provide information on the total leakage (or average leakage per LUV) of the internal contents (e.g., water-soluble fluorescent probes and ions) from all LUVs as a function of time and as a function of peptide concentration. The amount and rate of leaked content allow the evaluation of the degree of damage to the LUV membrane induced by the peptide to be evaluated. However, the cause of leakage (such as pore formation, membrane fusion, large curvature change, local rupture, burst vesicles) cannot be identified, and the elementary processes of leakage cannot be elucidated due to the ensemble averaging of the measured values (Yamazaki 2008). The entry of fluorescent probe-labeled CPPs into vesicles has also been examined using the LUV suspension method (Drin et al. 2001; Binder and Lindblom 2003; Thorén et al. 2004; Persson et al. 2004; Walrant et al. 2013; Swiecicki et al. 2014). However, again, this method does not provide conclusive evidence that the observed changes in signal are due to the entry

of CPPs or to aggregation and membrane fusion of LUVs, and the elementary processes of entry of CPPs cannot be observed (Islam et al. 2018).

Unlike LUVs, the size and shape of giant unilamellar vesicles (GUVs) with diameters larger than 10 μ m can be observed using various optical microscopes in real time. We recently developed a single GUV method to investigate the interaction of AMPs and CPPs with lipid bilayers (Tamba and Yamazaki 2005, 2009; Islam et al. 2014a). In this method, we observe changes in the structure and physical attributes of a single GUV as a function of time as it interacts with peptides/proteins. The experiment is repeated with many “individual GUVs” under the same conditions; then, we analyze the results statistically. This single GUV method has enabled us to elucidate details regarding the elementary processes of individual events, such as the membrane permeation of fluorescent dyes and the entry of CPPs into the vesicle lumen, and to determine the rate constants of these elementary processes. The single GUV method has been successfully applied to several AMPs such as magainin 2 (Mag) (Tamba and Yamazaki 2005, 2009; Tamba et al. 2010; Karal et al. 2015a; Hasan et al. 2018a), lactoferricin B (LfcinB) (Moniruzzaman et al. 2015), and PGLa (Parvez et al. 2018); to several CPPs such as transportan 10 (TP10) (Islam et al. 2014b, 2017; Moghal et al. 2018), oligo-arginine (Sharmin et al. 2016), and LfcinB (4–9) (Moniruzzaman et al. 2017); to the pore-forming toxin (PFT) lysenin (Alam et al. 2012); and to the antibacterial compound epigallocatechin gallate (EGCg) (Tamba et al. 2007).

Several reviews describe the single GUV method (Yamazaki 2008; Islam et al. 2014b, 2018; Hasan and Yamazaki 2019); their contents include the characteristics of the method (Yamazaki 2008; Islam et al. 2014b), its application to AMP studies (several modes of action of AMPs and the elementary processes of AMP-induced damage in lipid bilayers) (Hasan and Yamazaki 2019), and the application of this method to CPP studies (the elementary processes underlying the entry of CPPs into the vesicle lumen) (Islam et al. 2018). In this review, we focus on the use of the single GUV approach to investigate the role of membrane tension in the action of AMPs and CPPs and also on the mechanism of action of these peptides. There are many AMPs and CPPs, and thus, we can expect that these peptides exhibit various modes of action. In this review, we focus on Mag as a representative AMP and TP10 as a representative CPP. Mag is found in the African clawed frog *Xenopus laevis* (Zasloff 1987) and has been extensively investigated using various biophysical techniques (Matsuzaki et al. 1995, 1996, 1998; Ludtke et al. 1996; Yang et al. 2000; Gregory et al. 2009), and TP10 is an artificial designed CPP (Soomets et al. 2000) with practical applications (EL-Andaloussi et al. 2005; Stalmans et al. 2015; Fadzen et al. 2017; Rusiecka et al. 2019; Ruczyński et al. 2019). First, we briefly describe the characteristics of the

single GUV method and then summarize recent insights into the effect of membrane tension on lipid bilayers. Finally, we describe the role of membrane tension in the action of Mag and TP10 and the mechanisms of their actions.

Characteristics of the single GUV method

In the single GUV method, a target GUV is selected under an optical microscope and a peptide solution is slowly introduced into its vicinity through a micropipette, allowing continuous observation of the interaction of the peptide with a single GUV from its start. The peptide concentration near the GUV remains unchanged during the interaction because peptide solution is continuously added. Figure 1a shows an example of the interaction of an AMP (here, Mag) with a single GUV whose lipid bilayer is composed of dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidylcholine (DOPC) (4/6: molar ratio) as observed using confocal laser scanning microscopy (CLSM) (Karal et al. 2015a). The interior of this GUV (the lumen) contains the water-soluble fluorescent dye Alexafluor647 hydrazide (AF647). The fluorescence intensity (FI) of the GUV lumen due to AF647 (Fig. 1a (1)) is proportional to the AF647 concentration in the lumen. The FI of the GUV lumen due to AF647 remains almost constant over the first 149 s, after which the FI gradually decreases (red squares in Fig. 1b), indicating that Mag induces pore formation in the lipid bilayer of the GUV at 149 s, allowing AF647 to diffuse outside of the GUV (i.e., membrane permeation) (Fig. 1b). Prior to pore formation, there is no membrane permeation of AF647 and no structural changes in the GUV, and this state of the GUV is called the intact state. This figure (red squares in Fig. 1b) provides two pieces of information: the time at which pore formation begins and the rate of membrane permeation of AF647 from the GUV through the pore. The latter information is useful for evaluating the size of the pore and the evolution of the pore over time (Tamba et al. 2010; Alam et al. 2012). The Mag solution in the example described here contains a low concentration of the fluorescent dye carboxyfluorescein (CF)-labeled Mag (CF-Mag) to reveal the location of the peptide. The FI of the GUV membrane (i.e., the rim intensity) due to CF-Mag is proportional to the CF-Mag concentration in the membrane if there is no fluorescence quenching. Figures 1a (2) and b (green triangles) show that the rim intensity rapidly increases to reach a steady value, I_1 , at 50 s, and remains constant for ~ 90 s until pore formation starts, and then the rim intensity rapidly increases to another steady value, I_2 . The average value of I_2/I_1 in this example was approximately 2. These results indicate that Mag binds to the outer leaflet of the GUV and attains a steady binding state at 50 s; then the surface concentration of Mag, X , in the outer leaflet remains constant for an extended time until pore formation begins; and finally (at the

time of pore formation), Mag transfers from the outer leaflet to the inner leaflet until X in both monolayers is equal. This result indicates that Mag locates only in the outer leaflet just before pore formation. Solution NMR and solid-state NMR revealed that Mag molecules form an α -helix in the bilayer interface and orient parallel with the membrane surface (i.e., the tilt angle between the helix axis and the bilayer normal is close to 90°) (Bechniger et al. 1993; Gesell et al. 1997; Strandberg et al. 2009). Based on these results, we can reasonably infer that in the intact state before pore formation, Mag locates in the membrane interface of the outer leaflet (Fig. 1c).

In this method, we repeat the same experiment using many “individual GUVs” and analyze the results statistically. Figure 1d shows the time courses of the FI of lumens of several individual DOPG/DOPC (6/4)-GUVs containing another water-soluble fluorescent dye, calcein, during the interaction with Mag (note that the Mag solution in the experiment shown in Fig. 1d does not contain CF-Mag). Each curve corresponds to the result obtained with an individual GUV. This figure indicates clearly that the membrane permeation of calcein starts at different times in all GUVs, i.e., pore formation occurs in the lipid bilayer at a different time, but after pore formation, the time courses of the FIs are similar (i.e., the rates of leakage are similar). The probability or the fraction of intact GUVs among all examined GUVs, $P_{\text{intact}}(t)$, is useful for analyzing such stochastic phenomena (Tamba and Yamazaki 2005, 2009). Figure 1e shows that $P_{\text{intact}}(t)$ decreases monotonically with time for various Mag concentrations. Based on the results of Fig. 1a, b, we can reasonably infer that an irreversible two-state transition from the intact state (Fig. 1c) to the pore state occurs in Mag-induced pore formation, and thus, we obtain the following theoretical equation for $P_{\text{intact}}(t)$:

$$P_{\text{intact}}(t) = \exp\{-k_P(t-t_{\text{eq}})\} \quad (1)$$

where k_P is the rate constant of the two-state transition, regarded as the rate constant of Mag-induced pore formation at the initial stage, and t_{eq} is a fitting parameter, which implies the time required to achieve binding equilibrium of Mag to the GUV membrane. The k_P value determined by fitting Eq. 1 to the experimental data for $P_{\text{intact}}(t)$ obtained using DOPG/DOPC (6/4)-GUVs increases with an increase in Mag concentration in aqueous solution (black squares in Fig. 1f). Figure 1e indicates that after equilibrium of the binding of Mag to the GUV membrane at t_{eq} Mag-induced pore formation occurs in each GUV stochastically, but the behavior of pore formation in many GUVs follows an irreversible two-state transition described in Eq. 1. It is considered that in the thermal energy-induced two-state transitions individual events occur stochastically, because it is necessary to overcome the energy barrier (or activation energy) to make a transition from an initial state to a final state using thermal energy. It is well known that the transition between open and closed states of

Fig. 1 Characteristics of pore formation in single GUVs induced by their interaction with Mag. **a** Interaction of 31 μM Mag/CF-Mag with a single DOPG/DOPC (4/6)-GUVs containing AF647 in the GUV lumen. CF-Mag (0.16 μM) is included. CLSM images of AF647 (1) and CF-Mag (2). The numbers below each image represent the interaction time (s) with Mag/CF-Mag. The bar indicates 30 μm . **b** Time course of the change in FI of the GUV shown in **a**. The normalized FI of AF647 in the GUV lumen (red filled square) and the FI of CF-Mag in the rim of the GUV (green filled triangle), respectively. **c** A scheme of binding of Mag to a lipid bilayer. **d** Time courses of the FI due to calcein in the lumen of several “single DOPG/DOPC (6/4)-GUVs” during the interaction with

3 μM Mag. **e** Time course of P_{intact} of DOPG/DOPC (6/4)-GUVs interacting with various Mag concentration: (open circle) 5, (filled square) 3, (open triangle) 2.5, and (filled circle) 1 μM . The curves represent the best fit ones of Eq. 1. **f** Dependence of the rate constant of pore formation, k_p , on Mag concentration in buffer, C . (Filled square): DOPG/DOPC (6/4)-, (green filled circle): DOPG/DOPC (5/5)-, (blue filled circle): DOPG/DOPC (4/6)-, and (red filled triangle): DOPG/DOPC (3/7)-GUVs. (**g**) Dependence of k_p on X . (Blue filled circle): DOPG/DOPC (4/6)-, and (red filled triangle): DOPG/DOPC (3/7)-GUVs. **a**, **b**, **g** and **d–f** are reprinted from Karal et al. (2015a) and Tamba and Yamazaki (2009), respectively, with permission from the American Chemical Society

single ion channels (Sakmann and Neher 1995) and the force-induced unfolding of single proteins (Yamazaki et al. 2002) follow such two-state transition. We describe the mechanism of the Mag-induced two-state transition or pore formation in “Mag-induced stretching of lipid bilayers (membrane tension) and induced pore formation” and “Effect of trans-bilayer asymmetric lipid packing on Mag-induced pore formation.”

Electrostatic interactions likely play a key role in the preferential binding of AMPs to the bacterial plasma membrane (Zaslhoff 2002). Generally, in biological systems in aqueous solution, surface charge density and ion concentration (or ionic strength) determine the electrostatic interactions (Israelachvili 1992; Li et al. 2001). We can examine the effect of electrostatic interactions between Mag and GUVs on

Mag-induced pore formation by preparing GUVs with different surface charge densities of lipid bilayers by changing the negatively charged DOPG concentration in the DOPG/DOPC bilayer. As the surface charge density of the lipid bilayer increases, the k_p value increases for the same Mag concentration in aqueous solution; consequently, lower concentrations of Mag can induce rapid pore formation in lipid bilayers with larger surface charge density (Fig. 1f) (Tamba and Yamazaki 2009). The Mag surface concentration in the membrane, X (mol/mol; the molar ratio of Mag bound to the membrane interface to the lipid in the outer leaflet of a GUV), can be obtained from its aqueous concentration, C , using the intrinsic binding constant of Mag to the same lipid bilayer and the theory of electrostatic interaction (Poisson–Boltzmann

Fig. 2 Characterization of interaction of CF-TP10 with single GUVs and its entry to their lumen. **a, b** Interaction of CF-TP10 with single DOPG/DOPC (2/8)-GUVs containing AF647 and DOPG/DOPC-LUVs in the GUV lumen. CLSM images of AF647 (1) and CF-TP10 (2). The numbers above each image represent the interaction time (s) with 1.0 μM (**a**) and 2.0 μM (**b**) CF-TP10. The bars indicate 30 μm . **c** and **d** are time course of the change in FI of the GUV shown in **a** and **b**, respectively. The

normalized FI of AF647 in the GUV lumen (red filled square), the normalized FI of CF-TP10 in the rim of the GUV (green filled square), and I_{lumen} due to CF-TP10 (open square). (**E**) Dependence of I_{lumen} (6 min) (blue open square) and P_{entry} (6 min) (red filled circle) on CF-TP10 concentration. These figures are reprinted from Moghal et al. (2018) with permission from Elsevier (Fig. 2e is a little modified)

equation) (Tamba and Yamazaki 2009). Using this technique, we obtained the relationship between k_p and X for DOPG/DOPC (4/6)-GUVs and DOPG/DOPC (3/7)-GUVs (Fig. 1g). The dependencies of k_p on X for both GUVs are almost the same, indicating that the Mag surface concentration, X , is a main determinant of k_p .

The single GUV method enables us to measure simultaneously the time courses of several physical characteristics of single vesicles during their interactions with peptides/proteins to obtain the relationship between these characteristics using CLSM. For example, we can determine the time courses of changes in the peptide concentration in the vesicle membrane, the entry of peptides into the vesicle lumen, and peptide-induced pore formation after initiating the interaction of peptides with single vesicles. Figure 2 shows an example of the interaction of the fluorescent dye CF-labeled TP10 (i.e., CF-TP10) with single DOPG/DOPC (2/8)-GUVs containing AF647 and DOPG/DOPC LUVs (diameter: 100 nm) in the GUV lumen (Moghal et al. 2018). During the interaction of 1.0 μM CF-TP10, the FI in the GUV lumen due to AF647

does not change over 360 s, indicating that no pores are formed through which AF647 can leak (Fig. 2a (1), red squares in Fig. 2c). Figure 2a (2) and c (green squares) shows that the rim intensity due to CF-TP10 gradually increases to reach a steady value at 220 s. On the other hand, initially, the FI of the GUV lumen due to CF-TP10 bound to the LUVs, I_{lumen} , is almost 0, but I_{lumen} starts to increase at 220 s and becomes large ($I_{\text{lumen}} = 515$) at 360 s (black squares in Fig. 2c), indicating that a significant amount of CF-TP10 binds to LUVs without pore formation. It is noteworthy that the presence of LUVs in a GUV lumen greatly enhances the sensitivity of detection of CF-TP10 in the GUV lumen (Moghal et al. 2018). Based on the results of the correlations between three physical characteristics (CF-TP10 concentration in the GUV membrane and in the lumen, and AF647 concentration in the lumen), one can conclude that CF-TP10 translocates across the GUV membrane from the outside, enters the lumen (aqueous solution), and then binds to the membranes of LUVs in the lumen without inducing pore formation. Figure 2b (1) shows that during the interaction of a higher concentration (2.0 μM)

of CF-TP10, the FI of the GUV lumen due to AF647 does not change over the first 240 s, then gradually decreases (red squares in Fig. 2d), indicating that at 240 s, CF-TP10 induces pores in the GUV bilayer through which AF647 leaks from the lumen. Figure 2b (2) shows that the rim intensity due to CF-TP10 gradually increases to reach a steady value at 200 s and remains unchanged after pore formation (green squares in Fig. 2d), indicating that CF-TP10 achieves binding equilibrium to both the outer and inner leaflets at 200 s. This symmetric transmembrane distribution of CF-TP10 is different from the asymmetric transmembrane distribution of Mag (Fig. 1c). On the other hand, initially, the FI of the GUV lumen due to CF-TP10, I_{lumen} , is almost 0, but I_{lumen} starts to increase at 80 s and becomes large before AF647 starts to leak (black squares in Fig. 2d), indicating that a significant amount of CF-TP10 binds to LUVs before pore formation. Based on these results, one can conclude that CF-TP10 permeates through the GUV membrane from the outside, then enters the GUV lumen to bind to the LUV membranes without pore formation, and after a lag period AF647 begins to permeate through the membrane (i.e., pore formation occurs).

This method provides several parameters regarding the rate of entry of CF-TP10 into a GUV lumen, V_{entry} . One parameter is I_{lumen} (6 min), which is proportional to the amount of CF-TP10 which has entered a GUV lumen at a specific time (here 6 min). I_{lumen} (6 min) increases with CF-TP10 concentration (blue squares in Fig. 2e), indicating that V_{entry} increases as the concentration of CF-TP10 outside the GUV increases. Using this method, one can judge that CF-TP10 enters a GUV lumen if I_{lumen} is above a threshold intensity (under this condition, 50). There are several reports that the entry of CPPs occurs stochastically (Islam et al. 2014a; Sharmin et al. 2016; Islam et al. 2017; Moniruzzaman et al. 2017), and thus, the fraction of GUVs which CPPs enter before a specific time t among the total examined GUVs (i.e., the fraction of entered GUVs), $P_{\text{entry}}(t)$, can also be used as the measure of the rate of entry of CF-TP10 (Islam et al. 2014a; Moghal et al. 2018). Figure 2e (red circles) shows that at or below 0.50 μM CF-TP10, P_{entry} (6 min) = 0, and above 0.50 μM , P_{entry} (6 min) increases with CF-TP10 concentration. The values of P_{entry} (6 min) and its dependence on CF-TP10 concentration are the same as those obtained using another method to detect the entry of CPPs using single GUVs containing smaller GUVs (1–10 μm in diameter) in the mother GUV lumen (see “Effect of mechanical properties on the entry of CF-TP10 into the GUV lumen and its translocation across the lipid bilayer” and Fig. 8a) (Islam et al. 2014a) and provide further evidence that V_{entry} increases with CF-TP10 concentration. The advantage of the method using single GUVs containing LUVs in their lumen over using single GUVs containing smaller GUVs is that this method enables us to perform continuous measurement of entry of CPPs and CPP concentration in their lumen as a function of time (Fig. 2c, d) (Moghal et al. 2018).

The analysis of the rim intensity due to fluorescent dye-labeled CPPs of single GUVs not containing vesicles in their lumen provides the rate constant of binding of CPPs to the lipid monolayer from aqueous solution, k_{ON} , and that of unbinding of CPPs from the monolayer to aqueous solution, k_{OFF} , and thus the binding constant of CPPs to the membrane, $K_{\text{B}} (= k_{\text{ON}}/k_{\text{OFF}})$, which are useful to elucidate the mechanism of their entry (Islam et al. 2014a, 2017; Sharmin et al. 2016; Moniruzzaman et al. 2017).

Effects of membrane tension on the physical properties of lipid bilayers

The application of various external forces to eukaryotic cells and lipid vesicles causes stretching or compression of the plasma and vesicle membranes, respectively, and lateral tension (i.e., membrane tension) is concomitantly induced (Sandre et al. 1999; Evans et al. 2003). Other factors such as electric fields (Portet and Dimova 2010) and osmotic pressure (Shibly et al. 2016) also produce lateral tension in membranes. Large stretching induces lipid vesicle rupture and cell lysis, resulting in cell death, and stretching of the plasma membrane plays a key role in the functions of membrane proteins such as the gating of mechano-sensitive channels (Sachs 2010; Sukharev et al. 1994; Levina et al. 1999). Thus, it is indispensable to elucidate the mechanism of tension-induced rupture and the effect of stretching on the physical characteristics of lipid bilayers.

Lipid bilayer GUVs have been used to examine quantitatively the stretching- or tension-induced rupture of lipid bilayer vesicles. For example, measurement of the elastic modulus of GUVs using the micropipette aspiration method has long provided information on the mechanical properties of lipid bilayers, including the rupture of GUVs. In this measurement, as the tension in a GUV membrane increases, the fractional area change increases, and then at critical tension, the GUV ruptures. This critical tension is used as a measure of the strength of a lipid bilayer (Kwok and Evans 1981; Needham and Nunn 1990). Using fluorescence microscopy, Brochard-Wyart and colleagues observed a micrometer-sized pore in a GUV which formed due to stretching induced by strong optical illumination and analyzed the time course decrease in the diameter of the pore (Sandre et al. 1999; Karatekin et al. 2003). Their results provide direct evidence of the existence of only a single large pore in a vesicle membrane after the stretching-induced pore formation and also quantitative information on the dynamics of closure of a single pore in the membrane. One important insight is that the rate of closure of a pore greatly depends on the line tension (or line free energy per unit length), Γ , of a large pore in a lipid bilayer. They found that the value of Γ for a DOPC bilayer is in the range 6.9–20.7 pN, depending on the manufacturer of the

DOPC (Karatekin et al. 2003). Subsequently, various experimental methods have been used to investigate the dynamics of the closing of large pores induced by stretching (Srividya and Muralidharan 2008; Portet and Dimova 2010; Ryham et al. 2011), leading to several theories to explain the observed dynamics (Brochard-Wyart et al. 2000; Ryham et al. 2011). Furthermore, Evans et al. increased tension at various rates on a GUV and analyzed the rupture of the GUV (Evans et al. 2003; Evans and Smith 2011). They found that the rupture tension of a GUV increases as the tension loading rate increases and proposed this as a mechanism worthy of further study.

Recently, a simple experimental method for the constant tension-induced rupture of GUVs was developed (Levadny et al. 2013). First, a constant tension is applied to a GUV using a micropipette; then, the GUV is observed under constant tension using differential interference contrast (DIC) microscopy and CLSM (Fig. 3a). Initially, there is no change in the GUV, but after a lag time, the GUV is suddenly aspirated into the micropipette within 1 s (Fig. 3a) (Levadny et al. 2013). This aspiration of a GUV into the micropipette is interpreted as follows. Constant tension induces a pore in the GUV membrane, resulting in rupture and then complete aspiration of the GUV into the micropipette because the pressure outside the micropipette is greater than that inside. When we repeat the same experiment with many “individual GUVs,” the results indicate that the rupture of GUVs under constant tension occurs at different times in all GUVs. This stochastic event is analyzed using the probability or the fraction of intact (non-ruptured) GUVs among all examined GUVs, $P_{\text{intact}}(t)$. Figure 3b shows the time course of $P_{\text{intact}}(t)$ of DOPG/DOPC (4/6)-GUVs under constant tensions of 5 mN/m and 7 mN/m (Levadny et al. 2013). From a theoretical point of view, this constant tension-induced rupture of GUVs is considered as an irreversible two-state transition from the intact state to the ruptured state, and therefore, $P_{\text{intact}}(t)$ is given by the following:

$$P_{\text{intact}}(t) = \exp(-k_R t) \quad (2)$$

where k_R represents the rate constant of tension-induced rupture of a GUV, regarded as the rate constant of tension-induced pore formation. The time courses of $P_{\text{intact}}(t)$ fit well to Eq. 2 (Fig. 3b), and the best fit provides the values of k_R (Levadny et al. 2013; Karal et al. 2015b; Karal and Yamazaki 2015; Karal et al. 2016; Shibly et al. 2016; Sharmin et al. 2016). Figure 3c shows the dependence of k_R on tension, σ , for DOPG/DOPC-GUVs whose membranes contain various concentrations of DOPG (Levadny et al. 2013; Karal et al. 2015b). The figure indicates that k_R increases with tension for all GUVs, regardless of the concentration of DOPG, and as the concentration in DOPG increases (i.e., the surface charge density of the membrane increases), the lower the

Fig. 3 Rate constant of rupture of DOPG/DOPC-GUVs induced by constant tension. **a** A scheme of tension-induced rupture of GUV. First, a GUV is aspirated by a micropipette which induces a constant tension in the GUV membrane. When pore formation occurs suddenly in the GUV membrane, inducing rupture of GUV, then the GUV is aspirated into the micropipette completely. **b** Time course of the change in fraction of intact DOPG/DOPC (4/6)-GUVs, $P_{\text{intact}}(t)$, under tension of 5.0 (open circle) and 7.0 mN/m (open square). The solid curves represent the best fit ones of Eq. 2. **c** The dependence of k_R on tension σ for GUVs composed of various DOPG/DOPC membranes containing different DOPG concentration. Buffer contains 150 mM NaCl. DOPC (filled square), DOPG/DOPC (1/9) (open square), DOPG/DOPC (4/6) (filled circle), and DOPG/DOPC (7/3) (open circle). The solid curves show the best-fit theoretical ones obtained by MFPT. **b** is reprinted from Levadny et al. (2013) with permission from the American Chemical Society. **c** is reprinted from Karal et al. (2015b) with permission from the American Physical Society

tension required to induce rupture of the GUV. Moreover, as NaCl concentration decreases, lower tensions induce rupture of the GUV. These results indicate that electrostatic interactions elevate the rate of constant tension-induced rupture of GUVs (Karal et al. 2015b).

The fluidity of lipid bilayers (related to the diffusion coefficient of lipid molecules) and the molecular motions of lipid molecules in the liquid crystalline (L_α) phase or liquid-disordered phase are large. Therefore, we can reasonably consider that various regions of rarefaction (i.e., nanodomains of lower lipid density) and of condensation (i.e., nanodomains of higher lipid density) form transiently due to large fluctuations in the lipid bilayers driven by thermal forces. Here we define rarefactions in lipid bilayers as pre-pores. Several pre-pore structures have been proposed (Glaser et al. 1988; Tieleman et al. 2003; Tolpekina et al. 2004; Wang and Frenkel 2005; Wohlert et al. 2006; Gozen and Dommersnes 2014; Akimov et al. 2017a, b) and can be categorized into two types: hydrophobic pre-pores and hydrophilic pre-pores. In a hydrophobic pre-pore, the orientational change of lipid molecules is small and hence the pre-pore wall is comprised of lipid hydrocarbon chains which contact water inside the pre-pore. The line tension of this pre-pore is mainly due to the hydrophobic interactions between the hydrocarbon chains and water. In contrast, a hydrophilic pre-pore has a toroidal structure, where the bent outer and inner leaflets are connected to each other at the pre-pore, greatly changing the orientational order of the lipid molecules at the pre-pore so that its inner wall is composed of lipid headgroups in contact with water in a water channel (Fig. 4a). We note that a micrometer-sized pore in a GUV is also considered to have a toroidal structure (Sandre et al. 1999; Karatekin et al. 2003). The line tension of a

hydrophilic pre-pore is determined mainly by the bending energy of the monolayer. However, a part of the hydrocarbon chains of the lipids in the pre-pore wall tend to contact water due to large deformations in the lipid structure, and this hydrophobic interaction increases the line tension. Intuitively we can consider that as the pre-pore radius initially increases, a hydrophobic pre-pore is formed and then converts into a hydrophilic pre-pore (Glaser et al. 1988). However, atomistic molecular dynamics (MD) simulation results indicate that whereas larger radius hydrophilic pre-pores are generally observed, smaller radius, hydrophobic pre-pores are not observed when the pre-pore radius is increased gradually from zero in simulations (Tolpekina et al. 2004; Wohlert et al. 2006). Therefore, here we consider that only hydrophilic pre-pores exist. The difference between pores and pre-pores is defined here as follows. Pores have a water channel in a lipid bilayer through which fluorescent dyes can pass through (i.e., the diameters of the pores are larger than the diameters of the dyes and the lifetime of an open pore is relatively long so that dye molecules permeate significantly), whereas pre-pores have a narrow channel through which dyes cannot pass significantly and their lifetimes are very short. Based on several models of tension-induced pore formation in lipid bilayers (Lister 1975; Glaser et al. 1988; Fuertes et al. 2011), when a pre-pore is formed in a bilayer, the free energy of the bilayer increases due to the free energy of the pre-pore, $U(r)$, where r is the radius of the pre-pore. Several physical factors affect $U(r)$. One factor is the line tension, Γ , which increases $U(r)$ by $2\pi r\Gamma$. The formation of a pre-pore in a stretched membrane decreases the area of the membrane by πr^2 , which decreases its elastic energy by $\pi r^2\sigma$. In a charged membrane, electrostatic interactions decrease $U(r)$ by πBr^2 , where B is a parameter for the electrostatic interaction and depends on the surface charges of the bilayer, the Debye length, geometrical parameters such as the thickness of the membrane and area per lipid, and other parameters (for electrically neutral lipid membranes such as DOPC, $B = 0$) (Karal et al. 2015b, 2016). Hence, $U(r)$ is a function of r as follows:

$$U(r) = 2\pi\Gamma r - \pi(\sigma + B)r^2 + U_0 \quad (3)$$

where U_0 is the nucleation free energy required to form a hydrophilic pre-pore (Karal and Yamazaki 2015; Karal et al. 2016). The maximum value of $U(r)$, U_a , occurs at the critical radius, $r_c = \Gamma/(\sigma + B)$, which is the energy barrier for tension-induced GUV rupture, and is expressed as a function of tension as follows:

$$U_a = U_0 + \pi\Gamma^2/(\sigma + B) \quad (4)$$

Figure 4b shows the free energy landscape of a pre-pore. Thermal fluctuation of the lipid bilayer results in the formation of a pre-pore with radius r , but the pre-pore tends to close due

Fig. 4 A pre-pore in a lipid bilayer. **a** Illustration of a hydrophilic pre-pore with toroidal structure. **b** Free energy landscape of a hydrophilic pre-pore with radius r , $U(r)$, for various tensions. Curve A: $\sigma = 0$; B: 4.0; C: 6.0, and D: 8.0 mN/m. Curves of $U(r)$ are drawn based on Eq. 3 using $B = 1.8$ mN/m, $U_0 = 2.2$ kT, and $\Gamma = 12.4$ pN

to the large line tension if r is less than r_c . Consequently, the radius of the pre-pore fluctuates rapidly when r is less than r_c , and when a large fluctuation in r results in it becoming greater than r_c , pore formation occurs. Figure 4b indicates that U_a decreases with tension, indicating that GUVs rupture rapidly at higher tensions. This theoretical prediction reasonably explains the experimental results for the tension dependence of k_R shown in Fig. 3c. Thus, U_a can be regarded as the activation energy of constant tension-induced GUV rupture or constant tension-induced pore formation in lipid bilayers. Theoretically, the rate constant for the tension-induced rupture of GUVs, k_R , is calculated using the mean first passage time (MFPT) approach (Levadny et al. 2013; Karal et al. 2015b). This approach is based on Brownian motion theory (Hänggi et al. 1990; Gardiner 1990), and the fluctuation in the radius of a pre-pore is treated as diffusive motion of a particle in r -space between two walls at $r=0$ and at $r=r_c$. The lifetime of the intact GUV, τ , is determined as the time when the first particle arrives at $r=r_c$. Since $k_R=1/\tau$, we obtain a theoretical equation for k_R as a function of σ , which fits well to experimental data (Fig. 3c). This fitting allows determination of the values of Γ .

The activation energy of constant tension-induced GUV rupture was recently determined by measuring the temperature dependence of k_R (Karal and Yamazaki 2015; Karal et al. 2016). The results verify the relationship described in Eq. 4 (i.e., $U_a \propto 1/(\sigma + B)$), partially validating Eq. 3. The values of line tension Γ are determined from the slopes of the relationship between U_a and $1/(\sigma + B)$: Γ of a pre-pore in a DOPC bilayer is 11.6 pN (Karal and Yamazaki 2015), which is very similar to that determined by different methods: 11.5 pN from dynamic tension spectroscopy measurements (Evans et al. 2003), 10.5 pN from MFPT analysis of the tension dependence of k_R (Levadny et al. 2013), and 6.9–20.7 pN for large pores obtained using closure dynamics (Karatekin et al. 2003).

We can observe the structure of tension-induced micrometer-diameter pores in lipid bilayers using optical microscopy (Sandre et al. 1999; Brochard-Wyart et al. 2000; Karatekin et al. 2003), but pre-pores in lipid bilayers have not been observed to date due to their extremely small size and short lifetime. Experimental results supporting the existence of pre-pores are limited, although fluctuations in electrical current in planar membranes are supportive of their presence (Melikov et al. 2001). Recently, new experimental evidence for the existence of transient hydrophilic pre-pores was found as follows. For this purpose, first, a new method was developed to measure the rate constant of trans-bilayer movement (or flip-flop) (k_{FF}) of fluorescent dye-labeled lipids in a GUV (Hasan et al. 2018b). This method enables us to examine the effects of membrane tension on the k_{FF} of lipids, because the membrane tension of a GUV can be controlled precisely (but it is difficult for LUVs) as described in “Effects of membrane tension on the physical properties of lipid bilayers.” The k_{FF} of lipid

molecules with charged polar headgroups is very small ($\sim 10^{-5} \text{ s}^{-1}$ depending on structures of lipids) due to the low dielectric constant of the hydrophobic core of the lipid membrane (McConnell and Kornberg 1971; Homan and Pownall 1988; Wimley and Thompson 1990; Bai and Pagano 1997; Moreno et al. 2006; Nakano et al. 2009). In this method, we first generate GUVs with an asymmetric distribution of the fluorescent probe (NBD)-labeled PG (i.e., 18:1–06:0 NBD-PG, hereafter NBD-PG) using the method described in “Mag-induced stretching of lipid bilayers (membrane tension) and induced pore formation.” The inner leaflet comprises DOPG/DOPC/NBD-PG, and the outer leaflet comprises DOPG/DOPC, and AF647 is present in the lumen. Then, we measured the rim intensity of single GUVs using CLSM under various tensions (applied externally) as a function of time. As time goes on, NBD-PG transfers from the inner leaflet to the outer leaflet in a GUV and then to aqueous solution outside the GUV. The former step (i.e., the trans-bilayer movement of NBD-PG) is a rate-determining step, and thus, the rim intensity of the GUV is determined by the concentration of NBD-PG in the inner leaflet (Hasan et al. 2018b). It is well known that the trans-bilayer movement of lipids follows a first-order reaction. Therefore, the rim intensity, $I(t)$, can be described by $I(t) = I(0) \exp(-k_{FF}t)$, where $I(0)$ is the rim intensity at $t=0$. Fitting of this equation to the experimental data of $I(t)$ determines the value of k_{FF} . During application of constant tension to a GUV due to aspiration by a micropipette, we observe the time course change in the rim intensity due to NBD-PG, $I(t)$, and in the FI of the GUV lumen due to AF647 (Fig. 5a). The results indicate that the k_{FF} of NBD-PG greatly increases with tension without membrane permeation of AF647 from the GUV lumen (i.e., no pore formation) (Fig. 5b) (Hasan et al. 2018b). This result indicates that the increase in k_{FF} is not due to the transfer of NBD-PG through a pore. Moreover, it is difficult to infer that tension changes greatly the dielectric constant of the hydrophobic core. Therefore, it is inferred that the plausible mechanism for the increase in k_{FF} with tension is due to rapid lateral diffusion of NBD-PG through the combined monolayers in a pre-pore with a toroidal structure (Fig. 5c). This inference supports the existence of hydrophilic pre-pores in lipid bilayers. The results also indicate that the frequency of pre-pore formation and the sizes of pre-pores increase with tension, and as a result, k_{FF} increases with tension. This phenomenon may be related to the effect of tension on the membrane permeation of CPPs (see “Effect of mechanical properties on the entry of CF-TP10 into the GUV lumen and its translocation across the lipid bilayer”).

The number of MD simulation studies of tension-induced pore formation in lipid bilayers has increased over the past 15 years (e.g., Tolpekina et al. 2004; Wohllert et al. 2006; Shigematsu et al. 2015, 2016; Awasthi and Hub 2016; Akimov et al. 2017a). Special sampling techniques such as the umbrella sampling method have been used to obtain the

Fig. 5 Effect of tension on trans-bilayer movement of NBD-PG in single GUVs. **a** CLSM images of a DOPG/DOPC/NBD-PG (40/59/1; inner leaflet)-DOPG/DOPC (40/59; outer leaflet)-GUV aspirated by a micropipette. (I) DIC, (II) NBD-PG, and (III) AF647. The bar corresponds to 20 μm . **b** Effect of tension on k_{FF} of NBD-PG at 25 $^{\circ}\text{C}$. **c** A scheme of lateral diffusion of NBD-PG through a connected monolayer in a transient, pre-pore with toroidal structure from the inner leaflet to the outer leaflet. Black arrows indicate the direction of the lateral diffusion of NBD-PG molecule indicated by purple headgroup. **a** and **b** are reprinted from Hasan et al. (2018b) with permission from the American Institute of Physics

free energy profile for tension-induced pore formation using MD simulations, but the results obtained greatly depend on the reaction coordinates selected. Moreover, all-atom MD simulations have not yet provided results supporting the fluctuation of pre-pore radii because current simulations are very

limited in terms of time length and membrane area (Buch et al. 2010).

Mag-induced stretching of lipid bilayers (membrane tension) and induced pore formation

Revealing the mechanism of AMP-induced damage in lipid bilayers requires information on the effect of AMP binding on the physical properties of lipid bilayers. Among their various physical properties, AMP-induced area changes of lipid bilayers from their optimal areas are important because these changes vary their mechanical state greatly. To examine the AMP-induced area change of GUV membrane, one can use the micropipette aspiration method (Rawicz et al. 2000). Here we explain a case of Mag (Karal et al. 2015a). First, a GUV is fixed at the tip of a micropipette using a slight aspiration (inducing a small tension of 0.5 mN/m), and the GUV is equilibrated for a few minutes to eliminate an excess area of the GUV (Levadny et al. 2013; Shibly et al. 2016). Upon initiating the interaction of Mag with a GUV at the tip of a micropipette, the fractional change in the area of the GUV membrane, $\delta(t) = (A(t) - A_0) / A_0$, where $A(t)$ and A_0 are the total membrane area of a GUV at time t after starting the addition of Mag solution and $t = 0$, respectively) increases rapidly with time. Within a short time (less than 60 s, depending on conditions), δ reaches a steady value (δ^{ST}), which remains constant for a long time until the GUV is aspirated into the micropipette due to Mag-induced pore formation (e.g., open triangles in Fig. 6a). δ^{ST} is almost proportional to the Mag surface concentration, X (i.e., $\delta^{\text{ST}} = 0.58X$) (Fig. 6b). The same experiment performed using Mag solution containing a low concentration of CF-Mag shows that the time course of the rim intensity due to CF-Mag (proportional to X) is almost the same as that of $\delta(t)$ (Fig. 6a), which supports the result of Fig. 6b. By combining the results of Fig. 1g (i.e., k_p increases with X above its threshold value) and Fig. 6a ($\delta^{\text{ST}} \propto X$), one can draw a conclusion that k_p increases greatly with an increase in δ^{ST} above its threshold value. On the other hand, the presence of membrane tension due to external force increases the value of k_p (Karal et al. 2015a). These results indicate that Mag-induced pore is activated by stretching lipid bilayers.

The results of Figs. 1 and 6 suggest that when Mag binds to the outer leaflet, the pure lipid monolayer in the inner leaflet is stretched, resulting in pore formation. The membrane tension in the inner leaflet (σ_{IM}) during this stretching can be estimated as follows: $\sigma_{\text{IM}} = K_M \delta_{\text{IM}}$, where K_M is the elastic modulus of the monolayer (70.5 mN/m) and δ_{IM} is the fractional change in the area of the inner leaflet. When Mag binding attains steady state, $\delta_{\text{IM}} = \delta^{\text{ST}} = 0.58X$ and thus $\sigma_{\text{IM}} = 41X$ (mN/m). This analysis indicates that the binding of Mag to the outer

Fig. 6 Mag-induced area change and Mag-induced pore formation. **a** Time course of the change in FI of a DOPG/DOPC (4/6)-GUV at the tip of a micropipette and the change in fractional change in the area of the same GUV during the interaction of 15 μ M Mag/CF-Mag. The normalized FI of AF647 in the GUV lumen (red line, left axis). δ (open triangle, right axis) and the normalized rim intensity due to CF-Mag (green filled triangle, left axis). **(B)** Dependence of δ on Mag surface concentration in the membrane interface, X (mmol/mol). (Blue filled circle) DOPG/DOPC (4/6)-GUV, and (red filled triangle) DOPG/DOPC (3/7)-GUV. **(C)** The comparison between the rate constant of Mag-induced pore formation, k_p (blue filled circle) and the rate constant of constant-tension-induced GUV rupture, k_R , (red filled square) of DOPG/DOPC (4/6)-GUV as a function of σ_{IM} . **a**, **b**, and **c** are reprinted from Karal et al. (2015a) and Hasan et al. (2018a), respectively, with permission from the American Chemical Society

leaflet. Based on this model, one can reasonably infer that a Mag-induced pore has a toroidal structure (see “Effects of membrane tension on the physical properties of lipid bilayers”) since the constant tension in the inner leaflet induces pore formation. It has been suggested that Mag-induced pore is a toroidal pore (Matsuzaki et al. 1996; Ludtke et al. 1996; Yang et al. 2000): in this structure, several α -helical peptides locate at the wall of the toroidal pore formed by bent lipid monolayer, but there is no interactions between these peptides, and hence, the surface of pre-pore wall is composed of headgroups of lipids and peptides (Qian et al. 2008). Therefore, the structure of a toroidal pore is different from the structure of the barrel-stave (or the helix-bundle) type of pore found in ion channels such as acetylcholine receptor, where several α -helices strongly interact with each other (Hille 1992). Experiments using the single GUV method revealed clearly that the k_{FF} of the trans-bilayer movement of fluorescent dye-labeled lipids in a single DOPG/DOPC (4/6)-GUV greatly increases when Mag induces pore formation in a GUV and the value of k_{FF} was greater than that in the presence of tension (Fig. 5b), whereas before pore formation (i.e., only the binding of Mag) k_{FF} does not increase in the same single GUV. This result indicates that this pore has a toroidal structure, because rapid lateral diffusion of the lipid through the combined monolayers in a pore with toroidal structure can enhance the k_{FF} (“Effects of membrane tension on the physical properties of lipid bilayers”) and the lifetime of a pore is much longer than that of a pre-pore (Hasan et al. 2018a). On the basis of these results, the structure of a Mag-induced pore also supports our model describing pore formation.

In a different model of peptide-induced pore formation (Lee et al. 2004; Huang 2009), based on the equilibrium state of the membrane, peptides bind to both leaflets, distorting the lipid hydrocarbon chains and thus decreasing membrane thickness. Above a critical peptide to lipid ratio, pore formation occurs. In contrast, our model described above is a kinetic model of the initial stage of Mag-induced pore formation and allows us to calculate the threshold concentration of AMPs to

leaflet induces a constant tension in the inner leaflet, σ_{IM} , for an extended time. By combining this analysis with the results shown in Figs. 1g and 6a, we can conclude that k_p increases with σ_{IM} (blue circle in Fig. 6c) (Hasan et al. 2018a). For comparison, we plot the rate constant of constant tension-induced rupture of GUV of the same lipid composition, k_R , as a function of σ_{IM} (red solid squares) in Fig. 6c and showed that the σ_{IM} dependencies of both k_p and k_R are similar. This comparison supports the validity of the model that Mag-induced pore formation is induced by stretching of the inner

induce pore formation, which depends on the time scale (Hasan et al. 2018a). This initial stage of pore formation is sufficient to leak the internal contents of vesicles or cells and to remove the membrane potential of the plasma membrane. Consequently, pore formation in bacterial membranes and resulting leakage of substantial amounts of internal contents from cytoplasm can inhibit the growth of or kill bacteria, as described in the “Introduction.”

Effect of trans-bilayer asymmetric lipid packing on Mag-induced pore formation

To elucidate the role of Mag-induced stretching of the inner leaflet in pore formation, a new method has been developed for preparing GUVs with an asymmetric transmembrane lipid distribution, with specific lipids present only in the inner leaflet of the GUV (Hasan et al. 2018a, b). These specific lipids (Y) have a high critical micelle concentration (e.g., lyso-phosphatidylcholine (hereafter LPC), 18:1-NBD-lyso-PE (hereafter NBD-LPE), and NBD-PG) and thus can be easily removed from the membrane. These asymmetric GUVs are prepared as follows (Fig. 7a). First, we prepare DOPG/DOPC/Y (40/(60-x)/x; molar ratio)-GUVs and then remove lipid Y from the outer leaflet by changing the surrounding buffer to a new buffer continuously for a given time (e.g., 1 h) using the membrane-filtering method (Tamba et al. 2011), originally developed for purifying GUVs by removing LUVs, smaller GUVs, and fluorescent dyes. Figure 7b shows a handmade apparatus used for this method. Figure 7c (I) and (II) shows typical CLSM images of a DOPG/DOPC/NBD-LPE (40/59/1)-GUV before and after purification, respectively. The rim intensity of the GUV due to NBD-LPE after purification is approximately half that before purification, indicating that NBD-LPE is removed from the outer leaflet completely to produce DOPG/DOPC/NBD-LPE (40/59/1; inner leaflet)-DOPG/DOPC (40/59; outer leaflet)-GUVs (Hasan et al. 2018a, where the detailed characterization of the asymmetric GUVs is described). The GUVs are spherical with no local shape anomalies, and the number of lipids in the inner leaflet is greater than that in the outer leaflet. Hence, we can reasonably expect that lipid packing in the inner leaflet is larger (i.e., transmembrane asymmetric packing of lipids).

Using these asymmetric GUVs, one can investigate the effect of asymmetric lipid packing on the interaction of AMPs with single GUVs. Figure 7d shows the results of the interaction of Mag with asymmetric PG/PC/LPC (40/(60-x)/x; inner leaflet)-PG/PC (40/(60-x); outer leaflet)-GUVs (Hasan et al. 2018a). With an increase in LPC concentration in the inner leaflet, the rate constant of Mag-induced pore formation (k_p) decreases and Mag-induced increase in δ^{ST} decreases. As discussed in “Mag-induced stretching of lipid bilayers (membrane tension) and induced pore formation,” some

experimental results suggest that the binding of Mag in the outer leaflet induces stretching of the inner leaflet, which may be the main determinant of Mag-induced pore formation. The decrease in Mag-induced stretching of inner leaflet due to the asymmetric lipid packing can explain the results of Fig. 7d reasonably, which supports the above mechanism of Mag-induced pore formation.

Asymmetric transmembrane distribution of lipids in biomembranes is well known. However, the effect of transmembrane asymmetric packing of lipids on function of

Fig. 7 Preparation of GUVs with asymmetric lipid packing and the effect of asymmetric lipid packing on Mag-induced pore formation. **a** A scheme of conversion of a GUV of symmetric lipid distribution to a GUV of transmembrane asymmetric lipid distribution, where lipid Y exists only in the inner leaflet. **b** A picture of handmade apparatus for the membrane filtering method. See Tamba et al. (2011) for the details of the methodology using this apparatus. **c** CLSM images of a DOPG/DOPC/NBD-LPE (40/59/1)-GUV before purification (I) and after purification (II). The bar indicates 20 μm . **d** Dependence of the rate constant, k_p , of Mag-induced pore formation in asymmetric DOPG/DOPC/LPC (40/(60- x)/ x ; inner leaflet)-DOPG/DOPC (40/(60- x); outer leaflet)-GUVs on LPC concentration in the inner leaflet (mol%), x . (Blue filled circle) 62 μM and (red filled triangle) 31 μM Mag. **b** is reprinted from Tamba et al. (2011) with permission from Elsevier. **c** and **d** are reprinted from Hasan et al. (2018a) with permission from the American Chemical Society

peptides and proteins has not been well recognized. One of the reasons is that there was no membrane system whose asymmetric lipid packing is well characterized. Using the new method described above, this effect on various functions in biomembranes would be investigated further in near future.

Effect of mechanical properties on the entry of CF-TP10 into the GUV lumen and its translocation across the lipid bilayer

As described in “[Characteristics of the single GUV method](#),” CF-TP10 can translocate from the outer leaflet to the inner leaflet to reach a steady surface concentration in a GUV and then enter the GUV lumen without pore formation. This symmetric distribution of CF-TP10 without pore formation contrasts greatly with the asymmetric distribution of Mag before pore formation. To elucidate the mechanism of entry of CF-TP10 into the lumen of single GUVs without pore formation, information on the effect of membrane tension due to an external force on the entry of CF-TP10 into the vesicle lumen is important. Figure 8a shows an example of the interaction of 0.50 μM CF-TP10 with a DOPG/DOPC (2/8)-GUV containing smaller GUVs (1–10 μm in diameter) in the mother GUV lumen being aspirated over time at the tip of a micropipette which applies a constant tension of 1.0 mN/m to the membrane (Islam et al. 2017). After starting the interaction, the rim intensity due to CF-TP10 gradually increases and reaches a steady value at 150 s and remains constant until total aspiration of the GUV due to pore formation at 263 s. Initially, there is no fluorescence due to CF-TP10 in the GUV lumen, but after 210 s some fluorescent, small open circles can be observed in the lumen before aspiration of the GUV, which corresponds to the smaller GUVs bound with CF-TP10 in the mother GUV lumen (Fig. 8a (2)), indicating that CF-TP10 enters the GUV lumen before pore formation. Under this condition ($\sigma = 1.0$ mN/m, 0.50 μM CF-TP10), the fraction of entered GUVs, P_{entry} (6 min), is 0.53 ± 0.03 , whereas in the absence of tension, no CF-TP10 enters the lumen

(i.e., P_{entry} (6 min) = 0). These results indicate that a 1.0-mN/m tension increases the rate of entry. Figure 8b shows that low concentrations of CF-TP10 (0.30 μM and 0.50 μM) enter the DOPG/DOPC (2/8)-GUV lumen in the presence of membrane tension and that P_{entry} (t) increases with increasing applied membrane tension. At higher tension (1.5 mN/m), P_{entry} (6 min) of 0.50 μM CF-TP10 apparently decreases, due to the increase in the rate constant of CF-TP10-induced rupture of GUVs. We can simultaneously measure the fractional change in the area of a GUV membrane, δ (t). Figure 8a shows that the length of the cylindrical part of the GUV in the micropipette (i.e., projection length) increases with time during the interaction of CF-TP10 with the GUV until GUV aspiration, indicating that δ (t) increases with time until pore formation in the GUV. The time course of δ (t) is different from that of the rim intensity due to CF-TP10, which is different from that of Mag shown in Fig. 6a (Islam et al. 2017). Before steady binding of CF-TP10 to both leaflets is achieved, one can reasonably expect that the CF-TP10 concentration in the outer leaflet is higher than that in the inner leaflet. This imbalance can induce membrane tension in the inner leaflet, but the value of this tension cannot be obtained because the elastic moduli of lipid monolayer may change due to the binding of various concentrations of CF-TP10 to the membrane.

Several models have been proposed for the entry of CPPs into the lumen of vesicles. One is the “pore model” in which CPPs induce pores in a lipid bilayer, followed by CPP permeation through the pores (Mishra et al. 2008; Ciobanasi et al. 2010; Mishra et al. 2011) and another is the “inverted micelle model” in which CPPs induce an inverted micelle in a lipid bilayer by binding with negatively charged lipids at a stoichiometric ratio, followed by CPP translocation across the lipid bilayer (Berlose et al. 1996; Kawamoto et al. 2011; Swiecicki et al. 2014). We have proposed the “pre-pore model” in which CPPs translocate across the lipid bilayer using transient pre-pores (Sharmin et al. 2016; Islam et al. 2017, 2018). As described in “[Effects of membrane tension on the physical properties of lipid bilayers](#),” pre-pores form in lipid bilayers due to thermal fluctuation, but close rapidly due to large line tension at the pre-pore edge. However, if CPPs in the outer leaflet can bind to the edge of a pre-pore, they stabilize the edge and decrease its free energy (and hence decrease the line tension) by reducing the bending energy and hydrophobic interactions. This stabilization increases the frequency of pre-pore formation and their lifetime. Since the hydrophilic pre-pore has a toroidal pore structure, CPPs bound to lipids in the outer leaflet can diffuse laterally to the inner leaflet through the combined monolayers in a pre-pore (Fig. 4a). This point is clarified in Fig. 8c using the free energy landscape of a pre-pore, where it is assumed that the membrane tension induced by the binding of CPPs to a lipid bilayer is 3 mN/m; furthermore, for simplicity, $B = 0$ and $U_0 = 0$ are used because there is no experimental data for the activation energy in DOPG/DOPC

Fig. 8 Effects of membrane tension on the entry of CF-TP10 into single GUVs. **a** CLSM images of a DOPG/DOPC (2/8)-GUVs containing smaller GUVs due to (1) AF647 and (2) CF-TP10 during the interaction of 0.50 μM CF-TP10. The membrane tension is controlled at 1.0 mN/m. The numbers above each image represent the time (s) after CF-TP10 was started to add. The bar is 20 μm . **b** Effect of tension on the fraction of entered GUVs before pore formation at 6 min, P_{entry} (6 min). Values 0.50 μM (red open square) and 0.30 μM (open triangle) CF-TP10. **c**

Free energy landscape of a pre-pore, $U(r)$, for various line tensions, Γ . **a** 6.0 pN. **b** 12 pN. **c** 18 pN. $U(r)$ is determined based on to Eq. 3 using $\sigma = 3.0$ mN/m, $B = 0$, and $U_0 = 0$. **d** A scheme of binding of TP10 to a lipid bilayer, based on the structure of TP10 determined by NMR spectroscopy. **a**, **b**, and **c** are reprinted from Islam et al. (2017) and Sabrina et al. (2016), respectively, with permission from the American Chemical Society

(2/8)-GUVs. Note that these assumptions do not change the main point of the effect of line tension on the free energy landscape. Curve B corresponds to the line tension (e.g., $\Gamma = 12$ pN) of a pre-pore in a lipid bilayer in the absence of CPPs, whereas curve A corresponds to that in the presence of CPPs (e.g., $\Gamma = 6$ pN). As line tension is reduced, the maximum value of $U(r)$ decreases, and hence, the initial slope of $U(r)$ near $t = 0$ also decreases. Since the fluctuation of thermal force induces pre-pore formation, the smaller the initial slope, the greater the fluctuation of the radii of the pre-pores, and thus the higher the frequency of pre-pore formation and the larger the sizes of the pre-pores.

As described above, the binding of CF-TP10 increases the area of the bilayer, inducing membrane tension. If we apply membrane tension through an external force, the total membrane tension increases further and the total tension is described by the summation of the tension due to the binding of CF-TP10 and the tension due to the external force (Islam et al. 2017). As described in “Effects of membrane tension on the physical properties of lipid bilayers,” an increase in total tension decreases the maximum value of $U(r)$, and hence, the initial slope of $U(r)$ near $t = 0$ also decreases. Therefore, the frequency of pre-pore formation and the sizes of the pre-pores increase with tension, which explains the result shown in Fig. 8b.

In contrast, the presence of a high concentration of cholesterol (chol) in a lipid bilayer suppresses the entry of CF-TP10 (e.g., P_{entry} (6 min) = 0 at concentrations of CF-TP10 at and below 1.0 μM for DOPG/DOPC/chol (2/6/4)-GUVs (Islam et al. 2017). Other experimental results indicate that the high concentration of cholesterol suppresses the translocation of CF-TP10 from the outer leaflet to the inner one in the GUVs, which is the main cause of the decrease in entry of CF-TP10 to these GUV lumens. The effect of cholesterol on the translocation of CF-TP10 can be explained using the free energy landscape of a pre-pore (Fig. 8c) as follows. It is reported that cholesterol increases line tension of a pore in PC bilayer (Karatekin et al. 2003). Curve C corresponds to a line tension in lipid bilayers containing high cholesterol concentration (e.g., $\Gamma = 18$ pN), indicating that the initial slope is very large. As a result, pre-pore formation is suppressed greatly, which prevents translocation of CF-TP10 across the bilayer within a limited time.

As described above, Mag and TP10 behave very differently, perhaps reflecting differences in the conformation and orientation of these peptides. In a lipid bilayer, the C-terminal half of TP10 forms an α -helix (56% helicity) which inserts into the outer leaflet with a tilt angle of 55°, and the other half of the peptide is

unstructured (Fig. 8d) (Fanghänel et al. 2014). In contrast, at the interface of a lipid bilayer, Mag is completely in an α -helix conformation and orients parallel with the membrane surface (Fig. 1c). This difference in structure may allow TP10 to easily bind to a transiently formed pre-pore and translocate across the bilayer. Further studies are required to reveal the mechanistic difference between these peptides.

Conclusion

In this review, we summarized and discussed the present status of our understanding of the effect of membrane tension on the physical properties of lipid bilayers and on the actions of AMPs and CPPs and also their elementary processes and the mechanisms of AMP-induced pore formation and the entry of CPPs into vesicle lumen, focusing on Mag and TP10. Due to complexity of lipid bilayers and their interactions of peptides, we have not yet drawn final conclusions about these mechanisms. For this purpose, we need new experimental techniques to obtain direct evidence of change in physical properties of lipid bilayers and behaviors of peptides in the bilayers. Along with more experimental efforts, further progress in molecular dynamics based simulations is also required. Here we described mainly the interactions of Mag and TP10 with lipid bilayers of PG/PC membranes. It is recognized that there are various modes of action of AMPs and CPPs (Hasan and Yamazaki 2019; Islam et al. 2018). If we consider the diversity of biological systems, these various modes of action of AMPs and CPPs are favorable and strategically correct. Therefore, it is indispensable to examine the modes of action of the other AMPs and CPPs using the various methods described in this review. Moreover, it is also considered that lipid compositions in lipid bilayers affect the action of AMPs and CPPs (Tamba and Yamazaki 2009; Sharmin et al. 2016; Islam et al. 2018). Due to the existence of significant lipid composition diversity in biomembranes, the mode of action of AMPs and CPPs with membranes of other lipid composition needs to be addressed.

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Compliance with ethical standards

Conflict of interest Moynul Hasan declares that he has no conflict of interest. Md. Mizanur Rahman Moghal declares that he has no conflict of interest. Samiron Kumar Saha declares that he has no conflict of interest. Masahito Yamazaki declares that he has no conflict of interest.

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