Poisson-Boltzmann Calculations

To address the issue of the relative stability of anionic lipids in each leaflet of the membrane we have turned to electrostatic calculations. Interactions with the charged silica substrate can be described in a mean-field approximation by the Poisson-Boltzmann equation.[1]

$$\nabla \cdot [\epsilon(\mathbf{r})\nabla\phi(\mathbf{r})] - \frac{\phi(\mathbf{r})}{\lambda^2(\mathbf{r})} = -4\pi\rho(\mathbf{r}),$$

where φ, ϵ and ρ are the electrostatic potential, dielectric constant, and fixed charged density at position r, respectively. The equation also includes a concentration (c)-dependent Debye screening length, λ ~ 3.04/sqrt(c) (in bulk water with dielectric constant 78.6)[2], which describes the exponential decay of electrostatic potentials due to screening by salts or electrolytes.

We have carried out numerical solutions to Eq. 1, using the complete supported membrane geometry illustrated in Fig.2A of the main text. The model consists of a 40 Å thick membrane made up of a 25 Å thick hydrocarbon core[3] of dielectric constant 2 with no Debye screening. This is surrounded by two 7.5 Å thick interfacial regions that contain polar head groups and water molecules which can be considered to be at least as polarizable as bulk water.[4] The membrane is surrounded by a symmetric 1:1 electrolyte, with water between the silica and the membrane is assumed to be bulk like. The silica substrate is a low dielectric (4.5)[2] insulator, (50 Å thick) that is coated with a periodic lattice of negative silanol charges 1.4-3.0 Å above the silica-water interface, and placed 10 or 15 Å below the lower membrane interface. We represent an infinite 2D lattice of silanol charges with 49 explicit atoms (7×7) in this periodic box (corresponding to a charge density of -1e per 30.25 Å^2).

The PBEQ module of the CHARMM version 32b2 program[5] was used for all calculations. The non-linear Poisson-Boltzmann equation[6] was solved with an under-relaxation algorithm.[7] The system was placed in a 38.5 ×38.5 ×175Å^3 box (grid spacing 0.25) with periodic boundary conditions imposed. Results were found to be invariant to small changes in the lattice size and grid dimensions. Charges on the silica surface have been placed at varying distances of 1.4-3.0 Å above a silica slab, with results exhibiting almost no sensitivity. Each charge is represented by a single oxygen atom, for simplicity, with associated Born radius.[8] A water-sized (1.4 Å) reentrant probe was used for the assignment of dielectric constants. Average potentials (expressed as kcal/mol for a positive test charge) at each interface of the membrane are reported within the range 16-22 Å, above or below the membrane center, based on head group P atom distributions typical of POPC or POPE bilayers.[9] Potentials varied by ~0.1 kcal/mol within this range, and by ~0.03 kcal/mol with lateral position (parallel to the membrane surface) thus allowing for straightforward averaging.

Cholera toxin (B_5 subunit) binding analysis

The question to be addressed is whether, at equilibrium, the concentrations of CTB and GM1 in the leaflet asymmetry experiments are such that the amount of CTB bound will depend on the GM1 concentration. What is observed experimentally is a higher concentration of CTB bound to the bilayer regions. If concentrations are in a regime where CTB binding depends on receptor concentration, then this result would strongly suggest an enrichment of GM1 into the outer leaflet of the bilayer region.

For estimates of binding affinities (and cross-linking, i.e., binding of CTB to multiple GM1 molecules), we will use the results of Lauer et al. (*Biochem.* **41** (2002) 1742). In order to account for uncertainties, a range of affinities for the monovalent binding of FITC-CTB (hereafter, just CTB) to GM1 will be used, from $1x10^7$ M^{-1} to $8x10^7$ M^{-1}.

To perform the analysis, we follow the procedure put forth in Lauer et al., and consider the following set of equations (Equations 3 through 8 of Lauer et al.). In their notation, the concentration of GM1 is referred to as "receptor", or R, and the concentration of CTB is referred to as C. For conservation equations, one has

$$R_T = R + B_1 + B_2 + B_3 + B_4 + B_5 \tag{10}$$

$$C_T = C + f \cdot (B_1 + B_2 + B_3 + B_4 + B_5) \tag{11}$$

where R_T is the total receptor (GM1) concentration expressed in number of molecules per cm^2 of area, R is the concentration of "free" GM1 molecules/cm^2 (i.e., GM1 molecules not bound to a CTB), B_i is the number of CTB-GM1 complexes of valency i (i.e., B_i has i GM1 molecules bound to a CTB molecule) in number of complexes per cm^2 of area, C_T is the total CTB concentration expressed, for convenience in nM, and f is a factor depending on experimental geometry that converts surface concentrations of molecules/cm^2 to volume concentrations of nM.

In our case, we can calculate f as follows. The surface-bound molecules are in a concentration of #/cm^2, we assume the total surface area to be 4 cm^2, yielding a total number of molecules, N, to be (#/cm^2)(4 cm^2). Then, N is converted to nM by multiplying by (1 mole/$6.023x10^{23}$ molecules), dividing by the volume (assumed to be 3 ml in our case) and converting ml to liters. The final conversion factor is f = $2.215x10^{-12}$.

As explained in Lauer et al., one may express the values of B_i in terms of C, R and the equilibrium constants K_1 and K_{xl}, where K_1 is the fundamental association constant and K_{xl} is the surface aggregation or cross-linking constant (K_{xl} is K_2 of Lauer et al.). Thus, $B_1 = 5K_1CR$, $B_2 = (4/2)K_{xl}RB_1$, $B_3 = (3/3)K_{xl}RB_2$, $B_4 = (2/4)K_{xl}RB_3$, and $B_5 = (1/5)K_{xl}RB_4$. These expressions account for the degeneracy factors associated with having five binding sites on each CTB molecule. Thus, for example, if K_1 is defined as the ratio of the fundamental rate constants for the binding/release of a single domain of the CTB molecule and a GM1 molecule, then the equilibrium relation involving B_1 can be written as

$$K_1 = \frac{B_1}{5C \cdot R} \tag{12}$$

where the factor of 5 accounts for the presence of five empty binding sites on each CTB molecule. Likewise, the equilibrium for the initial cross linking step (conversion of B_1 to B_2) can be described as

$$K_{xl} = \frac{2B_2}{4B_1 \cdot R} \tag{13}$$

recognizing the singly bound complex retains four open sites for additional receptor binding, while the doubly bound complex has two sites for receptor release. Similar considerations yield the remaining relationships.

Continuing to follow Lauer et al. by denoting $X = K_{xl}R$, one obtains,

$$B = B_1 + B_2 + B_3 + B_4 + B_5 = K_1 CR(5 + 10X + 10X^2 + 5X^3 + X^4) \tag{14}$$

where B is the total amount of bound CTB (in molecules/cm^2), which is the quantity measured experimentally. Then, from Equations (10) and (11),

$$R_T = R + K_1 CR(5 + 20X + 30X^2 + 20X^3 + 5X^4) \tag{15}$$

and

$$C_T = C + f \cdot K_1 CR(5 + 10X + 10X^2 + 5X^3 + X^4) \tag{16}$$

where f is as calculated above. Combining these equations to eliminate C,

$$R_T = R + \frac{K_1 C_T R(5 + 20X + 30X^2 + 20X^3 + 5X^4)}{1 + f \cdot K_1 R(5 + 10X + 10X^2 + 5X^3 + X^4)} \tag{17}$$

which is an expression of one unknown (R). An Igor macro was written to calculate the function g(R), where

$$g(R) = R_T - R - \frac{K_1 C_T R(5 + 20X + 30X^2 + 20X^3 + 5X^4)}{1 + f \cdot K_1 R(5 + 10X + 10X^2 + 5X^3 + X^4)} \tag{18}$$

and the zeros of g(R) were found numerically (using the "findroots" command of Igor) for fixed values of K_1, K_{xl}, C_T and R_T. This process determines R that solves Equation (17). Once R is determined, then C can be found from Equation (16), and with both C and R known, B can be found from Equation (14), or alternatively calculated as $(C_T\text{-}C)/f$.

In performing these calculations, the values used for K_1, K_{xl}, C_T and R_T were chosen as follows. Values of R_T were chosen to correspond to GM1 concentrations ranging from 0.1 mole% to 4

mole%. For example, for GM1 present at 1 mole%, $R_T = 1.6\text{x}10^{12}$ molecules/cm^2 (see above, just after Equation (3)). Preparation of CTB solution involves using 60 µl of 0.5 mg/ml concentration stock solution, and diluting to 3 ml. CTB molecular weight is 58000 g/mole (Lai, *J. Biol. Chem.* **252** (1977) 7249). Using these numbers, we obtain

$$[CTB] = \left(\frac{\left(\frac{0.5\ mg}{0.001\ l}\right)\left(\frac{1\ g}{1000\ mg}\right)(6\times10^{-5}\ l)}{0.003\ l} \right)\left(\frac{1\ mole}{58000\ g}\right) = 1.7\times10^{-7}\ M \qquad (12)$$

so C = 170 (nM). K_{xl} was taken from Lauer et al. to be $1.1\text{x}10^{-12}$ cm^2. A range of K_1 values are discussed in Lauer et al, so calculations were done for K_1 ranging from 0.01 nM^{-1} to 0.08 nM^{-1} (i.e., $1\text{x}10^7$ M^{-1} to $8\text{x}10^7$ M^{-1}).

For each set of fixed values, B, the bound CTB concentration in molecules/cm^2 was calculated. Results are shown in Figure 2.

There are two important results from this set of calculations. First, as apparent in Figure 2, in the concentration regimes where these experiments were performed (e.g., GM1 concentrations of 1 mole%, potentially enriched up to 2 mole% in the bilayer region), the amount of bound CTB varies approximately linearly with GM1 concentration (only slight deviation from linearity is observed for even the smallest value of the fundamental affinity constant). Second, taking the cross-sectional area of a CTB molecule to be 25 nm^2 (corresponding to a circle of radius 2.8 nm), a full close-packed monolayer of CTB would correspond to a surface coverage of ≈$4\text{x}10^{12}$ molecules/cm^2, so the predicted coverages at 1 mole% GM1 are about 0.3 of a monolayer, while at 2 mole % GM1, the coverage is about 0.6 monolayer (exact values depend on the choice of K_1). These estimates of surface coverage are quite consistent with the observed coverages inferred from elipsometry data, lending further support to the analysis provided here.

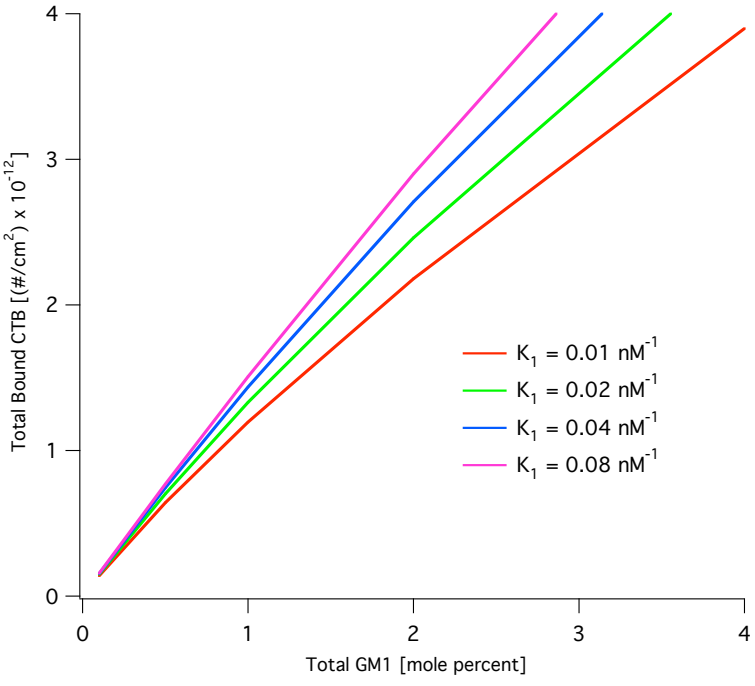

Figure 2. Concentration of CTB bound to the surface as a function of GM1 concentration in supported membrane for a range of K_1 values.

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