Articles

Surface Potential of Charged Liposomes Determined by Second Harmonic Generation

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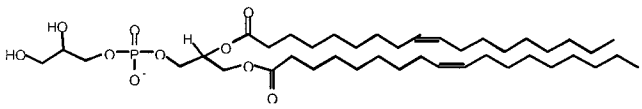
We demonstrate that the surface potential of charged liposomes can be determined by second harmonic generation. The Gouy−Chapman model is used to describe the electric double layer. From the experimental data, we obtained the surface charge density and the surface potential of liposomes consisting of the negatively charged phospholipid dioleoylphosphatidylglycerol. The surface potential was found to be in the range of 20−100 mV depending on the concentration and valence of the electrolyte (NaCl or $MgSO_4$) in solution. The charge density was found to be $\sim 1.3 \times 10^{14}$ per cm^2, corresponding to ~ 70 Å^2/charge, which is comparable to the area per phospholipid headgroup.[4]

1. Introduction

Surface charge density and surface potential are important properties of colloidal microscopic particles.[1] For example, the populations and orientation of the polar and charged species near the interface region of the particles depend on the surface charges and surface potential. As a consequence, the rate of chemical reactions occurring at the particle surfaces and the interactions between particles can be affected by the surface potentials. Liposomes, having enclosed bilayer structures, are excellent model systems for biomembranes and drug delivery systems.[2] We recently found that the amount of organic cations adsorbed onto negatively charged liposomes and the transport rate of the ion across the negatively charged liposome bilayers depend linearly on the surface charge density of the liposomes.[3] These results suggest that the electrostatic interaction between the adsorbates and the surface of the liposome is a determining factor for both the adsorption and transport processes of charged species. In the present studies, we demonstrate that second harmonic generation (SHG) can be used to measure the surface charge density of the liposomes and thereby the surface potential. The liposomes were made of dioleoylphosphatidylglycerol (DOPG); see Chart 1. The surface potential of the liposomes was found to be in the range of 20−100 mV depending on the valence and the concentration of the electrolyte. The inverse of the charge density σ^{-1} was found to be ~ 70 Å^2/charge, which is consistent with the area per headgroup in liposomes.[4]

Other methods have been applied to study the surface potential of liposomes. Fluorescent probes such as cyanine and oxanol molecules incorporated in the liposome bilayers

Chart 1



DOPG

can be used to study the surface potential.[5−7] Electron paramagnetic resonance (EPR) can be used to study the surface potential of liposomes, which is done by measuring the redistribution of the spin probes bound to the bilayers and free in the bulk solutions.[8] By measurement of the mobility of charged liposomes in an externally applied electric field, the zeta potential ζ at the shear plane of the liposomes can be obtained by electrophoresis from which the surface potential can be estimated. However, assumptions have to be made in order to get the surface potential from ζ[9−12] because it is not precisely known where the shear surface is located. It is also possible that the double layer and the shape of the liposomes are perturbed by both the motion of the liposomes and the externally applied electric field. Studies of the surface potential with these various methods used a mixture of charged and neutral phospholipids. The values obtained range from 0 to 50 mV. In the second harmonic studies, the liposomes consisted only of charged phospholipids.

The SHG method, being an optical technique, does not perturb the system. Because the SHG signal directly responds to the water molecules polarized by the charged liposome surfaces, no external probe molecules need to be introduced to the system. Because SHG is surface

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specific,[13−15] it is directly sensitive to the charged plane. Therefore, it may serve as a complementary technique to other methods in investigating the electrostatic properties of liposomes.

We previously reported[16] that the surface potential and surface charge density of microparticles, such as polymer beads and oil/water emulsions, can be obtained by the SHG method. It is known that the second harmonic field, E_{SHG}, from a charged interface has two contributions, namely, $\chi^{(2)}$ and $\chi^{(3)}$ contributions:[16−19]

$$E_{SHG} \propto \chi^{(2)} + \chi^{(3)}\Phi_s \tag{1}$$

where Φ_s is the surface potential, $\chi^{(2)}$ is the second-order susceptibility originating from the noncentrosymmetry of the interface, and $\chi^{(3)}$ is the third-order susceptibility originating from the bulk solvent molecules polarized by the static electric field due to the surface charges. As we increase the electrolyte concentration of the aqueous phase, the counterions screen the electric field extending from the surface into the bulk, leading to a decrease in the strength and number of solvent molecules polarized by the electric field. The Gouy−Chapman model describes the dependence of the surface potential on the bulk electrolyte concentration:

$$\Phi_s = \frac{2kT}{Ze}\sinh^{-1}\left(\sigma\sqrt{\frac{\pi}{2\epsilon kTc}}\right) \tag{2}$$

where Φ_s is the surface potential, k is the Boltzmann constant, T is the Kelvin temperature, Z is the valence of the electrolyte, σ is the surface charge density, ϵ is the dielectric constant of the medium, and c is the electrolyte concentration in the bulk solution. Therefore, the second harmonic field can be expressed as follows:[16−19]

$$E_{SHG} = A + B\frac{2kT}{Ze}\sinh^{-1}\left(\sigma\sqrt{\frac{\pi}{2\epsilon kTc}}\right) \tag{3}$$

where A and B contain $\chi^{(2)}$ and $\chi^{(3)}$, respectively. Using A, B, and σ as the fitting parameters, we have demonstrated in the previous studies that the surface charge density can be obtained and the surface potential at each electrolyte concentration can be calculated.[16] This method has been successfully applied to study the interfaces of the polystyrene sulfate microparticles and the sodium dodecyl sulfate (SDS) oil/water emulsions.[16]

In the present studies, the method is extended to liposomes. The surface of liposomes is negatively charged because of the negatively charged headgroup of DOPG. The difference between a liposome and a particle such as a polymer bead is that liposomes have two interfaces: the interface of the outer layer of the liposome bilayer with the external aqueous phase and the interface of the inner layer of the liposome bilayer with the interior aqueous phase. For symmetry reasons, the molecules adsorbed on the opposite surfaces of a bilayer are oppositely oriented.[20,21] Therefore, $\chi^{(2)}_{in}$ has the opposite phase of $\chi^{(2)}_{out}$. The second harmonic field from a charged liposome is the sum of the second harmonic field from the inner and the outer surfaces.

$$E_{SHG} \propto \chi^{(2)}_{out} + \chi^{(2)}_{in} + \chi^{(3)}_{out}\Phi_{out} + \chi^{(3)}_{in}\Phi_{in} \tag{4}$$

The experiment was designed to keep the inner aqueous phase of the liposomes unchanged. Only the electrolyte concentration of the outer aqueous phase was varied. For small inorganic ions, the rate of the transport across the liposome bilayer is very slow, in the time scale of ~10 h.[2] Thus, for our experimental time scale (<10 min) we assume that the change of electrolyte concentration in the inner aqueous phase because of the ion diffusion across the bilayers is negligible. As the electrolyte concentration in the external aqueous phase changes, the only term that changes in eq 4 is Φ_{out}. Hence, it can be written as

$$E_{SHG} = A' + B\Phi_{out}(c) \tag{5}$$

where A' scales with $(\chi^{(2)}_{out} + \chi^{(2)}_{in} + \chi^{(3)}_{in}\Phi_{in})$ and B scales with $\chi^{(3)}_{out}$. Equation 2.5 can then be used to fit the dependence of E_{SHG} on the electrolyte concentration, c, using A', B, and σ as fitting parameters. It is to be noted that E_{SHG} depends not only on the electrolyte concentration but also on the valence of the electrolyte. Using both 1:1 NaCl and 2:2 $MgSO_4$ as the electrolytes, we obtained two independent sets of experimental data. We found that a unique set of A', B, and σ can fit both sets of the data. The surface charge density can be obtained from the fitting, and the surface potential can be calculated at different electrolyte concentrations.

2. Experiment

A powder form of sodium dioleoylphosphatidylglycerol (DOPG) lipid with purity 99% was obtained from Avanti. Large unilamellar vesicles of DOPG were prepared by the extrusion technique.[22] Briefly, the lipid powder (Avanti, 99%), total weight 20 mg, was weighed into a round-bottom flask and then dissolved in ~10 mL of chloroform. The solvent was evaporated under vacuum in a rotavapor setup for more than 2 h. We observed that evaporation for 2 h or overnight did not affect the results of our liposome experiments. The thin film of lipids obtained was hydrated in 10 mL of 250 mM sucrose solution by vigorous vortexing. The multilamellar vesicles formed in this way were extruded 10 times through a double stacked 0.2 μm polycarbonate filter under N_2 at a pressure of 100−120 psi. The sizes of the liposomes were estimated from UV/vis turbidity measurements[23] and were found to be 107 ± 5 nm in diameter. We determined by ^{31}P NMR experiments that more than 90% of the liposomes are unilamellar.[24] The DOPG concentration in the liposomes after mixing with various solutions of electrolytes was kept constant at 0.2 mg/mL, which corresponds to a particle density of 1.6×10^{12} liposomes/mL. The salts of sodium chloride and magnesium sulfate were obtained from Aldrich and put in an oven at 500 °C overnight before the preparation of the solutions. All the aqueous solutions were prepared in double-distilled water.

To keep the inner and outer osmotic pressure constant, sucrose was added to the systems. The concentration of sucrose in the inner solution is kept at 250 mM. The concentration of sucrose in the outer solution was adjusted such that the total solute (cations, anions, and sucrose) concentration is 250 mM, that is,

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$$2 \times [\text{salt}] + [\text{sucrose}] = 250 \text{ mM}$$

In this way, the shrinking and swelling of liposomes because of changes in osmotic pressure across the bilayer induced by changing the electrolyte concentration in the outer solution is eliminated. The ranges of the electrolyte concentration in the outer solution were 0.6−13 mM for NaCl and 0.6−4 mM for $MgSO_4$.

The stability of the liposome was also of concern, and the size was monitored by UV/vis measurements at different electrolyte concentrations. We found that the optical density of the samples did not change during the time of the SHG measurement. This indicates that the samples were stable and no fusion or aggregation of liposomes occurred.

The SHG measurements were carried out in a 90° geometry setup, which is described in refs 20 and 21. Briefly, an argon ion laser pumped Ti:Sapphire oscillator yielded 100 fs pulses at 830 nm at a rep rate of 82 MHz, with an energy of 10 nJ per pulse. After passing through a half-wave plate and a polarizer, the light is gently focused into the sample in a 1-cm rectangular cuvette. The SH photons generated from the sample were collected at an angle of 90° to the incident light by lenses and focused into the monochromator. A computer connected with a photomultiplier tube and a single photon counter was used to record the signal. The polarizations of the incident light and the output signal were both horizontal. This polarization combination is chosen to minimize the hyper-Rayleigh background.

In the experiment, the physical quantity we measured is the total intensity at frequency 2ω, $I_{\text{total}}^{2\omega}$, which contains not only the coherent SHG signal from the liposome surface but also the incoherent hyper-Rayleigh (HR) scattering from bulk water. The HR scattering originates from orientation and density fluctuations of water molecules.[25] To obtain the second harmonic field, E_{SHG}, a correction is needed:

$$E_{\text{SHG}} = \sqrt{I_{\text{SHG}}} = \sqrt{I_{\text{total}}^{2\omega} - I_{\text{HR}}} \tag{6}$$

where I_{SHG} is the SHG intensity from the surface of liposomes and I_{HR} is the hyper-Rayleigh intensity from bulk water.

3. Results and Discussion

The second harmonic field, E_{SHG}, from the charged surface of DOPG liposomes was measured as a function of electrolyte concentration of NaCl and $MgSO_4$, shown as circles and triangles in Figure 1. The experimental data was fitted to eq 5 using A', B, and σ as fitting parameters, shown as a solid line in Figure 1. The results of the fitting are summarized in Table 1.

Table 1 shows the values of A', B, and σ obtained from the independent measurements using two electrolytes, NaCl and $MgSO_4$. The ratio of BkT/Ze of NaCl to that of $MgSO_4$ is 2. That is consistent with the prediction of the Gouy−Chapman theory because the valence (Z) of $MgSO_4$ is twice that of NaCl. The inverse of charge density obtained from the fitting is about 70 Å^2/charge, which is comparable to the area per headgroup in the liposome.[4] With A', B, and σ known from the fitting, the surface potentials can be obtained and are shown as circles for NaCl and triangles for $MgSO_4$ in Figure 1. The solid line in Figure 1 is the surface potential calculated by the Gouy−Chapman model, eq 2, using the value of σ obtained from the fitting. The surface potential measured by the SHG method is in the range of 20−100 mV depending on the valence and the concentration of the electrolytes.

Equation 2 can be substituted into eq 5, leading to the following expression:

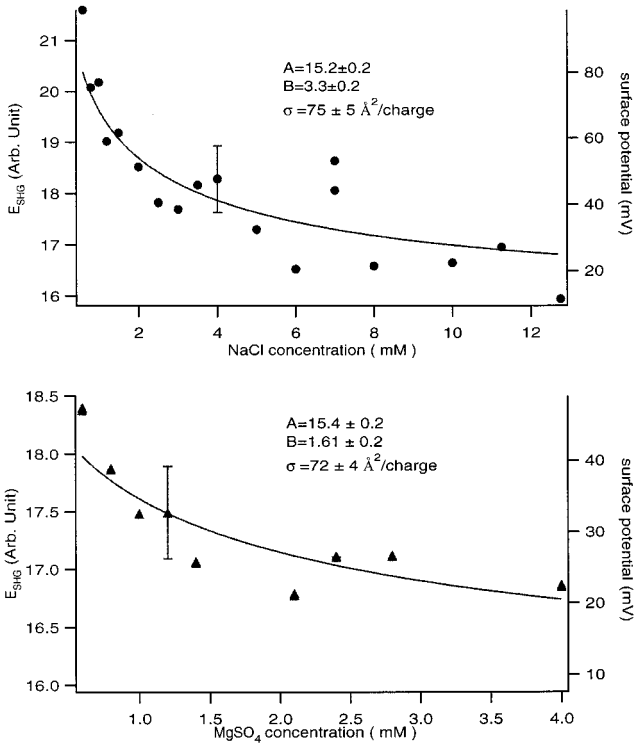

Figure 1. The dependence of the second harmonic field, E_{SHG}, on the concentration of the univalent electrolyte NaCl (●) and the divalent electrolyte $MgSO_4$ (▲) is plotted on the left axis. The solid lines are the fits to eq 5 with A', B, and σ as fitting parameters. The surface potential of the DOPG liposomes is calculated from eq 2 using the value of σ obtained from the fitting, which is plotted on the right axis.

Table 1. Fitting Parameters for the Curves of E_{SHG} versus c Using the Gouy−Chapman Model

	NaCl	$MgSO_4$
A' (arb unit)	15.2 ± 0.2	15.4 ± 0.2
BkT/Ze	1.6 ± 0.1	0.8 ± 0.1
σ^{-1} (Å^2/charge)	75 ± 5	72 ± 4

$$E_{\text{SHG}} = A' + B\frac{2kT}{Ze}\sinh^{-1}\left(\sigma\sqrt{\frac{\pi}{2\epsilon kTc_{\text{out}}}}\right) \tag{7}$$

where c_{out} is the electrolyte concentration in the outer solution of the liposomes. A manipulation of eq 7 further illustrates that experimental results are in good agreement with the Gouy−Chapman model. It is known that $\sinh^{-1}(x)$ can be approximated by the $\ln(2x)$ with ≤1% error when $x \geq 4$. This approximation can be applied to our experimental data because the value of $\sigma(\pi/2\epsilon kTc)^{1/2}$ is always much greater than 4 for our experimental conditions. Using the approximation, we can separate the fitting parameter σ and the variable c, leading to the following equation:

$$E_{\text{SHG}} = A' + \frac{2BkT}{Ze}\ln\left(2\sigma\sqrt{\frac{\pi}{2\epsilon kT}}\right) - \frac{BkT}{Ze}\ln c = D - \frac{BkT}{Ze}\ln c \tag{8}$$

where D is a constant in the experiment. It is expected that E_{SHG} is a linear function of $\ln(c)$ and the slope is inversely proportional to the valence of the electrolyte. Figure 2 shows the plots of E_{SHG} versus $\ln(c)$ for both NaCl and $MgSO_4$. Two straight lines are obtained for the two electrolytes. The slope for NaCl is −1.6 ± 0.2, and the

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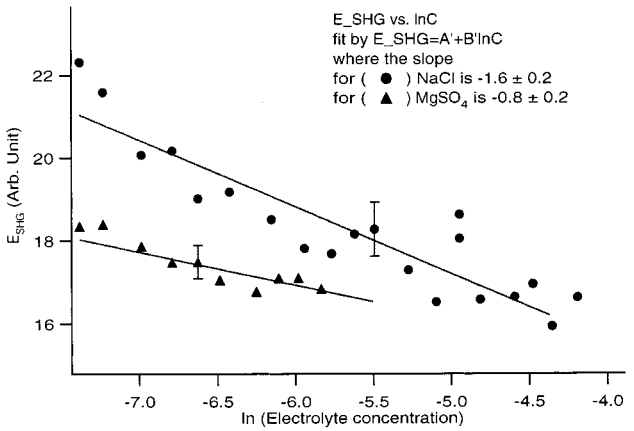

Figure 2. The linear dependence of the second harmonic field, E_{SHG}, on the log scale of the concentration of the univalent electrolyte NaCl (●) and the divalent electrolyte MgSO4 (▲). The solid lines are the fit to eq 8. The slope of NaCl is twice the slope of $MgSO_4$, as predicted from the Gouy−Chapman model.

slope for $MgSO_4$ is -0.8 ± 0.2. The experimental ratio of the slope of NaCl to that of $MgSO_4$ is 2.0, in agreement with the theoretical value of 2.0 predicted by the Gouy−Chapman model.

Though the Gouy−Chapman model is derived strictly for a charged planar surface, it can still be applied to the curved charged surface of the liposomes used in these studies. When a planar charged surface is considered, the Poisson−Boltzmann equation can be solved analytically for symmetric electrolytes (i.e., 1:1 such as NaCl or 2:2 such as $MgSO_4$), yielding the Gouy−Chapman model as described by eq 2. For spherical particles, there is no analytical solution of the Poisson−Boltzmann equation. However, the equation has been solved numerically and empirically expressed by the following equation:[26]

$$\sigma = \sqrt{\frac{2\epsilon\epsilon_0 kTc}{\pi}}\left[\sinh\left(\frac{e\Phi_s}{2kT}\right) + \frac{2}{\kappa a}\tanh\left(\frac{e\Phi_s}{4kT}\right)\right] \quad (9)$$

where Φ_s is the surface potential, κ^{-1} is the electrical double-layer thickness, and a is the radius of the particle. Therefore, eq 9 serves as a very good approximation to describe the surface potential of the charged spherical surface. If the conditions of $\Phi_s \geq 25$ mV and $\kappa a \geq 10$ are satisfied, the second term in the bracket can be dropped, yielding an error less than 5%. For ∼100 nm diameter particles, if the electrolyte concentration is in the range of 0.5−50 mM the above conditions are well satisfied. Then, the second term in the bracket of eq 9 can be neglected, yielding eq 2, which is the Gouy−Chapman model.

In our experiments, adsorption of the small inorganic cations to the surface of the negatively charged liposomes is negligible. The equilibrium constant for the adsorption of Na^+ onto negatively charged surfaces of liposomes is about 1 M^{-1}.[2] The electrolyte concentration of NaCl was varied from 0.6 to 13 mM in the experiments. It follows that the surface coverage of Na^+ to the liposome surface is less than 1.3%. The divalent cation of Mg^{2+} has a slightly higher affinity to the liposome. The adsorption equilibrium is about 6 M^{-1}.[2] The electrolyte concentration of $MgSO_4$ was varied from 0.6 to 4 mM in the experiments. The surface coverage is again less than 2.4%.

In the data analysis, an assumption is made that the electrolytes (NaCl and $MgSO_4$) added externally to the outer solution of the liposomes do not diffuse across the bilayers in the time scale of the SHG measurements. It is based on this assumption that the inner surface potential of the liposomes remains constant upon changing the outer electrolyte concentration so that eq 5 remains valid. This assumption is examined by comparing the time scale of electrolyte crossing the bilayers and the time scale of the SHG measurements. Na^+ has a permeability coefficient of 10^{-14} cm/s and Cl^- has a permeability coefficient of 10^{-11} cm/s for egg PC liposome.[2] The permeability of divalent ions such as Mg^{2+} or SO_4^{2-} is orders of magnitude smaller than that of univalent ions.[27] The time scale of these inorganic ions crossing the liposome bilayers is of the order of 10 h, which is substantially longer than the time scale of the SHG measurements (10 min). We therefore consider the liposome bilayers to be acting as a partition separating two unrelated solutions during the time of the measurements. Therefore, there will be no equilibrium Nernst potential or steady-state diffusion potential across the membrane.[28]

4. Conclusions

We have demonstrated that the outer surface potential and surface charge density of the DOPG liposomes can be obtained by the SHG technique. The experimental data show excellent agreement with the Gouy−Chapman model. The surface potential measured is in the range of 20−100 mV, depending on the valence and concentration of the electrolyte. The charge density was found to be $\sim 1.3 \times 10^{14}$ per cm^2, corresponding to ∼70 $Å^2$/charge, which is consistent with the area of a lipid headgroup.[4] Because SHG is an optical surface-specific technique, it has the advantages of being nonperturbative and directly sensitive to the charged plane. Therefore, it is complementary to conventional techniques used in investigating the electrostatic properties of liposomes.

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