24 March 2000

ELSEVIER

Chemical Physics Letters 319 (2000) 435–439

CHEMICAL PHYSICS LETTERS

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Second harmonic spectroscopy: detection and orientation of molecules at a biomembrane interface

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Received 23 November 1999; accepted 17 January 2000

Abstract

The adsorption and orientation of a dye molecule to a planar, glass-supported phospholipid bilayer, a widely-used model membrane for biological studies, was measured with second harmonic spectroscopy. The results indicate that the positively charged dye binds at an angle of 19 degrees from the normal to the membrane plane. These results open the possibility of obtaining structural information on a variety of other adsorbed molecules at phospholipid membranes, including proteins, organic molecules and drugs. The nonlinear optical properties of the dye molecule are also demonstrated for the first time and indicate that others in the oxaxole family may be useful for second harmonic generation studies.

1. Introduction

Second harmonic generation (SHG) is a powerful technique for studying interfacial regions of molecular thickness. It has been useful in studying electron-transfer reactions occurring at interfaces [1], electrostatic properties of interfaces [2], studies of dye molecules adsorbed to liposomes [3], a charge-transfer spectrum of a semiconductor–dye surface complex [4], and others. Supported phospholipid bilayers are single-bilayer membranes which are electrostatically pinned to the surface of some substrate such as glass and are formed by the fusion of liposomes to the substrate [5–7]. Lipid molecules in the supported bilayers are highly mobile in the plane of the membrane. The liposomes may also contain protein components which retain their structural and functional integrity, and orientation, within the supported bilayer [7]. The supported bilayers have proven highly useful model systems for studying fundamental issues in biology such as cell-binding to protein components in membranes [5], protein–protein interactions, such as with cytochrome *c* and the photosynthetic reaction center [7], and others. Supported bilayers are also structurally robust and attractive candidates for biochemical sensor devices which rely, for example, on the use of a signal generated by a membrane protein [8].

We demonstrate for the first time that SHG can be applied to a study involving the supported bilayers. In particular, we use the planar geometry of the supported bilayer to study the orientation of a dye molecule adsorbed to the membrane surface. Our results demonstrate not only that structural information at the bilayer interface can be obtained, but also

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0009-2614/00/$ - see front matter
PII: S0009-2614(00)00116-0

prepare the way for other biological studies with SHG.

2. Theoretical background

The production of second harmonic light can be described in general by the following equation:

$$I(2\omega) = \frac{32\pi^3\omega^2\sec^2\Theta}{c^3\,\varepsilon(\omega)\,\varepsilon^{1/2}(2\omega)} \times|\boldsymbol{e}(2\omega)\cdot\chi^{(2)}:\boldsymbol{e}(\omega)\,\boldsymbol{e}(\omega)|^2 I^2(\omega) \quad (1)$$

where $I(2\omega)$ and $I(\omega)$ are the intensity of the harmonic and fundamental light, respectively, $\chi^{(2)}$ is the second-order nonlinear susceptibility tensor, $\boldsymbol{e}(\omega)$ and $\boldsymbol{e}(2\omega)$ are the products of the Fresnel factors and the polarization vectors for the light beams, and Θ is the angle between the reflected harmonic light and the surface plane [9]. The detailed derivation of Eq. (1) relies on a model of the interface in which a thin dielectric sheet lies between the two different phases; all light beams in the system interact with or originate from the nonlinear properties of this sheet. In our experimental set-up, the incident fundamental beam (45° polarization; $E_s = E_p$) is totally internally reflected at a glass–water interface. The orientation of a dye molecule adsorbed to the glass surface, or to another plane within this region, i.e. the supported bilayer, can be determined using a null-angle measurement in which the harmonic light (2ω) is nulled by an analyzer inserted before the monochromator. By blocking the SHG output, we ensure that:

$$\boldsymbol{e}(2\omega)\cdot\chi^{(2)}:\boldsymbol{e}(\omega)\,\boldsymbol{e}(\omega) = 0 \quad (4)$$

from Eq. (1), where $\alpha^{(2)}_{\xi\xi\xi}$ is the molecular hyperpolarizablity tensor, and θ is the angle between the molecular-frame ξ-axis and the surface normal. Here we assume that a single element of $\alpha^{(2)}_{\xi\xi\xi}$ dominates the second harmonic response. This behavior is expected for rod-like molecules such as the one we have chosen to study, in which the transition moment for excitation likely lies along the molecule's principal bond axis. By taking into account the Fresnel factors for transmission of the incident fundamental light and the coordinate transformations from lab-frame to molecular-frame, we have the following expressions for the macroscopic polarization:

$$\begin{aligned} P^{(2)}_x &= \langle\cos\theta\sin^2\theta\rangle N\alpha^{(2)}_{\xi\xi\xi}\,E'_x(\omega)\,E'_z(\omega)\\ P^{(2)}_y &= \langle\cos\theta\sin^2\theta\rangle N\alpha^{(2)}_{\xi\xi\xi}\,E'_y(\omega)\,E'_z(\omega) \quad (5)\\ P^{(2)}_z &= \tfrac{1}{2}\langle\cos\theta\sin^2\theta\rangle N\alpha^{(2)}_{\xi\xi\xi}\left(E'_y(\omega)^2\,E'_y(\omega)^2\right)\\ &\quad + \langle\cos^3\theta\rangle N_s\,\alpha^{(2)}_{\xi\xi\xi}\,E'_z(\omega)^2 \end{aligned}$$

where θ is the angle that $\alpha^{(2)}_{\xi\xi\xi}$ makes with the normal to the surface in the molecular frame, N denotes the number of dye molecules at the interface, and the brackets denote an orientational average. The polarization radiates as a sheet to produce the harmonic light, which itself has s- and p-component polarizations. The output polarization angle, $\Phi_{out} = \tan|E_p/E_s|$, is 90° rotated from the null angle and an expression for the null angle as a function of molecular orientation θ can be determined using Ref. [10].

3. Experimental methods

Liposomes were prepared by the freeze-thaw method using a mixture of phospholipids (4:1 DOPC:DOPG) (1,2-Dioleoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] and (1,2-Dioleoyl-sn-Glycero-3-phosphocholine) doped with 1 mole % of a fluorescent dye-lipid – Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (Texas Red, DHPE; Molecular Probes), as described previously [7]. The addition of 25 mole % of the negatively charged lipid (DOPG) corresponds to a bilayer surface charge density of 3.5×10^5 charges per cm^2 using an average molecular area per lipid of 70 Å^2. Phospholipid bilayers were formed from these on the underside of a glass Dove prism (type BK-7; Melles Griot) mounted in a piece of teflon and suspended over a well, by filling the well with the liposome solution and rinsing out after several min as described in Ref. [7]. Fluorescence recovery after photobleaching (FRAP) experiments were performed using a Biorad MRC-600 confocal fluorescence microscope with appropriate filters for the Texas Red dye, and focused on the membrane plane. The prism was previously cleaned

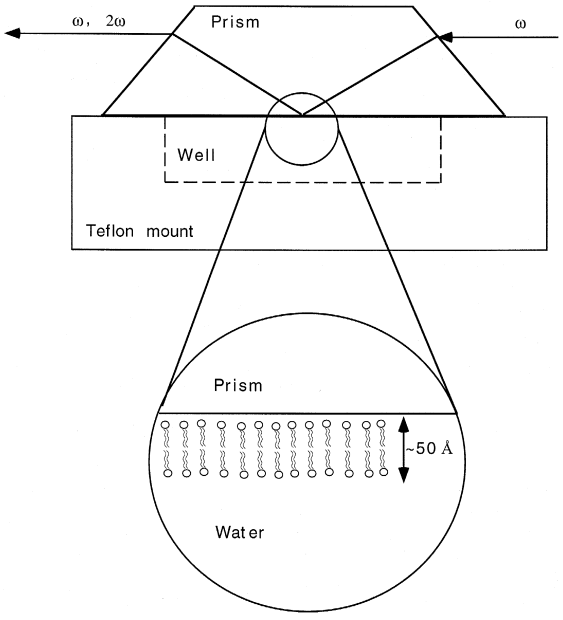

Fig. 1. Cross-section schematic of the prism cell. The Dove prism (10.5×5.0 mm high) is mounted into a groove in a piece of teflon. Incoming light (ω) is refracted and totally internally reflected at the glass–water interface with an incident angle of 30° to the surface. Outgoing light (ω, 2ω) is filtered to remove the fundamental component and residual dye-probe fluorescence. The polarization of the light *E*-field vector is −45° from the normal to the laser table. In the magnified region, the prism interface is shown with the supported phospholipid bilayer (not to scale). There is a gap of ~10–20 Å between the membrane and the glass surface.

by soaking for 45 min at 90°C in a mixture of freshly prepared 30:70 H_2O_2:H_2SO_4 (by vol., 'Piranha solution'). The well underneath the prism, with or without the supported bilayer present, was filled with double-distilled deionized water (pH 7) and the underside of the prism was never exposed to air after formation of the supported bilayer. The SHG probe dye used in these experiments is 4-[5-methoxyphenyl)-2-oxazolyl]pyridinium methanesulfonate (also known as 4PyMPO–MeMs) and is water-soluble and photostable at neutral pH. The dye is also positively charged (+1) and is expected to interact electrostatically with the glass and the supported bilayer which are both negatively charged. The second harmonic generation set-up has been described previously in detail (Ref. [2] and references therein). Briefly, the beam of an argon ion laser (10.5 W) is directed into the cavity of a titanium sapphire mode-locking laser (Tsunami). The output serves as the fundamental (ω) and occurs at a repetition rate of 82 MHz with ~150 ps pulse duration (800–834 nm) and 1.1–1.3 mW power. For all experiments, the polarization of the fundamental was set to −45° from the normal of the laser table. The fundamental beam was directed on to one face of the Dove prism where it is refracted and totally internally reflects at the glass–water interface, as shown in Fig. 1. Detection of the second harmonic photons at 2ω was accomplished using a series of focusing lenses, a monochromator, and a photomultiplier tube in single photon-counting mode.

4. Results and discussion

Separate experiments were performed with the prism alone and with the supported bilayer present. To test the integrity and uniformity of supported bilayers formed from the liposomes, supported bilayers were prepared on the underside of a 18-mm square glass coverslip, suspended over a microscope slide with a concave well (as described in Ref. [7]), and imaged using epifluorescence microscopy of the Texas red dye–lipid probe: the supported bilayers formed are uniform and the lipid molecules fluid as evidenced by the rapid recovery of the dye–lipid

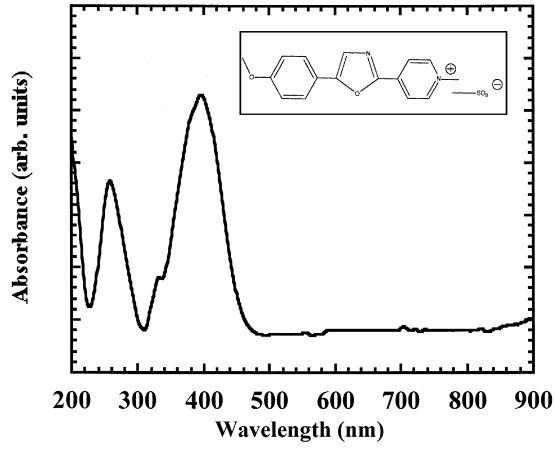

Fig. 2. Absorption spectrum of the oxaxole dye in water at pH 7. The structure of the dye is shown in the inset. The dye is second-harmonic active at pH 7 and photostable.

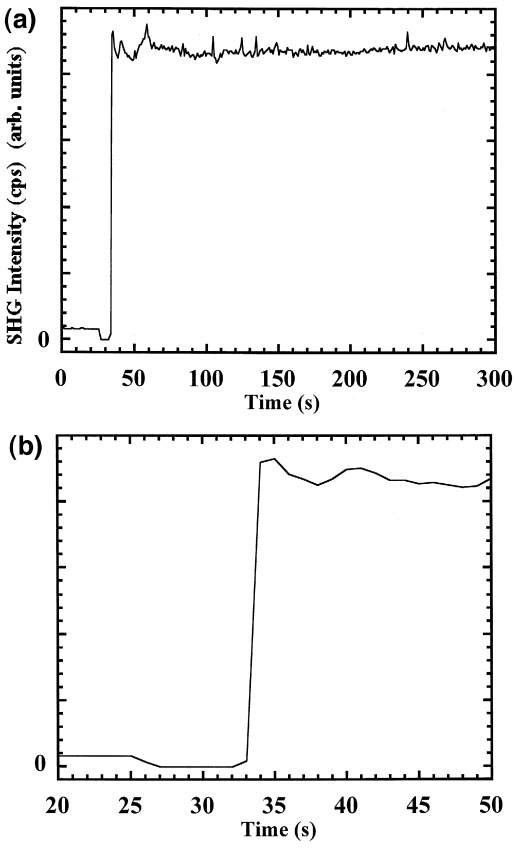

Fig. 3. Kinetics of the 2ω signal following addition (10 μM) of the dye to the well at $t = 33$ s. The increase in the signal is immediate (see inset) and remains stable over a period of at least 5 min. The slight dip in the intensity at $t \cong 30$ s is due to blocking of the beam just before adding the dye.

fluorescence following photobleaching, which occurred within a few minutes. The absorption of the oxaxole dye is shown in Fig. 2 and its molecular structure in the Fig. inset. It is evident from the absorption spectrum that a strong resonant enhancement effect is present at our fundamental wavelengths ($2\omega \cong 400$ nm). Although the fluorescence of the dye, due to two-photon absorption, is easily visible as a greenish-yellow glow, the Dove prism collects only a small portion of that emitted within the evanescent region at the interface (~ 100 nm). In addition, the fluorescence of the dye is spectrally well separated (Stokes shift of ~ 130 nm) from the 2ω signal, which further reduces the amount of detected fluorescence to near-background levels. To the best of our knowledge, this is the first report of the SHG capabilities of this oxaxole dye and suggests further uses of such dyes in biological systems, which often require a near-neutral pH.

The glass–water interface of the Dove prism produces a signal (order of 10^3–10^4 counts per second) due to both the $\chi^{(2)}$ and $\chi^{(3)}$ processes at the interface: the former is due to the intrinsic second-harmonic generating properties of the terminal silica groups of the glass and the water at the silica interface, and the latter is due to the water molecules polarized by the charged silica groups [11]. The signals are strongly enhanced by the use of the prism in the total internal reflection geometry. Upon addition of the probe dye, the signal increases immediately and remains constant during the course of the experiment (Fig. 3). To test whether the dye is capable of crossing the phospholipid bilayer, we measured the SHG signals due to the dye with DOPG and DOPC:DOPG (4:1) liposomes. Upon addition of the dye, the SHG signal increases immediately and remains constant, indicating that the dye molecule does not cross the bilayer but remains adsorbed to the outer surface. This behavior is identical to what we observe with our supported bilayer system and indicates that the dye is adsorbed only to the bilayer and does not cross to the glass surface underneath; the supported bilayer acts as a molecu-

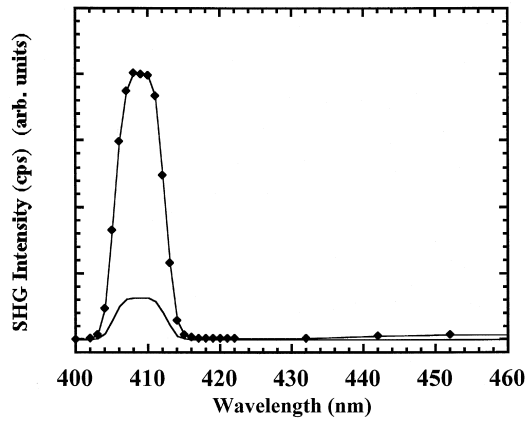

Fig. 4. The intensity (counts per second) of the second harmonic light (2ω) collected as a function of wavelength for both the background (without dye) and with dye (10 μM) (♦) for the glass-supported bilayer system. The peak in the signal occurs at the 2ω wavelength of the fundamental (ω).

larly thin barrier between the silica interface and the bulk solution. A typical spectrum of the second harmonic light, in this case with the supported bilayer present, and with and without the oxaxole dye, is shown in Fig. 4 indicating the characteristic peak at the second harmonic wavelength.

The orientation of the dye molecule at the interface was investigated by inserting an analyzer after the sample and measuring the null angle of the 2ω signal. For the prism without supported bilayer, a null angle Φ_{null} of $111^\circ \pm 2^\circ$ was measured. With the supported bilayer present $\Phi_{\text{null}} = 96^\circ \pm 2^\circ$. The molecular-frame angle can be determined assuming a sharp distribution in the dye orientation. In the presence of supported bilayer, the dye molecules are oriented at $\theta = 19^\circ \pm 2^\circ$ to the surface normal. Without the supported bilayer present, the dye is oriented at $\theta = 33^\circ \pm 2^\circ$ to the surface normal. These calculations were made using indexes of refraction $n_{\text{water}} = 1.3$ and $n_{\text{prism}} = 1.5$ (Melles Griot) and assuming that the index of refraction of the interfacial region is their average, $n' = 1.4$. An uncertainty of $\sim 10\%$ in the values of the indexes of refraction has a negligible effect on the calculated θ angles. The question of whether the dye is partly adsorbed to the glass in the presence of the phospholipid bilayer, or remains adsorbed only to the membrane, is an important one. To address this further we added detergent (Triton X-100) to both dye-glass and dye-glass-membrane samples and monitored the orientation of the dye molecule in time. The effect of the detergent is to solubilize the lipid membrane, removing it from the glass surface. Upon addition of the detergent to the dye-glass system, we saw no change in the orientation as measured by the null angle. However, addition of the detergent to the dye-glass-membrane system had an immediate effect on the orientation which changed over a period of about minutes and reached an equilibrium value of $\Phi_{\text{null}} = 110^\circ \pm 2^\circ$. This is the same value we measure for the dye-glass system and indicates that when the bilayer membrane is present, the dye remains adsorbed only to it and not to the glass surface.

5. Conclusions

We have demonstrated that the SHG technique can be used to probe the orientation of organic molecules (oxaxole dye) adsorbed to a supported phospholipid bilayer interface using polarized SHG. The presence of the dye adsorbed to either the glass or the bilayer was measured and its orientation determined using the null-angle technique. In addition, we demonstrate that the supported bilayer acts as a molecularly thin, impermeable barrier to the dye. The supported bilayer system is growing in use as a biological model membrane for addressing a wide range of issues involving protein–protein recognition, protein function, and others. Our work, therefore, opens the possibility for further applications of SHG to studying the bilayer interface and, with it, fundamental biological questions.

Acknowledgements

We gratefully acknowledge a generous gift of the oxaxole dye from Dr. J.M. Kauffman of the University of the Sciences in Philadelphia and thank E. Macagno of the Department of Biological Sciences, Columbia University for the use of the confocal fluorescence microscope. We also acknowledge the generous support of the Division of Chemical Science, Office of Basic Energy Sciences of the Department of Energy, and the National Science Foundation for their generous equipment support.

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