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Kinetics of molecular transport across a liposome bilayer

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Abstract

Transport of the organic molecule malachite green, across a bilayer of dioleoylphosphatidylglycerol liposome in aqueous solution, has been observed using a new method, namely second harmonic generation (SHG). Following the rapid mixing of liposome and malachite green solutions, a decay of the SHG signal in time is observed as the malachite green bound to the outer liposome surface crosses to the inner liposome surface. The effect of liquid-crystalline versus gel liposome structures on the transport across the bilayer is reported also. © 1998 Elsevier Science B.V. All rights reserved.

1. Introduction

A primary role of biological membranes is to regulate the passage of chemical species into and out of cells. Because the basic structure of the biological membrane is the phospholipid bilayer matrix [1], the study of self-assembling phospholipid molecules into bilayer structures, called liposomes [2–4], has proven to be of great importance. Identification and determination of the energetics and local environment of molecules binding to the liposome surface are necessary for the description of equilibrium and dynamic chemical and physical processes involving lipid-bilayer systems [5–8]. It is worth noting that bilayer structures, liposomes and vesicles, are not only of interest as biological models but also as a method for capturing pollutants in environmental remediation programs and as a drug-delivery method [9–13]. In this Letter we show that the non-invasive method of second harmonic spectroscopy provides a new method to measure in real time the transit of molecules across a liposome bilayer. Unlike other methods there is no need to introduce extraneous probes, quenchers, shift reagents or other species to differentiate between molecules present in the bulk solution from those molecules in the region of interest, namely the bilayer. The reason for this is that second harmonic generation (SHG) senses molecules that are located in the bilayer region only and not those in the bulk aqueous regions, either internal or external to the liposome. Thus if the molecules of interest are present in the bilayer and have a reasonable non-linearity, which can be greatly enhanced ($\sim 10^6$) by tuning the incident lasers to the molecules one or two photon electronic or vibrational resonances, then they can be selectively detected in the bilayer by SHG or SFG (sum frequency generation).

Most importantly we have demonstrated [14] that second harmonic spectroscopy can selectively probe the surface of centrosymmetric microscopic structures, which constitute a class of natural and man-made materials of fundamental scientific and technological significance. We have studied up to this time particles as small as 0.06 μm in diameter with no

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upper limit to larger-sized particles. The non-linear optical methods of SHG and SFG sense the molecules adsorbed at the interface of the microscopic particles and do not sense, and are therefore not overwhelmed by, the large population of molecules present in the bulk medium in which the microscopic particles are suspended [14]. This method has proven to be successful in studies of microscopic systems, among which are polymer beads, semiconductor colloidal particles, naturally occurring clay particles, oil droplet/water emulsions, bilayer structures that are composed of surfactants (vesicles) and the phospholipid liposomes [14,15], and microsomes made from rat liver cell membranes [16]. In these studies the driving force for adsorption of molecules to the surface of microscopic particles, i.e. the adsorption free energies, as well as the absolute number of adsorbed molecules [15], and the surface electrostatic potential [17] of both charged polymer beads and the charged interface of oil droplets in an oil/water emulsion have been obtained.

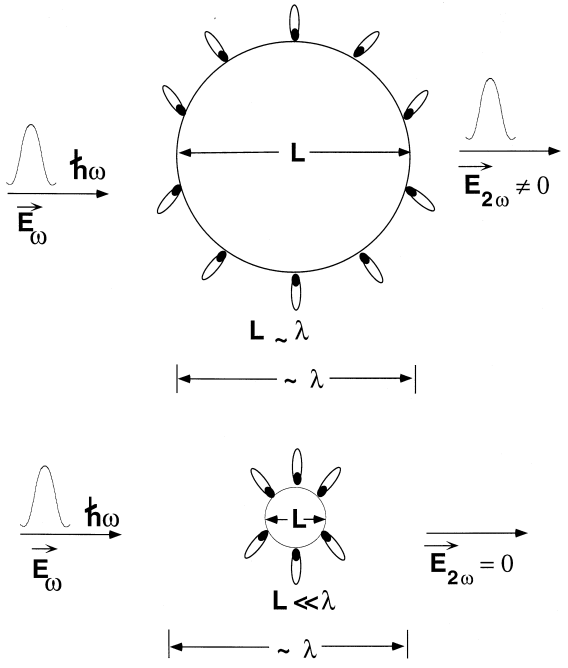

Fig. 1. SHG is dipole allowed from a centrosymmetric particle of size of the order of the wavelength of light (more precisely the coherence length of the SHG), but is dipole forbidden for small particles.

2. Second harmonic generation from the surfaces of centrosymmetric microscopic structures

Because SHG and SFG are described as electric dipole forbidden in centrosymmetric media, there is no SHG or SFG generated in such media. (For simplicity we will refer only to SHG in this Letter although the same ideas apply to SFG which we have observed at 278 nm using IR (834 nm) and VIS (417 nm) input pulses.

If SHG is forbidden in centrosymmetric media, as stated above, how is it that SHG is generated from the surface of microscopic centrosymmetric particles in a bulk isotropic medium [14]? The answer is that SHG is dipole forbidden in centrosymmetric systems provided the particle is centrosymmetric on length scales much less that the coherence length of the process (Fig. 1). The coherence length (L_c) of the process is given by the relation $\Delta k \cdot L_c \sim \pi$, where $\Delta k = k_{2\omega} - k_\omega$ and $k_{2\omega}, k_\omega$ are the light propagation vectors for the SHG and the fundamental light, and are inversely proportional to their respective wavelengths in the medium. For the experiments described in this work the coherence length is typically about 2 μm. Although the microscopic particle shown is centrosymmetric on a macroscopic scale, its surface is not locally centrosymmetric. With respect to oppositely oriented molecules located at opposite positions of the microscopic particle surface of the microscopic particle surface there is no cancellation of their signals. The oppositely oriented molecules on the surface of the microscopic particle are separated by a distance of the order of the coherence length of the SHG process and therefore do not necessarily have opposite phases. Thus the second-order polarization generated at the opposing parts of the centrosymmetric particles surface can add constructively to give a SHG signal. Strong SHG signals have been observed and reported for various microparticle systems [14].

3. Molecular transport across a bilayer

We report in this Letter on the application of SHG to include time dependent processes occurring at the surface of microscopic particles. In particular we

have used second harmonic spectroscopy to observe in real time the transport of molecules across the bilayer of a phospholipid liposome that is made up of a membrane phospholipid, dioleoylphosphatidylglycerol (DOPG) [18,19], in aqueous solution (Fig. 2). The experiment consists of rapidly mixing together an aqueous solution containing the DOPG liposomes, prepared by an extrusion method [20] using 0.2 μm filters, with an aqueous solution containing organic molecules. The liposomes are unilamellar, 236 nm mean diameter as determined by dynamic light scattering, and are at a density of 10^{10} liposomes/cm^3. The concentration of the organic molecule malachite green (MG chloride) which is a triphenylmethane dye commonly used to stain tissue [21–23] is 5×10^{-6} M in the final solution. The laser used in this experiment is an 80 MHz rep rate Ti:Sapphire laser that generates 10 nJ pulses of 100 fs duration at 832 nm. The incident light irradiates the solution of liposomes thereby generating the SH signal, which is detected by a single photon-counting system. This is shown in Fig. 2. The SH signal is due to the adsorbed MG molecules. The non-linearity of the phospholipid DOPG is very small compared with that of MG and does not contribute to the SH signal under the conditions of the experiment. The adsorption of the dye molecule onto the outer liposome surface is very fast, as shown by an immediate rise (< 1 s) in the SH signal upon mixing (Fig. 3). Following the rise in the SH signal we observed

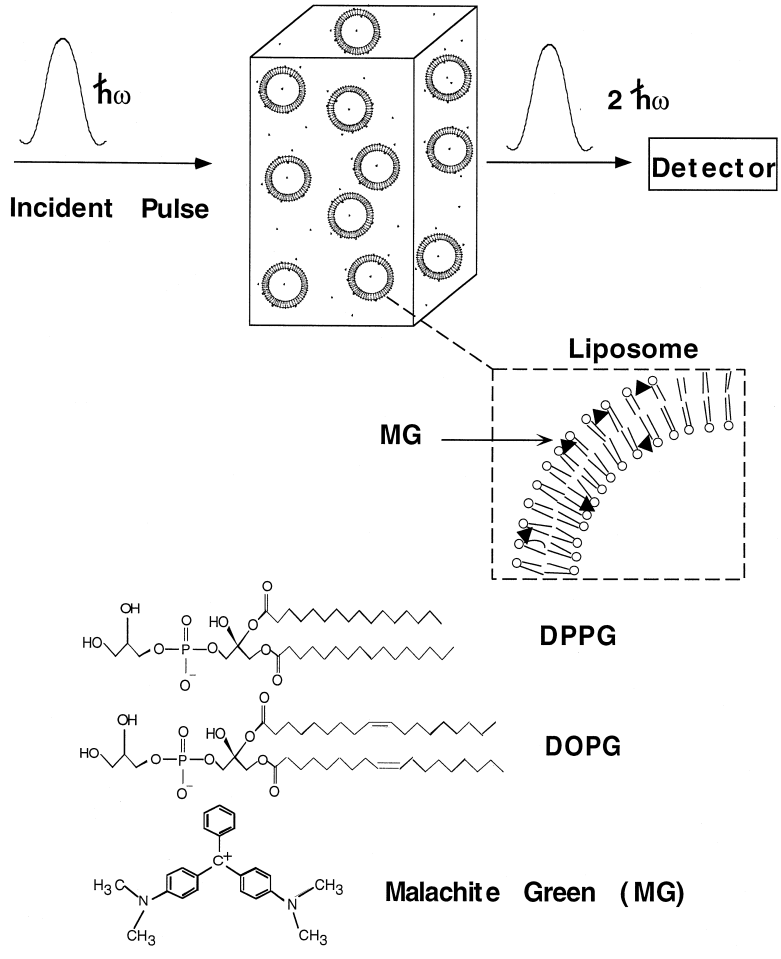

Fig. 2. Schematic of experiment and structures of DOPG and DPPG phospholipids, and malachite green.

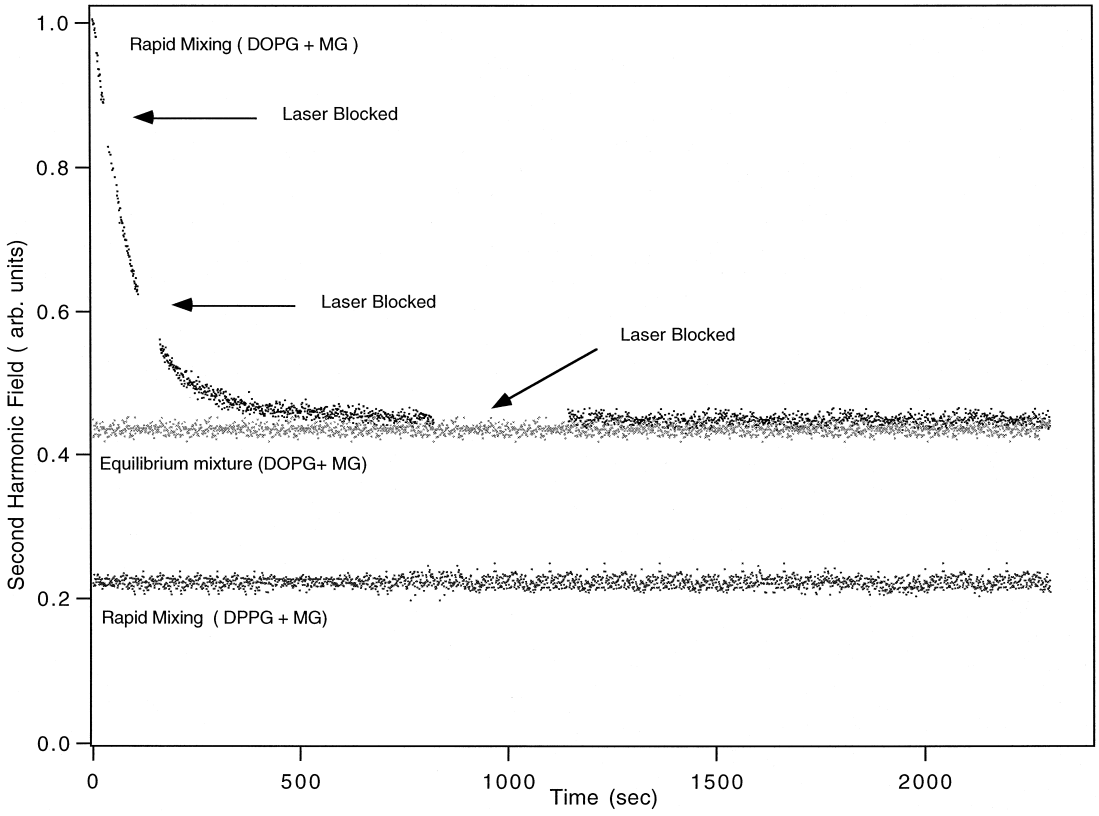

Fig. 3. Second harmonic field versus time. [MG] = 5 μM. Transport is seen on rapid mixing of DOPG and MG solution. The long-time SH field following rapid mixing is equal to the SH field from DOPG liposome prepared with MG, i.e. the equilibrium mixture. No transport is observed across DPPG liposome bilayer.

an exponential decay in the signal to a final constant level. The decay of the SH signal was due to the transport of the MG, which was bound to the outer surface, across the bilayer where the MG then binds to the inner liposome surface. Those MG molecules that pass across the bilayer that do not bind to the inner surface, but rather enter the bulk water phase interior to the liposome, do not contribute to the SH signal. Based on symmetry arguments, the MG molecules that bind to the interior water/bilayer surface are oriented oppositely to the MG molecules bound at the outer bilayer/water surface. Because the separation between the inner and outer bound organic molecules is small ($\sim$ 5 nm) compared with the wavelength of the laser light, they contribute with opposite signs to the SH signal and thus cancel. This results in a decay of the SH signal as the inner liposome surface because populated with MG. In order to determine if the observed decay process was the result of some laser-induced process caused by the laser pulses used to monitor the kinetics, the laser was blocked and then unblocked (Fig. 3). The results demonstrated that the decay kinetics continues along the same decay curve independent of the interruptions by the blocking and unblocking of the incident probe laser. The decay process is thus seen to be a thermal and not a light induced process. From Fig. 3 it is seen that the SH signal does not go to zero at long times when equilibration of the MG populations on the inner and outer surfaces has occurred. The reason that the SH signal does not decay to zero is that there are fewer sites and thus fewer molecules adsorbed on the inner interface because of its smaller area. The outer area is roughly 10% larger than the inner area for the 236 nm liposome. In order to establish that the SH signal decays to its equilibrium

value, which is determined by the populations of MG bound to the outer and inner liposome surfaces reaching their equilibrium values, a separate experiment was performed. In this experiment, we mixed the MG with the phospholipids before they had self-assembled into liposomes. This results in an equilibrium distribution of MG bound to both the inside and outside liposome surfaces in the process of self-assembly. The concentration of MG and the density of the DOPG liposomes was the same as in the time-dependent experiments. In the latter premixed MG with phospholipid experiment, the SH signal is time independent (Fig. 3) because the populations of MG bound to the outer and inner surfaces of the liposome are at equilibrium at the start of the SH measurements. The SH signal is equal in magnitude to the SH signal obtained in the mixing experiment following the SH decay to its long time value. This suggests that the final state in the mixing experiment of MG solution with liposome suspension is an equilibrium distribution of MG bound to both the inside and outside liposome surfaces, and supports the interpretation that the decay is a result of the approach to equilibrium as the MG molecules move from the outside to the inside liposome surface. The time constant of the SH decay in the mixing experiment ranged from 40 to 400 s depending on the concentration of MG used in the experiment. To our knowledge, this is the first kinetic study for MG transport across the DOPG liposome bilayer. The timescale for the transport of MG across the DOPG liposome bilayer, obtained by SHG, is consistent with fluorescent experimental results for transport of organic molecules across liposome bilayers, such as Oxonol V crossing, the dioleoylphosphatidylcholine (DOPC) bilayer [24], and 1-anilino-8-napthalene sulfonate (ANS-), crossing, the dimyristolphosphatidylcholine (DMPC) bilayer [25]. There have also been ESR studies that used spin labeled phosphonium ions to follow the crossing of an egg PC bilayer [26]. The transport kinetics of different molecules across different liposome bilayers are of course different. However, for the range of aromatic molecules and liposomes used in the SHG, fluorescence and ESR studies, the crossing times are in the range of minutes. It should be noted that in both the fluorescence and ESR methods the contributions from molecules in the bilayer must be differentiated from the large background signals of the same molecules in the bulk solution. The introduction of quenchers and shift reagents into the bulk solution and, when feasible, the analysis of spectral changes associated with the presence of liposomes are used to minimize the background signals. An appealing feature of the SHG method is that it detects molecules in the bilayer region only and not molecules in the bulk solution external or internal to the liposomes. Furthermore, molecules that adsorb to the liposome can be detected with the only requirement that the molecule be non-centrosymmetric or that the molecule creates an electric field, e.g. adsorbed ionic species, that polarize the solvent molecules. Thus no extraneous molecule, e.g. spin label, shift reagent, strong fluorescer or quencher, which can perturb the system, need be introduced. An additional important feature is that SHG is sensitive to the absolute orientation of a molecule. Thus molecules of opposite orientation generate opposing SH signals whereas fluorescence and ESR do not differentiate the absolute orientation. Therefore molecules bound to the outside of a liposome, which by symmetry have the opposite orientation to those bound to the inner surface of the bilayer, can be differentiated from each other. This latter feature is the basis for observing by SHG the transport of molecules, which are initially bound to the outer surface, across the bilayer and ending up bound to the inner surface. It should be emphasized that the different methods for studying bilayers and transport across bilayers have complementary features that make each of them valuable.

4. Liposome structure and molecular transport

To investigate the effect of modifications in the phospholipid structure on transport across the bilayer, we selected the phospholipid DPPG, dipalmitoylphosphatidyl glycerol to form the liposome (Fig. 2). DPPG has the same headgroup as DOPG but differs in not having a double bond in each chain and in having two fewer CH_2 groups in the hydrocarbon chain. The mean diameter of the unilamellar DPPG liposomes was 148 nm. Unlike the mixing experiments of DOPG with MG, where bilayer transport was observed, we found no transport of MG across the bilayer of the DPPG liposome. Following the

mixing of DPPG with MG a rapid rise in the SH signal was observed indicating adsorption to the outer surface of the liposome. It was immediately followed by a constant SH signal out to the longest measurement time of 2400 s (Fig. 3). The impermeability of the DPPG liposome bilayer to the MG molecule versus the permeability of the DOPG liposome is attributed to the different structural states of the DOPG and DPPG liposomes. At room temperature the DOPG liposome is in a liquid crystalline state whereas the DPPG liposome is in the gel state [27,28]. In the gel state the phospholipid acyl chains are packed in a highly ordered arrangement with only restricted motions of the chain. For the liquid crystalline state the bilayer is more fluid because the chains are more disordered and are free to undergo fast rotational motions unlike the gel state. It is this difference in the packing of the acyl chains in DOPG and DPPG that is the origin of the permeability of MG across the DOPG bilayer and its impermeability across the DPPG bilayer. The populations of MG adsorbed to the inner and outer surface of the DOPG liposome, the population of MG adsorbed to the outer surface of the DPPG liposome, and their respective adsorption free energies will be reported in a future publication.

5. Conclusions

A new method has been used to observe in real time the transport of molecules across the bilayer of a phospholipid liposome. The method is the surface selective technique of SHG. The motion of the triphenylmethane dye, malachite green (MG), across the bilayer of dioleoylphosphatidylglycerol (DOPG), unilamellar liposomes of 236 nm diameter served to demonstrate the feasibility of this technique to study dynamics across bilayer structures. The populations of MG in the aqueous bulk solutions exterior or interior to the liposome are not sensed by SHG and thus do not contribute to the observed SH signal. As the MG bound to the outer surface crosses the bilayer and binds to the inner liposome surface an exponential decay in the SH signal is observed. The SH time-dependent signal is dependent on the difference in the populations of MG bound to the outer and inner surfaces of the liposomes. The decay time varied from 40 to 400 s depending on the MG concentration. Unlike the transport observed in the DOPG liposome, there was no observed transport of MG across the liposome bilayer of dipalmitoylphosphatidyl glycerol (DPPG). The distinctly different behavior was attributed to the DOPG liposome being in the liquid-crystalline state where there is disorder and fluidity in the chain packing, and the DPPG liposome being in the gel state, which is a highly ordered restricted arrangement of the hydrocarbons chains. Unlike other methods, the SH method does not require the introduction of extraneous probes, quenchers, shift reagents or other foreign species in order to differentiate between the molecules of interest located in the bilayer vs. those located in the bulk solution either external or internal to the liposome. The SHG method is sensitive to the molecules located in the bilayer only and not to bulk molecules, and is thus a method that has some advantages complementary to existing methods.

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