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Concentration-dependent behavior of nisin interaction with supported bilayer lipid membrane

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Abstract

Nisin is a positively charged antibacterial peptide that binds to the negatively charged membranes of gram-positive bacteria. The initial interaction of the peptide with the model membrane of negatively charged DPPG (dipalmitoylphosphatidylglycerol) was studied by cyclic voltammetry and a.c. impedance spectroscopy. Nisin could induce pores in the supported bilayer lipid membrane, thus, it led to the marker ions $Fe(CN)_6^{3-/4-}$ crossing the lipid membrane and giving the redox reaction on the glassy carbon electrode (GCE). Experimental results suggested that the pore formation on supported bilayer lipid membrane was dependent on the concentration of nisin and it included three main concentration stages: low, middling, high concentration.

Keywords: Nisin; Supported bilayer lipid membrane; a.c. Impedance; Cyclic votammetry; Marker ions

1. Introduction

The interest in forming phospholipid bilayers on solid substrates is to identify a model system that exhibits membrane mimetic behavior like Langmuir–Blodgett (LB) films and black (bilayer) lipid membranes, but with the additional advantages of ease and reproducibility of preparation, long-term stability, and formation on a support that is conducive to surface analysis and is electrically conductive.

The lipid membranes of cells are the sites of biological activity for many natural and synthetic cytolytic peptides. One particular class of peptides includes positively charged amphipathic molecules, which bind to negatively charged lipid membranes in a non-specific way. This class includes a number of toxins and antibacterial peptides, which exercise their lytic action via the formation of non-selective *trans*-membrane pores. Melittin [1–3] and magainin [4], for example, have been studied extensively and have become models for the design of a number of synthetic analogues, amphipathic in nature and positively

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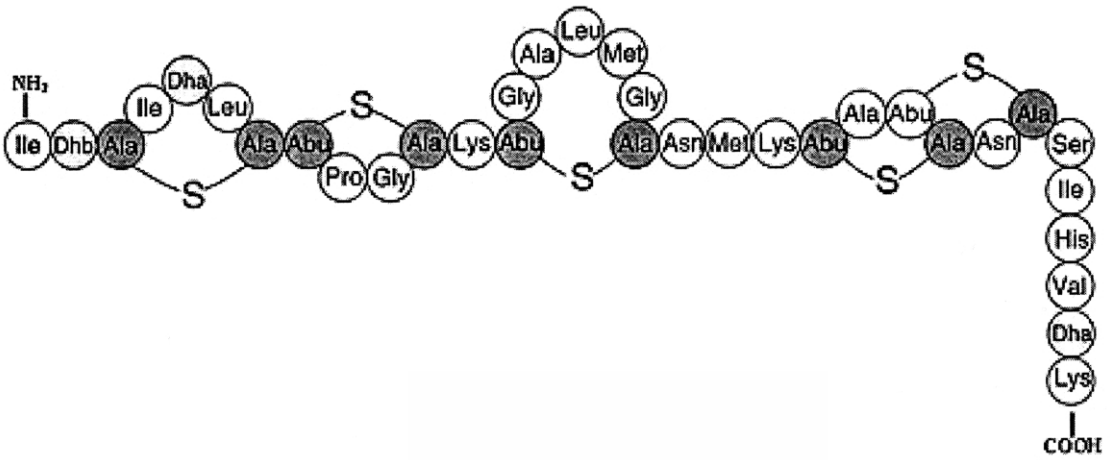

Fig. 1. The primary structure of nisin Z. The dark gray for lanthionine residues and light gray for dehydrated residues in lanthionins. Dha, dehydroalanine; Dhb, dehydrobutyrine, Ala-*S*-Ala, lanthionine; Abu-*S*-Ala, -methyllanthionine.

charged, with enhanced cytolytic or bactericidal activity [5].

Of all the antimicrobial peptides known, only a very few of them are actually allowed to be used either as a preservative in the food industry or as an antibiotic in health care. The 34-residue-long peptide nisin is one example that has been used as a food preservative for a long time. There are two naturally occurring forms of nisin, nisin A and nisin Z, differing in only one residue (His or Asn at position 27) and with very similar bactericidal activities. The peptide shares similar characteristics with other antimicrobial peptides. It is overall positively charged (+4) and its structure possesses amphipathic properties. However, some structural properties make nisin rather special. Nisin is post-translationally modified such that serine and threonine residues are dehydrated to become dehydroalanine and dehydrobutyrine. Subsequently, five of the dehydrated residues are coupled to upstream cysteines, thus, forming the thioether bonds that produce the characteristic lanthionine rings (Fig. 1). The thioether bonds give nisin two rigid ring systems, one N-terminally and one C-terminally located. A hinge region (residues 20–22), which is often found in antimicrobial peptides, separates the ring systems. Nisin kills its target by pore formation in the target membrane. It inhibits the growth of a wide range of Gram-positive microorganisms and also inhibits the germination and/or outgrowth of spores of *Bacillus* and *Clostridium* species. It has been widely used as a preservative in the food industry for a number of years [6,7]. Nisin activity is not well understood. Its microbial action is due to an interaction with the phospholipid membrane [8–11]. But it does not seem to require a specific membrane receptor. The phospholipid composition of the membrane influences nisin activity [11–15]. The molecular details of the pore-forming mechanism of nisin remain unclear. The pore formation in bilayer lipid membrane induced by nisin has been reported already in studies using planar bilayer (black) lipid membrane by detecting the change of conductance of lipid membranes [16–18]. The black lipid membranes are especially sensitive to the modification of electrical properties such as conductance, dielectric constant of the membrane or surface charges. However, the black lipid membranes are only held by the small lateral tension which, unfortunately, can only be varied within a very small limit. Too small tensions cause enormous fluctuation and lead to the rupture of the black lipid membranes. The softness and instability of the bilayer lipid membranes can be overcome by forming the film on a supported solid that exhibits membrane mimetic behavior like Langmuir–Blodgett (LB) films and black (bilayer) lipid mem-

branes, but with the additional advantages of ease and reproducibility of preparation and long-term stability.

In this paper, we used glassy carbon electrode supporting bilayer lipid membrane as a biomimetic membrane model to study the interaction between nisin and the model membrane. The current study was undertaken in an attempt to better understanding the molecular mechanism of the interaction of nisin with cell membranes. We found that nisin interacted with negatively charged lipid membrane by concentration-dependant mode and it made pores in the supported bilayer lipid membrane in its middle concentration stage. Pore formation produced by nisin in the supported bilayer lipid membrane made $Fe(CN)_6^{3-/4-}$ cross the biomembrane easily. Moreover, nisin could remove and break the lipid membrane on the GCE acting as surfactant in its high concentration stage. The mechanism of interaction between nisin and GCE supported bilayer lipid membrane has been predicted.

2. Experimental

2.1. Reagents

DPPG was purchased from Sigma (USA) and used without further purification. Nisin was obtained from Aplin and Barrett (Trowbridge, UK). Other chemicals were of the highest quality as possible as obtained and all chemicals were used without further purification. Pure water obtained by means of a Millipore Q (USA) water purification set was used throughout.

2.2. Apparatus

All cyclic voltammetric experiments were carried out with a model CS-1087 Cypress Systems (Lawrence, USA) connected PC for control and data storage. The apparatus used for electrochemical impedance measurement was composed of Autolab with potentiostat/galvanostat PGSTA30 and the Frequency Response Analysis system software (FRA) (Eco Chemie B.V. Utrecht, The Netherlands), to provide fully computer-controlled electrochemical impedance spectroscopy. Impedance measurement was performed in the frequency range from 0.1 to 10 000 Hz with a signal amplitude of 5 mV. A standard three-electrode cell was employed for the electrochemical experiment. A glassy carbon electrode (diameter 1 mm) was used as the substrate electrode and the counter electrode is a platinum wire. All potentials reported in the paper are referenced to an Ag|AgCl (KCl saturated) electrode.

Impedance measurement was performed in the presence of a 1.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1 M KCl as marker ions at the potential of the system, $E^\circ = 305$ mV in 10 mM phosphate buffer at pH 6.0. All experiments were carried out at room temperature. The buffer and sample solution were deaerated with purified nitrogen for 15 min at least for removing oxygen prior to the beginning of a series of experiments.

2.3. Method for supported bilayer lipid membrane formation

DPPG was dissolved in a chloroform/methanol (2/1) system to give a final concentration of 2 mg ml^{-1}. Prior to supported bilayer lipid membrane formation, a glassy carbon electrode was polished with 1.0, 0.3, and 0.05 μm alumina slurry, respectively, and then sonicated for 1 min in deionized water and acetone successively. Then, the GCE was immersed in the 0.1 M NaOH solution, and the potential was held at 1500 mV for 3 min in order to polarize the electrode. After the electrode was polarized, it was dried under purified nitrogen. Subsequently, a 5-μl aliquot of the lipid solution was dropped onto the surface of the electrode by a microsyringe and the electrode was immediately transferred into the 0.1 M KCl solution. Thirty minutes after transformation of the GCE coated with lipid into the bathing solution, a bilayer lipid membrane was formed on the substrate. Our technique for forming a lipid bilayer membrane on GCE is based on the interaction between the hydrophilic polarized GCE surface and amphipathic lipid which is the same as described before [19,20].

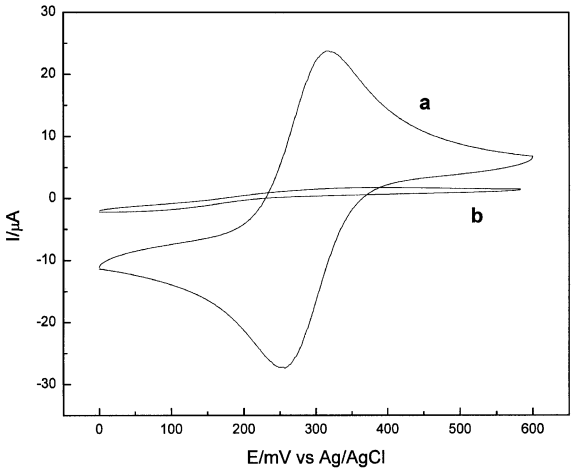

Fig. 2. Cyclic voltammorgrans of GCE in 1 mM $K_3[Fe(CN)_6]$ solution containing 0.1 M KCl, 10 mM phosphate buffer at pH 6.0 (a) bare GCE; (b) GCE coated with lipid membrane. Scan rate, 100 mV s^{-1}.

3. Results and discussion

3.1. Formation and characterization of supported bilayer lipid membrane

The formation of supported bilayer lipid membrane on the GCE surface was judged by capacitance and cyclic voltammorgams before and after the electrode was coated with a bilayer lipid membrane. Fig. 2 shows the cyclic voltammograms of the bare GCE (Fig. 2a) and the GCE coated with lipid bilayer (Fig. 2b) in the presence of $Fe(CN)_6^{3-}$, Comparing Fig. 2b with Fig. 2a, we could find before GCE was coated with bilayer lipid membrane, a pair of well-defined reversible waves of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ couple was obtained. After it was coated, there were no waves observed, a cyclic voltammogram of only the capacitor's charging and discharging, which featured solid substrate supported bilayer lipid membrane electrode, was obtained. This result shows that bilayer lipid membrane self-assembled on GCE dramatically slows down the electron transfer between the solution and the GCE, which is consistent with previous results [21–23].

Impedance spectroscopy is an effective method for probing the features of a surface-modified electrode. Fig. 3 illustrates the results of impedance spectroscopy on a bare electrode (a) and a modified electrode (b) with supported lipid membranes in the presence of 1 mM $Fe(CN)_6^{3-/4-}$, measured at the formal potential of $Fe(CN)_6^{3-/4-}$. The impedance plots of supported bilayer lipid membrane are characterized by a single semicircle in the high frequency domain followed by a Warburg-like mass transfer impedance in the low frequency portion. These results indicate that the equivalent circuit of the GCE supported bilayer lipid membrane system could be represented by a membrane bulk impedance in series with an impedance consisting of the charge transfer resistance, the double layer capacitance, and intramembrane mass transfer impedance [23]. It could be seen that the presence of the bilayer lipid membrane caused the charge transfer resistance to increase and the double layer capacitance to decrease compared with that of the bare GCE, further proving

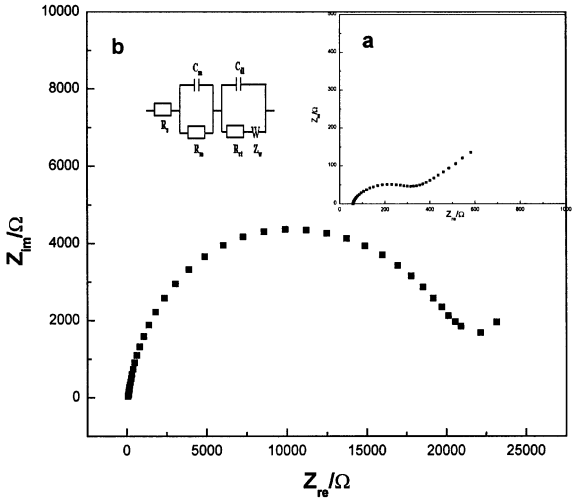

Fig. 3. a.c. impedance spectroscopy in 1 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture containing 0.1 M KCl, 10 mM phosphate buffer, pH 6.0 at (a) a bare GCE; (b) a modified electrode with supported lipid membrane, Frequency range: 0.1–100 000 Hz. Inset: modified Randle's equivalent circuit used to model impedance data in the presence of redox couples. R_s denoting the electrolyte resistance, C_m denoting the lipid membrane capacitance, R_m denoting the lipid membrane resistance, C_{dl} denoting the double-layer capacitance, R_{ct} denoting charge-transfer resistance and Z_w denoting Warburg element, respectively.

the formation of the supported bilayer lipid membrane. It has been known that defects in the lipid bilayer could provide locations where ions may be able to approach more closely to the electrode surface and to accumulate. The presence of a single semicircle in the high frequency domain following a Warburg-like mass transfer impedance in the low frequency portion indicated that the GCE supported bilayer lipid membrane was not sufficiently defect-free to entirely eliminate direct electron transfer between redox species and the bilayer lipid membrane modified GCE.

In order to give more detail information about the impedance property of the membranes, a modified Randle's equivalent circuit (inset of Fig. 3b) was chosen to fit the measured results [19,24] Then we can get the value of Cm as 0.26 u F cm^{-2} by using FRA system software.

To determine the thickness of the lipid membranes, the capacitance was chosen to show this feature. With the capacitance value of the lipid membrane and an estimate of its dielectric constant k, one can estimate the thickness d of the lipid membrane [25]:

$$C_m = \varepsilon_0 k/d$$

where ε_0 is the permittivity of free space (8.85×10^{-14} F cm^{-1}), k is the relative dielectric constant with the hydrocarbon region of BLM (2.05) [26].

From the equation, we calculated that the d-value of the lipid membranes is approximately 7 nm. It is very close to the value of 6.24 nm, which is the thickness of the bilayer of DPPG [27]. It could be concluded that the membranes of DPPG formed on the surface of the GC electrode were bilayer membranes. Obviously, we constructed an ideal model of a biological membrane on the surface of the GC electrode successfully.

3.2. Pore formation in the supported bilayer lipid membranes

In order to investigate the interaction between nisin and supported bilayer lipid membrane, we choose $Fe(CN)_6^{3-/4-}$ as the marker ions. The amperometric response of the marker ions on supported bilayer lipid membrane at different concentrations of nisin was shown in Fig. 4. At first, there was no obvious amperometric response of the marker ions on supported bilayer lipid membrane when the concentration of nisin was below 150 μM (Fig. 4, curve a), except for the characteristic of a cyclic voltammogram of only capacitor's charging and discharging. However, when the concentration of nisin was above 150 μM, an amperometric response of $Fe(CN)_6^{3-/4-}$ complex was found. With increasing the concentration of nisin, the enhanced amperometric response of $Fe(CN)_6^{3-/4-}$ was found gradually (Fig. 4, curve b–f). With the concentration of nisin reaching 0.75 mM, a dramatic enhanced amperometric response of $Fe(CN)_6^{3-/4-}$ was found. Above the value, no obvious enhanced amperometric response of the marker ions was found any more. Increasing the concentration of nisin continuously to 1 mM, the characteristic of amperometric response of $Fe(CN)_6^{3-/4-}$ on bare GCE appeared (Fig. 4, curve g) and a pair of well-defined reversible waves of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ couple was obtained again. It indicated that nisin corrupt the lipid membrane totally,

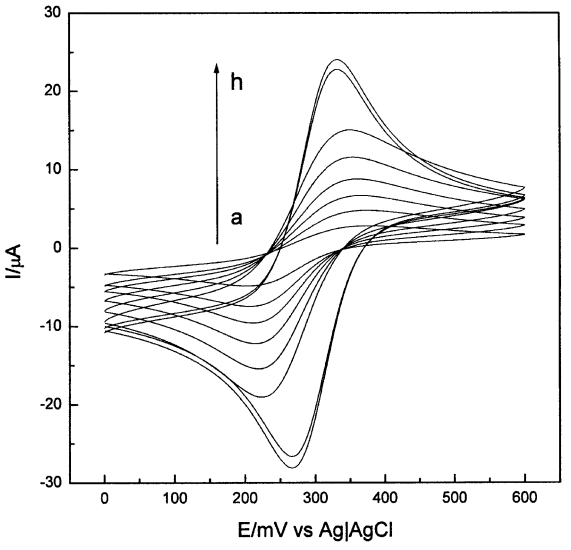

Fig. 4. Cyclic voltammeric responses of 1 mM $Fe(CN)_6^{3-/4-}$ at the GCE coated with BLM with 0.1 M KCl, 10 mM phosphate buffer at pH 6.0 in the presence of different concentration of nisin (a) 0 (b) 150 (c) 250 (d) 300 (e) 500 (f) 700 (g) 750 (h) 1000 μM. Scan rate, 100 mV s^{-1}.

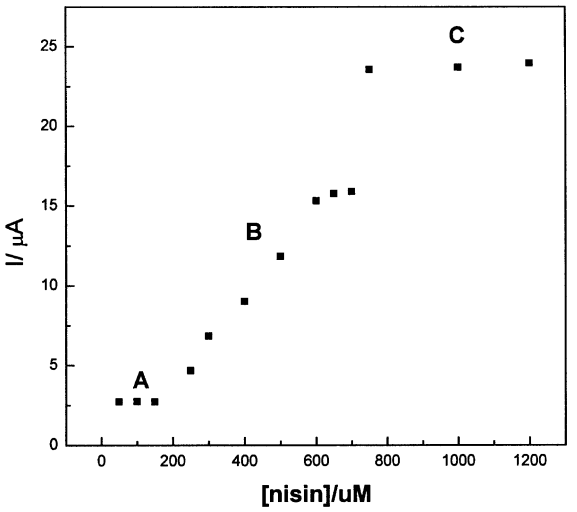

Fig. 5. The plot of the peak current I vs. [nisin]/μM in the presence 1 mM $Fe(CN)_6^{3-/4-}$ in 0.1 M KCl, 10 mM phosphate buffer at pH 6.0, Scan rate, 100 mV s^{-1}.

which was built on the GCE surface previously. The behavior of nisin acted as some kind of surfactant that removed the lipid molecules from the GCE resulted in the marker ions flushing to the GCE without any hindrance. Diao [28] had analyzed the pore formation induced by Ca^{2+} on the supported bilayer lipid membrane. Comparing to Ca^{2+}, nisin has larger molecule volume and more charge. Nisin could contact with much more lipid molecule and give the lipid membrane stronger disturbance to make the surface of the lipid membrane more inhomogeneous and remove the lipid molecule from the GCE finally.

Fig. 5 shows nisin concentration-dependent changes against the reduction peak current of the marker ions. It contained three different domains: low, middling and high concentration domains (stage A, B and C). At low concentration domains, the current value for the reduction peaks showed almost a small flat roof with only capacitor's charging and discharging of supported bilayer lipid membrane. It was because that small amount of nisin molecules were immersed in the 'sea' of large amount lipid molecules and they could not affect the lipid membrane greatly. That was to say, nisin could not induce pores in the lipid membrane so the marker ions had no chance to cross the lipid membrane. At middling concentration domains, the current value for the reduction peaks showed almost a linear relationship with the concentration of nisin in the range 150–600 μM. The peak current initially increases with the concentration of nisin, then increases linearly and finally reaches a plateau. These results may be explained as follows: nisin molecules accumulated and aggregated to form hydrophilic pores of molecular dimensions for marker ions in the hydrophobic membrane surrounding and it led to forming the pores in the supported bilayer lipid membrane. In the same time, the extent of mismatched bilayer regions between nisin-associated and nisin-free lipids was intensified by the electrostatic attraction between nisin and acidic headgroups of lipids. However, at high concentration domains, the current value for the reduction peaks increased sharply to a plateau whose shape was almost the same with that obtained on the bare GCE. It indicated that the lipid membrane on GCE collapsed and was removed out of the substrate by relatively high concentration of nisin except for the remaining small amount of separated lipid molecules on the surface of GCE.

4. Discussion

Nisin activity is depending upon lipids [9,11,13–15,29]. It was demonstrated that nisin associates with negatively charged lipids and the presence of phosphatidylglycerol (PG) is essential for nisin binding, insertion and pore formation [30] This could be due to electrostatic attraction between lysine residues and negatively charged polar lipid heads. This would increase the nisin binding resulting in a direct role of lipids in the nisin insertion and pore formation. Pore formation by nisin is most likely a cooperative process, which is preceded and/or accompanied by aggregation of peptides in the membrane. The shapes of the binding isotherms of nisin in the presence of anionic lipid-containing membranes were indicative of a process where the peptides aggregated once inside the membrane [31]. The fluorescence of the tryptophan variants of nisin displayed self-quenching most likely caused by aggregation of

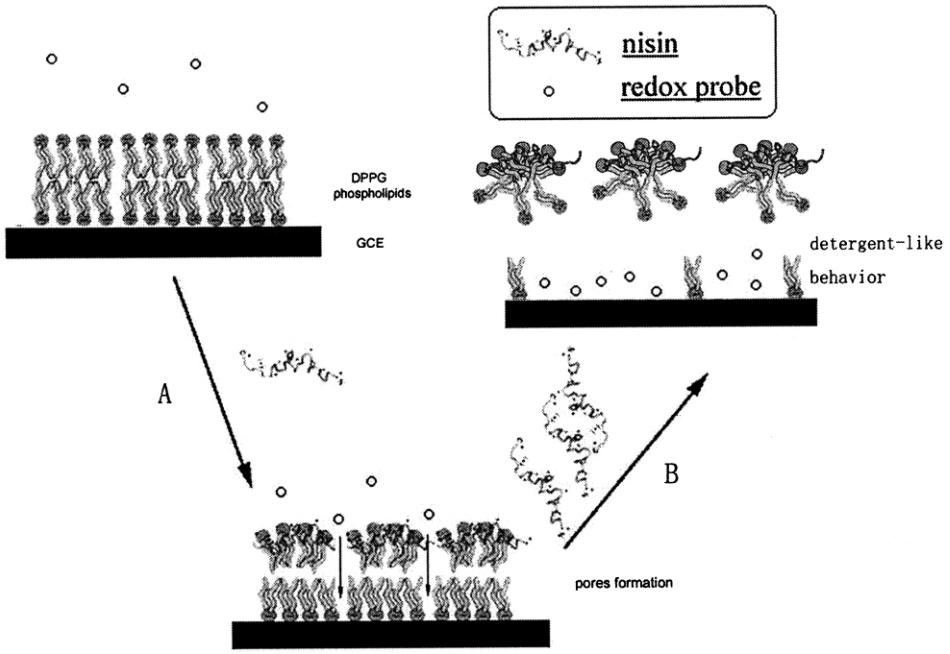

Fig. 6. Schematic illustrations of the antibiotic peptide nisin interacting with GCE supported bilayer lipid membrane. (a) pore formation behavior on supported bilayer lipid membrane at middling concentration of nisin. (b) detergent-like behavior on supported bilayer lipid membrane at high concentration of nisin.

the peptides [27]. Results from planar lipid bilayer studies also suggested that aggregation of nisin monomers occurred in the membrane [29].

The solid supported bilayer lipid membrane has the advantage of ease and reproducibility of preparation, long-term-stability and the possibility to use an electrically conductive support. This kind of model membrane has been widely used to investigate ion channel behavior of some kinds of ions, such as Ca^{2+}, ClO_4^-, etc. [19,20,28].

A number of tentative models have been proposed for the mechanism of pore formation by lantibiotics, but as yet, very little is known about the in vivo situation [32]. The mechanism of interaction between nisin and GCE supported bilayer lipid membrane has been predicted. Nisin first binds with the anionic head group of DPPG via electrostatic interactions. Upon insertion into the membrane, nisin might locally disturb the phospholipids in such a way that it induces a positive interface curvature. Up to this, the negatively charged marker ions could not cross the membrane yet (Fig. 5 stage A). As nisin is a small peptide that can span the membrane only once, it was assumed that several molecules associated with the membrane to form a pore. Whether this aggregation of molecules occurred prior to insertion or in the membrane after insertion was unknown. However, it was also unknown how many monomers were required to form a pore. The peptides were thought to align around a central channel, with the hydrophobic faces towards the lipid bilayer and the hydrophilic faces towards the pore center. In the same time, the marker ions crossed the membrane through the water filled pores to reach the surface of GCE and exhibited the amperometric response. Moreover, the amount of pores increased with increasing the concentration of nisin, so more and more marker ions reached the GCE surface to give the intensified amperometric response and got to be saturated finally (Fig. 5, stage B). The relatively high peptide concentration on the outer leaflet of the bilayer would translate the inner leaflet of the

bilayer connecting with the substrate. Nisin had broken the structure of the underlying lipid membrane with removing the lipid membrane out of GCE. (Fig. 5, stage C). The possible process of interaction between nisin and GCE supported bilayer lipid membrane was shown in Fig. 6.

In conclusion, the interaction of nisin with DPPG membrane has been investigated by using $Fe(CN)_6^{3-/4-}$ couples as electrochemical marker ions. Nisin interacted with negatively charged lipid membrane strongly. Electrostatic attraction between nisin and lipid membrane and self-aggregation of nisin in the presence of membrane caused pore formation and resulted in marker ions crossing the supported bilayer lipid membrane and reaching the surface of GCE. Although the process is somewhat speculative, it does provide a simple physical picture that suggests how nisin may act in medical treatment pertaining to biological membrane.

Acknowledgments

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