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Effect of Cholesterol on Molecular Transport of Organic Cations across Liposome Bilayers Probed by Second Harmonic Generation

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ABSTRACT The effect of cholesterol on the molecular transport of an organic cation, malachite green (MG), across large unilamellar dioleolyphosphatidylglycerol (DOPG) liposome bilayers with 0–50 mol% cholesterol was studied by second harmonic generation (SHG). Because SHG is a surface-specific technique, it requires no labeled molecule, quencher, or shifting agent to distinguish the location of the solute molecules. An additional important feature of SHG is that it is sensitive only to the probe molecules bound to the liposome, whereas other methods can only differentiate between molecules that are outside and those inside the liposome. The transport kinetics of MG across the liposome bilayers was observed in real time, and the results show that cholesterol retards the rate of transport of MG across liposome bilayers. The rate was found to decrease by six times for 50 mol% cholesterol content compared with cholesterol-free liposomes. This demonstrates the applicability of SHG to investigation of the effect of liposome composition on the transport kinetics across the liposome bilayers.

INTRODUCTION

Cholesterol is an essential constituent of plasma membranes in mammalian cells found in many biomembranes at very high concentration (Yeagle, 1993; Gennis, 1989; Yeagle, 1985). It has a pronounced effect on the physical properties of membranes, particularly on the structure of the phospholipid bilayers (Yeagle et al., 1977; Forbes et al., 1988; Sankaram and Thompson, 1990; Mukherjee and Chattopadhyay, 1996; DuFourc et al., 1984; Robinson et al., 1995; Lasic, 1993). The effect of cholesterol on transport kinetics across liposome bilayers is important to the application of liposomes as drug delivery systems because cholesterol is often added to optimize the permeability of the liposome bilayers (Janoff, 1999; Lasic and Papahadjopoulos, 1998). In this study we investigate the effect of cholesterol on the molecular transport kinetics of an organic cation, malachite green (MG), across unilamellar liposome bilayers composed of negatively charged dioleolyphosphatidylglycerol (DOPG), the pK_a of which is below 3 (see Scheme 1). The cholesterol content changed from 0 to 50 mol%. A surface-specific technique of second harmonic generation (SHG) (Shen, 1989; Eisenthal, 1996a,b; Corn and Higgins, 1994; Shen 1999) was used in these studies, which has been proved to be useful for the observation of molecular transport across liposome bilayers in real time (Srivastava and Eisenthal, 1998). The experimental results show that cholesterol retards the rate of transport of MG across DOPG liposome bilayers at room temperature.

The effect of cholesterol on the permeabilities of various chemical species across lipid bilayers has been studied systematically since the 1970s. Demel et al. showed that cholesterol reduced the permeability of glucose, glycerol, and Rb^+ across the phosphatidylcholine liposome (Demel et al., 1972). Papahadjopoulos et al. found that the permeability of K^+, Na^+, Cl^-, and glucose across negative, neutral, and positively charged phospholipid bilayers decreased with cholesterol content (Papahadjopoulos et al., 1971). Szabo performed experiments to measure the conductance of organic ions across a membrane and found that cholesterol, on one hand, decreased the permeability of organic cations and, on the other hand, increased the permeability of organic anions (Szabo, 1974). Other molecules such as water (Martial and Ripoche, 1991; Carruthers and Melchior, 1983), fluorescence probe (Bittman et al., 1981), acetic acid (Xiang and Anderson, 1995), Na^+-ionophore complex (Wehrli et al., 1992), and Ca^{2+} (Bittman et al., 1981) were also found to cross membranes at a slower rate when cholesterol is incorporated into the membranes.

We recently showed that the SHG method can be used to study molecular transport across liposome bilayers in real time (Srivastava and Eisenthal, 1998). Because SHG is a surface technique sensitive to both the inner and outer surfaces of the bilayer, unlike other methods, there is no need to introduce extraneous probes, quenchers, shift reagents, or other species to differentiate molecules present in the bulk solution from those molecules in the region of interest, namely the interface of bilayer and water. The observation of the effect of cholesterol on the transport kinetics of MG across DOPG liposome by SHG demonstrates the applicability of SHG to investigation of the physical properties of liposomes with mixed composition.

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THE SECOND HARMONIC METHOD

Second harmonic generation (SHG) is a surface-specific technique (Shen, 1989; Eisenthal, 1996a,b; Corn and Higgins, 1994; Shen, 1999) that involves the conversion of

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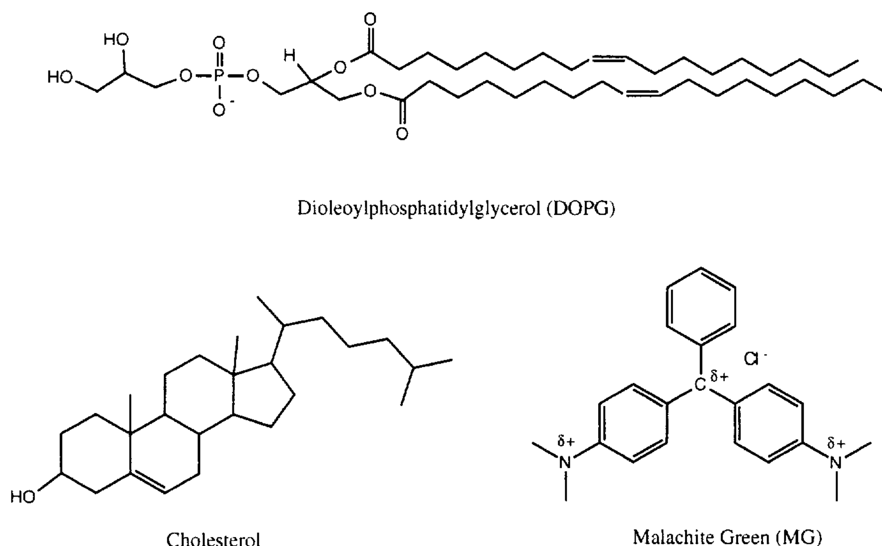

Scheme I

incident light at a fundamental frequency (ω) to light at twice the frequency (2ω) via a nonlinear interaction with the medium. It is often described as electric dipole forbidden in centrosymmetric media. In centrosymmetric media, for every molecule oriented in one direction there is one nearby oriented in the opposite direction, as shown schematically in Fig. 1 *a*. The second-order polarizations induced in these oppositely oriented molecules are of opposite phases and thus cancel, yielding no SHG from the bulk media. Unlike the molecules in the bulk liquid, the molecules at interfaces are not centrosymmetrically distributed because of the asymmetrical forces acting on them (Fig. 1 *b*). Therefore, the second-order polarizations induced at the interface due to incident light at frequency ω do not cancel and lead to a second harmonic field, $E_{2\omega}$, radiated at frequency 2ω from the interface (Fig. 1 *b*), given by

$$E_{2\omega} \sim P_{2\omega}^{(2)} = N_s \langle \alpha^{(2)} \rangle E_\omega E_\omega \qquad (1)$$

where E_ω is the electric field of the incident light, N_s is the surface density, and ${P_{2\omega}}^{(2)}$ is the second-order polarization. The second-order polarizability, $\alpha^{(2)}$, contains the information for the chemical species at the interface, and the brackets indicate an average over the molecular orientations. The second harmonic field, $E_{2\omega}$, which is ultimately detected, is directly related to the chemical composition and structural arrangement of the molecules at the interface.

SHG has recently been shown to be a new method for the study of microparticle surfaces (Wang et al., 1996). Because the local regions of the surfaces of microparticles are noncentrosymmetrical, even when the microparticle is centrosymmetrical, the second harmonic field, $E_{2\omega}$, can be generated by the irradiation of fundamental light at frequency ω (Fig. 1 *c*). If the size of the microparticle is comparable to the wavelength of the light, the second harmonic field, $E_{2\omega}$, generated locally from the opposite sides of the centrosymmetrical microparticle can add constructively, yielding a SHG signal. Taking advantage of both the surface specificity and spectroscopic selectivity of SHG, we

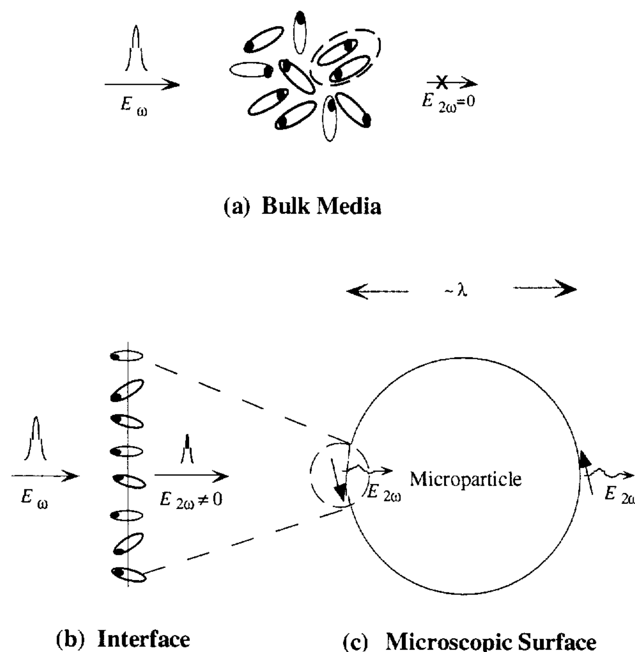

FIGURE 1 (*a*) In bulk media, for every molecule oriented in one direction there is one oriented in the opposite direction. Therefore the second-order polarizations cancel and give no SHG signal. (*b*) At the interface, molecules are specifically oriented because of asymmetrical forces acting on them. Therefore the second harmonic fields, $E_{2\omega}$, can be generated locally at the interface. (*c*) At the microscopic surface, the $E_{2\omega}$ generated at opposite surfaces of a particle with a diameter on the scale of the wavelength of the light can add coherently and give a SHG signal.

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have studied aqueous suspensions of polystyrene beads (Wang et al., 1996, 1998; Yan et al., 1998), liposomes (Srivastava and Eisenthal, 1996), semiconductor particles (Liu et al., 1999), oil/water emulsions (Wang et al., 1998; Yan et al., 1998), and clay particles (Yan and Eisenthal, 1999) to obtain information on the interface adsorbate populations, the free energies of adsorption (Wang et al., 1998), the surface potential of the charged microparticles (Yan et al., 1998), the surface spectrum of a charge transfer complex on semiconductor particles (Liu et at., 1999), and the real-time measurement of molecular transport across a bilayer of liposomes (Srivastava and Eisenthal, 1998).

We have reported in our recent study that SHG can be used to follow the transport kinetics of molecules crossing liposome bilayers (Srivastava and Eisenthal, 1996). The basic structure of a liposome is an enclosed membrane that has two lipid/aqueous interfaces (Fig. 2). The thickness of the membranes is several nanometers, which is much smaller than the wavelength of the light. When the molecules are added externally to the liposome solutions, the molecule adsorbing to the outer surface will interact with the fundamental light and give a SHG signal (Fig. 2 *a*), whereas the large population of unadsorbed bulk molecules will not. When the molecules start crossing the bilayer and adsorb to the inner surfaces, the adsorbed molecules on the inner and outer surfaces of a liposome will induce opposite-phase second-order polarizations because they have opposite orientations due to the symmetry (Fig. 2 *b*). Because their separation is much smaller than the wavelength of the light, the SH fields generated by the opposing molecules on the inner and outer surfaces cancel each other. Consequently, $E_{2\omega}$ generated from the liposome is proportional to the difference of the population of the solute molecules adsorbed on the outer surface, N_o, and the inner surface, N_i,

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

Therefore, we can observe in real time the molecular transport across the liposome bilayers by the SHG method. It should be noted that at equilibrium the SH field does not vanish because the surface populations on the inner and outer surfaces of a liposome are not equal. Thus measuring the final signal, i.e., $E_{2\omega}(\infty)$, at different MG concentrations, we are able to obtained an adsorption isotherm (Liu, Yan, and Eisenthal, in preparation).

EXPERIMENTAL PROCEDURE

The setup for second harmonic measurement is described in detail elsewhere (Wang et al., 1998; Yan et al., 1998). Briefly, it consists of a Ti:sapphire oscillator, which provides 100-fs pulses at 846 nm at a repetition rate of 82 MHz. The fundamental light is focused into a sample cell, and the second-harmonic photons at 423 nm are detected at 90° with respect to the incident light by a single photon counting system.

The liposome systems used in the studies were made of phospholipid, dioleoylphosphatidylglycerol (DOPG) (Avanti Polar Lipids), with different cholesterol (Sigma) contents, and the solute molecule used was malachite green (Aldrich). All aqueous solutions were prepared in 10 mM citric acid/12 mM NaOH buffer solution at pH 4.2. The individual solutions of DOPG lipid and cholesterol were initially prepared at 1.25 mM in chloroform. The DOPG and cholesterol solutions were mixed in different ratios to give 10-ml mixtures with different mole percentages of cholesterol, 0–50 mol%. The chloroform was then removed to give thin films of lipid and cholesterol mixtures, which were hydrated in 5 ml buffer solution and were extruded at room temperature and a pressure of 120 psi through 0.2-μm filters 10 times to yield a stock unilamellar liposome suspension (Hope et al., 1985). We determined by ^{31}P NMR experiment that more than 90% of the liposomes are unilamellar (Mayer et al., 1986). After the extrusion, the liposome suspensions were diluted 100-fold. The SHG measurements were made immediately after the preparation of the liposomes. The change of size upon aging was of concern, and the size was monitored by UV/VIS measurement of the stock solution. We found that the optical density of the samples did not change during the time of the SHG measurement. This indicates the samples were stable and no liposome fusion occurred, because this process would change the Mie scattering of the liposome suspensions by changing the size of the liposomes. The size of the liposomes was also measured by dynamic light scattering 1 day after

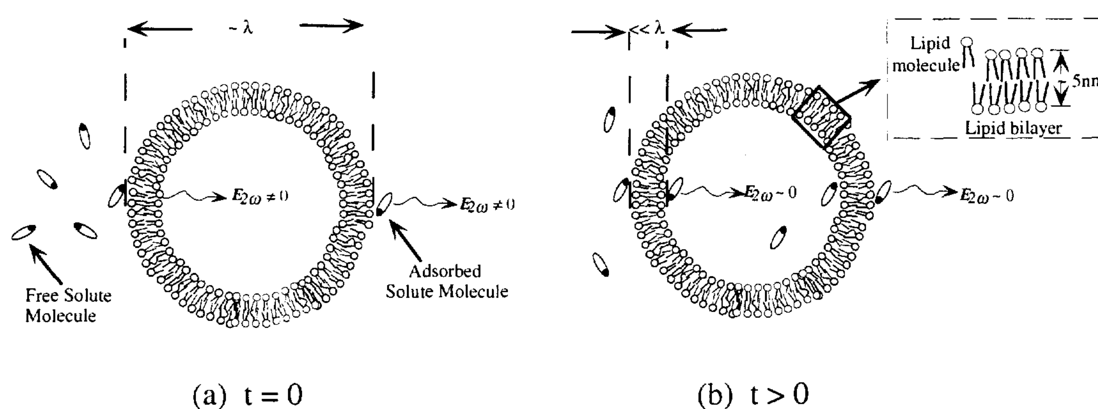

FIGURE 2 Solute molecules are added externally to a liposome suspension. (*a*) At the early time, the molecules adsorb only to the outer surface of the liposome. Because the size of the liposome is comparable to the wavelength of the light, the second field from the outer surface of the liposome can add coherently and a SHG signal can be observed. (*b*) When the solute molecules start diffusing into the liposome and adsorb to the inner surface, the second fields, $E_{2\omega}$, generated at the opposite surface of the bilayer, are separated by the thickness of the bilayer, which is on a length scale much smaller than the wavelength of the light. Therefore they cancel and the second harmonic field, $E_{2\omega}$, decreases.

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the sample preparation and was found to be 180 nm with a narrow distribution. The variation in size for different cholesterol contents was within 10%.

Malachite green (MG) chloride (Aldrich) was used as the solute molecule. We choose pH 4 because MG is in cationic form at this pH, which has an absorption at 427 nm, which is in resonance with the second-harmonic frequency of the fundamental laser input and therefore gives resonance enhancement of the SHG signal. At room temperature (22°C) the MG solution was injected into an equal volume of DOPG-cholesterol liposome suspension with a syringe. The injections were completed within 1 s. The second harmonic field, $E_{2\omega}$, was monitored as a function of time. The final concentration of MG is 10 μM, and the concentration of DOPG lipid plus cholesterol was 12.5 μM for all liposome samples.

RESULTS AND DISCUSSION

We observed that with the addition of MG solution at 10 μM to the liposome suspensions, the SHG signal increased immediately because of the adsorption of MG on the outer surface of the liposomes. After the initial increase in the SHG signal, there is a decay in the signal (shown in Fig. 3). The decay was fit empirically to single exponential functions. It is to be noted that fitting the data to a double-exponential function gives two very similar time constants with negligible improvement in the fitting residue. The decay times are summarized in Table 1. The SHG signals shown in Fig. 3 indicate a decrease in the signal-to-noise ratio as the cholesterol content is increased. This is because the absolute SHG signal decreases as the cholesterol content increases. We found from an adsorption study that the interaction between the positively charged MG and negatively charged PG liposome is primarily electrostatic and that the surface population scales linearly with the amount of negatively charged lipid (Liu et al., 2000). Because we keep the total lipid plus cholesterol concentration the same, the negatively charged PG content decreases as the cholesterol content increases. This results in a lower surface population, a lower absolute SHG signal, and thereby a lower signal-to-noise ratio.

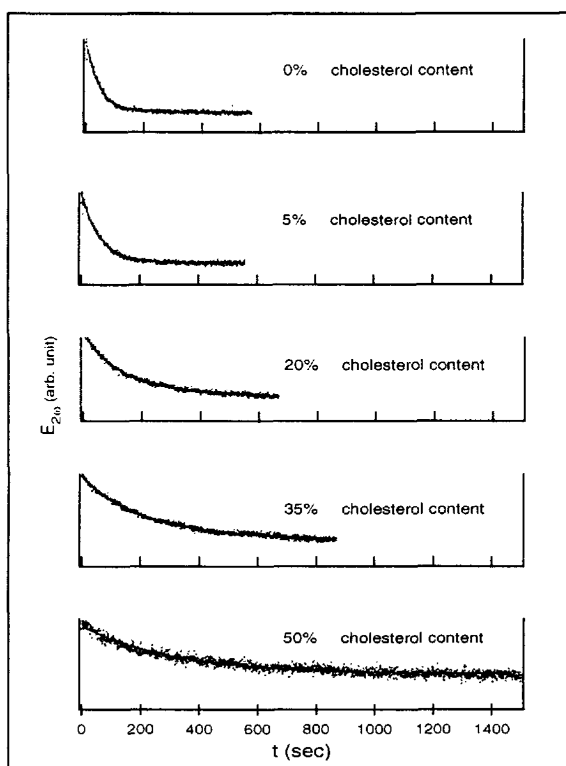

FIGURE 3 The SHG signals decay exponentially with time after a 10 μM malachite green (MG) solution is mixed rapidly with the suspensions of liposomes with different cholesterol contents. The total lipid and cholesterol concentration of the samples is kept constant at 12.5 μM. The solid lines are the single exponential fit.

We have found that the higher the cholesterol content, the slower the transport rate. This result is consistent with the structural modification of the bilayers by cholesterol. It is known that cholesterol decreases the permeability of bilayers in the liquid crystalline state (Demel et al., 1972; Papahadjopoulos et al., 1971; Szabo, 1974; Martial and Ripoche, 1991; Carruthers and Melchior, 1983). In the experiments reported here, the temperature of the samples is 22°C, at which the DOPG liposomes are in the liquid crystalline state. The cholesterol molecules incorporated into the bilayers make the upper portions of the lipid hydrocarbon chains adopt a more *trans* configuration, which decreases the number of kinks in the bilayers and increases the order of the lipid molecules. Therefore the permeability of the bilayers decreases (Yeagle, 1993; Gennis, 1989).

The rate of MG transport across DOPG liposome is found to be very sensitive to the cholesterol content of the bilayers. There is a fivefold decrease in the time constant for 35 mol% cholesterol content and a sixfold decrease for 50 mol% cholesterol content, compared with cholesterol-free liposomes (see Table 1). These results are consistent with other experimental findings on organic solutes. It was found by NMR that the permeability of acetic acid decreases 14 times when the cholesterol content of large unilamellar DMPC liposomes reaches 30 mol% (Xiang and Anderson, 1995) and the permeability of organic cations, tetraphenyl phosphonium, and 3,3′-dipropyloxadicarbocyanine ob-

TABLE 1 The time constants obtained by fitting the decays of the second harmonic field, $E_{2\omega}$, with single exponential functions for different cholesterol contents of the DOPG liposome, shown in Fig. 3

Cholesterol content (mol%)	τ (s)
0	46.5 ± 0.6
5	63.7 ± 1.5
20	135 ± 2
35	224 ± 5
50	278 ± 5

The total lipid and cholesterol concentration of the samples is kept constant at 12.5 μM.

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tained from fluorescence quenching decreases five times when the cholesterol content reaches 50% (Szabo, 1974). Moreover, the rate of carboxyfluorescein crossing the bilayers was halved when the cholesterol content of the phosphatidylcholine bilayers was 50 mol% (Bittman et al., 1981). The cholesterol content of mammalian plasma membrane can be as high as 35–45 mol% (Yeagle, 1993; Gennis, 1989). The high sensitivity of the rate of molecular transport of MG across the liposome bilayer found in this study supports the important role of cholesterol in the cellular uptake of organic ions.

Although, the desorption and adsorption processes happening on both inner and outer surfaces can change the surface population, we found experimentally that these processes are not important for the observed decay of the SH field. Experimentally, we found that the increase in the signal happens immediately, i.e., within the instrument respond time. This respond time is 1 s, which is much shorter than the decay time of the second harmonic signal. This indicates that the adsorption of MG on the liposome surface is very fast compared with the time scale of MG crossing the bilayers.

Moreover, the adsorption and desorption process of MG inside the liposome can be neglected because the enclosed volume of the liposome is very small, $\sim 3 \times 10^{-18}$ liter. Thus the number of MGs in the interior bulk solution at a concentration equal to that in the exterior solution, 10 μM, is less than 10 molecules. It is estimated from the adsorption study (Liu et al., 2000) that the surface population of MG is on the order of 10^4 molecules per liposome, which is much larger than the number of MGs in the interior bulk solution.

It is important to note that the molecules bound to the inner and outer surfaces of the liposome have opposite directions due to symmetry. SHG is only sensitive to chemical species bound to the outer and inner interfaces of the liposome, where there is a net orientation, e.g., an unequal number of molecules on the inner and outer surfaces. Randomly oriented molecules present in the bulk interior and exterior regions as well as in the bilayer region would not contribute to the SH signal. On the other hand, the other methods do not differentiate between inner bulk and inner surface adsorption, but only indicate whether the molecule is outside or inside the liposome. Thus SHG can be complementary to these other methods.

The fluidity of the biomembranes has been the subject of much speculation, but it has no precise meaning in the context of cell membrane structure (Yeagle, 1993). Fluidity has only been quantified by parameters derived from the spectroscopic properties of small probe molecules. These parameters include the rotational correlation time obtained by electron spin resonance and fluorescence, the order parameter measured by NMR and electron spin resonance, the anisotropy of the fluorescent probes, and the permeability of certain probes between the membrane and aqueous medium (Gennis, 1989). The present study, which uses SHG to observe in real time the transport of MG across the liposome bilayer, may provide a supplementary method for characterizing the fluidity of bilayers.

CONCLUSION

The effect of cholesterol on the molecular transport of MG, an organic cation, across large unilamellar dioleoylphosphatidylglycerol (DOPG) liposomes has been investigated by second harmonic generation (SHG). We have found that cholesterol retards the rate of transport of MG across DOPG liposome bilayers. The high sensitivity of the rate of transmembrane motion of MG to the cholesterol contents of the liposome bilayers supports the important role of cholesterol in the cellular uptake of organic ions in biological systems.

Because SHG is a second-order spectroscopic technique, it responds directly to the second-order susceptibility of the solute molecules adsorbed on the surfaces of the bilayer. Therefore it does not suffer from the background due to the large population of unadsorbed molecules in the bulk solution. Moreover, SHG requires no labeled molecule, quencher, or shifting agent to distinguish the location of the solute molecules on the liposome surfaces and is therefore free from the perturbation induced by these molecules. Unlike other methods, SHG is sensitive only to molecules adsorbed to the outer and inner surfaces of the liposome, not to the interior and exterior bulk populations, which is what other methods detect. SHG can measure in real time the transport of molecules from the outer surface to the inner surface. This is the first application of SHG to the study of the effect of the composition of liposome bilayer on the rate of molecular transport. The success of observing the effect of cholesterol on the transport rate of MG across a DOPG liposome demonstrates the applicability of SHG to the investigation of the effect of the composition of liposome on the transport kinetics across bilayers.

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