Antibiotic assisted molecular ion transport across a membrane in real time

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The transport of an organic cation across a 4–5 nm liposome bilayer is observed in real time using second harmonic generation. It is proposed that an electrostatic barrier between the inside and outside of the liposome develops as the cation crosses the bilayer. This would explain why the SHG signal does not approach zero at long times. To test this mechanism, the antibiotic valinomycin, which can transport alkali ions across a phospholipid bilayer, is introduced into the system. It is found that the transport time is reduced by a factor of three from 90 ± 2 s to 30 ± 1 s with 1.25×10^{-8} M valinomycin concentration, and a factor of fifteen to 6.2 ± 0.2 s with 1.25×10^{-8} M valinomycin concentration. In addition, the SHG signal approaches zero, which further supports the presence of an electrostatic barrier that can be eliminated by the alkali ion transporter valinomycin.

1. Introduction

Phospholipid bilayers are the basic structural feature of biological membranes, which most importantly serve to control the passage of chemical species between the inner enclosed aqueous region of the cell and the outer aqueous solution.[1] Structures similar to membranes, such as liposomes, can be prepared in the laboratory. These membrane mimetic structures, consisting of a bilayer, which are made up of amphiphiles, such as phospholipids, have been extensively used as simplified models of biological membranes.[1] Basic research studies of various chemical and physical phenomena as well as applications such as solar energy conversion, catalysis, cosmetics and drug delivery are areas of considerable interest.[2,3] An issue of special importance is the transport of chemical species, ranging from inorganic and organic ions to neutral species, across the bilayer of a biomembrane.[4–13] With liposomes one can investigate the transport of chemical species across bilayers of differing phospholipid composition and net charge. This latter point is of interest because many biological membranes are negatively charged due to negatively charged phospholipids in the bilayer.

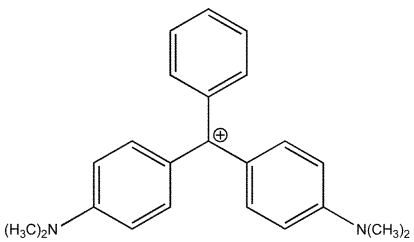
In earlier studies we have shown that the nonlinear spectroscopic method of second harmonic generation (SHG) can be used to selectively probe the surfaces of both centrosymmetric and asymmetric microscopic particles and nanoparticles.[14–22] Of relevance to this paper is the real time measurement of the transport kinetics of an organic cation, malachite green (MG), across a unilamellar (only one bilayer) liposome.[14–17] Unlike other optical methods the second harmonic method does not require the addition of quenching or other marker molecules in order to

differentiate the molecules transported across the bilayer from the much larger population of the same molecules in the external bulk solution. It should be noted that SHG, being a second order optical effect is electric dipole forbidden in centrosymmetric systems. Despite this apparent prohibition we have shown that SHG is electric dipole allowed and can be generated at the interface of centrosymmetric structures when the surface is not locally centrosymmetric. In a series of experiments on centrosymmetric microparticles,[14–22] including polymer beads, emulsions and liposomes, we have demonstrated that second harmonic light can be generated selectively from their surfaces provided the size of the microparticle is not much smaller than the coherence length of SHG, roughly the wavelength of the incident light. For SHG measurements the molecules in the bulk solution do not generate a coherent second harmonic signal because they are randomly oriented. It is only the adsorbed molecules, which being polar, align themselves in a preferred orientation at the interface and thereby can generate an SHG signal. The underlying idea of the SHG method to study the transport kinetics of molecules across a liposome bilayer is as follows. On rapidly adding ($\leq$1 s) the molecules of interest to a solution containing liposomes, a very rapid rise in the SHG signal is achieved as the molecules rapidly adsorb onto the outer surface of the liposome. If the liposome is permeable to the adsorbed molecules they will migrate across the bilayer and adsorb to the inner surface of the bilayer. By symmetry the adsorbed molecules at the inner and outer surfaces of the liposome are oppositely oriented. Because the thickness of the bilayer is 4–5 nm, which is much less than the SHG coherence length, the second order polarizations induced by the incident light have opposite phases for the oppositely oriented molecules and therefore cancel. Consequently the second harmonic field $E_{2\omega}$ generated by the light field E_{ω}, incident on a liposome is proportional to the second order susceptibility $\chi^{(2)}$, which contains information of the absorbate population. For liposomes the second harmonic field $E_{2\omega}$ is proportional to the difference in the populations of the molecules located on the outer surface, $N_0(t)$, and the inner surface, $N_i(t)$, at a time t after mixing the liposome solution with the MG solution, containing the solute molecules of interest. The generated second harmonic field at 2ω is given by,

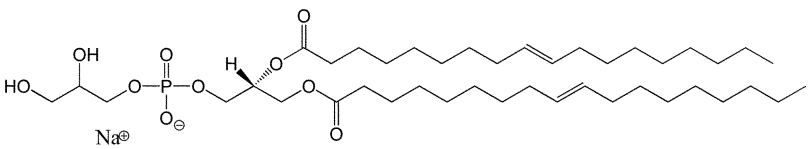
$$E_{2\omega} \propto [N_o(t) - N_i(t)]E_{\omega}E_{\omega} \quad (1)$$

Thus by monitoring the SHG signal, which is proportional to the square of the second harmonic field, $E_{2\omega}$, makes it possible to observe the transport of molecules across the bilayer in real time.

In this report we demonstrate that the transport of the positively charged cation malachite green (MG) across the bilayer of the negatively charged dioleoylphosphatidylglycerol (DOPG) liposome

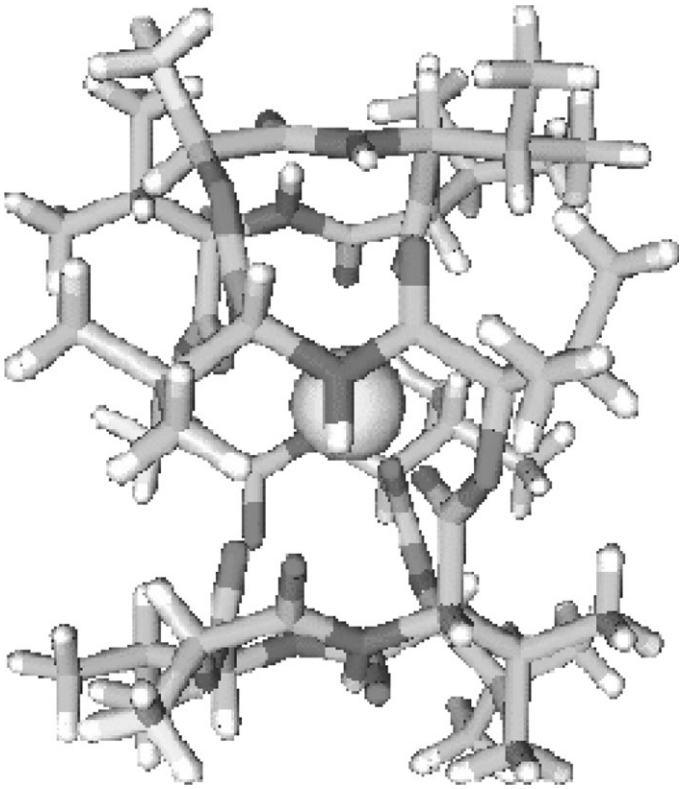


Malachite green cation (MG)



DOPG

Scheme 1 The structures of MG and DOPG.



Scheme 2 The structure of valinomycin.

is incomplete because of electrostatic forces. The molecular structures of MG and DOPG are shown in Scheme 1. To support this electrostatic mechanism, we use the antibiotic valinomycin (Scheme 2), which can carry alkali ions across a liposome bilayer. As MG crosses to the liposome interior, a relatively negative potential external to the liposome is generated, which can serve as the driving force for valinomycin to transport a net population of Na^+ to the exterior. In this way, the positive potential that would otherwise have been generated by MG crossing to the inner compartment of the liposome can be reduced or eliminated. As a result, more MG molecules would then cross the bilayer, which would reduce the SHG signal to a value approaching zero.

2. Experimental

2.1 Sample preparation

DOPG phospholipid (Avanti Polar Lipids, Inc.) was used to make a negatively charged liposome. The procedures to prepare the liposome are described in detail elsewhere.[14] The sizes of liposomes were determined by UV-vis turbidity or dynamic light scattering measurements. All the liposome samples used in this study were very close in size, ranging from 100 to 104 nm in diameter.

The probe molecule, malachite green chloride (Aldrich) was used as received after the purity was checked by the HPLC method. The malachite green chloride solutions were adjusted to a pH of 4 and the NaCl concentration was 10 mM. The concentration of malachite green chloride in all experiments was 20 μM. The liposome density was calculated to be 1.8×10^{11} cm^{-3}. The valinomycin purchased from Molecular Probes, Inc. was added to the liposome solution before mixing with the MG solution. The transport of MG across the liposome bilayer was initiated by mixing the liposome solution containing valinomycin with an equal volume of MG solution. After mixing, the concentrations of both lipid and MG, diluted by a factor of 2, are 12.5 μM and

10 μM, respectively. For all experiments, we kept the same concentration of MG, NaCl and lipid, whereas the concentration of valinomycin in the liposome solution was varied.

2.2 SHG setup

The experimental configuration of SHG measurements has been described in detail previously.[15] Briefly, a $Nd:YVO_4$ solid state laser (Spectral-physics, Millennia Vs) pumped Ti : sapphire laser (KMLab) provided 30 fs pulses with an energy of 4 nJ $pulse^{-1}$ at 840 nm at a repetition rate of 82 MHz. The femtosecond laser pulse train passes through a polarizer, and a color filter used to block all the stray light at twice the frequency of the laser light. It was then focused into the sample in a 1 cm rectangular cuvette by a lens with a focal length of 5 cm. A pair of extra-cavity prisms was used to compensate for dispersion due to the sample measurement optics. The insertion of the glass prisms were optimized by maximizing the SHG signal. The incident light was *S* polarized. The generated SHG signal was collected at 90° relative to the incident light direction and sent into a monochromator. Before the monochromator, an analyzer was used to allow for selectively detecting the signal with *S* polarization. The data acquisition system includes a PC connected to a PMT and single photon counter. The injection of the liposome solution into the MG solution was completed within one second.

3. Results and discussion

A. Kinetics of MG crossing the bilayer in the absence of valinomycin

Fig. 1(a) shows a typical time profile of the SH intensity before and after a liposome solution was injected into a MG solution in the absence of valinomycin. The initial constant signal level is from the MG solution alone and is dominated by the two-photon excited fluorescence of MG. This conclusion is supported by the experimental evidence that the two-photon fluorescence tail of MG (500 counts s^{-1}) at the second harmonic frequency 2ω, is much larger than the intensity of the hyper-Rayleigh scattering of water ($\sim$20 counts s^{-1}).

Immediately after the liposome solution was injected into the MG solution, a sharp increase ($\sim$1 s) in the SHG signal was observed. This indicates that adsorption of MG molecules onto the outer surface of the liposome bilayer is faster than the mixing process, which is limited by the injection time. Following the initial increase of the SHG signal, there is a decrease until equilibrium is reached. The decrease of the SHG signal is the result of MG molecules migrating across the liposome bilayers and adsorbing at the inner surface with an orientation opposite to that at the outer surface.

The analysis of the experimental data involves the background correction and calculation of the SH field, $E_{2\omega}$. It can be described by the following equation

$$E_{2\omega}(t) = \sqrt{I_{\mathrm{MG+lipsome}}(t) - I_{\mathrm{background}}} \tag{2}$$

where $I_{\mathrm{MG+liposome}}(t)$ is the total signal detected at twice the frequency of the fundamental incident laser, 2ω, at time t after mixing of the MG solution and the liposome solution. $I_{\mathrm{background}}$ represents the contributions from the factors other than the SH field generated by the MG adsorbed on the DOPG liposome bilayer.

The data shown in Fig 1(b) is fitted to a single exponential function,

$$E_{2\omega}(t) = a_0 + a_1 \exp(-t/\tau_1) \tag{3}$$

A time constant of 90 ± 2 s is obtained, which is in agreement with our previous result of 107 ± 11 s.[14]

A notable feature of the SHG decay kinetics is the observation that the decay levels off at a SHG value that is well above zero. This indicates that there is a significantly greater population of MG at the outer surface than the inner surface at the conclusion of the MG transport kinetics. This result can be expressed quantitatively using the fact that the SHG kinetics contains information about the MG population at the bilayer interfaces as well as the transport rate constant. Combining eqns. (1) and (3) and the initial condition that there is no MG at the inner surface at $t = 0$, *i.e.* $N_i(t = 0) = 0$, one obtains

$$\frac{a_0}{a_0 + a_1} = \frac{N_O - N_i}{N_O} \tag{4}$$

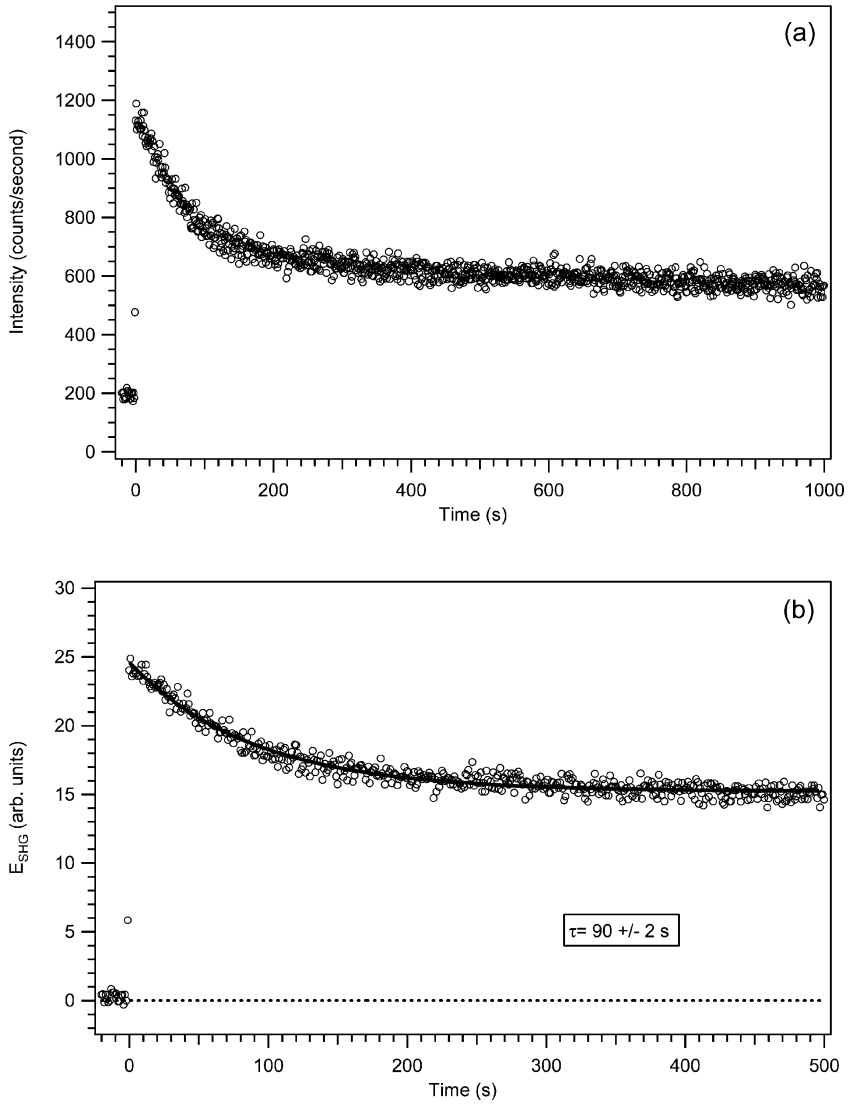


Fig. 1 Temporal profile of SHG intensity (a) and E field (b) before and after injection of liposome into MG solution. The data shown is for an aqueous 10 mM solution of NaCl at pH 4.0. The solid line represents the biexponential fit to the data.

where N_o is the number of MG molecules at the outer surface and N_i at the inner surface after the transport has been completed. It should be noted that at the bulk concentration of 10 μM the number of MG at the outer surface is time independent and equal to its maximum value. This is inferred from our observations that the adsorption of MG at the outer surface is saturated at the MG concentration of 10 μM.[23] The fit of the data shown in Fig. 1(b) gives $a_0/(a_0 + a_1) = 0.62$, which leads to $N_i/N_o = 0.38$. Thus, it is clear that there is a difference in the number of adsorption sites at the outer and inner interfaces. This disparity cannot be due to the difference in the areas of the surfaces, which differ by roughly 10%. Why then does MG not continue to cross the bilayer to yield an SHG signal that is within 10% of zero?

A key element to understand these results is the progressive buildup of a positive potential at the inner surface relative to the outer surface, as the positively charged MG crosses the bilayer. This

increasing positive potential opposes the transport of additional MG molecules to the inner surface of the bilayer. The transport of MG across the bilayer would then cease when the concentration gradient driving MG to cross to the inner liposome region comes into balance with the opposing inner positive potential generated by the MG that have already crossed the bilayer. We can calculate this potential using Gauss's law. We find that the potential developed by the MG that have crossed the bilayer in the elapsed time t after the addition of MG to the liposome solution is given by

$$V_{\text{in}} - V_{\text{out}} = \Delta V(t) = \frac{N_{\text{i}}(t)e}{4\pi\varepsilon_0\varepsilon_r}\left(\frac{1}{r_{\text{in}}} - \frac{1}{r_{\text{out}}}\right) \tag{5}$$

where e is the magnitude of the electron charge, ε_0 is the permittivity of the vacuum, ε_r is the dielectric constant inside the bilayer, r_{in} and r_{out} are the radius of the inner and outer surfaces, respectively, and $N_{\text{i}}(t)$ is the number of MG inside the liposome at time t. Eqn. (5) shows that ΔV is proportional to the number of MG at the inner surface. Furthermore we see that as the population of $N_{\text{i}}(t)$ increases with time the positive potential inside the liposome increases, which in turn more strongly opposes the transport of MG across the bilayer. From this description it follows that the SHG signal would not approach zero, as we have experimentally observed.

One way to determine if this explanation is correct would be to effect the transfer of a positive ion, *e.g.* Na^+, across the bilayer from the inner compartment of the liposome as the positively charged MG crosses the bilayer to the inner region of the liposome. The driving force for a positive ion such as Na^+ to transfer from inside the liposome is the relative negative potential outside the liposome that is produced as MG crosses the bilayer to the inner compartment of the liposome. If Na^+ could cross the bilayer from the inner compartment there would not be a buildup of a relative positive potential as MG crosses to the inner compartment. Thus additional MG molecules could cross to the inner region and the SHG signal would approach zero as the inner and outer surface populations of MG approach equality. However, because Na^+ is strongly hydrophilic it cannot cross the bilayer on the time scale of these experiments. To circumvent this problem we can use a Na^+ transporter to ferry Na^+ across the bilayer. Such alkali ion transporters are well known. Macrocyclic antibiotics, such as valinomycin and monactin, have been shown to increase alkali ion permeability by several orders of magnitude.[24,25] These macrocyclic transporters share common structural features, which are that the interior is hydrophilic and can accommodate an alkali ion whereas the exterior is hydrophobic, which permits the antibiotic to cross the hydrophobic alkyl chain region of the bilayer.

In the next section, we report on how the presence of valinomycin affects the transport of MG across a liposome bilayer and indeed find that our results support the idea that an increasing relative positive potential that develops as MG crosses the bilayer inhibits the crossing of additional MG molecules.

B. Effect of valinomycin on transport kinetics

The SH electric field $E_{2\omega}$ as a function of time t in the presence of valinomycin at two concentrations are shown in Figs. 2 and 3, respectively. Similar to the experiment without valinomycin, the SHG kinetics exhibits a sharp spike immediately after adding the solution containing liposomes and valinomycin (*i.e.*, $t = 0$) followed by a decay. A control experiment shows that valinomycin itself in liposome solution does not generate an observable SHG signal. However, the SHG kinetics of MG crossing the bilayer are seen to be strongly dependent on the presence of valinomycin. Figs. 1–3.

The MG transport kinetics with and without valinomycin were fitted to single exponential decays Figs. 1–3. The fit results are summarized in Table 1. For comparison, Table 1 also lists the fitting parameters for MG transport kinetics in the absence of valinomycin. Our results show that the presence of the alkali ion carrier, valinomycin, has significant effects on the MG transport across the DOPG liposome bilayer. At a valinomycin concentration of 1.25×10^{-8} M, the MG transport time ($\tau = 30\text{ s} \pm 1\text{ s}$) is three times faster than in the absence of valinomycin, *i.e.* $\tau = 90\text{ s} \pm 2\text{ s}$. Increasing the valinomycin concentration by a factor of ten decreases the transit time ($\tau = 6.2 \pm 0.2$ s) by an additional factor of five. The number of valinomycin carriers per liposome that are present in the bilayer or near the liposome surfaces is roughly estimated to be 20 and 200 at the two concentrations.

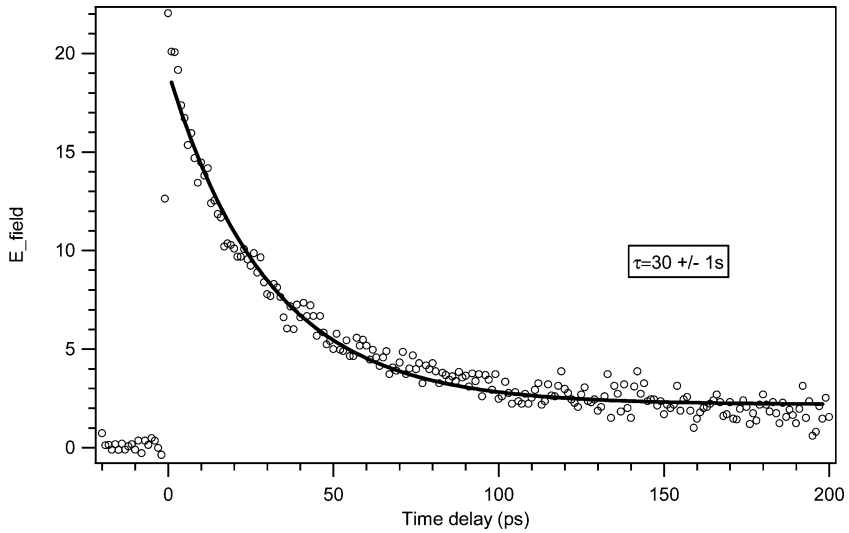


Fig. 2 Measured and fitted time evolution (solid line) of SH electric field upon injection of liposomes (with low concentration of valinomycin 1.25×10^{-8} M) into a MG solution at 10 mM of NaCl at pH 4.0; (a) and (b) are from the same data but drawn in different time scales.

In addition Figs. 2 and 3 show that the SHG electric field $E_{2\omega}$ decays to values much closer to the background in the presence of valinomycin which is contrary to the decay in the absence of valinomycin. These findings suggest that in the presence of valinomycin, not only is the transport more rapid, but also that more MG molecules cross the bilayer. With regard to the magnitude of the $E_{2\omega}$ decay we can calculate the ratio of the MG population at the inner surface to that at the outer surface with the fitting parameters, a_0 and a_1, as described earlier. The ratios of 0.88 for $C_{\mathrm{Val}} = 1.25 \times 10^{-8}$ M and 0.89 for $C_{\mathrm{Val}} = 1.25 \times 10^{-7}$ M are much larger than that in the absence of valinomycin (0.38). They are comparable to the ratio of the inner surface area to outer surface

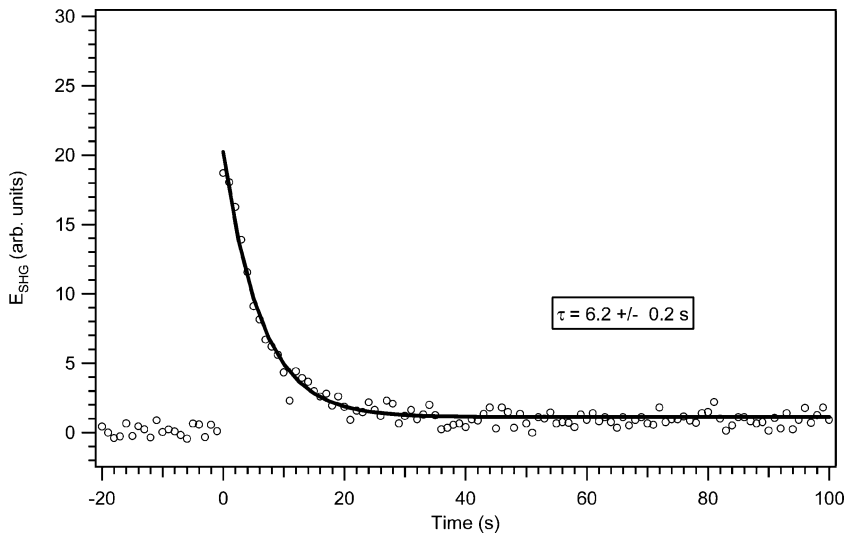


Fig. 3 Measured and fitted time evolution (solid line) of SH electric field upon injection of liposomes (with low concentration of valinomycin 1.25×10^{-7} M) into a MG solution at 10 mM of NaCl at pH 4.0; (a) and (b) are from the same data but drawn in different time scales.

Table 1 Summary of the fit parameters to the SHG kinetics

$C_{\text{valinomycin}}$	τ/s	N_i/N_o
0	90 ± 2	0.3836
1.25×10^{-8} M	30 ± 1	0.88
1.25×10^{-7} M	6.2 ± 0.2	0.89

area (0.85). These numbers are consistent with the description that valinomycin assists the MG to cross the bilayers and occupy the adsorption sites at the inner liposome surface.

Although the valinomycin–Na^+ complex may be formed at both surfaces of the bilayer, the direction of net complex transport depends on the potential between the two surfaces. Before the MG solution is added to the liposome valinomycin solution, there is no potential across the bilayer. The transport of Na^+ per unit time by the valinomycin carrier is equal in the opposing directions. Following the addition of the MG solution, a positive inner potential relative to the outer surface develops as MG crosses the bilayer. This initial development of a negative potential at the outer surface relative to the inner surface acts as the driving force for a net transfer of the Na^+–valinomycin complex from the inner surface to the outer surface. As a result, valinomycin can move a net population of Na^+ from the inner compartment to the outer aqueous solution. This net transport of Na^+ outward reduces or eliminates the development of an inner positive potential, which is the key factor in retarding and limiting the population of MG that crosses the bilayer. At time t, the potential at the inner surface relative to the outer surface can be written as,

$$\Delta V(t) = \frac{(N_i(t) - N_{Na^+}(t))e}{4\pi\varepsilon_0\varepsilon_r}\left(\frac{1}{r_{in}} - \frac{1}{r_{out}}\right). \quad (6)$$

where $N_{Na^+}(t)$ is the net number of sodium ions that have crossed from the inner aqueous compartment to the outer aqueous solution up to time t, and $N_i(t)$ is the net number of MG that crossed the bilayer to the inner liposome compartment up to time t. From eqn. (6) we see that the decrease in the net number of cations in the inner compartment lowers the positive potential developed by the transport of the MG molecules. Therefore the MG transport rate across the bilayer would be expected to be faster in the presence of valinomycin as we have observed.

We are currently developing a quantitative model based on the mechanism proposed in this paper to describe the transport of MG across a phospholipid bilayer. With no valinomycin, the model includes both the concentration gradient that drives MG to cross the bilayer and the development of an electrostatic barrier that retards and limits the further transport of MG. With valinomycin the model is modified to include the valinomycin mediated Na^+ transport.

Conclusion

The transport of the organic cation, malachite green (MG), from the bulk aqueous solution across a dioleoylphosphatidylglycerol liposome bilayer (4–5 nm) to the inner aqueous compartment is observed in real time using second harmonic spectroscopy. It is proposed that both the transport kinetics and the total number of MG located in the inner liposome compartment are limited by the positive potential that develops as MG crosses the bilayer. The inner positive potential opposes additional MG crossing the bilayer. To investigate this proposal the alkali ion carrier valinomycin, which is known to carry alkali ions across phospholipid bilayers, is introduced into the system. As MG crosses the bilayer, valinomycin can transport Na^+ ions in the opposite direction, driven by the outer negative potential due to MG that have crossed to the interior. In this way any positive potential due to MG inside the liposome is reduced or eliminated by the outward passage of Na^+ ions. At a valinomycin concentration of 1.25×10^{-8} M, the MG transit time (30 ± 1 s) is reduced by a factor of three compared with the transit time (90 ± 2 s) in the absence of valinomycin. At a higher valinomycin concentration, 1.25×10^{-7} M, the MG transit time (6.2 ± 0.2 s) is reduced by a factor of 15 with respect to the transit time when valinomycin is absent. At long times the second harmonic signal approaches zero ($\sim$10% above) due to the cancellation of the second order polarization of MG at the outer surface by a slightly smaller number of MG oriented in the opposite direction at the inner interface. In summary, the observation of more rapid transport kinetics of the organic

cation MG and reduction of the SHG signal in the presence of valinomycin, supports the mechanism of an electrostatic "barrier" limiting MG transport. This barrier can be eliminated by a small alkali ion carrier, such as valinomycin.

Acknowledgements

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