## Instability Phenomena in Lipid Bilayers and Lipid-DNA Complexes

Thesis submitted in partial fulfillment of the requirements for the degree of "DOCTOR OF PHILOSOPHY"

by

Yotam Yosef Avital

Submitted to the Senate of Ben-Gurion University of the Negev

Be'er-Sheva October, 11, 2015

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Approved by the advisor, Prof. Oded Farago				
Signature	Date			
Approved by the Dean of the Kreitman School of Advanced Graduate Studies				

Signature Date

Be'er-Sheva October, 11, 2015 This work was carried out under the supervision of Prof. Oded Farago In the Ilsa Kats Institute of Nanoscale Science and Technology and in the Department of Biomedical Engineering, Faculty of Engineering

### Research-Student's Affidavit when Submitting the Doctoral Thesis for Judgment

I <u>Yotam Yosef Avital</u> whose signature appears below, hereby declare that (Please mark the appropriate statements):

\_\_\_\_\_ I have written this Thesis by myself, except for the help and guidance offered by my Thesis Advisors.

\_\_\_\_\_ The scientific materials included in this Thesis are products of my own research, culled from the period during which I was a research student.

\_\_\_\_\_ This Thesis incorporates research materials produced in cooperation with others, excluding the technical help commonly received during experimental work. Therefore, I am attaching another affidavit stating the contributions made by myself and the other participants in this research, which has been approved by them and submitted with their approval.

Date October, 11, 2015 Student's name <u>Yotam Yosef Avital</u> Signature: \_\_\_\_\_

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#### Abstract

Lipid bilayers membranes, positioned between two solutions, are the barrier of the cell and its internal components (organelles). Membranes define the cell limit and, by allowing the existence of two different solutions in close proximity, enable their functions. Membranes participate in various biological processes such as cell-cell adhesion, controlled ions transfer, and signal transduction. The membrane role in these processes is not necessarily carried by the membrane as a bulk, but rather via proteins incorporated into it, or a fraction of the consisting lipids that have a unique property (e.g., charged).

Biological membranes experience forces that may cause the membrane instabilities, e.g., pore formation, buckling, and membranes fusion. Such instabilities take place in processes such as endocytosis or cytokinesis, which are part of the cell function, or through an external damaging factor, such as pore-forming toxins. A physical understanding of membrane instabilities is of interest as it might be applied in many field of research and medicine e.g., causing membrane rupture in cancer cells. Membrane instabilities are also of interest in the context of artificial membranes since they dictate working and experimental parameters. Furthermore, they may have a practical use, e.g., drug release.

The mechanical properties of membranes are traditionally studied through the framework of Helfrich's effective Hamiltonian. While this model has proved to be very useful for stable membranes, instabilities are not well understood. Many insights from the Helfrich Hamiltonian are gained for membranes that are stable, namely, weakly undulating intact membranes. This thesis discussed instabilities in membranes outside of this regime, that is, buckled or ruptured membranes. Computer simulations allow for such studies, but due to their nature, simulations of lipid membranes require large amount of water molecules which. Due to the high cost (in computation terms) associated with water molecules, the time and length scales of such simulations are limited. High coarse-grained computer simulations, such as those used in this thesis, treat the aqueous region implicitly (via effective hydrophobic interactions). This allows us to overcome the limitations of "traditional" atomistic simulations.

Chapter 2 discusses instabilities of membranes under positive and negative surface tension, chapter 3 discusses instabilities in charged membranes, and chapter 4 discusses a lipid-DNA complex degradation. The thread line of the results reported in this thesis is the instabilities of lipid-based systems, reported in all three chapters. However, even though the reported instabilities can be roughly divided into pore formation and strong undulations or buckling, the cause of the instabilities and their "fine details" have some important differences. Studying such instabilities allows for a better understanding of the biological, physical, and chemical mechanisms that involve lipid membranes. This, in turn, may be exploited for practical purposes (e.g., drug delivery), where such instabilities may be essential.

Chapter 2 presents a simulation study of bilayer membrane response to the application of a negative (compressive) mechanical tension. We used the simulation results to develop a simple free energy model for membranes under negative tension. Instabilities of membranes due to compressive surface tension is size dependent. It is known that negative tension destabilizes the long wavelength undulation modes of giant vesicles, but such tension can be sustained when small membranes and vesicles are considered. The negative tension simulation results reveal two regimes: (i) a weak negative tension regime characterized by stretching-dominated elasticity and (ii) a strong negative tension regime featuring bending-dominated elastic behavior. This resembles the findings of the classic Evans and Rawicz micropipette aspiration experiment in giant unilamellar vesicles (GUVs) [E. Evans and W. Rawicz, Phys. Rev. Lett. 64, 2094 (1990)]. However, in GUVs, the crossover between the two elasticity regimes occurs at a small positive surface tension, while in smaller membranes it takes place at a moderate negative tension. Another interesting observation concerning the response of a small membrane to negative surface tension concerns the relationship between the mechanical and fluctuation tensions, which are equal to each other for non-negative values. When the tension decreases to negative values, the fluctuation tension  $\gamma$  drops somewhat faster than the mechanical tension  $\tau$  in the small negative tension regime, before it saturates (and becomes larger than  $\tau$ ) for large negative tensions. The bending modulus exhibits an "opposite" trend. It remains almost unchanged in the stretching-dominated elastic regime and decreases in the bendingdominated regime. Both the amplitudes of the thermal height undulations and the projected area variations diverge at the onset of mechanical instability.

In chapter 3, the same coarse-grained molecular model is used for studying the elastic properties of charged membranes in solutions of monovalent and pentavalent counterions. The simulation results of the two cases reveal trends opposite to each other. The bending rigidity and projected area increase with the membrane charge density for monovalent counterions, while they decrease for the pentavalent ions. These observations can be related to the degree that the counterions screen the lipid. While the monovalent counterions only weakly screen the Coulomb interactions, which implies a repulsive Coulomb system, the multivalent counterions condense on the membrane and, through spatial charge correlations, the overall effective interactions due to the charged lipids become attractive. The differences in the elastic properties of the charged membranes in monovalent and multivalent counterion solutions are reflected in the mechanisms leading to their mechanical instability at high charge densities. In the former case, the membranes develop pores to relieve the electrostatic tensile stresses, while in the latter case, the membrane exhibits large wavelength bending instability.

In chapter 4, we study the physics of complexes (supermolecular assemblies) of cationic membranes and DNA molecules. Such lipid-DNA complexes (lipoplexes) have attracted much interest as gene delivery vectors because they are non-pathogenic and they are self-assembled under conditions of thermal equilibrium. In the chapter, we focus on the driving forces governing the release of DNA molecules from a lipoplex trapped inside an endosome. The release of DNA molecules is thought to be the limiting stage in the transfection process, which is viewed as a three-stage process: (i) endocytosis, (ii) lipoplex breakdown, and (iii) DNA release followed by gene expression. As successful transfection requires lipoplex degradation, it tends to be hindered by the lipoplex thermodynamic stability; nevertheless, it is known that the transfection process may proceed spontaneously. The relevant results chapter in this thesis uses a simplified model to study the thermodynamic driving forces governing transfection. It is demonstrated that after endocytosis [stage (i)], the lipoplex becomes inherently unstable. This instability, which is triggered by interactions between the cationic lipids of the lipoplex and the anionic lipids of the enveloping plasma membrane, is entropically controlled involving both remixing of the lipids and positional entropy gain of counterions initially confined to the surfaces. The detailed calculation in this chapter shows that the free energy gain during stage (ii) is approximately linear in  $\Phi_+$ , the mole fraction of cationic lipids in the lipoplex. This free energy gain,  $\Delta F$ , reduces the barrier for fusion between the enveloping and the lipoplex bilayers, which produces a hole allowing for DNA release [stage (iii)]. The linear relationship between  $\Delta F$  and the fraction of cationic lipids explains the experimentally observed exponential increase of transfection efficiency with  $\Phi_+$  in lamellar lipoplexes.

Chapter 5 summarizes this thesis. In this chapter, the similarities and differences between the instabilities in the different systems are underlined. Insights from this comparison are concluded with a possible future simulation study.

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### Chapter 1

## Introduction

### 1.1 Physical Understanding of Lipid Membranes

Lipid bilayer membranes allow biological systems to separate two chemical solutions which are of distinctly different chemical composition. The most obvious example of that is the cell plasma membrane which separates the (acidic) cytoplasm from the external (alkaline) environment. Lipid membranes are formed spontaneously due the hydrophobic nature of the lipid tails that are shielded from water molecules when the membrane is formed [1, 2]. Composed of two monolayers that face each other and expel water molecule from the aliphatic tails, bilayer membranes may assume several structures: planer, cylindrical, and spherical. Furthermore, the bilayer phase occupies a small part of a rather complex phase diagram which includes further morphologies [3]. The structure of the lipid bilayer is controlled by the characteristics of its constituting lipids e.g., tail length, tail rigidity, and hydrophilic head size [1]. At room temperature, biological membranes are fluid: the lipids tails are disordered and they can diffuse within the monolayer. Also in fluid membranes, lipids can *slowly* transfer between the two monolayers in a process known as "flip-flop." In their fluid state, membrane might develop instabilities that hinder their function; the two most commonly known are buckling and pore formation. Understanding membrane instabilities is important as they commonly occur when the membrane is participating in a far from equilibrium processes. In addition, there are cases, such in drug and gene delivery applications, where—upon a certain condition (e.g., endocytosis by the target cell)—it is desirable to trigger instabilities.

Theoretical studies of bilayer membranes commonly use Helfrich's effective Hamiltonian

which describes 2D manifolds, such as membranes, in 3D space [4]

$$\mathcal{H} = \int_{A} dS \left[ \sigma_{0} + \frac{1}{2} \kappa_{0} \left( c_{1} + c_{2} - 2c_{0} \right)^{2} + \bar{\kappa}_{0} c_{1} c_{2} \right], \qquad (1.1)$$

where the integration is carried over the membrane's total area, A. In this model, the constants are the surface tension,  $\sigma_0$ , the bending modulus,  $\kappa_0$ , the saddle splay,  $\bar{\kappa}_0$ , and the spontaneous curvature,  $c_0$ . The variables  $c_1$  and  $c_2$ , are the local principal curvatures. The discussion in this thesis is limited for conditions where  $c_0 = 0$  (symmetric membrane) and to membrane deformations that preserve the membrane topology, which means that the total energy of the contribution due to the last term is constant. Under these conditions, one can simplify eq. (1.1)

$$\mathcal{H} = \int_{A} dS \left[ \sigma_{0} + \frac{1}{2} \kappa_{0} \left( c_{1} + c_{2} \right)^{2} \right] = \sigma_{0} A + \frac{1}{2} \kappa_{0} J^{2}, \qquad (1.2)$$

where J, defined via  $J^2 = \int dS (c_1 + c_2)^2$ , is the integrated square total curvature, and A is the total area of the membrane. Helfrich Hamiltonian provides a successful framework for describing many features of bilayer membranes and vesicles, including their large-scale shapes and the transformations between them [3], membrane-membrane interactions [5], and membrane-mediated forces between proteins ("inclusions") [6]. Equations (1.1) and (1.2) are applicable when studying the elastic properties of a nearly flat membrane with low curvature. This thesis discusses some instabilities and phenomena that are not always described properly by these equations. Chapters 2 and 3 of this thesis discuss phenomena that occur as a result of compression [7], and inclusion of charged lipids in the membrane [8]. In chapter 4, a possible mechanism for DNA release from lipid-DNA complexes is proposed [9]. The DNA release requires both a high bending degree and pore formation in regimes are relevant to those discussed in chapters 2 and 3.

The remainder of this introduction provides a general background relevant to this thesis research. Surface tension in the context of bilayer membranes, a general view of charged membranes in solution, the effect of electrostatic interactions on the bending modulus, and finally, the role of charged membranes in drug delivery. This chapter is followed by three results chapters; the response of small membranes to negative surface tension, the effect of electrostatics on the stability of membranes, and a thermodynamic analysis of lipoplexes in various stages of the gene delivery process. Following these, chapter 5 summarizes the entire thesis and underlines the similarities and differences between the various instabilities phenomena.

List	of	Sym	bol	$\mathbf{l}$ S
	OI	Sym	001	D

A	Membrane total area	$\overline{N}$	Number of lipids in monolayer
$A_0$	Membrane preffered area	$\Phi_x$	Iinital mole fraction of $x$
$A_p$	Membrane projected area	$\phi^x$	Mole fraction of $x$
$a_l$	Porjected area per lipid	$b_l$	Simulation interaction distance
L	Simulation "box" size	$\vec{q}$	Fourier's mode wave vector
l	Simulation grid cell size	$\vec{n}$	Fourier's mode wave number
$\sigma_0$	Surface tension	$\psi$	Electric potential
$\sigma$	Renormalized surface tension	u i	Number density of ion $i$
au	Frame tension	$\lambda_D$	Debye length
$K_A$	Area stretching modulus	$\lambda_{GC}$	Gouy-Chapman length
$\kappa_0$	Bending rigidity	$\lambda_B$	Bjerrum length
$\kappa$	Renormalized bending rigidity	$\langle x \rangle$	Mean value of $x$
$\delta \kappa_e$	Electrostatic contribution to $\kappa$	$h_x$	Amplitude of $x$
$\gamma$	Fluctuation tension	e	Electron unit charge
F	Arrhenius free energy	$\epsilon_0$	Vacuum permitivity
$F_e$	Electrostatic contribution to $F$	$\epsilon_w$	Dielectric constant of water
f	F per unit of area	$\epsilon_l$	Dielectric constant of oil
G	Gibbs free energy	$\epsilon$	Dielectric constant of vacuum
$\sigma_e$	Surface charge density	$k_B$	Boltzmann's constant
$q_l$	DNA:cationic lipids charge ratio	Т	Temperature

### **1.2** Membranes Under Surface Tension

The surface tension,  $\sigma$  appearing in eqs. (1.1) and (1.2), originates from similar concepts to liquid phases context, though it has different, less intuitive, physical interpretation The definition of surface tension for two liquid phases that are in contact with each other, like a water-oil interface, is the energy per unit area required to maintain the area of contact [10]. It is calculated by the energetic cost of interaction between the two solutions per unit area compared to their energy in the respective bulk phases. Lipid bilayers mark the barrier between two solutions and as such, they appear to be applicable to this reasoning. However, applying it to derive the surface tension term in eq. (1.1),  $\sigma_0$ , is not straightforward [7]. Increasing the area of a membrane can be achieved by adding more lipid molecules into the membrane, while maintaining the area density of the lipids fixed. However, lipids concentration in aqueous solution is low ( $\sim 10^{-6}$ ). An alternative interpretation is the increase of the lipid area density, which greatly differs from the meaning "surface tension" in a liquid phase context.

To understand surface tension in the context of lipid membranes, it is important to recognize that  $\sigma_0$ ,  $\kappa_0$  are material properties and they are coupled with the membrane *total* area, A, and square total curvature,  $J^2$ , respectively. It is possible to renormalize  $\sigma_0$  and  $\kappa_0$  into  $\sigma$  and  $\kappa$ respectively using Helfrich's free energy

$$F = \sigma \bar{A} + \frac{1}{2} \kappa \bar{J}^2 \tag{1.3}$$

where  $\bar{A}$  and  $\bar{J}$  are the area and the squared total curvature of the *mean* profile of the membrane. Membranes with a flat profile (i.e., not subjected to bending forces) feature  $\bar{J} = 0$  and  $\bar{A}$  is equal to the projected area  $A_p$ . In this case, eq. (1.3) takes the form of  $F = \sigma A_p$  and  $\sigma = \partial F/\partial A_p$ . Generally, the property coupled with the membrane projected area is the frame tension,  $\tau = \partial F/\partial A_p$ , and for symmetric membranes  $\tau = \sigma$ . Unlike  $A, A_p$  is well defined, and insensitive to small protrusion, thus its measurements, and that of the  $\tau$  are simpler. Another quantity that can be identified as the membrane surface tension is the, so called,  $q^2$  coefficient  $\gamma$ , also known as the fluctuation tension. The fluctuation tension can be measured from the Fourier spectrum of the membrane height function with respect to the plane projection (see also chapter 2). For a membrane with a mean flat profile, the thermal average of the amplitude of a Fourier mode with wave vector  $\vec{q}$  satisfies:  $\langle h_{\vec{q}} \rangle = 0$  and

$$\langle |h_{\vec{q}}| \rangle = \frac{k_B T A_p}{l^4 \left(\gamma q^2 + \kappa q^4 + \mathcal{O}q^6\right)} \tag{1.4}$$

where  $k_B$  is Boltzmann constant, T is the temperature, and l is a microscopic cutoff length. Membranes with flat profiles under non negative surface tension exhibit  $\tau = \gamma = \sigma$  [11, 12].

The Giant Unilamellar Vesicles (GUV) experiment [13] provided some of the key insights about surface tension in the context of membranes. The experiment, carried out by measuring the projected area of vesicle stretch to various degrees, revealed two regimes of surface tension which are made distinct by the response of the projected area to stretching. When relatively low surface tension is applied to GUVs in the entropy dominated regime, the membrane is stretched by reducing the fluctuation amplitudes. When  $\tau$  is low, the membrane exhibits a strong increase in  $A_p$  in response to small changes in surface tension. With regard to the high surface tension regime, on the other hand, the membrane exhibits direct elastic compliance. In this regime, the membrane exhibits a linear increase of the projected area with surface tension. This is due to the stretching of the membrane which "irons" out its undulation and an increase in the membrane area occurs through increase in the total area per lipid of the membrane.

In chapter 2, we present the results of Monte-Carlo simulations of small membranes under negative surface tension. The reported results feature properties that resemble those of GUVs under positive tension. Equation (1.4) predicts that under negative surface tension, the Fourier modes of long wave length (small wave number) will develop mechanical instabilities. However, small membranes with linear size  $L < (2\pi\sqrt{\kappa/\tau})$ , should have a stability range in the negative tension regime. The results section of chapter 2 explores the features of stable membranes under moderate negative surface tension, as well as the buckling which occurs when strong negative tension is applied to the membrane.

### 1.3 Membranes in Charged Environments: The Poisson-Boltzmann Theory

Biological membranes are charged due to presence of charged lipids and proteins. The membranes are surrounded by ionic aqueous solutions, e.g., the cytoplasm, and electrostatic interactions affect their stability and mechanical properties. Furthermore, electrostatic interactions take place when two membranes interact, e.g., in cell-cell adhesion. While not accurate, the mean field approximation is convenient to gain the essential properties of charged membranes. Within this approximation, the charges included in the membrane are "smeared" to give surface charge density  $\sigma_e$ , and the concentration of ions in solution is taken as continuous. The relation between the potential  $\psi(\vec{r})$  and the charge distribution  $\rho(\vec{r}) = \sum \zeta_i \nu_i$  is given by the Poisson equation

$$\nabla^2 \psi\left(\vec{r}\right) = -\frac{e}{\epsilon_w \epsilon_0} \sum_i \zeta_i \nu_i,\tag{1.5}$$

where  $\epsilon_w \simeq 80$  is the water dielectric constant,  $\epsilon_0$  is the vacuum permittivity, e is the electron unit charge, and for specie i,  $\nu_i$  and  $\zeta_i$  are number density and valency, respectively. For fixed charges,  $\rho(\vec{r})$  is known, and eq. (1.5) determines the electric potential. However, ions in solutions are mobile and thus, even for fixed surface charge density  $\sigma_e$ , ions will adjust their positions to minimize the system's free energy. At equilibrium, the concentration of the mobile ions is described by the Boltzmann distribution. Assuming that the only positional energy is the electrostatic one, the number density  $\nu_i(\vec{r})$  of ion i reads

$$\nu_i = \nu_0^{(i)} \exp\left(\frac{-e\zeta_i\psi}{k_BT}\right). \tag{1.6}$$

Thus, the Poisson-Boltzmann equation is derived by combining eqs. (1.5) and (1.6)

$$\nabla^2 \psi\left(\vec{r}\right) = -\frac{e}{\epsilon_w \epsilon_0} \sum_i \zeta_i \nu_0^{(i)} \exp\left(\frac{-e\zeta_i \psi\left(\vec{r}\right)}{k_B T}\right).$$
(1.7)

This equation provides a good approximation of many settings that are relevant to physiological conditions, specifically when the  $\zeta_i = \pm 1$  [14]. For a system at contact to electrolyte reservoir with a 1:1 salt and  $\zeta_i = \pm 1$  ratio, for example negatively charged membrane in NaCl solution, eq. (1.7) is simplified to be

$$\nabla^2 \psi\left(\vec{r}\right) = -\frac{2\nu_0 e}{\epsilon_w \epsilon_0} \sinh\left(\frac{e\psi\left(\vec{r}\right)}{k_B T}\right). \tag{1.8}$$

where  $\nu_0$  is the number charge density of the salt at the reservoir (far away from the membrane). For a small surface potential, or high concentration of salt eq. (1.8), can be linearized to the Debye-Hückel equation

$$\nabla^2 \psi\left(\vec{r}\right) = \lambda_D^{-2} \psi, \tag{1.9}$$

where  $\lambda_D = (2\nu_0 e^2/\epsilon_w \epsilon_0 k_B T)^{-1/2}$  is the Debye screening length. At distances much greater than  $\lambda_D$ , the electric field of the membrane is screened by the cloud of counterions that surrounds the membrane.

It is also possible to solve eq. (1.7) when there is no added salt and  $\lambda_D$  diverges. This scenario is relevant to the simulation results reported in chapter 3, which correspond to negatively charged membrane in solution of cations with no added electrolytes. To consider this in eq. (1.7), one can set  $\zeta = 1$ , and  $\nu_0$  is the reference ions number density where  $\psi = 0$ . The total number of ions should be set so that the system will remain neutral. Without added salt, eq. (1.7) is simplified

$$\nabla^2 \psi\left(\vec{r}\right) = -\frac{e\nu_0}{\epsilon_w \epsilon_0} \exp\left(\frac{-e\psi\left(\vec{r}\right)}{k_B T}\right). \tag{1.10}$$

Since there is no inherit difference between positive and negative charges in the mean field picture, setting  $\sigma_e$  to be positive would require negative ions but would result in the same equation.

Equations (1.8) and (1.10) are solvable in several geometries applicable to membranes, the most simple of which is that of a flat surface of charge density  $\sigma_e$ . The surface is placed in the x - y plane at z = 0, and the field is allowed only at the positive part of z by setting  $\epsilon_{z>0} = \epsilon_w$  and in the oily part of the membrane  $\epsilon_{z<0} = 0$ . Under these conditions, and assuming overall neutral system, the electric field vanishes as  $z \to \infty$ . At the surface, the electric field has to

satisfy as well

$$\left. \frac{\partial \psi\left(z\right)}{\partial z} \right|_{z=0} = -\frac{\sigma_e}{\epsilon_w \epsilon_0}.$$
(1.11)

The dielectric discontinuity in the lipid tails part is most easily taken by considering two completely decoupled monolayers, namely where the electric field is excluded from the hydrophobic region. This is specifically justified for thick membranes [15, 16]. Assuming decoupled monolayers, the dielectric constant in the oil part is set to 0, thus excluding the electric field from it. Under these conditions, the potential and the counterion concentration without added electrolyte can be derived from eq. (1.10). The electric potential reads

$$\psi(z) = \frac{2k_BT}{e} \log\left[\sqrt{\frac{\nu_0\lambda_B}{2}} \left(z + \lambda_{GC}\right)\right] = \frac{2k_BT}{e} \log\left(z + \lambda_{GC}\right) + \psi_0 \tag{1.12}$$

where  $\psi_0$  is the reference potential that is dictated by  $\nu_0$  in eq. (1.10). The characteristic lengths  $\lambda_{GC} = 2k_B T \epsilon_w \epsilon_0 / e |\sigma_e|$  (Gouy-Chapman), and  $\lambda_B = e^2 / 4\pi k_B T \epsilon_w \epsilon_0 = \simeq 0.7$ nm (Bjerrum) are defined as the distance where an elementary charge experiences an electric force of magnitude  $1k_B T$  due to surface of charge density  $\sigma_e$  (Gouy-Chapman) or another elementary charge (Bjerrum). These two length scales arise naturally when electrostatic interactions are discussed since the former is a measure of the surface charge density and the latter defines the strength of electrostatic interactions in comparison to other forces. Note that the log function used here, and throughout this thesis, is the natural logarithm. The ions distribution, calculated by applying eq. (1.12) to eq. (1.6), reads

$$\nu\left(z\right) = \frac{1}{2\pi\lambda_B \left(z + \lambda_{GC}\right)^2}.\tag{1.13}$$

Applying the same conditions to a membrane in contact with a reservoir of monovalent electrolytes, the electric potential can be derived from eq. (1.8)

$$\psi(z) = -\frac{2k_BT}{e} \log\left(\frac{1+\eta e^{-z/\lambda_D}}{1-\eta e^{-z/\lambda_D}}\right)$$
(1.14)

where the parameter  $\eta$  is the positive root of the quadratic equation

$$\eta^{2} + \frac{2\lambda_{GC}}{\lambda_{D}}\eta - 1 = 0$$
$$\eta = -\frac{\lambda_{GC}}{\lambda_{D}} + \sqrt{\frac{\lambda_{GC}^{2}}{\lambda_{D}^{2}} + 1}$$

Using eq. (1.14), deriving the ions number density is straightforward,

$$\nu_{+}(z) = \nu_0 \left(\frac{1 + \eta \exp\left(-z/\lambda_D\right)}{1 - \eta \exp\left(-z/\lambda_D\right)}\right)^2 \tag{1.15}$$

$$\nu_{-}(z) = \nu_{0} \left( \frac{1 - \eta \exp\left(-z/\lambda_{D}\right)}{1 + \eta \exp\left(-z/\lambda_{D}\right)} \right)^{2}.$$
(1.16)

The concentration of the ions in the solutions is affected by the presence of charged membranes, as one intuitively expects. The membrane counterions are attracted to it resulting with condensation of ions on the surface that neutralize its charge [eq. (1.15)]. The membrane coions are repelled from the it although it is not 0 next to the surface [as described by eq. (1.16)]. Due to the appearance of  $\eta$  in eqs. (1.15) and (1.16), the equations are hard to understand intuitively. Generally speaking,  $\eta$  determines the level of deviation from the bulk concentration: At the weak screening regime, where the  $\lambda_D \gg \lambda_{GC}$  (low  $\nu_0$  or high  $\sigma_e$ ),  $\eta \to 1$  and the concentration of the ions next to the surface deviate significantly from their bulk concentration. At the strong screening regime, where  $\lambda_D \ll \lambda_{GC}$  (high  $\nu_0$  or low  $\sigma_e$ ),  $\eta \to 0$  and the concentration of the ions is fixed at  $\nu_0$  in the entire solution.

Completely coupled flat surfaces, where  $\epsilon_l = \epsilon_w$  are not discussed in this thesis <sup>1</sup>. Note though that this system is solvable as well using the same methods. This is so because the electric field of the bilayer is a linear sum of the electric field of the two monolayers. Thus, the equations above are applicable after correcting the surface charge density of each monolayer  $\sigma_e$  by adding the one of the other monolayer. As demonstrated in section 3.2, for symmetric systems, where the surface charge density of each monolayer is the same, and the total ion sum on each charge is the same, the electric field does not penetrate the oily part due to the symmetry.

The electrostatic free energy per unit are,  $f_e$ , of flat charge surface in ionic solution reads

$$f_e = \frac{\epsilon_w \epsilon_0}{2} \int \left(\frac{\partial \psi}{\partial z}\right)^2 dz + k_B T \int \left[\sum_i \nu_i \log\left(\frac{\nu_i}{\nu_0}\right) - (\nu_i - \nu_0)\right] dz, \qquad (1.17)$$

where the first term is the electrostatic energy and the second term is the entropy loss of ions due to their deviation from uniform concentration, composed of nonuniform concentration term and "counting" term. The latter term, is the local charge density in the solution, and the integral  $\int (\sum_i \nu_i - \nu_0) dz$  is equal to  $\sigma_e$  The integration over the latter term results in the charge density of the membrane. Appendix A demonstrates how for any flat surface, the

<sup>&</sup>lt;sup>1</sup>The simulations in chapter 3 are handled under such conditions; however, due to the symmetry of the bilayers and the ion solution above and below it, the electric field inside the membrane is negligible as in the decoupled case.

$$\frac{\epsilon_w \epsilon_0}{2} \int \left(\frac{\partial \psi}{\partial z}\right)^2 dz = k_B T \int \sum_i \nu_i \left(\nu_i - \nu_0\right) dz.$$
(1.18)

In a salt-free solution, these two terms are equal to  $1k_BT$ , which is the usually attributed cost of bound counterions. This equality indicates that bound counterions maintain some of their configurational entropy at the cost of electrostatic energy. Neutralizing this charge by macro molecules would allow the counterions to leave without electrostatic penalty. This counterions release mechanism is the driving force of many biological processes [17].

## 1.4 Membranes in Charged Environments: Beyond Mean Field

Thus far, the discussion was limited to the mean field framework where both charge densities and ion densities are smeared, and fluctuations in charge and ion densities were ignored. Assuming such fluctuations are not correlated, this approach is valid. However, when such correlations do exist, e.g., when the charge carriers in the electrolyte are multivalent, the mean field picture is not necessarily valid [18]. Such correlations result with the rather surprising phenomena, termed like-charged attraction [19, 20, 21, 22, 23], where two macromolecules of the same charge sign attract each other through mutual attraction to the counterions in the intermediating solutions. The key to understanding this phenomena lies in the analysis of charged surfaces and counterion condensation which reveals a coupling parameter  $\Xi = 2\pi \zeta^3 \lambda_B$ ,  $\sigma_e$  [18]. This coupling parameter distinguishes between the weak coupling regime, where  $\Xi \ll 1$ , and the mean field approximation holds true, to the strong coupling regime, where  $\Xi \gg 1$  and charge charge correlations might become significant.

The physical origin of the coupling parameter is the opposite influence the charged surface and the ions have on an individual ion [24]. While all the ions are attracted to the surface, they also repel each other. If all the ions adhere to the surface, the average area per ion is  $\zeta/\sigma_e$ , with the characteristic length  $\alpha = \sqrt{\zeta/\sigma_e}$ . When the ions are multivalent, and  $\Xi \gg 1$ , the ions condense on the surface in a structure of a hexagonal two dimensional crystal [18] that maximizes the distances between the ions. In this configuration, the electric field of the ions almost vanishes due to symmetry, and each ion experiences mostly the field of the charged surface.

Consider now two such surfaces that are close proximity to one another. The surfaces are

flat and parallel to each other, separated along the z axis by distance  $d \ll \alpha$ . Due to the short distance between the surfaces, the counterions on each surface correlate their position to form a Wigner crystal where the area per ion is halved (compared to the Wigner crystal on a single surface) and the Wigner crystal characteristic length is updated to be  $\alpha' = \alpha/\sqrt{2}$ . It turns out that when  $d/\alpha \ll \Xi^{1/4}$ , the interaction between ions in their crystal structure is still negligible while the combined potential between the two surfaces is uniform. Unlike the x - y positioning of the ions which is still fixed, the ion distribution in the z direction changes. The ions can now unbind from the surface and explore the space between the two surfaces without electrical energy cost. This allows the ions to unbind from the surfaces and adopt uniform distribution between them [25]. It is important to note that the criterion  $d/\alpha \ll \Xi^{1/4}$  may be satisfied when the distance between the two plates is greater than  $\lambda_{GC}$ .

The unbound ions in the water slab cast inter-plates pressure, P, on the surfaces

$$\frac{P}{k_B T} = 2\frac{\sigma_e}{\zeta d} - \frac{2\pi\sigma_e^2 e^2}{\epsilon_w \epsilon_0 k_B T} = 2\pi\lambda_B \sigma_e^2 \left(\frac{2\lambda_{GC}}{d} - 1\right).$$
(1.19)

In addition to the ideal gas-like pressure of the ions, which push the plates further apart, away from each other (increasing d), the ions also attract the two plates (decreasing d). When  $d > 2\lambda_{GC}$ , the pressure between the plate is negative and the plates are attracted to one another. This attraction stems from correlated local charge arrangement on the two surfaces in the x - y plane and cannot be accounted for within the mean field approach.

### **1.5** Elastic Properties of Charged Membranes

The elastic moduli appearing in eqs. (1.1) to (1.4) are primarily governed by the short range intermolecular forces between the lipids [11]. Electrostatic interactions, however, are long ranged and are expected to make a contribution both to the bending rigidity ( $\kappa_e = \kappa + \delta \kappa_e$ ) and to the saddle splay ( $\bar{\kappa}_e = \bar{\kappa} + \delta \bar{\kappa}_e$ ). The contributions  $\delta \kappa_e$  and  $\bar{\kappa}_e$  are governed by the electrolytes behaviour [20]. For example, the free energy barrier for membrane fusion includes the high energy associated with strongly-bent membranes. This barrier is reduced when the two lipid membranes are oppositely charged due to the strong attraction between the two surfaces. A possible presence of counterions in the space between the two surface will change the degree of reduction of the energy barrier. Listed here are some of the key insights about the effect of electrostatic interactions on the bending energy <sup>2</sup>.

<sup>&</sup>lt;sup>2</sup>The results in chapter 3 discuss only the effect on bending rigidity,  $\delta \kappa_e$  and as such, the effect on the saddle splay is not discussed here.

The simplest method to obtain  $\delta \kappa_e$  is solving the Poisson-Boltzmann equation for specific geometries, and calculating the free energy using eq. (1.17). Alternatively, one can find the free energy associated with charging the surface

$$f_e = \int_0^{\sigma_e} \psi_s\left(\sigma_e'\right) d\sigma_e',\tag{1.20}$$

where  $\psi_s$  is the surface potential. Note that the charging energy in eq. (1.20) includes implicit contributions resulting from the increase of electrolyte concentration in the solution as the system remains charge neutral. Once the free energy is obtained, one can rearrange the terms to resemble the free energy of neutral membrane which has a bending-like,  $\delta \kappa_e$  term and a curvature-like term. The electrostatic contribution to the bending modulus in the case of spherical or cylindrical membranes was calculated using a perturbation technique to a second order on the Poisson-Boltzmann equation [26]. In these geometries, the charge-free bending energy of a charge-free membrane reads [26]

$$f_{bend}^{sphere} = \frac{1}{2} \kappa \left(\frac{2}{R} - 2c_0\right)^2 \tag{1.21}$$

$$f_{bend}^{cylinder} = \frac{1}{2} \kappa \left(\frac{1}{R} - c_0\right)^2, \qquad (1.22)$$

where R is the sphere or cylinder radius. The general contribution to  $\delta \kappa_e$  obtained for these geometries is

$$\delta\kappa_{e} = \frac{1k_{B}T\lambda_{D}}{2\pi\lambda_{B}} \frac{\left(\sqrt{1+p_{l}^{2}}-1\right)\left(\sqrt{1+p_{l}^{2}}+2\right)}{\left(\sqrt{1+p_{l}^{2}}+1\right)\sqrt{1+p_{l}^{2}}}$$
(1.23)

where  $p_l$  is related, through some variable changes, to boundary condition (1.11)

$$p_l = \frac{2\pi\lambda_B\lambda_D \left|\sigma_e\right|}{e} \sim \frac{\sigma_e}{\nu_0^{1/2}}.$$
(1.24)

Equation (1.23) is applicable in any general condition of electrolyte concentration,  $\nu_0$ , and surface charge density,  $\sigma_e$ . Specifically, for cases when  $p_l \ll 1$  (low  $|\sigma_e|$  or large  $\nu_0$ ), or  $p_l \gg 1$ (large  $|\sigma_e|$  or low  $\nu_0$ ),  $\delta \kappa_e$  is simplified to

$$\delta\kappa_e = \frac{k_B T 3\pi \lambda_B \lambda_D^3 \sigma_e^2}{2e^2}, \qquad p_l \ll 1 \qquad (1.25)$$

$$\delta\kappa_e = \frac{k_B T \lambda_D}{2\pi \lambda_B}, \qquad p_l \gg 1. \tag{1.26}$$

Within the mean field approach, the electrostatic contribution in both cases, and in the general

case, is always positive, implying that the membrane become stiffer upon charging. The same conclusion for these geometries was found for the linearized Poisson-Boltzmann equation, for both fully coupled and fully decoupled geometries [16, 15].

A geometry more relevant to the studies reported in this thesis, which is harder to solve, is that of a flat but undulating bilayer. In this case, it is more convenient to consider the Fourier spectrum of the membrane height function, as in eq. (1.4), where the bending energy associated with each mod  $\vec{q}$  of amplitude  $h_{\vec{q}}$  is

$$f_{bend} = \frac{1}{4} \kappa_0 h_{\vec{q}} q^4.$$
 (1.27)

The electrostatic effect on the bending modulus was derived by a method similar to the one used to derive eqs. (1.25) and (1.26): One calculates the free energy using eq. (1.17) or eq. (1.20) and looks at the coefficients of  $q^4$  in the Fourier space. For decoupled membranes, where  $\epsilon_l \rightarrow 0$ , and for long wavelengths ( $\kappa q \gg 2\pi$ ), after redefining  $p_l$  to be  $\lambda_{GC}/\lambda_D$ ,  $\delta\kappa_e$  is approximated to be [27]

$$\delta\kappa_e = \frac{\lambda_D k_B T}{2\pi\lambda_B} \left( 1 - 2p_l^2 + \frac{2p_l^3}{\sqrt{1 + p_l^2}} \right). \tag{1.28}$$

Note that, similarly to the spherical and cylindrical geometries, the  $\delta \kappa_e$  is always positive and the bending modulus increases with  $\sigma_e$ . The same holds for the coupled system where the electric field is allowed to penetrate the membrane under the assumption that it is constant inside the membrane [28]. Once again, we reach the conclusion that within the mean field approximation—and regardless of their electric permeability, ions concentration, or charge density—membranes become stiffer when charged.

The discussion in this section is limited for solutions of delocalized charge that are applicable only to monovalent charge. Chapter 3 presents computer simulations of charged membranes in salt-free solution (in the presence of counterions). The simulated system allowed for undulation and considered the cases of monovalent ( $\zeta = 1$ ) and multivalent( $\zeta = 5$ ) counterions. The quality of measurement in the simulation isn't good enough for a quantitative comparison to the equations above, but they show the same qualitative trend; an increase in the bending rigidity upon membrane charging when monovalent counterions are present. When multivalent counterions are presents, the opposite trend is observed and the bending rigidity decreases upon membrane charging. The difference in the trends stems from the different behaviour of the counterions. When the counterions are monovalent, they don't fully condense on the membrane and they don't fully mask its charge. Bending the membrane brings charges closer and thus, the membrane is stiffened. On the other hand, pentavalent counterions are adsorbed to the membrane which allow for charge-charge and charge-height correlations leading to membrane softening.

### 1.6 Lipid-DNA Complexes

Complexes constituting DNA and lipids (both neutral and charged) are an example where electrostatic interactions affect the mechanical properties of lipids. Such complexes might be applicable for gene therapy, a medical approach that aims to replace a damaged gene with a properly functioning one. This holds great promise for future medical applications including, for example, new treatments for various inherited diseases and cancers [29]. The core of the process, called transfection, includes the key steps of transferring foreign DNA into a target cell, followed by the expression of the genetic information. Lipid-DNA complexes, designated lipoplexes, or CL-DNA complexes constitute one of the most promising non-viral gene delivery systems [30, 31, 32]. Although their transfection efficiency (TE) is, in general, inferior to that of viral vectors, lipoplexes have the advantage of triggering minimal immune response and being non-pathogenic [32, 33, 34, 35]. Furthermore, lipoplexes allow for the transfer of larger DNA segments. Their production does not require sophisticated engineering since they form spontaneously in aqueous solutions when DNA molecules are mixed with cationic and neutral lipids (NLs) [36, 37]. X-ray diffraction experiments have revealed several liquid crystalline phases of CL-DNA complexes.

Figure 1.1 depicts the most prominent structures: (i) a lamellar phase  $(L_{\alpha}^{C})$ , with 2D smectic array of DNA within lipid bilayers [36], and (ii) an inverted hexagonal phase  $(H_{II}^{C})$ , where the DNA rods are packed in a hexagonal lattice and the lipids form monolayers around them [37]. The lipoplex structure is largely determined by the bending rigidity and spontaneous curvature of the lipids [38]. The main thermodynamic driving force for lipoplex formation is the entropic gain stemming from the release of the tightly bound counterions from the DNA and the lipid bilayers, as mentioned in section 1.3.

Isoelectric complexes, where the total charge on the DNA molecules exactly matches the total charge of the CLs, are the most stable ones because they enable nearly complete counterions release [40]. Thermodynamically stable lipoplexes are easier to produce and maintain their integrity while outside the cell, but once inside the cell, lipoplex degradation is required but slowed due to its stability. The thermodynamic stability of a lipoplex is not the only property of lipoplexes that affect their TEs. The lipoplex liquid crystalline structure and its charge



Figure 1.1: The two most prominent structures of lipid DNA complexes. Left, the lamellar phase,  $L_{\alpha}^{C}$ , where the DNA molecules are ordered in smectic array between the lipid bilayers. Right, the inverted hexagonal phase,  $H_{II}^{C}$ , where the DNA rods are packed in a hexagonal phase lattice and the lipids "fill the gaps" between them. Images are taken from [39].

density (per unit area) are also of importance [39, 41]. Generally speaking,  $H_{II}^C$  complexes exhibit higher TEs than  $L_{\alpha}^C$  complexes and increasing the lipoplex charge density also results in higher TEs.

In spite of the insights about the transfection efficiency, the mechanism is not yet known. A key question is why the stable lipoplex becomes unstable after endocytosis, as evident by the spontaneous DNA release from the trapped lipoplex? Chapter 4 analyzes the essential thermodynamic driving forces of the three stages of lipoplex-based cell delivery [39, 42, 43]: (i) endocytosis, (ii) lipoplex breakdown that involves membrane fusion, and (iii) DNA release. Endocytosis, stage (i), is triggered by the adhesion of the lipoplex to the plasma membrane which occurs via electrostatic interactions and counterion release. Stage (i) results in a system that is thermodynamically different from a lipoplex in solution. The two membranes, the negatively charged plasma membrane and the positively charged lipoplex external membrane, are in close proximity. This allows for free energy gain due to mixing of lipids between the lipoplex and the plasma membrane. This free energy gain reduces the free energy barrier to membrane fusion which allows for stage (iii) DNA release. The release occurs through electrostatic interactions between the DNA molecules and the oppositely charged macromolecules that reside in the cytoplasm.

### Chapter 2

# Small Membranes Under Negative Surface Tension.

### 2.1 Background

In chapter 1, the Helfrich's effective Hamiltonian was introduced

$$\mathcal{H} = \int_{A} dS \left[ \sigma_{0} + \frac{1}{2} \kappa_{0} \left( c_{1} + c_{2} - 2c_{0} \right)^{2} + \bar{\kappa}_{0} c_{1} c_{2} \right].$$
(1.1)

For symmetric membranes that maintain their topology, the Hamiltonian is simplified

$$\mathcal{H} = \int_{A} dS \left[ \sigma_{0} + \frac{1}{2} \kappa_{0} \left( c_{1} + c_{2} \right)^{2} \right] = \sigma_{0} A + \frac{1}{2} \kappa_{0} J^{2}, \qquad (1.2)$$

Where  $\sigma_0$  and  $\kappa_0$  are the surface tension and bending rigidity of the membrane respectively,  $c_1$  and  $c_2$  are the main curvature, A is the membrane total area, and  $J^2 = \int dS (c_1 + c_2)^2$  is the integrated square total curvature. Measurement of  $\sigma_0$ , which is a material property, has proved to be problematic as it is coupled to the membrane total area, a property that is not well defined due undulations and molecular-size protrusions. The property coupled with the membrane projected area  $A_p$  is defined via the Helfrich free energy

$$F = \sigma \bar{A} + \frac{1}{2} \kappa \bar{J}^2 \tag{1.3}$$

where  $\sigma$  and  $\kappa$  are, the renormalized surface tension and bending rigidity of the membrane, respectively,  $\bar{A}$  is the mean area, and  $\bar{J}^2$  is the mean square curvature. For symmetric membranes  $\bar{A} = A_p$  and  $\bar{J}^2 = 0$ , the free energy takes the form  $F = \sigma A_p$  and the surface tension is equal to frame mechanical tension  $\sigma = \tau = \partial F / \partial A_p$  which is the force per unit length exerted on an edge of a bilayer.

Another quantity of interest is the fluctuation tension,  $\gamma$ , which is derived from the Fourier expansion of a weakly undulating membrane. The 2D Fourier transform of such membrane reads

$$h_{\vec{q}} = l/L \sum_{\vec{r}} h\left(\vec{r}\right) \exp\left(-i\vec{q}\cdot\vec{r}\right),\tag{2.1}$$

where the wave vector  $\vec{q} = 2\pi \vec{n}/L$ ,  $\vec{n} = (n_x, n_y)$ , with  $n_x, n_y = -4, -3, \dots, 2, 3$ , and  $\vec{r}$  being the two dimensional position vector. After the transform, Hamiltonian 1.2 is given by

$$\mathcal{H} = \frac{l^2}{2} \sum_{\vec{q}} \left( \gamma \left| \vec{q} \right|^2 + \kappa \left| \vec{q} \right|^4 \right) \left| h_{\vec{q}} \right|^2,$$
(2.2)

and applying the equipartition theorem results with

$$\langle |h_{\vec{q}}| \rangle = \frac{k_B T A_p}{l^4 \left(\gamma q^2 + \kappa q^4 + \mathcal{O}q^6\right)}.$$
(1.4)

This fluctuation tension in eqs. (1.4) and (2.2),  $\gamma$ , can also be identified as the membrane surface tension, and for a symmetric membrane under positive surface tension it is found that  $\gamma = \tau = \sigma$  [11, 12]. This equality holds true for non-negative values of  $\sigma$ . When the surface tension vanishes,  $\sigma = 0$ , the membrane is "free to choose" the equilibrium projected area  $A_p$  that minimizes the free energy eq. (1.3). This chapter concerns the instabilities and elastic response of the membrane to a further decrease in the frame area, which involves the application of a negative surface tension. Based on eq. (1.4), one may argue that for  $\gamma < 0$ , the membrane always becomes mechanically unstable because the amplitude of any mode with  $q < \sqrt{-\gamma/\kappa}$  diverges. But such modes exist only in sufficiently large membranes; hence, small membranes can always sustain some negative surface tension. For instance, consider a square membrane of linear size L with  $\kappa = 25k_BT \simeq 10^{19}J$ . From eq. (1.4), one finds that such a membrane can withstand negative surface tension of size  $\gamma = 5 \times 10^3 N/m$  [which is comparable in magnitude to the typical positive rupture tension [13]], provided that  $L < (2\pi)\sqrt{\kappa/\gamma} \simeq 30$ nm. This is the characteristic size of actual small liposomes and of bilayers in highly coarsegrained simulations. Thus, the above estimation highlights the fact that the question of elastic response to negative surface tension is not only interesting for its theoretical aspects, but is relevant to current experimental and computational studies.

The derivation of eq. (1.4), and the proof that the fluctuation and mechanical tensions coincide with each other, involves several assumptions that do not necessarily remain valid when  $\sigma$  becomes negative. Specifically, it is based on the investigation of the linear response of a mechanically-stable flat membrane to small normal forces and is restricted to configurations with smooth (twice differentiable) height functions  $h(\vec{r})$ . But when  $\sigma < 0$ , the membrane can relieve the free energy cost of compression by buckling. Note that in the *absence of normal forces* (which is the case under consideration here), the system is not expected to undergo spontaneous symmetry breaking similar to that occurring, in e.g., Ising spin systems below the critical point. The reason is that the membrane height profile  $h(\vec{r})$  is a continuous field and, therefore the transition between different buckled configurations (e.g., from buckled "upward" to "downward") does not require the crossing of a free energy barrier. Thus, the system remains ergodic for negative tension, and due to the symmetry of the bilayer,  $\langle h_{\vec{q}} \rangle = 0$  for all the Fourier modes. The questions that remain are as follows.

1. Does eq. (1.4) still hold for  $\sigma < 0$ ? It is not expected to remain valid for strongly compressed membranes since the quadratic approximation of Helfrich's Hamiltonian [eq. (1.1)] is not valid. However, considering the fact that it holds for  $\sigma = 0$ , there is no apparent reason why it should not hold for small negative  $\sigma$ .

2. Are the mechanical and fluctuation tensions still equal to each other? (Obviously, this question is relevant only if the answer to question no. 1 is "yes.") As noted above, the proof of this equality depends on the surface tension being positive. Now that it is negative, the membrane prefers more buckled configurations with larger mean squared amplitudes. Does this imply that the fluctuation tension  $\gamma$  drops faster (i.e., becomes more negative) than the mechanical tension  $\tau$ ?

3. What happens to the bending modulus  $\kappa$  under compression? The coefficient appearing in eq. (1.4) is the renormalized bending modulus which, just like the tension  $\gamma$ , may vary with the frame area  $A_p$ . For positive tensions, the variations in  $\kappa$  are usually negligible, but this may not be the case for negative tensions when the membrane becomes increasingly more buckled. Does the increase in the degree of buckling under larger compressive stresses involve a decrease in  $\kappa$ ?

4. Does the membrane exhibit linear (Hookean) elastic response to negative mechanical tension? In response to a positive tension, the membrane becomes stretched and the relationship between the change in the area (strain) and the stress is indeed linear. However, the lipids constitute a dense two-dimensional fluid and therefore, the membrane can be barely compressed below its most favorable physical area  $A_0$ . When, under the application of a negative tension, the physical area A reaches  $A_0$ , the negative tension causes the membrane to buckle and more and more area is "stored" in the out-of plane fluctuations. This could lead to a highly non-linear elastic response.

The following section provides the essential details about the Monte-Carlo simulation method employed for the results and discussion sections of this chapter.

#### 2.2 Monte Carlo Simulation Details

The Monte Carlo (MC) simulations reported here, a sample snapshot of which appears in fig. 2.1, constitute N = 1,000 lipids in each monolayer. To allow for simulation of these relatively large membranes, the Cooke-Deserno model [44, 7] was used. This highly coarsegrained model assumes each lipid to be a trimer consisting of one hydrophilic (head) and two hydrophobic (tail) beads of size  $b_l$ . The solvent is modeled implicitly and the hydrophobic pair potential [eq. (4) in [44]] parameters are  $\varepsilon = 1.05k_BT$  (potential depth) and  $\omega_c = 1.35b_l$ (potential length). As can be seen in the sample snapshot (taken under surface tension) in fig. 2.1, these parameters yield a soft membrane with  $\kappa \simeq 8k_BT$ . This value of  $\kappa$ , though it is lower than the typical value of biological membranes, allows greater sensitivity to changes in a membrane's physical properties while maintaining its stability. The simulation parameters also determine the unit of energy,  $k_BT$ , and the unit of length,  $b_l$ , which is the parameter of the repulsion potential [eq. (1) in [44]].

The membranes are simulated in a square box of size  $L = L_x = L_y$ , with periodic boundary conditions in the x - y plane. The lipids are placed randomly within two flat monolayers and



Figure 2.1: A sample snapshot of the simulated membrane with zero surface tension. The white beads are hydrophilic head particles and the gray ones the hydrophobic tail particles. The solvent is simulated implicitly.

allowed to equilibrate for  $1 \times 10^5$  MC units of time. On average, each MC time unit consists of N translation (with additional small intermolecular displacement) and rotation move attempts carried out on randomly chosen lipids. The membrane is simulated at constant frame tension  $\tau$ , which is accomplished by incorporating several collective move attempts, per time unit, to change the frame area,  $A_p$ , of the membrane [45]. Another collective move attempt in the simulations is the "mode excitation" attempt that accelerates the slow dynamics of the long-wavelength Fourier modes [46]. The quantities of interest, as detailed below, were sampled at intervals of 200MC over  $1.5 \times 10^6$  MC for each value of  $\tau$ .

The membrane simulations carried over the range  $-0.3 \leq \tau \leq 0.5$  (in  $k_B T/b_l^2$  units) where they were mechanically stable. Relation to physical units can be made by setting  $b_l = 0.65$  nm, which corresponds to membrane thickness of  $2 \times 3b_l \sim 4nm$  and gives the unit of the surface tension  $k_B T/b_l^2 \simeq 10$  mN/m. For  $\tau > 0.5$ , the membranes rupture, while for  $\tau < -0.3$ , they exhibit large normal undulations leading to the collapse of the membrane and the dissociation of lipids. The simulations included also measurements of the mean and variance of the projected area distribution ( $\langle A_p \rangle$ , and  $\langle \delta A_p^2 \rangle = \langle A_p^2 \rangle - \langle A_p \rangle^2$ , respectively).

The Fourier transform of the height undulations is also measured by dividing the membrane into  $8 \times 8$  grid cells and calculating the local mean height of the bilayer within each grid cell. The Fourier transform of  $h(\vec{r})$  in wavenumber space,  $\vec{n} = \vec{q}(L/2\pi)$ , where  $[\vec{n} = (n_x, n_y); n_x, n_y = -4, -3, \dots, 2, 3]$  is defined by

$$h_{\vec{n}} = \frac{1}{L} \sum_{\vec{r}} h\left(\vec{r}\right) \exp\left(-2\pi i \vec{n} \cdot \vec{r}/L\right)$$
(2.3)

Notice that to maintain constant frame tension in the simulations, the linear size of the frame L, appearing in the definition of  $\tilde{h}_{\vec{n}}$ , isn't constant, but rather fluctuates. Thus, at each measurement, the instantaneous value of L is used. Also notice that  $h_{\vec{n}}$  defined in eq. (2.3) is dimensionless, due to the  $L^{-1}$  prefactor that does not exist in the more commonly used  $h_{\vec{q}}$  of eq. (1.4). In terms of the variable  $h_{\vec{n}}$ , eq. (1.4) takes the form

$$\left\langle \left|h_{\vec{n}}\right|^{2}\right\rangle = \left(\frac{L}{l}\right)^{4} \frac{k_{B}T}{\gamma \left\langle A_{p}\right\rangle \left(2\pi n\right)^{2} + \kappa \left(2\pi n\right)^{4}}.$$
(2.4)

There are four different modes of corresponding to each value of  $|\vec{n}|$ . This number is reduced to two if  $|n_x| = |n_y|$  or if one of the components of  $\vec{n}$  is zero. The results in section 2.3, for  $|h_{\vec{n}}|$  (and other related quantities) represent averages over these distinct modes. In eq. (2.4), lis the grid size, which implies that L/l = 8, independently of the instantaneous value of L. Due to molecular-scale protrusion, the physical area of the membrane cannot be unambiguously determined. Therefore, the following approximation for  $\langle A \rangle$  is used:

$$\langle A \rangle = \langle A_p \rangle \left[ 1 + \frac{1}{2} \left( \frac{l}{L} \right)^4 \sum_{\vec{n}} (2\pi n) \left\langle |h_{\vec{n}}| \right\rangle \right]$$
(2.5)

which is the physical area "visible" up to the resolution of the grid. One can also define the effective area-stretch modulus of the membrane,  $K_A$ , by assuming that the free energy cost due to small variations in the projected area from  $\langle A_p \rangle$  can be approximated by the quadratic form

$$F_{stretch} = \frac{1}{2} K_A \frac{(A - A_p)^2}{A_p}.$$
 (2.6)

Under this approximation, the coefficient  $K_A$  can be extracted from the fluctuation statistics of  $K_A$  by using the equipartition theorem

$$K_A = \frac{k_B T \langle A_p \rangle}{\langle \delta A_p^2 \rangle}.$$
(2.7)

#### 2.3 Results and Discussion

Section 2.1 brought up several questions concerning the elastic and fluctuation behavior of membranes under negative mechanical tension. In this section, the results of a coarse-grained computer simulation address those questions.

The first question to be answered is the validity of eq. (1.4) [and eq. (2.4)] for negative frame tensions. Figure 2.2 displays the results for the fluctuation spectral intensity,  $\langle |h_{\vec{n}}| \rangle^2$ , as a function of  $n^2$  for membranes under three different mechanical tensions  $\tau = -0.24, 0$ , and 0.24. The fits of the computational results to eq. (2.4) are displayed with dotted-dashed lines. The quality of each fit in the range  $-0.3 \leq \tau \leq 0.5$  was quite good, and this dictates the range of stability. This demonstrates that, for stable membranes, eqs. (1.4) and (2.4) adequately describe the fluctuation behavior of bilayer membranes under both positive and negative tensions.

From the fitting curves, one can extract the values of the parameter  $\kappa$  and the product  $\gamma \langle A_p \rangle$ as a function of  $\tau$ . One can obtain the fluctuation tension  $\gamma$  by measuring  $\langle A_p \rangle$  independently. Attempts to use  $\kappa$  as a single fitting parameters by forcing  $\gamma = \tau$  (and using the measured value of  $\langle A_p \rangle$ ) resulted in poor fitting for negative tensions. This is due to the nonlinear response of  $\langle A_p \rangle$  to  $\tau$  as plotted in the inset of fig. 2.2. The observed increase in  $\langle A_p \rangle$  with  $\tau$  is anticipated



Figure 2.2: The spectral intensity as a function of the wavenumber for membranes under frame tension of  $\tau = -0.24$  (squares), 0 (circles), and 0.24 (triangles). The dotted-dashed curves represent the best fits of the results to eq. (2.4) over the first four modes. The inset shows the mean projected area per lipid as a function of  $\tau$ .

and will be discussed in detail later. Figure 2.3 depicts the fluctuation tension  $\gamma$  as a function of  $\tau$ . The values reported in fig. 2.3 are based on fitting analysis over the four longest fluctuation modes (smallest wave numbers), and the error bars represent the intervals over which the fitting parameters,  $\gamma$  and  $\kappa$ , can be (mutually) varied while still producing reasonable fits up to the accuracy of the computational results. For nonnegative tensions, the results in fig. 2.3 agree very well with the simple relationship  $\tau = \gamma$ . As noted in section 2.1, there is no reason for this equality to remain valid for negative tensions. The analysis summarized in fig. 2.3 reveals that, indeed,  $\gamma \neq \tau$  when the tensions are negative. Figure 2.3 demonstrates that  $\gamma < \tau$  and, as also argued above, it is likely that the more rapid decrease in  $\gamma$  compared to  $\tau$  is related to the tendency of the membrane to form buckled configurations under negative tensions. The equality between  $\gamma$  and  $\tau$  is regained for  $\tau \simeq -0.15$  and  $\gamma$  becomes larger than  $\tau$ .

A closer inspection of the behavior of  $\gamma$  vs.  $\tau$  curve depicted in fig. 2.3 reveals that it may be divided into three regimes: (i) a linear  $\gamma = \tau$  regime for  $\tau \ge 0$ , (ii) a non-linear regime where  $\gamma < \tau < 0$  for mildly negative frame tensions, and (iii) a plateau regime ( $\gamma \simeq const$ ) for larger negative values of  $\tau$ . Saturation of the negative tension for strongly compressed membranes was previously observed [47], and will be detailed along with the discussion about the physical area in fig. 2.5. The fluctuation tension in fig. 2.3 is extracted from eq. (2.4), where it appears in the coefficient  $4\pi^2 \gamma \langle A_p \rangle$  of the  $n^2$  term in the denominator. Naively, one may



Figure 2.3: The fluctuation tension  $\gamma$  as a function of the frame mechanical tension  $\tau$ . The solid line represents the equality  $\gamma = \tau$ , which is expected to hold for positive tensions. The inset shows the spectral intensity of the longest Fourier mode (n = 1),  $\langle |h_1|^2 \rangle$  as a function of  $\tau$ .

expect the saturation of the fluctuation tension  $\gamma$  to result in the leveling-off of the fluctuation spectral intensity  $|h_{\vec{n}}|^2$ . However, the computational results indicate that the amplitudes of the normal undulations continue to grow for decreasing values of  $\tau$ , as shown in the inset of fig. 2.3. This apparent discrepancy can be only partially resolved by the trend in  $\langle A_p \rangle$ , whose value is reduced by about 10% in the plateau regime of  $\gamma$ . The main factor explaining the increase in the undulation amplitude in the constant  $\gamma$  regime is the decrease in the effective bending modulus  $\kappa$ , the value of which is plotted in fig. 2.4. Note though,  $\kappa$  is not a material but rather a thermodynamic quantity. For a tensionless membrane, the thermal undulations reduce (renormalize) the bending rigidity by  $\delta \kappa = -(3/4\pi) k_B T \log (L/l)$ , which is a small correction [48]. For  $\tau < 0$ , the amplitude of the fluctuations increase and therefore, this correction term should become larger (in absolute value), which explains the drop in the value of  $\kappa$  seen in fig. 2.4. To state it in other words, just like the rapid decrease in  $\kappa$ , reported above in fig. 2.3 for membranes under negative tension, the reduction in  $\kappa$  is also related to the increasing thermal roughness of the membrane and the tendency of the membrane to form more buckled configurations.

The results of figs. 2.3 and 2.4 point to an interesting difference between the elastic coefficients  $\gamma$  and  $\kappa$ . The former decreases faster than  $\tau$  for small negative tensions and levels off at large negative tensions. The latter exhibits "opposite" behavior and remains fairly constant



Figure 2.4: The bending rigidity  $\kappa$  as a function of the frame tension  $\tau$ . The dotted-dashed line is a guide to the eye.

in the small negative tension regime, and then decreases for strongly compressed membranes. The crossover between the regimes occurs at  $\tau \simeq -0.15$ . Some light may be shed on these observations by the results of fig. 2.5 depicting the mean projected and total areas as a function of  $\tau$ . The results for the mean projected area,  $\langle A_p \rangle$ , were measured directly from the simulations, while the data for the mean total area,  $\langle A_p \rangle$ , were calculated using eq. (2.5). For  $\tau > 0$ , a nearly linear dependence of both  $\langle A_p \rangle$  (see also the dotted-dashed line) and  $\langle A \rangle$  on  $\tau$ is observed.

This behavior agrees very well with the experimental results of Evans and Rawicz, who also measured linear elastic response of giant unilamellar vesicles (GUVs) under positive mechanical tension[13]. Notice, however, an important difference between the origins of linear elasticity in GUVs and small bilayer membranes. In both cases, the linear elastic response is energetic in nature and dominated by the area elasticity of the membrane, while the entropy and bending energy of the height fluctuations play a secondary role in the response to stretching. In GUVs, this happens after the height fluctuations have been ironed by a very weak positive tension scaling inversely with  $A_p$ . In small membranes, the height fluctuations are not dumped and in fact, the simulation results in fig. 2.5 reveal that the excess area "stored" in the height fluctuations,  $\langle A \rangle - \langle A_p \rangle$ , decreases only weakly with  $\tau$ . This implies that the entropy and bending energy of small membranes do not vanish (as in GUVs under tension), but simply exhibit relatively weak dependence on the frame tension (and, therefore, contribute weakly to



Figure 2.5: Measured area as a function of the frame tension  $\tau$ . Solids circles and squares denote, the results for the frame and total areas, respectively. The former was measured directly from the simulation, while the latter was derived from the computed data for the spectral intensity, by using eq. (2.5). The open circle marks the optimal area of a flat tensionless membrane,  $A_0$ . The dotted-dashed line is a linear fit for the results for  $\langle A_p \rangle$ , while the horizontal dotted line marks  $A_0$ . All areas plotted in the figure are normalized per lipid.

the elastic response).

In addition to the simulations of fluctuating membranes, a flat, tensionless ( $\tau = 0$ ) membrane was simulated by running an MC code with moves allowing only local protrusions of lipids, but completely suppressing the longer scale bending modes (i.e., ensuring  $h_{\vec{n}} = 0$  for all n). For a flat membrane,  $A = A_p$ . The measured area of the flat tensionless membrane,  $A_0$ , is denoted by the open circle and the horizontal dotted line in fig. 2.5. This is the area that minimizes the elastic energy of the membrane. Figure 2.5 provides an interesting interpretation for the weak and strong negative tension regimes. The weak negative tension regime is essentially a continuation of the positive tension regime. The mean area of a tensionless fluctuating membrane is slightly larger than  $A_0$  which implies that, in fact, the membrane is stretched despite the negative mechanical tension. Therefore, the area-dependent elastic energy continues to decrease with  $\tau$  into the weak negative tension regime. The strong negative tension regime begins when the total physical area reaches  $A_0$ . Since the membrane constitutes a dense fluid of lipids, it cannot be much further compressed, and in order to maintain the total area at  $A_0$ , more area must be expelled into the height fluctuations. Notice that eq. (2.5) is actually the Taylor expansion of  $\langle A \rangle$  about weakly fluctuating membranes. In this regime, the relatively strong undulation cause greater deviation from the real value of  $\langle A \rangle$ . This partially explains the continued decrease of  $\langle A \rangle$  in this regime. At this point, the elastic response becomes dominated by the height fluctuations bending elasticity and entropy. This causes the apparent reduction in the effective bending modulus,  $\kappa$ , instead of the reduction in the now constant fluctuation tension  $\gamma$ . The saturation of the membrane's physical area, and its correlation with that of the surface tension, was previously reported [47]. Figure 2.5 demonstrates that this occurs when  $\langle A \rangle$  reaches the value of  $A_0$ , which provided an intuitive explanation for these observations. Notice that the rapid decrease in the projected area  $A_p$  with  $\tau$  in this regime occurs simultaneously with the increase in the projected area fluctuations. The resulting rapid decrease in the effective stretch modulus  $K_A$ , defined by eq. (2.7), is plotted in fig. 2.6. The increase in the membrane buckling in the plateau regime results also in the apparent decrease in  $K_A$ . Hence, the vanishing of  $K_A$  in fig. 2.6 should not be interpreted as changes in the material stiffness of the membrane, but rather as another signal for the onset of mechanical instability.

### 2.4 Conclusions

In this chapter, we used coarse-grained computer simulations to study the behaviour of small membranes under negative surface tension. Based on the results in section 2.3, one can identify two regimes of negative tension with distinct features:



Figure 2.6: The stretch modulus  $K_A$ , measured from eq. (2.7), as a function of the frame tension  $\tau$ . The dotted-dashed line is a guide to the eye.



Figure 2.7: The bending, stretching, and total free energies (see definitions in the text) per grid cell, as a function of mean projected area  $A_p$  per lipid. The data for the total free energy have been shifted vertically by 0.3 for clarity. The vertical dotted-dashed line marks the measured projected area for  $\tau = 0$ .

- (i) For weak negative tensions, the fluctuation tension γ drops somewhat faster than the mechanical tension τ. This behavior, which stand in contrast to the positive tension behavior: γ = τ, is attributed to the fact that in this regime, the membrane is still effectively stressed (⟨A⟩ > A₀). Under negative surface tension, the membrane tends to buckle, which is achieved by reducing the free energy associated with strong undulations.
- (ii) In the strong negative tension regime, while the fluctuation tension saturates, the effective bending rigidity begins to fall. Additionally, the total membrane area in the simulations reported here reaches the optimal value of  $A_0$  and does not continue to drop much.

The different response that membranes exhibit to negative values of  $\sigma$ , compared to positive ones, indicates that the elastic free energy (eq. (1.3) in section 1.2), isn't applicable in the negative regime. However, a free energy model for the negative regime can be rationalized in the same spirit by considering the sum of two terms associated with stretching and bending. The former is given by the quadratic form

$$F_{stretch} = (1/2)K_A[\langle A \rangle - A_0]^2 / A_0, \qquad (2.8)$$
while the latter may be evaluated by

$$F_{bend} = (1/2)\kappa \sum_{\vec{n}} n^4 \left\langle \left| h_{\vec{n}}^2 \right| \right\rangle.$$
(2.9)

These two contributions and their sum are plotted in fig. 2.7 as a function of  $\langle A_p \rangle$ . Notice that both  $F_{stretch}$  and  $F_{bend}$  depend on  $\langle A_p \rangle$  the former explicitly, and the latter implicitly through the Fourier modes calculations. The free energies in fig. 2.7 provide insight into the computational results in this chapter. First, the total free energy attains a minimum at  $\langle F \rangle /N \simeq 1.32$  (marked by the vertical dotted dashed line), which is the mean projected area measured for  $\tau = 0$ . This must be the case since  $\tau = \partial F / \partial A_p$ . Second, the bending free energy decreases with  $\tau$ , which considering fig. 2.2, is expected since, upon stretching, the thermal bending undulations tend to be suppressed. The stretching free energy increases with  $A_p$  in the weak negative and positive tensions regimes, i.e., when  $\langle A \rangle > A_0$ . Under strong negative tensions,  $F_{stretch}$  vanishes, which is associated with the observation that the total physical area remains at the optimal value and does not change in this regime.

A crossover from bending- to stretching-dominated membrane elasticity has also been observed in micropipette aspiration experiments on GUVs [13]. There are, however, several key differences between the elastic behaviors of small membranes, such as reported here, and large ones. The former can withstand a (size-dependent) negative tension while the latter are destabilized due to the strong undulations of the large bending modes. The negative surface tension that causes undulations in the small membranes may be comparable in magnitude to the positive rupture tension. In giant membranes, bending-dominated elasticity is limited to extremely small positive tensions that are typically two orders of magnitude smaller than the rupture tension. In small membranes, the crossover from bending-dominated to stretchingdominated elasticity is smoother and occurs at small negative tensions. In other words, the stretching-dominated elasticity regime extends into negative tensions, which stems from the fact that at zero tension, the membrane is still slightly stretched. Bending-dominated elasticity is observed at larger negative tensions. It is characterized by a decrease in the effective bending rigidity and stretch modulus of the membrane that ultimately leads to mechanical instability and membrane collapse.

### Chapter 3

### **Rigidity of Charged Membranes.**

#### **3.1** Background.

Section 1.5 in chapter 1 discusses the the electrostatic effects on the bending rigidity ( $\kappa_e = \kappa + \delta \kappa_e$ ) of a membranes upon charging while its other properties remain the same. That discussion was limited to the relatively well understood mean field picture and its underling conclusion was that  $\delta \kappa_e > 0$  regardless of the membrane morphology, the surface charge density, or the ions concentration in solution. This is so because without its counterions, charged membrane would become significantly more rigid ( $\delta \kappa_e \gg \kappa$ ) [20] and the counterions, due to their distribution, does not mask the membrane charges, and thus, not fully negate this effect.

In section 1.4, the charge-like attractions phenomena, which is not accounted for using the mean field approach, was discussed. The mean field solution ignores charge density fluctuations and their spatial correlations. While such correlations are negligible when monovalent counterions are discussed, this is no longer true with multivalent ions and lipids ( $z_i \ge 2$ ). Studying the effect of multivalent counterions proves to be rather challenging, partially due to the multiple predictions that, while they don't fully contradict each other, are not at full agreement either. Including charge fluctuations in the Poisson-Boltzmann equation may lead to a reduction in the bending modulus  $\delta \kappa_e < 0$  [21]. The same trend is predicted by the opposite assumption of "freezing" counterions in a Wigner crystal structure on a uniformly charged membrane and allowing for some correction terms [20]. Allowing for compressibility of the charge fluctuations resulted in a prediction of membrane buckling due to long-range electrostatic interactions [22]. Taking a different approach and applying a second order perturbation to a general two-body potential resulted in the possibility of membrane softening due to electrostatic interactions [49].

Similarly to the charge-like attraction phenomenon discussed in section 1.4, the appearance

of negative  $\delta \kappa_e$  in bilayer membranes is attributed to multivalent counterions condensation and their spatial correlation. Membrane softening involves both charge-charge and *height-charge* correlations. The details of this effect are not fully understood, but it is generally believed to be small and thus, hard to measure experimentally. Furthermore, the effect is overshadowed by intermolecular interactions which are not screened and  $\lambda_D$  is long [21, 22]. The results reported in this chapter were gained through coarse-grained computer simulations that allowed exploring the effect in the absence of excess salt and other influences. The reported results support the picture that spatial correlation in the charge density due to the presence of highly multivalent counterions tend to soften membranes and reduce their bending rigidity.

#### **3.2** Monte Carlo Simulation Details

The method of simulations and variable measurements in this chapter are the same as those detailed in section 2.2, with the addition of electrostatic calculations. To simulate a charged membrane, a fraction of the lipid  $\Phi_{-}$  is charged by introducing a charge of -e at the center of the lipid heads. To maintain overall charge neutrality of the system, monovalent or pentavalent counterions are placed in the simulation box with no added salt<sup>1</sup> In terms of simulation length unit  $(b_l = 6.3\text{\AA})$ , the Bjerrum length reads  $\lambda_B \simeq 7.1 \text{\AA} = 1.1 b_l$ . The short range potential used for lipids was applied for the ions as well, with the radius of interaction set to  $b_{ion-head} = 0.5b_l$ when interacting with the hydrophilic heads and  $b_{ion-tail} = 1.5b_l$  when interacting with the hydrophobic tail beads. This choice of parameters allows the ions to approach the surface of the head beads, while excluding them from the hydrophobic core of the bilayer. The membranes are simulated at zero surface tension using the same method as in section 2.2 [45]. The membranes are generated as two flat bilayers with N = 1,000 lipids in each monolayer. The monolayers are placed in the middle of the simulation box, with the counterions distributed evenly above and below the membrane. The simulation length and move attempts were as described in section 2.2 with the addition of ion displacement attempts. The thermalization period was  $10^5$ MC time units; and the simulation lasted for  $1.8 \times 10^6$  MC time unit; the quantities of interest were sampled at 50 MC time unit intervals.

Electrostatic interactions were computed using Lekner summations [50]. The dielectric constant of water,  $\epsilon_w = 80$ , is taken as the only dielectric constant in the simulation since it

<sup>&</sup>lt;sup>1</sup>We choose pentavalent counterions because the effects under investigation are quite weak, and can be computationally observed when the counterions are of high valency. We also include in fig. 3.3 some results obtained from simulations of trivalent counterions, which exhibit "intermediate" behaviour between the monovalent and pentavalent counterions.

is practically impossible to calculate image charges for a fluctuating surface. This neglect of discontinuities between the aqueous solution and the membrane interior, however, is justified for a nearly flat surface (as is the case here) due to symmetry. To understand this, consider two flat, uniformly charged surfaces (of charge density  $\sigma_e$ ) positioned at the x - y plains  $z_1$ and  $z_2$ . Ionic solutions surround the two surfaces and exactly neutralize the surfaces. For the conditions of the simulated system ( $\sigma_e < 0$  and positive solvated counterions), the electric field at position z is equal to  $E = d\psi/dz = \tilde{\sigma}_e/2\epsilon_w\epsilon_0$  where  $\tilde{\sigma}_e$  is equal to

$$\begin{split} \tilde{\sigma}_{e} &= \\ \int_{\infty}^{z} \left[ e \,\nu\left(z'\right) + \sum_{i=1}^{2} \sigma_{e,i} \delta\left(z' - z_{i}\right) \right] dz' - \int_{z}^{-\infty} \left[ e \,\nu\left(z'\right) + \sum_{i=1}^{2} \sigma_{e,i} \delta\left(z' - z_{i}\right) \right] dz' = \\ &= 2 \int_{\infty}^{z} \left[ e \,\nu\left(z'\right) - + \sum_{i=1}^{6} \sigma_{e,i} \delta\left(z' - z_{i}\right) \right] dz'. \end{split}$$

$$(3.1)$$

The systems as reported here are symmetric,  $\sigma_{e1} = \sigma_{e2}$ , and on average the *total* number of ions on each side of the membrane are equal. Thus, the electric field vanishes inside the membrane ( $z_1 < z < z_2$ ). The simulated membranes undulate and the electric field does not vanish inside the membrane. However, since the undulations are relatively small, the effect of neglecting the electric discontinuities should be small as well.



Figure 3.1: Equilibrium configurations of membranes with charge density  $\Phi_{-} = 0.08$  in solutions of monovalent (A) and pentavalent (B) counterions. The head and tail beads of the lipids appear in white and gray colors, respectively, while the ions are presented as black spheres

#### **3.3** Results and Discussion

Equilibrium configurations of membranes with charge density  $\Phi_{-} = 0.08$  in solutions of monovalent ( $\zeta = 1, A$ ) and pentavalent ( $\zeta = 5, B$ ) counterions are displayed in fig. 3.1. In the latter case, the ions tend to condense on the membrane, forming a thin Gouy-Chapmann "double layer" [51, 18].

Figure 3.2(A) depicts the spectral intensity [eq. (1.4)] of the membrane's thermal undulations computed for the bilayers with  $\Phi_{-} = 0.08$ , whose snapshots are shown in fig. 3.1 (the graphs have been vertically shifted for clarity). Both graphs exhibit the power law  $|h_{\vec{q}}|^2 \sim n^{-4}$  $(\vec{n} = \vec{q} (L/2\pi))$ , where  $[\vec{n} = (n_x, n_y); n_x, n_y = -4, -3, \ldots, 2, 3]$ ) in agreement with the form of eq. (1.4) for  $\gamma = 0$ . By fitting the simulation results to eq. (1.4), one can extract the value of  $\kappa$ . The charge fraction range  $0 \leq \Phi_{-} \leq 0.16$  are summarized in fig. 3.2(B), showing  $\kappa$  as a function of  $\Phi_{-}$  for membranes in solutions of monovalent (circles) and pentavalent (squares) counterions. The dashed line denotes the value of  $\kappa$  for a neutral membrane ( $\Phi_{-} = 0$ ). Note that the error bars on  $\kappa$  measurements are quite large, reflecting not only the difficulty in obtaining good statistics for the spectral intensity of the thermal undulations, but also uncertainties in fitting the data to the functional form of eq. (1.4). Therefore, it is impossible to draw quantitative conclusions from the data regarding the variations of  $\kappa$  with  $\Phi_{-}$ . Nevertheless, the



Figure 3.2: (A) Spectral intensity as a function the squared wave number  $n^2$ . Data has been obtained from simulations of charged membranes with surface charge density  $\Phi_- = 0.08$ in solutions of monovalent (circles) and pentavalent (squares) counterions. The solid lines indicates a fit to the power law  $\langle |h_{\vec{n}}|^2 \rangle \sim n^4$  based on the four largest Fourier modes. For clarity, the graph corresponding to the monovalent counterions simulations is vertically shifted by multiplying the spectral intensities by a factor of 5. (B) The bending rigidity  $\kappa$  as a function of the charge density  $\Phi_-$  for membranes with monovalent (circles) and pentavalent (squares) counterions. The horizontal dashed line indicates the value of  $\kappa$  for neutral membranes ( $\Phi_- = 0$ ).

data in fig. 3.2 clearly supports the picture that the bending modulus of charged membranes increases from its value for  $\Phi_{-} = 0$  when the counterions are monovalent. This observation is consistent with the Poisson-Boltzmann results as summarized in section 1.5, although it should be acknowledged that previous theoretical calculations of  $\delta \kappa_e$  were done for systems with extra salt and for stationary (non-undulating) membranes [14].

As mentioned in section 1.4, the Poisson-Boltzmann theory is expected to break down when the so-called dimensionless coupling parameter  $\Xi = 2\pi\zeta^3\lambda_B^2\Phi_-/a_l$  (where  $a_l$  is the area per lipid, and  $\zeta$  is the counterion valance) becomes much larger than unity. Given the strong dependence of  $\Xi$  on  $\zeta$ , it is not surprising that simulations with pentavalent counterions reveal a very different trend of reduction in  $\kappa$  due to electrostatic effects. As in the case of monovalent counterions, the large error bars preclude quantitative analysis of the variation of  $\kappa$  with  $\Phi_-$ . The observation that the bending modulus is reduced when the membrane is charged and suspended in a multivalent counterions solution agrees with previous theoretical studies [20, 21, 22, 49]. The fact that the magnitude of the negative electrostatic contribution to  $\kappa$  is fairly small ( $k_BT$ ) is also in general agreement with existing theoretical calculations. As discussed in section 3.1, the negative electrostatic contribution to  $\delta\kappa_e$  in pentavalent counterions solutions has been attributed to the attraction due to spatial charge correlations in the double layer, which allows the membrane to bend more easily [22].

The picture emerging from fig. 3.2(B) is also consistent with the measurements of the equilibrium projected area per lipid,  $a_l = A_p/N$ , depicted in fig. 3.3. In the presence of monovalent



Figure 3.3: Projected area per lipid  $a_l$  as a function of  $\Phi_-$  for membranes with monovalent (circles), trivalent (triangles), and pentavalent (squares) counterions.

counterions, the area per lipid increases linearly with  $\Phi_-$ . The increase in  $a_l$  arises from the repulsive electrostatic interactions between the charged lipids, the strength of which is enhanced with the increase in the density  $\Phi_-$  of the charged lipids. The pentavalent counterion simulations feature markedly different behavior, exhibiting a slight decrease in  $a_l$  with  $\Phi_-$ . The decrease in  $a_l$  in this case indicates that the effective electrostatic interactions between the lipids and counterions in the double layer become attractive due to spatial charge correlations. Also shown in the figure are results of similar simulations with trivalent counterions which exhibit intermediate behavior between the monovalent and pentavalent counterions. The observed increase in  $a_l$  may be attributed to the fact that the coupling parameter corresponding to the trivalent counterions simulations satisfies  $\Xi \leq 10$ , which is still within the range where, usually, mean-field theory still holds. The same trend of "intermediate" behavior of trivalent counterions is also observed in the results for the bending rigidity (data not shown in fig. 3.2(B)), in which the electrostatic contribution was found to be vanishingly small.

The increase in area per lipid reported in fig. 3.3 can be intuitively explained by recognizing that the electric contribution to the free energy,  $F_{el} = k_B T \Phi_- N$ , is *independent* of the surface area A. The repulsion arises from the entropy of the counterions which, to an approximation, can be viewed as confined within a volume of size  $V = A\lambda_{GC}$  around the surface. The associated free energy contribution is  $F_{en} \sim k_B T N \log (V) = -k_B T \Phi_- N \log (A^2/\phi N)$ , where the last equality is due to the fact that  $\lambda_{GC} \sim (N\Phi_-/A)^{-1}$ . Introducing the area per lipid  $a_l$ , and expanding the logarithm around  $a^* = a_l (\Phi_- = \pm 0)$ , results in the area-dependent part of this free energy is  $F_{el} = -ck_B T \Phi_- N (a_l - a^*)/a^*$ , where c is a numerical prefactor and the minus sign accounts for the fact that this free energy is repulsive. Adding this  $F_{el}$  to the elastic energy of the uncharged membrane [eq. (2.6)], yields the following expression for the elastic energy per lipid f = F/N:

$$f = \frac{1}{2}K_A \frac{(a_l - a^*)^2}{a^*} - ck_B T \Phi_- \frac{(a_l - a^*)}{a^*}.$$
(3.2)

This stretching free energy attains a minimum at the area per lipid

$$a_l^{min} = a^* + c\Phi_- \left(k_B T / K_A\right), \tag{3.3}$$

which grows linearly with  $\Phi_{-}$ , as depicted in fig. 3.3.

Interestingly, both experiments [52] and atomistic simulations [53] found the area of monovalently charged phosphatidylserine (PS) lipids to be smaller than the area of their neutral phosphattidylcholine (PC) analogs. This counterintuitive result was primarily attributed to the formation of transient intra-molecular hydrogen bonds between the amine and carboxylate



Figure 3.4: Top view of a simulated membrane with a pore. The membrane, with charge density  $\Phi_{-} = 0.2$ , is in contact with a solution of monovalent counterions (not displayed). Color coding is similar to fig. 3.1.

groups of the PS head-group. The course-grained model used here allows the hydrogen bonding effect to be "turned off" and for the "isolation" of the Coulombic contribution, which turns out to be repulsive in monovalent systems. In the course-grained simulations reported here, the repulsive electrostatic interactions are balanced by relatively soft hydrophobic interactions. Because of the weakness of these attractive interactions, for  $\Phi_{-} > 0.16$ , the areal strain in the monovalent counterions simulations exceeds the rupture strain of the bilayer membrane, leading to the formation of pores, as demonstrated in fig. 3.4. The rupture value of  $\Phi_{-}$  could be increased by including hydrogen bonding in the coarse-grained model or, alternatively, by strengthening the hydrophobic interactions, but this will also lead to an undesirable increase in  $\kappa$ . Real PS bilayers have  $\kappa \simeq 20 - 50 k_B T$  which is several times larger than that of the membrane simulated here. Assuming a linear relationship between the area stretch modulus  $K_A$  and bending rigidity  $\kappa$ :  $\kappa \sim K_A d^2$ , where d is the bilayer thickness [54], one can expect the stretch modulus of real bilayers to also be a few times larger than in simulations. This feature of real PS bilayers, together with the extra attractive interaction provided by the hydrogen bonds, explains their mechanical stability at all charge densities, including for  $\phi = 1$ . The magnitude of the hydrogen bonding interactions (per lipid) can be roughly estimated by adopting eqs. (3.2) and (3.3) derived for the case of repulsive electrostatic interactions, with a



Figure 3.5: Sample snapshots of strongly undulating charged bilayers with pentavalent counterions (not displayed). Color coding is similar to fig. 3.1.

modified (negative rather than positive) constant c. For fully-charged membrane ( $\Phi_{-} = 1$ ) PS bilayers, the area stretch modulus is typically  $K_A \simeq 0.15 J/m^2$ , and the H-bond interactions reduce the area per lipid from  $a^* \sim 0.72 \text{nm}^2$  to  $a_l \simeq 0.65 \text{nm}^2$  [52]. Substituting these values into eq. (3.3) yields  $c \simeq -2.5$ . Using this value of c in the second term on the r.h.s. of eq. (3.2) gives an estimate for the H-bonding free energy contribution which is  $f_{\text{H-b}} \simeq -0.25 k_B T$ .

Pore formation, as exhibited in fig. 3.4, is not observed when the charged membranes are simulated with pentavalent counterions. Therefore, such membranes can be simulated at much higher values of  $\Phi_{-}$ . However, the multivalent counterion simulations feature a different type of mechanical instability, which is directly related to the previously discussed reduction in  $\kappa$ . At high charge densities, the membranes in pentavalent counterion solutions begin to develop large wavelength bending instabilities, as illustrated in the series of snapshots in fig. 3.5, corresponding to membranes with  $0.2 \leq \Phi_{-} \leq 0.5$ . The growth in the amplitude of the undulations, observed in fig. 3.5, can also be inferred from the results of fig. 3.6 that plots the spectral intensity of the membranes whose snapshots are displayed in fig. 3.5. Clearly, there is poor agreement between the results in fig. 3.6 eq. (1.4). The deviation of the computational results from eq. (1.4) is expected because the power law  $\langle |h_{\vec{q}}|^2 \rangle \sim n^{-4}$  derived from the quadratic approximation of eq. (1.1), strictly speaking, is only applicable to weakly fluctuating membranes. The dashed lines represent attempts to fit eq. (1.4) to the data from the second  $(n^2 = 2)$  and third  $(n^2 = 4)$  largest modes. These lines highlight the rapid increase in the undulation amplitude of largest Fourier modes  $(n^2 = 1)$ , which are also the softest modes and the first to become unstable as  $\Phi_{-}$  increases. The onset of this bending instability can



Figure 3.6: The spectral intensity of membranes in solutions of multivalent counterions, with surface charge density  $\Phi_{-} = 0.2$  (circles), 0.3 (squares), 0.4 (triangles), and 0.5 (diamonds). The dashed line indicates attempts to fit the data from the second  $(n^2 = 2)$  and third  $(n^2) = 4$ ) largest modes to the power law form  $|h_{\vec{n}}^2| n^{-4}$ . The graphs have been shifted vertically for clarity.

thus be associated with the decline of the "apparent bending modulus" of the first mode,  $\kappa_1$ , which is the value of  $\kappa$  that solves eq. (1.4) for  $n^2 = 1$ . The results in fig. 3.6 correspond to  $\kappa_1/k_BT = 5.1 \pm 0.5, 5.2 \pm 0.7, 4.2 \pm 0.4$ , and  $3.8 \pm 0.4$  for  $\phi = 0.2, 0.3, 0.4$ , and 0.5, respectively. These values of  $\kappa_1$  are smaller than the values of  $\kappa$  reported in fig. 3.2(B) for low charge densities. At even larger charge densities ( $\phi > 0.5$ ), these undulations continue to grow and ultimately lead to the dissociation of the bilayer membranes.

The instabilities appearing in charged membranes are of similar nature to those reported for stretched and compressed membranes in chapter 2, namely pore formation and buckling. However, the "fine details" of the instabilities are different, which indicate that they are of different origin. The most obvious difference between the two cases is the fluctuation tension. In this chapter, the frame tension,  $\tau$ , was explicitly set to zero and this set  $\gamma = \tau = 0$ , as covered in section 1.2. The quality of the linear fits in fig. 3.2 confirm that this assumption holds true. This indicates that the pore formation reported in fig. 3.4 cannot be directly attributed to a frame tension or overall stretching of the membrane. Similarly, the buckling of the highly charged membrane with pentavalent counterions cannot be attributed to the overall compression forces applied to membrane. While it is not necessarily true that  $\gamma = \tau$ , there is no apparent reason to assume this is not the case. Moreover, the projected area that remains roughly constant in fig. 3.3 does not agree with the recognized drop in  $A_p$  when buckling occurs in projected membranes; as reported in fig. 2.5. The differences point to local interactions of relatively short range (compared to the membrane length scale) that induce "these instabilities" in the membrane.

#### 3.4 Conclusions

In this chapter, we investigated the elastic properties of charged membranes in contact with counterion solutions. Through computer simulations, we discovered that the cases of monovalent and multivalent (with  $\zeta = 5$ ) counterions exhibit distinctly different behaviors. In the former, both the bending rigidity and the equilibrium projected area increased with the membrane charge density. These observations suggest, in agreement with the Poisson-Boltzmann mean-field theory, that the repulsive forces between the lipid charges are only partially screened by the monovalent counterions. In the latter case, the trends are opposite namely, both  $\kappa$  and  $a_l$  show a slight decrease with increasing  $\Phi_{-}$ . These observations can be attributed to the formation of a thin layer of counterions around the membrane and the fact that the forces between spatially correlated charges within the "double layer" become attractive. More specifically, the presence of multivalent counterions creates regions within the double layer where local charge densities of opposite signs attract each other. The increase in the curvature undulations and decrease in the area per lipid represent mechanisms through which the distances between these correlated regions, especially those residing on the same side of the bilayer, are generally decreased [see illustration in fig. 1 of ref. [20]]. The different elastic properties of membranes in monovalent and multivalent solutions lead to different mechanical instabilities. In the former case, pores open to relieve the electrostatic tensile stresses, while the latter case is characterized by a growth in the amplitude of large wavelength bending modes. As a final note, we remind the reader that the elastic properties of real membranes may be affected by other intermolecular forces that can dominate the electrostatic effect on the bending rigidity. Several such "counter mechanisms" have been mentioned in the chapter, including hydrogenbonding interactions, screening by salt, ions-lipids excluded-volume interactions, and image charges that weaken the binding of the multivalent counterions to the membrane [55]. The coarse-grained simulations provide a framework for systematically exploring the effects of these additional interactions.

### Chapter 4

# The Thermodynamics of Endosomal Escape and DNA Release from Lipoplexes.

#### 4.1 Background

Section 1.6 brought forward complexes of cationic lipids (CLs), neutral lipids (NLs), and DNA (lipoplex or CL-DNA complexes), as examples of charged lipid systems of medicinal interest. Such complexes have therapeutic potential as gene delivery vectors that might be applied in somatic gene therapy. Compared to viral vectors, lipid-based vectors are safer to use and simpler to produce. However, their therapeutic efficiency is limited and their further improvement requires better understanding of their mechanism of transfection and the biophysical parameters of the CL-DNA complexes that influence it. Transfection, the core process of lipoplex gene delivery, is viewed as a three-stage process starting with adsorption and entry (via endocytosis) of the CL-DNA complex into the cell, followed by lipoplex degradation, and finally ending with the release of the DNA, making the latter available for expression [39, 42, 43].

The first stage is driven by electrostatic attraction between the oppositely charged plasma membrane and the lipoplex one. After endocytosis, the complex is within the cell, trapped inside an endosome. The second stage of the transfection process, which often emerges as the rate-limiting one, involves the breakdown of the CL-DNA complex. During this stage, the endosomal and the lipoplex external membranes fuse [39]. The improved Transfection Efficiency (TE) of hexagonal complexes over lamellar ones is likely to be related to the lower energy barrier of fusion in hexagonal complexes [39]. In the case of lamellar complexes, the fusion energy barrier decreases (and TE increases) when the mole fraction of the CLs increases. These observations suggest that the electrostatic attraction between the lipoplex and the endosomal membrane triggers thermodynamic instability, leading to morphologic changes. The third step, DNA release, is enabled by the second stage as the fusion of bilayers may cause pores that connect the lipoplex internal water region (next to the DNA) to the cytoplasm. This chapter explores the thermodynamic driving forces governing the transfection process from the stage of adhesion and endocytosis, up to the stage of DNA release.

CL-DNA complexes adhere to cell membranes due to considerations similar to those triggering their formation, namely counterion release. As illustrated in fig. 4.1(A), both the plasma membrane and the external bilayer of the lipoplex are covered with layers of tightly bound counterions. These counterions neutralize the lipid charges and exclude the electric field from the oily parts (see also discussion in section 1.3). The loss of the positional entropy of the bound counterions is significantly lower than the energetic cost of allowing an electric field to penetrate the low dielectric hydrophobic core. When the oppositely-charged surfaces are in close proximity, the anionic and cationic lipids can neutralize each other, which enables the release of counterion pairs. The positional entropy gained by the released counterions is the main driving force for cell-lipoplex adhesion, which initiates cellular entry via endocytosis.

Figure 4.1(B) shows, schematically, a small segment of a lipoplex trapped within an endosome. The entrapped lipoplex represents a thermodynamic system that is substantially different from the lipoplex originally residing outside the cell. The difference stems from the presence of anionic lipids (ALs) in the plasma membrane which can now mix with the CLs and NLs of the lipoplex [39]. The process of lipid mixing is slow since it requires the lipids to "flip-flop" between monolayers; nevertheless, it encompasses a large entropic reward. Moreover, a redistribution of the lipids, while protecting the hydrophobic cores of the bilayers from electric fields, dictates that the counterions "escort" the flip-flopping charged lipids. When the counterions move between the different aqueous layers of the system, they meet counterions of opposite charge, which allows them to mutually leave the system without affecting its charge neutrality.

In section 1.6, the thermodynamic stability of a lipoplex was discussed briefly with the understanding that it is easier to produce and handle stable lipoplexes. Entrapment of a lipoplex by the endosome introduces anionic lipids and positive counterions that may be sufficient to render it thermodynamically unstable. This is obviously a desirable feature since the ultimate goal of the transfection process is lipoplex disassembly and DNA release. To better understand the thermodynamics of transfection, a simplified model was developed. The model, which con-



Figure 4.1: (A) Schematics of a complex of CLs (head groups depicted as red circles), NLs (head groups - grey circles), and DNA rods (larger yellow circles), separated from the plasma membrane which is composed of ALs (head groups - blue circles) and NLs. The lipoplex attracts a layer of bound anions (shown as blue circles), while the plasma membrane is surrounded by bound cations (red circles). (B) The state of the system after adhesion and endocytosis, the formation of which is driven by cation-anion pairs release. (C) A simplified model of the system depicted in B (see detailed explanation in text). The model system consists of six uniformly charged plates with charge density  $\sigma_{e,i}$  and three water layers (shown in blue) where the ions reside. The yellow stripes represent hydrophobic regions that do not include ions, and at which the electric filed must vanish. Notice that the fifth charged plate, which represents the DNA array, allows the crossover of ions.

siders electrostatic interactions within the framework of a mean field approximation, depicts the membranes as uniformly charged planner sheets. The DNA arrays are also represented in the same manner [fig. 4.1(C)]. This grossly simplified model is presented in the following section. Using this model, it is demonstrated that a lipoplex entrapped inside an endosome is inherently unstable. This instability, triggered by interactions between the cationic lipids of the lipoplex and the anionic lipids of the enveloping plasma membrane, is entropically controlled involving both remixing of the lipids and counterions release. The relevance of this model is confirmed by relating the free energy behaviour reported to the experimentally observed increase in transfection efficiency (TE) and the associated free energy barrier [39].

#### 4.2 Lipoplex "Mean Field" Model

The model described here assumes the conditions in fig. 4.1(B) illustrating the entrapped lipoplex immediately after endocytosis. The system constitutes six charged layers. In reverse order [from number 6 to 1, see fig. 4.1(B)], these charged layers correspond to: 6 - the lipid monolayer "below" the DNA array, 5 (also denoted by D) - the DNA array, 4 - the lipid monolayer "above" the DNA array, 3 and 2 - the "intermediate" lipid monolayers, and 1 - the lipid monolayer facing the cytoplasm. The three aqueous environments in the system will be denoted by: 1 - the cytoplasm, 2 - the intermediate thin water layer between the endosomal membrane and the lipoplex, and 3 - the internal water surrounding the first DNA layer. At the initial state, the lipid composition in monolayers 1 and 2 is that of the cell plasma membrane. It consists of ALs and NLs only and, for simplicity, will be assumed to be symmetric. Similarly, surfaces 3 - 6 are in the equilibrium state of the self-assembled lipoplex and have the same CLs to NLs ratio. It is also assumed that the NLs of the plasma and lipoplex membranes are of the same type.

The three major contributions to the free energy of the system arise from electrostatic interactions, lipid mixing entropy, and the entropy loss of bound counterions. In the model system depicted by fig. 4.1(C), the lipid monolayers are replaced with uniformly charged flat surfaces of charge density  $\sigma_{e,i}$  (i=1,2,3,4,6). The aqueous solutions have a dielectric constant  $\epsilon_w \simeq 80$ , while that of the hydrophobic regions,  $\epsilon_l$ , is assumed to be vanishingly small. This precludes the penetration of electric fields into the hydrophobic regions due to the associated very large electrostatic energy [15, 56]. (We note that the cytoplasam is occupied with concentrated macromolecules. Their presence changes the inside relative permittivity to values ranging from about 50 to over 200 [57, 58], for which the assumption concerning the exclusion of the electric field is from the hydrophobic regions still holds.) A somewhat greater approximation is replacing the electric field of the DNA array with the electric field of a flat surface of charge density per unit area  $\sigma_{e,5} = \lambda_{DNA}/d_{DNA}$  where  $\lambda_{DNA} \simeq 1.7e/\text{\AA}$  linear (per unit length) charge density of the DNA rod, e being the electron charge, and  $d_{DNA}$  is the inter-DNA spacing. A more detailed mean field calculation, taking into account the geometry of the DNA rods, can be performed computationally [40]. Such a calculation, however, is not necessary here. In order to understand the "big picture," one only needs to recognize that the counterions must arrange themselves to minimize the electrostatic energy. Any appreciable deviation in the ions distribution will involve an energy cost much larger than the entropic components of the free energy. Specifically for the model system in fig. 4.1(C), the number of ions per unit area present in each aqueous environment will have to match the areal charge densities of the surfaces in a manner that eliminates the electric field from the low dielectric regions. Interestingly, these constraints dictated some equilibrium states with anions in the internal solution of the DNA rods. An electric field can be present in the aqueous regions and the associated energy can be derived by integrating over the electrostatic energy density. Under no-salt conditions, this precisely gives the free energy cost attributed to the bound counterions. An exact calculation (which requires the solution of the Poisson-Boltzmann equation) is not performed here, but instead a simple approximation is employed by assigning each bound counterion with a free energy of  $1k_BT$  [40, 59].

For each monolayer, i, which is located at  $z_i$ , the mole fractions of the cationic and anionic

lipids is denoted by  $\phi_i^+$  and  $\phi_i^-$  respectively. The area per lipid,  $a_l$ , is taken as identical for all three lipid types (CLs, ALs, and NLs). Additionally  $\nu_j^+$  and  $\nu_j^-$  are the number densities, per unit volume, in region j of the cations and anions, respectively. To make the mean field approximation applicable, only cases where all of the charged lipids, as well as the counterions, are monovalent are considered. Assuming ideal lipid mixing in the monolayers, the uniform charge density of each surface is  $\sigma_{e,i} = e \left(\phi_i^- - \phi_i^+\right) / a_l$ . Since the system has a planar symmetry in the x - y plane, the electric field at any point must be orthogonal to the plane, i.e., along the z axis. Moreover,  $\nu^+ = \nu_j^+(z)$ ,  $\nu^- = \nu_j^-(z)$ , and both vanish inside the hydrophobic parts of the membranes [colored in yellow in fig. 4.1(C)] where  $\epsilon_l \ll \epsilon_w$ . The electric field at a given coordinate z is given by  $E_z = \tilde{\sigma}_e/2\epsilon_z\epsilon_0$ , where

$$\begin{split} \tilde{\sigma}_{e} &= \\ e \int_{\infty}^{z} \left[ \nu^{+} \left( z' \right) - \nu^{-} \left( z' \right) + \sum_{i=1}^{6} \sigma_{e,i} \delta \left( z' - z_{i} \right) \right] dz' - e \int_{z}^{-\infty} \left[ \nu^{+} \left( z' \right) - \nu^{-} \left( z' \right) + \sum_{i=1}^{6} \sigma_{e,i} \delta \left( z' z_{i} \right) \right] dz \\ &= 2e \int_{\infty}^{z} \left[ \nu^{+} \left( z' \right) - \nu^{-} \left( z' \right) + \sum_{i=1}^{6} \sigma_{e,i} \delta \left( z' - z_{i} \right) \right] dz' \end{split}$$

$$(4.1)$$

and  $\epsilon_z$  is the dielectric constant at z. The second equality in eq. (4.1) is due to the overall charge neutrality of the system.

The requirement that the electric field vanishes inside the low dielectric regions of the bilayers can be used to determine the number of bound counterions,  $N_j^B$ , in the three aqueous solutions of the system (j = 1, 2, 3). For this, note that in each such region we expect to find only one type of counterions since pairs of oppositely charged counterions can be released without affecting the charge balance. Thus, the number of counterions in solution j can be defined by  $\sigma_{e,j,B} = \zeta_j e N_j^B / a_l$ , where  $\zeta_j$  is the valency of the counterion. The number of counterions bound to the endosome on its cytoplasmic side is obtained from

$$\sigma_{e,1,B} = -\sigma_{e,1}.\tag{4.2}$$

This relation ensures that the electric field between layers i = 1 and i = 2 vanishes. By the same logic, in the intermediate water layer

$$\sigma_{e,2,B} = -(\sigma_{e,1} + \sigma_{e,2} + \sigma_{e,3} + \sigma_{e,1,B}) = -(\sigma_{e,2} + \sigma_{e,3}), \qquad (4.3)$$

and in the internal water layer

$$\sigma_{e,3,B} = -(\sigma_{e,4} + \sigma_{e,5} + \sigma_{e,6}).$$
(4.4)

At short times after cellular intake, the surface charge densities of the endosome layers,  $\sigma_{e,i}$ , match those of the cell plasma membrane (i = 1, 2), and the lipoplex (i = 3 - 6). This initial state is, however, no longer the equilibrium state, since the anionic and cationic lipids can now mix with each other. This occurs via slow, but steady, "flip-flopping" events switching lipids between monolayers i = 1 - 4. Note that this explicitly assumes that monolayer i = 6 does not participate in the lipid mixing process. This is because the monolayer is separated from the two external bilayers (i = 1 - 4) by both the DNA array and the water layer. The redistribution of lipids between the participating monolayers not only increases the mixing entropy of the lipids within the layers, but may also allow further release of counterions whose densities within the aqueous solutions are simultaneously updated in order to satisfy the conditions of eqs. (4.2) to (4.4). Taking these considerations into account, the total free energy of the system, per unit area of the lipids  $a_l$ , is written as

$$\frac{F}{a_l k_B T} = \sum_{i=1}^{4} \left[ \phi_i^+ \log\left(\phi_i^+\right) + \phi_i^- \log\left(\phi_i^-\right) + \left(1 - \phi_i^+ - \phi_i^-\right) \log\left(1 - \phi_i^+ - \phi_i^-\right) \right] + \sum_{j=1}^{3} N_j^B \quad (4.5)$$

where  $\phi_i^{\pm}$  are the mole fractions of cationic (+) and anionic (-) lipids at the *i*-th layer, and  $N_j^B$  is the number of bound counterions per unit area a at the *j*-th water layer (see definitions also above). The first term in eq. (4.5) accounts for the mixing entropy of the lipids in each monolayer, while the second term represents the entropy cost of bound counterions. The former is based on the mean field assumption of ideal mixing. The latter employs the commonly used assumption of  $1k_BT$  per bound counterion.

Let  $\{\phi_{i,0}^{\pm}\}$  denote the initial mole fractions of the CLs and ALs. To find the equilibrium state, the free energy in eq. (4.5) needs to be minimized with respect to the variables  $\{\phi_i^{\pm}\}$ , under the constraints that  $\sum_{i=1}^{4} \phi_i^+ = \sum_{i=1}^{4} \phi_{i,0}^{\pm}$  representing the preservation of the total number of lipids of each type. The dependence of  $\{N_j^B\}$  on the variables  $\{\phi_i^{\pm}\}$  is given by eqs. (4.2) to (4.4), where  $N_j^B = (a_l/e) |\sigma_{e,j,B}|$ , and  $\sigma_{e,i} = e(\phi_i^+ - \phi_i^-)/a_l$ . Notice that in contrast to the lipids, the total number of bound counterions is not fixed but may vary by intake or release of ions from the cytoplasm.



Figure 4.2: Solid line - the free energy  $\Delta F$  (see text for definition), as a function of  $q = 2\Phi^+/\Phi_D$  with  $\Phi_D = 1$  and  $\Phi_- = 0.5$ . Dot-dashed and dotted lines show the partial contributions to  $\Delta F$  originating, respectively, from counterion release and lipid mixing.

#### 4.3 Free Energy Minimization of Trapped Lipoplex

The free energy  $\Delta F$ , per unit area  $a_l$ , that the system may gain during stage (ii) of the transfection process is given by the difference in F [eq. (4.5)] between the equilibrium and initial states. In the initial state, the distribution of lipids in the plasma membrane is given by  $\phi_{i,0}^- = \Phi_-$  and  $\phi_{i,0}^+ = 0$ , for i = 1, 2. In the lipoplex membranes  $(i = 3, 4, 6), \phi_{i,0}^- = 0$  and  $\phi_{i,0}^+ = \Phi_+$ . For convenience, the "mole fraction,"  $\Phi_D = -(a_l/e) \sigma_{e,5}$ , associated with the DNA array is defined here. Figure 4.2 plots the results for  $\Delta F$  as a function of  $q_l = 2\Phi^+/\Phi_D$ , which is the lipoplex charge ratio. The ratio  $q_l$  is varied by changing  $\Phi_+$ , while keeping  $\Phi_D = 1$  fixed. The initial anionic lipid mole fraction in the plasma membrane,  $\Phi_{-}$ , is set to 0.5. The data for  $\Delta F$  is plotted in the solid line, while the dotted and dashed curves show, respectively, the partial contributions due to lipid mixing first term in eq. (4.5) and the bound counterions (second term). The results reveal the existence of three different regimes. In regime (i), corresponding to  $q_l < 1$ , the decrease in  $\Delta F$  with  $q_l$  is very slow and arises exclusively from the lipid mixing term. In regime (ii), where  $1 < q_l < 4/3$ , the decrease in  $\Delta F$  is faster due to the additional contribution of counterion release. Finally, in regime (iii), where  $q_l > 4/3$ , lipid mixing again becomes a dominant factor, though there is a fixed gain of entropy due to counterion release.

The key to understanding the trends in fig. 4.2 is to correctly identify the transition points between the three different regimes. The transition from (i) to (ii) occurs at  $q_l = 1$ , which is the isoelectric point of the lipoplex, namely the point where the total cationic charge of

the lipids *exactly* matches the negative one of the DNA array:  $2\Phi_{+} = \Phi_{D}$ . Therefore, to satisfy eq. (4.4), the internal solution surrounding the DNA array [regime (i)] includes cations. Similarly, the external solution facing the cytoplasm and the intermediate solutions between the plasma membrane and the lipoplex also include cations only [eqs. (4.2) and (4.3)]. Since the system contains no anions, it is impossible to release cation-anion pairs, which explains why, in this regime, the only contribution to the free energy comes from the mixing of the lipids. Equilibrium is achieved when the lipids are evenly distributed between the four monolayers. This is depicted in regime (i) in fig. 4.3, which presents the equilibrium distribution of the lipids between the four monolayers. In contrast to regime (i), in regime (ii)  $(1 < q_l < 4/3)$  both the intermediate and the internal solutions include anions at the initial conditions. Therefore, the decrease in free energy now involves contributions of both lipid mixing and counterion release. Detailed calculation shows that in regime (ii), equilibrium is reached when all the anions are released, while the excess cations accumulate at the internal water layer around the DNA molecules. Moreover, to satisfy the conditions of eqs. (4.2) and (4.3), the net charge density  $\sigma_{e,i}$  in monolayers i = 1, 2, 3 must vanish, which means that the mole fractions of CLs and ALs in each of these layers are the same. The composition of layer i = 4 is different, which implies that lipid mixing is not optimized in regime (ii). Regime (ii) ends at  $q_l = 4/3$ , which is the point where the total charge of the system (including the ALs of the plasma membrane, the



Figure 4.3: The equilibrium distribution of CLs (solid lines) and ALs (dot-dashed lines) in monolayers i = 1 (black), i = 2, 3 (red) and i = 4 (yellow). The vertical dashed lines mark the transition points between the different regimes discussed in the text.

4.3

CLs of the lipoplex, and the DNA array) vanishes; i.e., when

$$3\Phi_{+} = \Phi_{D} + 2\Phi_{-}.$$
 (4.6)

Therefore, at this point, the total number of bound cations and anions is also the same. Further increasing  $q_l$ , by increasing the fraction of the CLs and the number of associated bound anions, enters the system into regime (iii). In this regime, the total gain of free energy due to counterion release saturates, since it is capped by the number of cations originally bound to the plasma membrane. The free energy  $\Delta F$  continues to decrease with  $q_l$  since the lipids can now mix better and attain a more even distribution between monolayers i = 1 - 4. Notice that in fig. 4.3, the composition of lipids in monolayers i = 2, 3 is always the same, which is anticipated since any exchange of lipids between these two monolayers will not influence the charge balance condition of eq. (4.3).

Figure 4.4(A) depicts the results of  $\Delta F$  for a lipoplex with more densely packed DNA rods ( $\Phi_D = 1.4$ ). The charge density of the plasma membrane is the same as in fig. 4.2,  $\Phi_- = 0.5$ . The characteristics of fig. 4.4(A) are very similar to the those observed in fig. 4.2. One noticeable difference is that regime (ii) starts below the isoelectric point  $q_l = 1$ , at  $q_l = \Phi_D^{-1} \simeq 0.71$ . As in the previously discussed case, in regime (i), the initial state of the system includes only cations. In regime (ii), the intermediate water layer contains anions, which are released upon reaching equilibrium. The kink appears at the isoelectric point, above which, when  $q_l > 1$ , the internal solution also contains anions. The transition between regions (ii) and (iii) is at  $q_l \simeq 1.14$ , as dictated by eq. (4.6). In regime (iii), the contribution of counterions release to  $\Delta F$  is fixed by the amount of cations present in the system.

Figure 4.4(B) depicts the results of  $\Delta F$  for a lipoplex with more loosely packed DNA rods  $(\Phi_D = 0.6)$ , with a plasma membrane of charge density  $\Phi_- = 0.5$ . Here, the transition from (i) to (ii) is at the isoelectric point  $q_l = 1$  which, as noted above, is where anions first appear at the internal layer next to the DNA. The kink happens at  $q_l = \Phi_D^{-1} \simeq 1.67$  above which, the intermediate water layer contains anions at the initial state. The transition from (ii) to (iii) occurs at  $q_l \simeq 1.78$ , which, similarly to the previous cases, is predicted by eq. (4.6). Note that in both cases in fig. 4.4, after the kink, the slope of the free energy doubles.

4.3



Figure 4.4: (A) The free energy  $\Delta F$  (solid line) and the partial contributions to  $\Delta F$  originating from counterions release (dot dashed line) and lipid mixing (dotted line). Results are for a lipoplex with densely packed DNA molecules ( $\Phi_D = 1.4$ ). The vertical dashed lines marks the transition points between the regimes discussed in the text. (B) Same as in (A) for a lipoplex with loosely packed DNA molecules ( $\Phi_D = 0.6$ ).

### 4.4 Membranes Fusion, Pore Formation, and DNA Release

The free energy calculations reported in figs. 4.2 and 4.4 demonstrate the inherent instability of the entrapped lipoplex, triggered by its interactions with the enveloping plasma membrane. The latter constitutes a reservoir of ALs that can mix with the CLs of the lipoplex. Lipid mixing occurs through "flip-flop" events which, in general, are slow, especially when lipids transfer between distinct bilayers (as opposed to lipids moving between monolayers of the same membrane, which is probably somewhat faster). The exchange of lipids between the plasma and lipoplex membranes may cause these two membranes to fuse; a scenario that thus far was not taken into account [39]. Fusion is thermodynamically favorable since it reduces the number of participating monolayers from i = 4 to i = 2 and thus, it further increased the lipid-mixing entropy. However, it comes with the (initial) cost of bending energy. Crossing the associated energy barrier is what primarily determines the rate of successful endosomal escape and sets the TE (transfection efficiency). Experimentally, it is known that the TE of lamellar complexes grows exponentially with the cationic charge density of the complex,  $\Phi_+ = (q_i \Phi_D)/2$ [39]. This observation supports the picture of activated fusion where TE~ exp  $(-\Delta F_{fuse}/k_BT)$ , and

$$\Delta F_{fuse} = a\kappa - b\Phi_+ + c, \tag{4.7}$$

where  $\kappa$  is the bending rigidity of the bilayers [as in eq. (1.1)], while *a*, *b*, and *c* are parameters, the parameters of which may depend on the molecular conditions inside the endosome. The first term in eq. (4.7) represents the curvature energy cost of the fusion which, to a good approximation, is independent of the charge densities. The second term has been previously attributed to the electrostatic attraction between the plasma membrane and the complex. The last term accounts for other effects, e.g. the capacity of the low-pH environment of the endosome to disrupt the lipid bilayer. The results in this chapter reveal that the origin of the second term is actually not energetic but entropic. The free energy gain  $\Delta F$  due to lipid mixing and the associated counterion release at the second stage of the transfection process (see solid curves in figs. 4.2 and 4.4) grows piecewise linearly with  $\Phi_+$ . This linear dependence is simply a reflection of the fact that when the lipoplex contains a higher fraction of CLs, the potential entropic gain involved in ideal mixing of lipids and counterions release is larger.

Once fusion occurs, a hole opens that connects the cytoplasm and the internal water layer containing the first DNA array of the lipoplex [fig. 4.5(A)]. Such a hole allows for the influx of



Figure 4.5: A schematic illustration of DNA release from trapped lipoplex after pore formation. This is viewed as a two step process which involves (A) the influx of macroions into the complex and (B) condensation out of the endosome. For ease of viewing, monovalent counterions which may be released during the condensation, are not displayed.

positively charged (macro)molecules, e.g., unstructured peptides, which are able to condense the DNA molecules and release them into the cytoplasm [fig. 4.5(B)]. Since such macroions also condense counterions about them, further counterion release may take part in this process as well. Removing the first DNA layer results in a smaller lipoplex whose composition is similar to the original one. Interactions of this positively charged complex with negatively charged components of the cell may cause renewed thermodynamic instability and lead to further degradation of the CL-DNA complex.

#### 4.5 Conclusions

In this chapter, a simplified model was used to study the transfection thermodynamics of CL-DNA complexes. The formation of these complexes is known to be driven by the increase in the translational entropy of the counterions that are released to the bulk solution when the oppositely charged membranes and DNA molecules associate together. The same counterion release mechanism is also responsible (at least partially) for the association of the lipoplex with the cell plasma membrane, which initiates the transfection process. In this chapter, it was argued that the contact between the lipoplex external bilayer and the plasma membrane triggers thermodynamic instability that leads to lipoplex degradation, which is essential for the transfection process to proceed.

The thermodynamic instability of the entrapped lipoplex is of entropic origin: It stems from the fact that the lipid composition of the lipoplex and the plasma membrane are different and, therefore, mixing of these lipids increases the configurational entropy of the system. Since the two membranes are oppositely charged, the mixing of lipids has another effect: It reduces the charge density of the membranes. This enables further counterion release and a further decrease in the free energy. Thus, the counterion release mechanism which has been identified as the thermodynamic driving force for formation of various supramolecular structures [17], is here used to explain the disassembly of such structures.

Despite the gross simplicity of the model presented here, and the fact that it ignores specific molecular details, it successfully predicts a roughly linear increase in the free energy gain with the mole fraction of CLs in the complex, which explains the observed exponential increase in transfection efficiency of lamellar complexes with the charge density [39]. The model is based on a mean-field picture and replaces the lipid monolayers with uniformly-charged flat surfaces. This modeling approach is routinely used in theoretical studies of electrostatic effects in soft matter systems. We avoid solving the Poisson-Boltzmann equation explicitly by simply assigning instead a fixed free energy gain of  $1k_BT$  for each released counterion. By solving the Poisson-Boltzmann equation, a more accurate value may be obtained (which may depend on the water region next to the DNA array where the counterion resides in a denser condition), but the result is only expected to be different by a factor of order unity. What might be the boldest approximation in the model is the replacement of the DNA array with a uniformly charged surface as well. By employing this picture, two entropic contributions of opposite signs are ignored: (i) The CLs in the monolayers facing the DNA arrays are expected to accumulate near the DNA rods, which lowers their mixing entropy; (ii) the space available to the ions surrounding the DNA molecules is quite small, which implies that the entropic gain involving in their release may be higher than assumed by the model. The order of magnitude of these effects is comparable to the other contributions discussed here. Therefore, even though it is not expected that these two entropic will cancel each other out, they are not expected to dominate the thermodynamic behavior and significantly modify it.

### Chapter 5

### Conclusions

This thesis reported on instabilities in three different lipid-based systems: Small membranes under positive or negative surface tension (chapter 2), charged membranes with monovalent or pentavalent counterions (chapter 3), and lipoplexes trapped inside an endosome (chapter 4). The first two systems were studied using coarse-grained simulations and presented instabilities under "extreme cases": When the membrane is ecumenically stretched, or when it is highly charged with *monovalent* counterions, pores are formed to relieve the excess elastic energy at the cost of line tension. When the membrane is compressed, or when it is highly charged with *pentavalent* counterions, buckling occurs, which also reduces the areal elastic energy at the cost of bending energy. Even though both system responded by the same apparent instabilities of pore formation and buckling, the mechanism of these instabilities is different when the membrane is charged or under surface tension:

- Pore formation is expected intuitively; pores relive the membrane tension energy by reducing its total projected area [at the cost of line tension [60]]. The origin of the area increase, when the membrane is stretched by positive frame tension is not exactly the same when it is done by charging the membrane. The fact that the surface tension was explicitly set to zero in the charge membrane case, and the quality of the fit in fig. 3.2(A), indicate that the pore is not formed by an indirect frame tension applied to the membrane (e.g., the ions' osmotic pressure), but rather due to the electrostatic repulsions which act against the hydrophobic attractive (cohesive) interactions between the lipids.
- Membrane buckling, while it looks similar in the two systems, is not the same. There are several indications for that:
  - Prior to the buckling of charged membrane in the presence of pentavalent counterions, the equality  $\gamma = \tau = 0$  still holds true (as in the monovalent counterions

case). When the membranes where compressed, however, the equality  $\gamma$  approached a constant value as  $\tau$  decreased.

- The buckling of charged membranes with pentavalent counterions occurs through a sharp decrease in the bending rigidity,  $\kappa$ , while for compressed membrane the decrease in  $\kappa$  is fairly moderate.
- The projected area,  $A_p$ , dropped with the increase in compression while that of the charged membrane with pentavalent counterions remained roughly constant.
- It is also important to highlight the difference between the monovalent and pentavalent counterions cases. While in the former case, the counterions formed a cloud around the membrane, in the latter, the counterions adhered to the membrane, effectively incorporating charges into the membrane. These charges caused attraction and repulsion between different parts of the membrane that might cause non-even amplification of the undulation modes.

The entrapped lipoplex discussed in chapter 4 also brings forward instability that is associated with electrostatic interactions and counterions behaviours. The lipoplex and the cell plasma membrane are attracted to each other through electrostatic interactions; that allows for counterion release. Once the lipoplex is entrapped, the mixing of anionic and cationic lipids from the cell and lipiplex membranes offers free energy gain as it allows for further counterion release and reduces the surface charge density of the membranes. This free energy gain reduces the free energy barrier of fusion, which allows the formation of a pore that connects the internal solution to the DNA array to the cytoplasm.

The discussion in chapter 4 is limited to a grossly simplified model. This model, while it provides initial insight about the importance of counterion release and lipid mixing in the transfection process, cannot contribute to the study about the fusion process itself. Highly coarse-grained simulations, such as those used in chapters 2 and 3 have been applied to demonstrate the self assembly of lipoplex [61]. Similar methods may be applied to study the dynamics of the lipoplex degradation problem and the fusion process specifically.

## Appendix A

### The Free Energy of Bound Counterions

This appendix presents an interesting relationship between the electrostatic energy and the number of counterions bound to a *flat surface*. The starting point for this proof is the Poisson-Boltzmann equation for a flat membrane in contact with a monovalent salt solution of bulk concentration  $n_0$ , which reads

$$\nabla^2 \psi = \frac{2\nu_0 e}{\epsilon_w \epsilon_0} \sinh\left(\frac{\mathrm{e}\psi}{k_B T}\right). \tag{A.1}$$

Since the surface is flat, the potential is invariant under translation in x and y directions and the Poisson-Boltzmann equation is reduced to

$$\psi'' = \frac{2e\nu_0}{\epsilon_w\epsilon_0} \sinh\left(\frac{\mathrm{e}\psi}{k_BT}\right),\tag{A.2}$$

where  $\psi'$  and  $\psi''$  denote the first and second derivative by z, respectively. Multiplying both sides by  $\psi'$  results in

$$\psi''\psi' = \frac{2e\nu_0}{\epsilon_w\epsilon_0}\sinh\left(\frac{\mathrm{e}\psi}{k_BT}\right)\psi'.$$
(A.3)

Notice that the left-hand side is half the derivative of  $(\psi')^2$ 

$$\left[\left(\psi'\right)^2\right]' = 2\psi''\psi',\tag{A.4}$$

and the right-hand side is

$$\cosh'\left(\frac{\mathrm{e}\psi}{k_BT}\right) = \sinh\left(\frac{\mathrm{e}\psi}{k_BT}\right)\frac{\mathrm{e}\psi'}{k_BT}.$$
(A.5)

Therefore eq. (A.3) is rewritten as

$$\frac{1}{2} \left[ \left( \psi' \right)^2 \right]' = \frac{2k_B T \nu_0}{\epsilon_w \epsilon_0} \cosh' \left( \frac{\mathrm{e}\psi}{k_B T} \right), \tag{A.6}$$

which can be integrated over z to yield

$$\frac{1}{2} \left(\psi'\right)^2 = \frac{2k_B T \nu_0}{\epsilon_w \epsilon_0} \cosh\left(\frac{\mathrm{e}\psi}{k_B T}\right) + C,\tag{A.7}$$

where C is an integration constant. The boundary conditions for a flat surface require that the electric field and potential would vanish far away from the surface  $(\psi_{z\to\infty} \to 0, \psi'_{z\to\infty} \to 0)$ . Using this, one can find the integration constant

$$\frac{1}{2} \left(\psi'\right)_{z \to \infty}^2 = \frac{2k_B T \nu_0}{\epsilon_w \epsilon_0} + C = 0$$

$$C = -\frac{2k_B T \nu_0}{\epsilon_w \epsilon_0}.$$
(A.8)

Thus eq. (A.7) reads

$$\frac{1}{2} (\psi')^2 = \frac{2k_B T \nu_0}{\epsilon_w \epsilon_0} \left[ \cosh\left(\frac{e\psi}{k_B T}\right) - 1 \right] \\ = \frac{k_B T}{\epsilon_w \epsilon_0} \left( \nu_0 \exp\left(-\frac{e\psi}{k_B T}\right) + \nu_0 \exp\left(\frac{e\psi}{k_B T}\right) - 2\nu_0 \right).$$
(A.9)

Notice though, that the first two terms are the equilibrium densities of the cations and anions repectively. Thus we get that

$$\frac{1}{2} (\psi')^2 = \frac{k_B T}{\epsilon_w \epsilon_0} (\nu_+ + \nu_- - 2\nu_0).$$
 (A.10)

Generally speaking, the electrostatic free energy per unit are,  $f_e$ , of flat charge surface in ionic solution reads

$$f_e = \frac{\epsilon_w \epsilon_0}{2} \int \left(\frac{\partial \psi}{\partial z}\right)^2 dz + k_B T \int \left[\sum_i \nu_i \log\left(\frac{\nu_i}{\nu_0}\right) - (\nu_i - \nu_0)\right] dz \tag{A.11}$$

where the first term is the electrostatic contribution and the second term is the entropy loss of the ions due to the deviation from uniform concentration. A simplified picture of monovalent 1:1 salt, the bulk free energy of charged surface (compared to a homogenous electrolyte reservoir) reads

$$F_{el} = \frac{\epsilon_w \epsilon_0}{2} \int_0^\infty (\psi')^2 dz + k_B T \int_0^\infty \left[ \nu_+ \log\left(\frac{\nu_+}{\nu_0}\right) + \nu_- \log\left(\frac{\nu_-}{\nu_0}\right) + \right] dz - k_B T \int_0^\infty (\nu_+ + \nu_- - 2\nu_0) dz.$$
(A.12)

The entropy term in eq. (A.12) is composed from a mixing term, which accounts for the nonuniform ion distribution, and a "counting" term, which measures the deviation of the local ion concentration from the uniform distribution. The integration over the last term results in the number of bound counterions and, dividing by the membrane area, results in the surface charge density.

Applying eq. (A.10) to eq. (A.11), the first and last terms cancel each other, and the bulk free energy reads

$$F = k_B T \int_0^\infty \left[ \nu_+ \log\left(\frac{\nu_+}{\nu_0}\right) + \nu_- \log\left(\frac{\nu_-}{\nu_0}\right) + \right] dz.$$
(A.13)

This suggests that the electrostatic energy per bound counterion is fixed at  $1k_BT$  regardless of the surface charge density. This is related to the Gouy-Chapman distance the ions are allowed to be separated from the surface. When another charged object approaches to a distance smaller than the Gouy-Chapman distance, the electrostatic interactions between the surface and the object are favourable and the counterion is released.

### Bibliography

- [1] J. Israelashivili, *Intermolecular and surface forces*, Academic Press, London (1985).
- [2] B. Alberts and et al, *Molecular Biology of the Cell*, Garland Science, New York (2002).
- U. Seifert and R. Lipowsky, Morphology of Vesicles, chapter 8, 403–463, Elsevier, Amsterdam (1995).
- [4] W. Helfrich, Elastic properties of lipif bilayers: theory and possible experiments, Z. Naturforch, 28C, 693 (1973).
- R. Lipowsky, Generic Interactions of Flexible Membranes, chapter 11, 521–602, Elsevier, Amsterdam (1995).
- [6] R. Bruinsma and P. Pincus, Protein aggregation in membranes, Curr. Opin. Solid State Mater. Sci., 1, 401 (1996).
- Y. Y. Avital and O. Farago, Small membranes under negative surface tension, J. Chem. Phys., 142, 124902 (2015).
- [8] Y. Y. Avital, N. Grønbech-Jensen and O. Farago, *Elasticity and mechanical instability of charged lipid bilayers in ionic solutions*, Eur. Phys. J. E, **37**, 69 (2014).
- [9] Yotam Y Avital, Niels Grønbech-Jensen and Oded Farago, The thermodynamics of endosomal escape and DNA release from lipoplexes, Phys. Chem. Chem. Phys., 18, 2591 (2016).
- [10] S. Rowlinson and B. Widom, *Molecular Theory of Capillary*, Clarendon Press, Oxford (1982).
- [11] O. Farago and P. Pincus, Statistical mechanics of bilayer membrane with a fixed projected area, J. Chem. Phys., 120, 2934 (2004).

- [12] O. Farago, Mechanical surface tension governs membrane thermal fluctuations, Phys Rev E, 84, 051914 (2011).
- [13] E. Evans and W. Rawicz, Entropy-driven tension and bending elasticity in condensed-fluid membranes, Phys. Rev. Lett., 64, 2094 (1990).
- [14] D. Andelman, , chapter 12, 603–642, Elsevier, Amsterdam (1995).
- [15] M. Kiometzis and H. Kleinert, *Electrostatic stiffness properties of charged bilayers*, Phys. Lett. A, 140, 520 (1989).
- [16] M. Winterhalter and W. Helfrich, Effect of surface charge on the curvature elasticity of membranes, J. Phys. Chem., 92, 6865 (1988).
- [17] D. Harries, S. May and A. Ben-Shaul, Counterion release in membrane-biopolymer interactions, Soft matter, 9, 9268 (2013).
- [18] A. G. Moreira and R. R. Netz, Simulations of counterions at charged plates, Eur. Phys, J. E, 8, 33 (2002).
- [19] N. Grønbech-Jensen, R. J. Mashl, R. F. Bruinsma and W. M. Gelbart, Counterion-induced attraction between rigid polyelectrolytes, Phys. Rev. Lett., 78, 2477 (1997).
- [20] T. T. Nguyen, I. Rouzina and B. I. Shklovskii, Negative electrostatic contribution to the bending rigidity of charged membranes and polyelectrolytes screened by multivalent counterions, Phys. Rev. E, 60, 7032 (1999).
- [21] A. W. C. Lau and P. Pincus, *Charge-Fluctuation-Induced Nonanalytic Bending Rigidity*, Phys. Rev. Lett., 81, 1338 (1998).
- [22] Y. W. Kim and W. Sung, Effects of charge and its fluctuation on membrane undulation and stability, Europhys. Lett, 58, 147 (2002).
- [23] A. W. C. Lau, D. B. Lukatsky, P. Pincus and S. A. Safran, *Charge fluctuations and counterion condensation*, Phys. Rev. E, 65, 051502 (2002).
- [24] E. Trizac and L. Šamaj, Like-charge colloidal attraction: A simple argument, 61, IOS (2012).
- [25] L. Šamaj and E. Trizac, Critical phenomena and phase sequence in a classical bilayer Wigner crystal at zero temperature, Phys. Rev. B, 85, 205131 (2012).

- [26] H. N. W. Lekkerkerker, Contribution of the electric double layer to the curvature elasticity of charged amphiphilic monolayers, Physica A, 159, 319 (1989).
- [27] A. Fogden, D. J. Mitchell and B. W. Ninham, Undulations of charged membranes, Langmuir, 6, 159 (1990).
- [28] A. Fogden and B. W. Ninham, *The bending modulus of ionic lamellar phases*, Langmuir, 7, 590 (1992).
- [29] N. Smyth-Templeton and D. D. Lasic, Gene Therapy. Therapeutic mechanisms and strategies, Marcel Dekker Inc., New York (2000).
- [30] P. L. Felgner, M. J. Heller, P. Lehn, J.-P. Behr and F. C. Szoka, Artificial Self-Assembling Systems for Gene Delivery, American Chemical Society, Washington, DC (1996).
- [31] A. D. Miller, *Cationic liposomes for gene therapy*, Angew. Chem. Int. Ed., **37**, 1768 (1998).
- [32] R. I. Mahato and S. W. Kim, *Pharmaceutical Perspectives of Nucleic Acid-Based Thera*peutics, Taylor and Francis, London and New York (2002).
- [33] L. Huang, M.-C. Hung and E. Wagner, Non-Viral Vectors for Gene Therapy, Elsevier, San Diego (2005).
- [34] K. Ewert, A. Ahmad, H. M. Evans and C. R. Safinya, Cationic lipid-DNA complexes for non-viral gene therapy: relating supramolecular structures to cellular pathways, Expert Opin. Biol. Ther., 5, 33 (2005).
- [35] H. Kamiya, H. Tsuchiya, J. Yamazaki and H. Harashima, Intracellular trafficking and transgene expression of viral and non-viral gene vectors, Adv. Drug Deliv. Rev., 52, 153 (2001).
- [36] J. O. R\"adler, I. Koltover, T. Salditt and C. R. Safinya, Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes, Science, 275, 810 (1997).
- [37] I. Koltover, T. Salditt, J. O. R\"adler and C. R. Safinya, An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery, Science, 281, 78 (1998).
- [38] C. R. Safinya, Structures of lipid-DNA complexes: supramolecular assembly and gene delivery, Curr. Opp. Stract. Biol., 11, 440 (2001).

- [39] K. Ewert, N. L. Slack, A. Ahmad, H. M. Evans, A. J. Lin, C. E. Samuel and C. R. Safinya, Cationic lipid-DNA complexes for gene therapy: Understanding the relationship between complex structure and gene delivery pathways at the molecular level, Curr. Med. Chem., 11, 133 (2004).
- [40] D. Harries, S. May, W. M. Gelbart and A. Ben-Shaul, Structure, Stability and Thermodynamics of Lamellar DNA-Lipid Complexes, Biophys. J., 75, 159 (1998).
- [41] A. Ahmad, H. M. Evans, K. Ewert, C. X. George, C. E. Samuel and C. R. Safinya, New multivalent cationic lipids reveal bell curve for transfection efficiency versus membrane charge density: lipid-DNA complexes for gene delivery, J. Gene Med., 7, 739 (2005).
- [42] S. Hubner, B. J. Battersby, R. Grimm and G. Cevc, Lipid-DNA Complex Formation: Reorganization and Rupture of Lipid Vesicles in the Presence of DNA As Observed by Cryoelectron Microscopy, Biophys. J., 76, 3158 (1999).
- [43] M. T. Kennedy, E. V. Pozharski, V. A. Rakhmanova and R. C. MacDonald, Factors governing the assembly of cationic phospholipid-DNA complexes, Biophys. J., 78, 1620 (2000).
- [44] I. R. Cooke, K. Kremer and M. Deserno, Tunable generic model for fluid bilayer membranes, Phys. Rev. E, 72, 011506 (2005).
- [45] O. Farago and N. Grønbech-Jensen, Computational and analytical modeling of cationic lipid-DNA complexes, Biophys. J., 92, 3228 (2007).
- [46] O. Farago, Mode excitation Monte Carlo simulations of mesoscopically large membranes, J. Chem. Phys., 128, 184105 (2008).
- [47] W. K. den Otter, Area compressibility and buckling of amphiphilic bilayers in molecular dynamics simulations, J. Chem. Phys., 123, 214906 (2005).
- [48] K. Kleinert, Thermal softening of curvature elasticity in membranes, Phys. Lett., 114, 263 (1986).
- [49] R. R. Netz, Buckling and nonlocal elasticity of charged membranes, Phys. Rev. E, 64, 051401 (2001).
- [50] N. Grønbech-Jensen, G. Hummer and K.M. Beardmore, Lekner summation of Coulomb interactions in partially periodic systems, Mol. Phys., 92, 941 (1997).

- [51] A. W. C. Lau and P. Pincus, Counterion Condensation and Fluctuation-Induced Attraction, Phys. Rev. E, 66, 041501 (2002).
- [52] H. I. Petrach, S. Tristram-Nagle, K. Gawrisch, D. Harries, V. A. Persegian and J. F. Nagle, Biophys. J., 86, 1574 (2004).
- [53] P. Mukhopadhyay, L. Monticelli and D. P. Tieleman, Molecular Dynamics Simulation of a Palmitoyl-Oleoyl Phosphatidylserine Bilayer with Na+ Counterions and NaCl, Biophys. J., 86, 1601 (2004).
- [54] W. Rawicz, K. C. Olbrich, T. McIntosh D., Needham and E. Evans, Effect of Chain Length and Unsaturation on Elasticity of Lipid Bilayers, Biophys. J., 79, 328 (2000).
- [55] S. Buyukdagli, M. Manghi and J. Palmeri, Variational approach for electrolyte solutions: From dielectric interfaces to charged nanopores, Phys. Rev. E, 81, 041601 (2010).
- [56] M. Winterhalter and W. Helfrich, Bending elasticity of electrically charged bilayers: coupled monolayers, neutral surfaces, and balancing stresses, J. Phys. Chem., 96, 327 (1992).
- [57] C. L. Davey D. B. Kell, The low-frequency dielectric properties of biological cells, chapter 5, 159–207, Birkhäuser, Zürich (1995).
- [58] J. Gimsa, T. Müller, T. Schnelle and G. Fuhr, Dielectric spectroscopy of single human erythrocytes at physiological ionic strength: dispersion of the cytoplasm, Biophys. J., 71, 495 (1996).
- [59] S. May, D. Haries and A. Ben-Shaul, The Phase Behavior of Cationic Lipid-DNA Complexes, Biophys. J., 78, 1681 (2000).
- [60] O. Farago and C. D. Santangelo, Pore formation in fluctuating membranes, J. Chem. Phys., 122, 044901 (2005).
- [61] O. Farago and N. Grønbech-Jensen, Simulation of self-assembly of cationic lipids and DNA into structured complexes, J. Am. Chem. Soc, 131, 2875 (2009).

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הוגש לסנאט של אוניברסטת בן גוריון בנגב

יותם יוסף אביטל

ע''י

מחקר לשם מילוי חלקי של הדרישות לקבלת תואר ''דוקטור לפילוסופיה''

# אי-יציבות של ממברנות ליפידיות וקומפלקסים של ליפידים ודנ<sup>יי</sup>א

באר שבע 2015 לאוקטובר, 2015 כ''ח בתשרי, ה'תשע''ו

ם על שם קרייטמן	אישור דיקן בית הספר ללימודי מחקר מתקדמינ
	חתימה

אישור המנחה: פרופ' עודד פרגו חתימה \_\_\_\_\_\_ תאריך\_\_\_\_\_\_

הוגש לסנאט של אוניברסטת בן גוריון בנגב

יותם יוסף אביטל

ע''י

# אי-יציבות של ממברנות ליפידיות וקומפלקסים של ליפידים ודנ''א
העבודה נעשתה בהדרכת פרופ' עודד פרגו במכון אילזה כץ למדע וטכנולוגיה בתחום הננומטרי, ובמחלקה להנדסה ביו רפואית, הפקולטה להנדסה.

## תקציר

ממברנות ליפידיות, הנמצאות בין שתי תמיסות, מגדירות את הגבול של התא החי ושל האיברונים הפנימיים שלו (אורגנלות). ממברנות מגדירות את קצה התא ומאפשרות את קיומן של שתי תמיסות שונות זו ליד זו, מצב הנחוץ לתפקוד התא. ממברנות משתתפות בתהליכים ביולוגיים שונים כגון הצמדות בין שני תאים, מעבר יונים מבוקר, ומעבר אותות. תפקיד הממברנה בתהליכים אלו אינו מתבצע בהכרח על ידי הממברנה בכללותה, אלא יכול להתבצע על ידי רכיבים הכלולים בה (למשל, חלבונים) או על ידי חלק מהיליפידים בעלי תכונה יחודית.

כוחות הפועלים על ממברנות ביולוגיות יכולים להפוך את הממברנה ללא יציבה ולגרום, לדוגמא, להופעה של חורים, עיוותי כיפוף חזקים (buckling), או מיזוג בין שתי ממברנות. אי-יציבויות כאלו מתרחשות בתהליכים כגון בליעה תאית או חלוקת התא, כחלק מתפקודו הרגיל של התא. כמו כן, אי-יציבויות יכולות להיגרם על ידי גורם חיצוני, כגון רעלנים הגורמים להופעת חורים בממברנה. הבנה פיזיקלית של אי-יציבויות בממברנות עשויה לשמש בתחומי מחקר ורפואה. למשל גרימה לקרע בממברנה של תאים סרטניים. בנוסף, ניתן לנצל אי-יציבויות לצרכים מעשיים כמו שחרור מבוקר של תרופות.

התכונות המכניות של ממברנות נחקרות באופן מסורתי באמצעות ההמילטוניאן של הלפריש (Helfrich's Hamiltonian). מודל זה משמש בדרך כלל להבנה ותיאור של ממברנות יציבות. תופעות של אי-יציבות מכנית מובנות פחות טוב. תיזה זו עוסקת בממברנות מכופפות בצורה חזקה או כאלו שנפער בהן חור. סימולציות מחשב מאפשרות לחקור ממברנות במצב של חוסר יציבות, אבל בגלל אופיין הכימי, סימולציות אטומיסטיות (atomistic simulations) של ממברנות ליפידיות דורשות מחיר גבוה (במונחי חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות מחיר גבוה (במונחי חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות מחיר גבוה (במונחי חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות מחיר גבוה למונחים חישוב) הכרוך בסימולציות שכאלו מגביל את סדרי הגודל והזמן של הסימולציה. בסימולציות מחיר גבוה כמולקולות פשוטות והתווך המימי מיוצג באופן עקיף (באמצעות אינטרקציות הידרופוביות אפקטיביות). באמצעות מודלים כאלו ניתן להתגבר על המחסומים של שיטות סימולציה "מסורתיות" ברמה האטומית.

פרק 2 בתיזה זו עוסק באי-יציבות של ממברנה תחת מתח פנים שלילי, פרק 3 דן באי-יציבויות של ממברנות טעונות, ופרק 4 דן בהתפרקות של קומפלקסים של ליפידים-ודנ''א. חוט השני המקשר בין התוצאות המדווחות בשלושת הפרקים של תיזה זו הוא אי-יציבות מכנית. יחד עם זאת, למרות שניתן לחלק באופן גס את המופעים של אי היציבות לחורים וקריסה, הגורמים לאותן אי-יציבויות וה''פרטים הקטנים'' שלהן, כוללים מספר הבדלים חשובים. הבנה של אי-יציבויות שכאלו תאפשר הבנה יותר טובה של תהליכים ביולוגים, כימיים, ופיסיקלים הקשורים בממברנות. ניתן לנצל תובנות אלו למטרות מעשיות במקרים בהם אי-יציבויות שכאלו הן חיוניות (מתן תרופות למשל). פרק 2 מציג מחקר באמצעות סימולציות של תגובה של ממברנות להפעלה של מתח פנים שלילי (דוחס). באמצעות תוצאות הסימולציה פיתחנו מודל פשוט של אנרגיה חופשית עבור ממברנות תחת מתח פנים שלילי. אי-יציבות בממברנות תחת דחיסה הוא תלוי גודל. ידוע כי מתח פנים שלילי גורם לאי-יציבות בתנודות בעלות אורך גל גבוה בווזיקולות (vesicles) גדולות, אבל נסבל כאשר מדובר בווזיקולות קטנות. תוצאות הסימולציות במתח פנים שלילי מגלות שני תחומים - (i) מתח שלילי חלש המאופיין באלסטיות הנשלטת על ידי מתיחה ו-(ii) מתח פנים שלילי חזק המציג התנהגות אלסטית - Rawicz ו Evans הנשלטת על ידי כיפוף. תוצאות אלו דומות לממצאים מהניסוי הקלאסי של E. Evans and W. Rawicz.1 - שאיבה של ווזיקולות ענק חד שכבתיות (GUV) דרר מיקרופיפטה [phys. Rev. Lett. **64**, 2094 (1990]. אולם, בGUV המעבר בין שני תחומי האלסטיות מתרחש במתח פנים חיובי חלש, בעוד שבממברנות קטנות המעבר מתרחש במתח פנים שלילי מתון. ממצא מעניין נוסף הקשור לתגובה של ממברנות קטנות למתח-פנים שלילי עוסק בקשר בין המתח המכאני והמתח התנודתי השווים זה לזה עבור ערכים לאי-שליליים. כאשר המתח יורד למתחים שלילים, המתח התנודתי,  $\gamma$ , יורד מעט מהר יותר מהמתח המכאני, au, בתחום המתח החלש, לפני שהוא מגיע לרוויה, והופך לגדול מau, עבור מתח שלילי חזק. מקדם הכיפוף מציג התנהגות "הפוכה", הוא נשאר כמעט ללא שינוי בתחום האלסטי הנשלט על ידי מתיחות, ויורד בתחום הנשלט על ידי כיפוף. הווריאציות באמפליטודה של התנודות התרמיות ושל שטח ההטלה (projected area) מתבדרות כאשר אי-יציבויות מתחילות להתפתח.

אותו מודל coarse-grained מולקולרי משמש בפרק 3 על-מנת לחקור את התכונות האלסטיות של ממברנות טעונות בתמיסה של יונים חד-ערכיים וחמש-ערכיים. תוצאות הסימולציה בשני המקרים מגלות מגמות הפוכות זו לזו. מקדם הכיפוף ושטח ההטלה גדלים עם צפיפות המטען על הממברנה כאשר היונים הנגדיים הם חד-ערכיים, שעה שהם קטנים כאשר היונים הם חמש-ערכיים. ניתן לקשר תצפיות אלו למידת מיסוך המטען של הליפידים על ידי היונים הנגדיים. יונים חד-ערכיים ממסכים את האינטראקציות רק במידה חלקית, מה שמרמז על מערכת דוחה קולומבית (Coulomb). מנגד, היונים הרב-ערכיים מתעבים על הממברנה ודרך קורלציות מטען מרחביות, מייצרים משיכה אפקטיבית הודות לכוחות המשיכה בין הליפידים החיוביים ליונים השליליים. ההבדלים בתכונות האלסטיות של ממברנות טעונות בתמיסות של יונים חד-ערכיים ורב-ערכיים משתקפות במנגנונים האלסטיות של ממברנות טעונות בתמיסות של יונים חד-ערכיים ורב-ערכיים משתקפות במנגנונים האלסטיות של ממברנות טעונות בתמיסות האיונים חד-ערכיים ורב-ערכיים משתקפות המנגנונים המובלים לאי היציבות המכנים שלהם בצפיפויות מטען גבוהות. במקרה הראשון, חורים מתחילים להופיע בממברנה בשביל להקל על המתיחה האלקטרוסטטית, במקרה השני, הממברנות מפתחות אי-יציבות של כיפוף באורכי הגל הארוכים.

בפרק 4 אנו חוקרים את הפיזיקה של קומפלקסים (אגד סופר-מולקולרי) של ממברנות קטיוניות ומולקולות דנ''א. קומפלקסים כאלו של ליפידים-דנ''א (ליפופלקסים) מעוררים עניין בתור ווקטורים להחדרת גנים לתאים, לצורך ריפוי גנטי, מאחר שהם אינם פתוגנים והם מתאספים באופן עצמאי בתנאים של שווי משקל תרמי. בפרק הזה אנו מתרכזים בכוחות המנייעים השולטים בשחרור של מולקולות דנ"א מליפופלקס הלכוד בתוך אנדוזום. נראה כי השחרור של מולקולות דנ"א הוא השלב המגביל בתהליך הטרסנפקציה (transfection - transfer followed by expression), אשר נתפס כתהליך תלת שלבי: (i) בליעה תאית, (ii) התפרקות של הליפופלקס, (iii) שיחרור של הדנ''א ולאחריו ביטוי של הגנים. כיוון שלטרנספקציה מוצלחת נדרשת התפרקותו של הליפופלקס, היציבות התרמודינמאית של הליפופלקס מעכבת אותה. יחד עם זאת, ידוע שתהליך הטרנספקציה יכול להתרחש ספונטנית. בפרק התוצאות המתאים של תיזה זו, אנו משתמשים במודל מופשט כדי לחקור את הכוחות התרמודינאמיים המניעים השולטים בתהליך הטרנספקציה. בפרק זה מודגם כי לאחר הבליעה התאית [שלב (i)], הליפולקס הופך להיות לא יציב. אי-יציבות זו, אשר נגרמת על ידי אינטראקציות בין ליפידים קטיונים בליפופלקס וליפידים אניונים בממברנת האנדוזום העוטף את הליפופלקס, נשלטת על ידי אנטרופיה הכוללת ערבוב מחדש של הליפידים ואנטרופית המיקום של יונים נגדיים אשר בתחילת התהליך ספוחים למשטחים. החישובים בפרק זה מראים שהרווח באנרגיה . החופשית בשלב (ii) הם לינארים בקירוב ב- $\Phi_+$ , השבר המולרי של הליפידים הקטיונים בליפופלקס. רווח זה באנרגיה החופשית,  $\Delta F$ , מוריד את המחסום למיזוג בין ממברנת הליפופלקס וממברנת  $\Delta F$  האנדוזום, ומביא להווצרות של חור שמאפשר שיחרור של דנ''א [שלב (iii)]. הקשר הלינארי בין והשבר המולי של הליפידים הקטיונים מסביר את הגידול האקספוננציאלי של יעילות הטרנספקציה . ב- $_+ \Phi_+$  אשר נצפתה באופן ניסיוני בקומפלקסים למלאריים.

פרק 5 מסכם תיזה זו. בפרק זה הדמיון והשוני בין אי היציבוייות המתוארות בפרקים השונים מודגשים. תובנות מהשוואות אלו מסוכמות בהצעה למחקר עתידי באמצעות סימולציות מחשב.

## הצהרת תלמיד המחקר עם הגשת עבודת הדוקטור לשיפוט

אני החתום מטה מצהיר/ה בזאת: (אנא סמן):

\_\_\_\_\_ חיברתי את חיבורי בעצמי, להוציא עזרת ההדרכה שקיבלתי מאת מנחה/ים.

\_\_\_\_\_ החומר המדעי הנכלל בעבודה זו הינו פרי מחקרי מתקופת היותי תלמיד/ת מחקר.

\_\_\_\_\_ בעבודה נכלל חומר מחקרי שהוא פרי שיתוף עם אחרים, למעט עזרה טכנית הנהוגה בעבודה ניסיונית. לפי כך מצורפת בזאת הצהרה על תרומתי ותרומת שותפי למחקר, שאושרה על ידם ומוגשת בהסכמתם.

תאריך 11 לאוקטובר, 2015 שם התלמיד/ה יותם יוסף אביטל

חתימה \_\_\_\_\_