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Utilizing zeta potential measurements to study the effective charge, membrane partitioning, and membrane permeation of the lipopeptide surfactin ☆

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ABSTRACT

The effective charge of membrane-active molecules such as the fungicidal lipopeptide surfactin (SF) is a crucial property governing solubility, membrane partitioning, and membrane permeability. We present zeta potential measurements of liposomes to measure the effective charge as well as membrane partitioning of SF by utilizing what we call an equi-activity analysis of several series of samples with different lipid concentrations. We observe an effective charge of −1.0 for SF at pH 8.5 and insignificantly lower at pH 7.4, illustrating that the effective charge may deviate strongly from the nominal value (−2 for 1 Asp, 1 Glu). The apparent partition coefficient decreases from roughly 100 to 20/mM with increasing membrane content of SF in agreement with the literature. Finally, by comparing zeta potentials measured soon after the addition of peptide to liposomes with those measured after a heat treatment to induce transmembrane equilibration of SF, we quantified the asymmetry of partitioning between the outer and inner leaflets. At very low concentration, SF binds exclusively to the outer leaflet. The onset of partial translocation to the inner leaflet occurs at about 5 mol-% SF in the membrane. This article is part of a Special Issue entitled: Interfacially Active Peptides and Proteins. Guest Editors: William C. Wimley and Kalina Hristova.

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1. Introduction

We are interested in the mechanisms that ensure high fungicidal activity, sensitivity, and synergy of lipopeptides of the surfactin, fengycin, and iturin families produced by *Bacillus subtilis* QST713. This strain is successfully used for crop protection against a variety of fungal pathogens and there is strong evidence that the mode of action involves the permeabilization of the target cell membrane [1–5]. One of the key properties governing the behavior of these lipopeptides is their charge. Charged molecules are much more soluble and usually not spontaneously membrane-permeant. On the one hand, asymmetric insertion of an impermeant additive into the membrane causes "bilayer couple" effects that give rise to bending stress and, possibly, transient mechanical failure of the membrane [6–8]. On the other hand, the initiation of membrane pores may require a lipopeptide to reside in both membrane leaflets and may thus be kinetically hindered for impermeant peptides [1,9].

We describe a new strategy to conduct and evaluate zeta potential (ζ) measurements that accounts for partitioning effects and sheds light on the effective charge and membrane permeation of peptides and ionic surfactants. Measurements of electrophoretic mobility and electrostatic models have provided a wealth of insight into the binding of proteins, peptides, and small molecules to lipid membranes [10]. McLaughlin and co-workers showed early on [11] that zeta potentials of vesicles containing anionic lipids can be described by the Stern equation if one allows for a specific adsorption of certain ions to the charged surface. Comparing measurements of electrophoretic mobility (yielding ζ) with the binding of an anionic dye to the vesicle surface, these authors have also established that zeta potentials of vesicles can be used to derive surface potentials if one assumes the plane of shear to be 2 Å away from the surface [11].

These fundamental insights have permitted studying the electrostatic binding of peptides, drugs, ions, and other additives to membranes using zeta potential measurements [12]. Zeta potential measurements also revealed that calmodulin inhibitors can induce the release of electrostatically anchored proteins from the membrane by lowering the potential of cationic membrane patches [13]. The traditional approach to interpret zeta data in terms of membrane partitioning has been to measure ζ as a function of the additive concentration at fixed lipid content. The resulting curve is fitted by a Langmuir isotherm [14–16] or a Langmuir–Hill curve [17] or alternatively, transformed to ζ versus square root of concentration for a linear fit [18]. The apparent partition coefficient or binding constant of an additive is thus derived

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based on its effective charge and additional, more or less physically meaningful parameters. Matos and coworkers compared such results with those of alternative partitioning experiments and found good agreement in most but not all cases [14,15,19]. Klasczyk et al. [20] obtained important, qualitative insight into metal ion binding to membranes but restrained themselves wisely from a quantitative evaluation, given the substantial experimental errors. This classical approach to interpret zeta data is limited by the problem that the effective charge number of a membrane-bound molecule may be affected by (de) protonation effects induced by intra- and intermolecular interactions and specific counterion and dipole effects that make it differ from the nominal value and render it hard to predict or measure. Such partial charge compensation has also been reported for SF before [21].

Since the surface potential affects the local concentration of an ionic surfactant or peptide at the membrane surface and, hence, the apparent partition coefficient from bulk, membrane binding data can provide information about the effective charges of membrane additives as well. A good example is the fit of isothermal titration calorimetry data on the basis of the Gouy–Chapman model [22–24]. Although this model makes a number of non-trivial assumptions, it was found to represent the binding isotherms astonishingly well. However, the effective charge corresponding to the best fit may deviate from the nominal charge. Insights into the charge state of membrane additives have also been obtained from NMR measurements detecting their effects on the orientation of neighboring lipid head groups (molecular voltmeter concept) [25].

We demonstrate that one can avoid any assumptions regarding the effective charge or partitioning model by what we refer to as an equiactivity analysis. It is based on quantifying how much more of an additive is needed to induce the same effect or membrane activity (e.g., induce a zeta potential of −20 mV) at higher lipid concentration. To our knowledge, this robust and largely model-independent approach has not been used so far to interpret zeta potential data. It is, however, very well established for fluorescence data from different dyes [26–28], leakage data [29,30], and phase transitions [31]; for a review see [32]. The only assumption it requires is that the observable is unequivocally related to the membrane composition represented, for example, by the additive-to-lipid mole ratio in the membrane, R_b. As a result of this procedure, one obtains the zeta potential as a function of R_b and, in turn, the effective charge numbers of additive and lipid. For additives with moderate apparent partition coefficients, the method provides also a model-free partitioning isotherm and, hence, composition-dependent apparent partition coefficient.

Another very interesting feature of zeta potential measurements is that they report, virtually exclusively, the concentration of peptide or surfactant in the outer leaflet of the liposomes. Comparing the local peptide or surfactant concentration in the outer leaflet after an addition with that after artificial transmembrane homogenization provides valuable insight into its membrane asymmetry and concentration-dependent membrane permeability.

2. Materials and methods

2.1. Materials

Large unilamellar vesicles were made from 1-palmitoyl-2-oleoyl-3-sn-glycero-phosphatidylcholine (POPC), which was a kind gift from Lipoid GmbH, Ludwigshafen (Germany). The surfactin fraction of the lipopeptides produced by *Bacillus subtilis* QST713 was kindly provided by Bayer CropScience, Davis CA.

All samples were made using Millipore water for preparing Tris buffer, 10 mM, including 100 mM NaCl and adjusted to pH 7.4 or 8.5. These materials were purchased from Sigma (Oakville, ON) or BioShop Canada Inc. (Burlington, ON) in the highest available purity.

Large unilamellar liposomes with a diameter of approximately 100 nm were produced by extrusion as described [1]. Briefly, an appropriate amount of lipid dissolved in chloroform was dried to a thin film by a gentle stream of nitrogen, followed by the exposure to vacuum overnight. Then, the appropriate amount of buffer was added, the lipid dispersed by vortexing, and the sample homogenized by 8 freeze-thaw cycles. Extrusion was done 15 times through Nuclepore polycarbonate filters of 100 nm pore size in a LIPEX Extruder (Northern Lipids, Burnaby BC) at room temperature.

The liposome size was monitored by dynamic light scattering using a Malvern Nano ZS. Previous phosphorus assays indicated that a gravimetric determination of the lipid has to take into account one water to remain bound per lipid (i.e., an effective molar mass of 778 g/mol POPC) and that the lipid concentration is essentially unchanged upon extrusion.

2.2. Zeta potential measurements

Measurements were made in a Malvern Nano ZS zeta sizer based on dynamic light scattering. The system works according to the PALS (phase analysis light scattering) principle, and the data are automatically evaluated on the basis of the Smoluchowski equation (the particle size of ≈100 nm is much larger than the Debye length, ≈1 nm). The sample was thermostated to 25°C by a built-in Peltier device. Measurements were made in standard disposable cuvettes using Malvern's dip cell.

Typically, a series of samples was prepared showing a constant lipid concentration and increasing concentrations of the lipopeptide. After adding appropriate amounts of a stock dispersion of lipid vesicles into a lipopeptide solution of the desired concentration, a sample was incubated for 1 h at 25°C. Then the zeta potential was measured to assess the state without additional equilibration procedures. Subsequently, transbilayer equilibration of the surfactant was realized by heat treatment, i.e., heating the sample under nitrogen to 65°C for 1 h, followed by cooling back to the experimental temperature, 25°C. There is good evidence that the enhanced temperature stimulates the flip-flop of membrane-bound surfactants across POPC bilayers, including ionic ones [23,33]. After the heat treatment, the zeta potential was measured again.

Each measurement was done in triplicate and the standard deviation of the results is shown as error bars in Fig. 1. It should be noted that the instrument may suffer from occasional outliers or systematic errors that build up and may cause biased yet reproducible and mutually consistent readings. Such effects may arise from deposits on the electrodes or contacts of the dip cell, air bubbles, or other problems (see also [17]). To ensure the validity of the data, a zeta standard was measured every 30 min. When the results showed poor reproducibility, unusual scatter compared to others in the same curve, or turned out to provide a poor resolution of $\zeta(c_S)$, additional samples were produced during the measurement to provide better statistics.

3. Theory

To model the experimental data, we need to derive a relationship between zeta potential and peptide content on the liposome surface. Charged head groups of the peptide (here, subscript SF for surfactin) and, in the general case, lipid on the surface of a liposome give rise to a surface charge density, σ [18]:

$$\sigma(X_b) = \frac{e_0 \cdot [X_b z_{SF} + (1-X_b) z_L]}{X_b A_{SF} + (1-X_b) A_L} = e_0 \frac{z_L + R_b z_{SF}}{A_L + R_b A_{SF}}. \quad (1)$$

Here, z_{SF} and z_L represent the signed charge numbers of peptide and lipid and R_b and X_b denote the mole ratio and mole fraction of the peptide within the membrane, respectively. Then, $1 - X_b$ gives the mole fraction of lipid (the mole fractions of lipid and peptide in a binary system add up to 1). Hence, the numerator of Eq. (1) represents the weighted average of the electrostatic charge per lipid molecule on the

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