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Lipids

Mechanistic Studies by Sum-Frequency Generation Spectroscopy: Hydrolysis of a Supported Phospholipid Bilayer by Phospholipase A_2**

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Phospholipase A_2 (PLA_2) is an enzyme that stereoselectively catalyzes the hydrolysis of the *sn*-2 ester linkage of L-phospholipids in biomembranes to produce a fatty acid and a lysophospholipid; importantly, it exhibits no catalytic activity on the enantiomeric D-phospholipids.[1] PLA_2 plays important roles in a variety of the functions in cell membranes, such as signal transduction and lipids metabolism, and has attracted tremendous interest from the pharmaceutical and biotechnology industries.[1,2] The PLA_2-catalyzed hydrolysis reaction consists of a slow interfacial-recognition process followed by a fast cleavage process, the so-called "lag-burst" behavior.[3–5] The catalytic activity of PLA_2 is strongly dependent on the organization of the phospholipids.[6] To understand the catalytic hydrolysis mechanism of PLA_2, it is essential to know the interfacial structures of the lipid membranes at a molecular level. Important advances have been made using a variety of modern analytical techniques, such as π-A isotherms, fluorescence spectroscopy[5] and microscopy,[6,7] grazing incidence X-ray diffraction (GIXD),[6] specular neutron reflectivity,[8] ellipsometry,[8,9] Brewster angle microscopy (BAM),[10] atomic force microscopy (AFM),[11] and infrared and Raman vibrational spectroscopy.[12] However, several essential mechanistic aspects are still not fully understood with regards to how the individual leaflet of a lipid bilayer interacts with PLA_2; for example, it is not clear whether PLA_2 hydrolyzes the two leaflets sequentially or simultaneously. Currently, there are still no reports on how the hydrolysis reaction of the leaflet that is away from the PLA_2 occurs.

Sum-frequency generation (SFG) vibrational spectroscopy is a second-order nonlinear optical technique that is particularly useful in surface science owing to the specific selection rule.[13] SFG has several advantages over conventional vibrational spectroscopic techniques, such as surface selectivity and sub-monolayer sensitivity. Recently, SFG has become a powerful tool in biophysics for the evaluation of the interfacial structure of lipids or proteins and the kinetics of many processes in model cell membranes, such as phase transitions, lipid–protein and lipid–metal interactions.[14–18] Using SFG, Conboy and Liu determined the translocation kinetics of label-free phospholipids during phase transition by monitoring the symmetry change of a supported lipid bilayer.[14] Chen et al. discussed the interaction mechanism of lipid/mellitin and lipid/antibiotic pairs by analyzing the SFG spectral changes of glycerol phospholipid bilayers.[15] Ye et al. reported the structural changes of fatty-acid bilayers that are induced by metal cations, and proposed a method to determine the orientation of individual layers in a multilayer system using interference effects.[17,18]

Herein, we report the first in situ SFG spectroscopy mechanistic study on the hydrolysis reaction of a planar supported-lipid bilayer that is catalyzed by the bee venom enzyme PLA_2; this hydrolysis reaction has been widely studied over the past three decades.[4] Our SFG observations confirm the lag-burst behavior in the hydrolysis process. In the lag-phase, the lipid molecules in both leaflets become more ordered, with a decrease of the tilt angle of the alkyl chain. In the burst-phase, although the apparent decay rates of the SFG signals for both leaflets of an L-lipid bilayer under PLA_2 catalysis seem to be similar, we are able to demonstrate that the hydrolysis starts from the leaflet of the bilayer that is adjacent to the solution by utilizing the enantioselectivity of PLA_2 for the L- and D-enantiomers. A "flip-flop" process is required for the lipid molecules in the other leaflet (substrate side) to be hydrolyzed by PLA_2.

A general introduction to SFG spectroscopy has been described elsewhere.[13] Importantly, the terminal CH_3 group of the lipid molecule can be used as an indicator to probe the structure and the symmetry of a well-defined lipid bilayer.[14–18] According to the selection rule for SFG spectroscopy, signals arising from the CH_3 groups from both leaflets of a symmetric lipid bilayer would cancel each other out. To overcome this problem, all of the lipid bilayers used in this study were prepared by fully deuterating lipid molecules in one of the leaflets.[17,18] This allowed us to monitor the change of lipid molecules in each leaflet of the bilayer simultaneously. However, the SFG signal from the ester group of the DPPC (DPPC = dipalmitoylphosphatidylcholine) in the asymmetric bilayer was still invisible because of the symmetry restrictions of the SFG technique.

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Figures 1a and 1b show the SFG spectra in the a) C–H, and b) C–D stretching regions for a supported L-DPPC (distal)/$[D]_{75}$-L-DPPC (proximal) bilayer (L-DPPC/$[D]_{75}$-L-DPPC) on a CaF_2 substrate; distal and proximal are defined with respect to the substrate. The bilayer was placed in a Ca^{2+}-containing Tris-buffer solution that contained 3.5 μM PLA_2 for different reaction times. All of the peaks are attributed to the CH_3 (CD_3) and CH_2 (CD_2) groups in the alkyl chains of DPPC molecules within the bilayer. The peak assignments have been reported elsewhere,[17,18] and are shown next to their associated peaks (Figure 1a and 1b). Importantly, the bilayer is comprised of an equal amount of L-DPPC and $[D]_{75}$-L-DPPC molecules for each leaflet, although the SFG spectral intensities in the C–D stretching region appear to be weaker than those in the C–H stretching region.[19] In order to inspect the spectral changes in detail, the SFG peak intensities were plotted on a logarithmic scale as a function of the reaction time for the r^+ and r^- modes of the distal (black) and proximal (red) leaflets (Figure 1c).

Before PLA_2 was introduced into the cell ($t = 0$ min), the peaks in both the C–H and C–D stretching regions decreased slowly with time (the half-life is estimated as (500 ± 5) min based on first-order kinetics[14]). This process can be ascribed to the flip-flop of the lipid molecules between the two leaflets, which can increase the symmetry of the initially asymmetric bilayer, and thereby decrease the SFG signal.[14] Once PLA_2 was introduced into the solution, the SFG peaks decreased quickly with time. Figure 1c appears to show a two-stage hydrolysis process. During the first 10 minutes after PLA_2 introduction, the r^- mode showed a significant change in the rate at which its intensity decreased, whilst the rate of the r^+ mode remained relatively constant. After that, the intensities of the two modes decreased rapidly in a similar fashion for both leaflets and finally disappeared after approximately 30 minutes (Figure 1a–b). Comparable SFG spectral changes and time profiles were observed for the lipid bilayer that was prepared in a reversed sequence, i.e. $[D]_{75}$-L-DPPC/L-DPPC (results not shown). The two-stage change of the SFG signals should be related to the lag-burst processes, as has been reported previously.[4,5]

In the lag-stage, the *gauche* defects, which are directly associated with the SFG intensities of the CH_2 (or CD_2) groups in the bilayer,[14,17] decreased quickly after addition of PLA_2 (see the Supplementary Information, Figure S1). Furthermore, the tilt angle of the hydrocarbon chains of DPPC, which is related to the relative intensities of r^+ and r^- modes, decreased from approximately 20° to being almost perpendicular to the surface at the end of the lag-stage (see the Supplementary Information). It should be noted that no additional *gauche* defects appeared and the tilt angle remained almost constant during the subsequent burst-stage. These results suggest that the DPPC molecules in the bilayer became more highly ordered during the lag-stage and remained constant during the burst-stage. These observations are consistent with those obtained by GIXD,[6] fluorescence microscopy,[7] BAM,[10] and AFM observations.[11] However, we found that the initial structural changes took place not only in the leaflet of the bilayer that is in contact with the solution, but also within the leaflet that is adjacent to the substrate. We considered two mechanistic possibilities for the structural changes during the lag-stage. Firstly, as reported by Dahmen-Levison et al., the structural changes could be

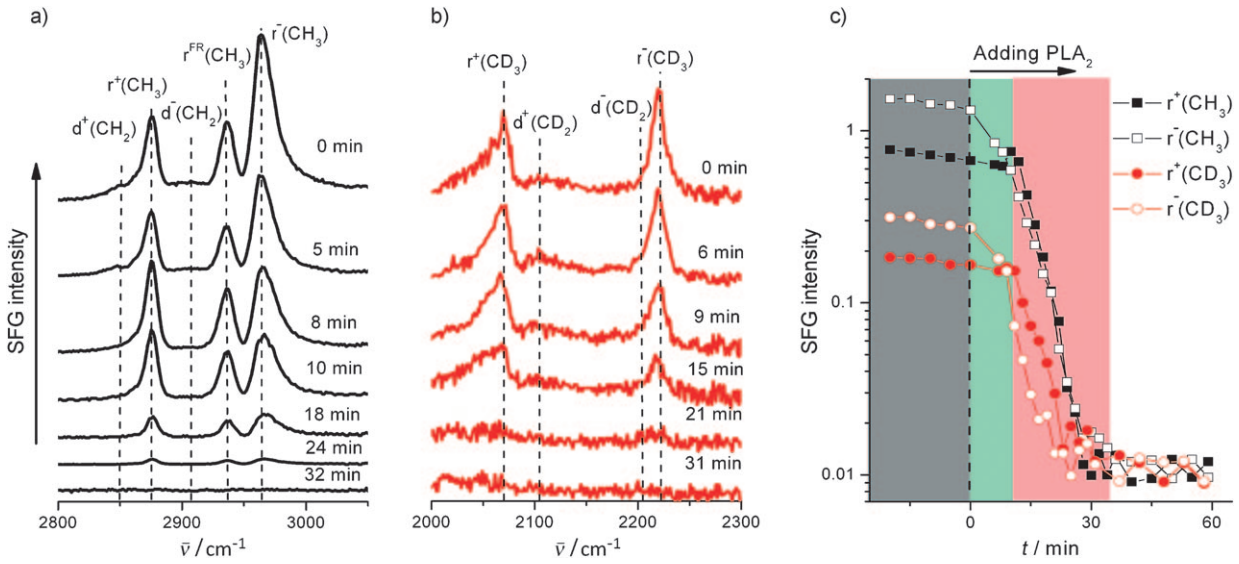

Figure 1. SFG spectra: a) C–H (black traces) and b) C–D (red traces) stretching regions of an L-DPPC (distal)/ $[D]_{75}$-L-DPPC (proximal)-supported bilayer in Tris-buffer solution with 3.5 μM PLA_2 for different reaction times. $r^+(CH_3$ or $CD_3)$, $r^{FR}(CH_3$ or $CD_3)$ and $r^-(CH_3$ or $CD_3)$ denote the C–H or C–D symmetric, Fermi resonance, and asymmetric stretching modes of the terminal methyl groups, respectively; $d^+(CH_2$ or $CD_2)$ and $d^-(CH_2$ or $CD_2)$ denote the symmetric and asymmetric C–H or C–D stretching modes of the methylene groups of the lipids molecules, respectively. The spectra are offset for clarity. c) SFG intensities of r^+ (solid) and r^- modes (empty) for the CH_3 (square) and CD_3 (circle) groups are shown as a function of reaction time. The gray, green, and red shadows indicate the periods before adding PLA_2, the lag-phase, and the burst phase of the hydrolysis reaction. The lines joining consecutive data points are shown for clarity. All the results were obtained using a *ppp* (i.e. *p*-polarized SFG, *p*-visible and *p*-IR) polarization combination.

ascribed to the adsorption of PLA_2 molecules onto the lipid monolayer.[6] The interaction between PLA_2 and the lipid monolayer could significantly increase the lipid packing density and reduce the tilt angle of the lipid alkyl chains. Conversely, the hydrolysis products, palmitic acid (PA), and lysophospholipid (lysoPC) could also improve the ordering and packing efficiency of the hydrocarbon chain of DPPC, as reported previously.[8,11] Recently, using an SFG study, Ma and Allen demonstrated that a small fraction of PA molecules in the DPPC/PA binary monolayer at the air/water interface exhibited a significant condensation effect on the alkyl chains of the DPPC molecules.[16d] A similar effect has also been observed using IR spectroscopy.[20] As no PLA_2 enzyme was included in solution for the above two studies, these results support the latter possibility described above.

The SFG intensities of the terminal CH_3 and CD_3 groups in both leaflets quickly decreased in the burst-stage (Figure 1c). The decay rates were much faster than those observed for the flip-flop and lag phases. As the DPPC and its hydrolysis products (PA and LysoPC) gave similar SFG peaks for the CH_3 and/or CD_3 groups, such a large decay of the SFG signals is not expected to occur if all of the products remain in the bilayer. The decay should be ascribed to the partial desorption of the hydrolysis products from the DPPC bilayer, as previously observed by AFM[11] and IR measurements.[12d] However, we were still unable to determine the amounts and fraction of the desorbed products, owing to the limitation of SFG measurements, as described above.

There appears to be a linear relationship between the rapidly decreasing SFG signal and the reaction time in the burst stage (Figure 1c), which suggests that the reaction can be analyzed using pseudo first-order reaction kinetics. Consequently, the gradient of the line can be used to quantitatively evaluate the reaction kinetics. The decay rates of the SFG signals for the two leaflets are comparable to each other (Figure 1c). Conversely, it is expected that PLA_2 would interact with the DPPC molecules in the distal leaflet, and therefore the hydrolysis rates for the two leaflets should be different. To understand the reaction mechanism of each leaflet in the bilayer, further SFG characterizations were carried out that employed the enantioselectivity of PLA_2 between L- and D-phospholipid enantiomers.

Figure 2 summarizes the SFG intensities of the r^+ mode for CH_3 and CD_3 groups as a function of the reaction time for two bilayer systems, D-DPPC/$[D]_{75}$-L-DPPC and $[D]_{75}$-L-DPPC/D-DPPC, with 0.7 μM PLA_2. The straight lines are shown as a guide. In the bilayer system, D-DPPC, which is PLA_2-inactive, was deposited on the distal or proximal layer instead of its L-enantiomer. For clarity, the peak intensities are normalized to the SFG intensity at $t=0$ for each bilayer. For reference, the pink triangles show a time profile for a D-DPPC/$[D]_{75}$-L-DPPC bilayer in a solution without PLA_2, which is mainly attributed to the flip-flop process as mentioned above (similar results were obtained with a $[D]_{75}$-L-DPPC/D-DPPC or $[D]_{75}$-L-DPPC/L-DPPC bilayer).[14] As indicated by the green area (Figure 2), the lag-phase was also observed for the two bilayers after PLA_2 introduction. In the case of the D-DPPC/$[D]_{75}$-L-DPPC, both the proximal (D-DPPC, blue squares) and distal leaflets ($[D]_{75}$-L-DPPC, black

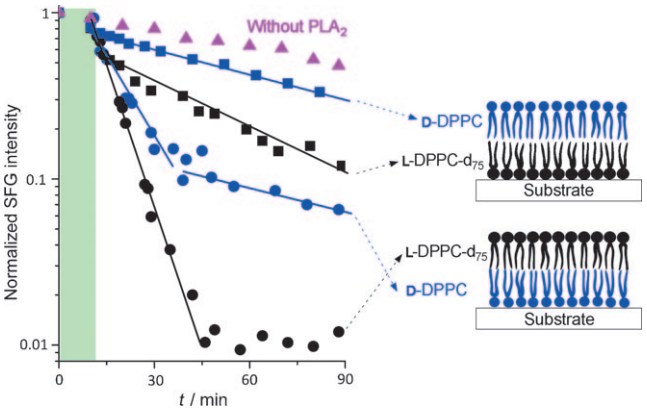

Figure 2. Left: the normalized SFG intensities for the r^+ mode of CH_3 (black) or CD_3 (blue) groups in the two bilayer systems of D-DPPC/$[D]_{75}$-L-DPPC (square) and $[D]_{75}$-L-DPPC/D-DPPC (circular) as a function of reaction time. As a reference, a time profile (purple) was obtained for a D-DPPC/$[D]_{75}$-L-DPPC bilayer in a solution without PLA_2. The green region indicates the lag-phase of the hydrolysis reaction. The straight lines are shown as a guide. Right: the schematic model for the two bilayer systems of D-DPPC/$[D]_{75}$-L-DPPC (top) and $[D]_{75}$-L-DPPC/D-DPPC (bottom). The SFG intensities were normalized to the SFG intensity at $t=0$ for clarity.

squares) decay slowly during the burst-phase, at a significantly slower rate than that observed for the L-enantiomer in L-DPPC/$[D]_{75}$-L-DPPC bilayer (Figure 1c) and $[D]_{75}$-L-DPPC/D-DPPC bilayer (black circle, Figure 2, see below). However, the decay is comparable to the flip-flop process for the same bilayer in the solution without PLA_2 (pink triangle, Figure 2). This result indicates that the catalytically inactive D-DPPC in the distal leaflet has dramatically blocked the hydrolysis reaction of the L-DPPC in the proximal leaflet. This clearly demonstrates that the hydrolysis reaction catalyzed by PLA_2 starts from the distal leaflet. In order to be hydrolyzed by PLA_2, L-DPPC lipids in the proximal leaflet have to flip-flop to the distal leaflet. Consequently, the reaction kinetics is controlled by the slow flip-flop process.

The "reverse" bilayer, $[D]_{75}$-L-DPPC/D-DPPC, exhibits a significantly different behavior. The $[D]_{75}$-L-DPPC distal leaflet (black circle) in the $[D]_{75}$-L-DPPC/D-DPPC bilayer decayed rapidly, and the SFG signals became indistinguishable after circa 30 minutes (Figure 2). This behavior is similar to that for the L-DPPC/$[D]_{75}$-L-DPPC bilayer (Figure 1), but the D-DPPC proximal leaflet (blue circle, Figure 2) in the $[D]_{75}$-L-DPPC/D-DPPC bilayer shows a more complex behavior. In the first 30 minutes the SFG signal decreases with time much faster than the usual flip-flop rate (pink triangles, Figure 2). Nevertheless, as soon as the hydrolysis of the L-enantiomeric distal leaflet was completed, the decay rate slowed significantly. As PLA_2 has no catalytic activity towards D-DPPC, the decrease in the SFG signals from D-DPPC in the $[D]_{75}$-L-DPPC/D-DPPC bilayer, which is observed during the burst phase (Figure 2), should be ascribed to organizational or structural changes that are induced by the hydrolysis of L-DPPC in the distal leaflet.

However, as soon as L-DPPC in the $[D]_{75}$-L-DPPC/D-DPPC is fully hydrolyzed, a flip-flop movement occurs

between the lipids or products in the distal and proximal leaflets, which again is a slow process. Although it is currently impossible to extract the SFG signals of the hydrolysis products from the SFG spectra, the presence of SFG signals from D-DPPC suggests that a certain asymmetry is still retained after complete hydrolysis of the distal L-enantiomeric lipids.

In the symmetric bilayer system containing only L-DDPC, hydrolysis of the distal leaflet caused the molecules in the proximal leaflet to flip-flop to the distal leaflet and be immediately hydrolyzed by PLA_2; this led to the observation that the two leaflets were apparently hydrolyzed simultaneously (Figure 1).

In conclusion, SFG spectroscopy has been applied to investigate the hydrolysis of a planar supported-DPPC bilayer that is catalyzed by bee venom enzyme PLA_2. Our observations have provided essential information at a molecular level about the structural changes and mechanism during the hydrolysis reaction. An ordered arrangement of the lipids in both leaflets seems to be required during the entire hydrolysis process. The L-enantiomer lipid bilayer apparently showed similar reaction behavior for each leaflet. However, by selectively hydrolyzing one enantiomer within the bilayer, we found that the hydrolysis starts from the distal leaflet. The SFG signals for the PLA_2 amide group, which were adsorbed onto the D-DPPC/D-DPPC bilayer, were very weak. This is probably attributed to disordered or random orientations of PLA_2 on the bilayer surface. Nevertheless, as a typical model for the cell membrane, these results on the supported bilayer are essential to understand the hydrolysis reaction on the real cell membrane catalyzed by PLA_2. This insight into hydrolysis mechanism will help understand the roles of this enzyme in cellular processes and will contribute to our knowledge of protein-lipid interaction and protein engineering.

Experimental Section

PLA_2 from bee venom[4] (Type III, 14 kDa) was purchased from Cayman Chemical. L- and D-dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma–Aldrich. To monitor the structural changes of each leaflet in lipid bilayers that are SFG inactive, an "asymmetric" bilayer[17,18] consisting of a per-deuterated $[D]_{75}$-L-DPPC (Avanti Polar Lipid) and a per-protonated DPPC were prepared. The proximal and distal leaflets of DPPC bilayer were deposited successively onto a flat CaF_2 hemicylindrical prism surface, at a surface pressure of 30 mN m^{-1} by vertical dipping and horizontal lifting methods in a Langmuir-Blodgett (LB) trough.[17,18] The surface of the CaF_2 substrate was pre-coated with a SiO_2 thin-film using a sol-gel method and cleaned by ozone before LB deposition. We are able to prepare a highly packed bilayer in the gel phase with an area of 0.44 nm^2 per DPPC. As reported previously, the lipid bilayer can be hydrolyzed by the bee venom PLA_2 under similar conditions.[11a,d] The supported bilayer was set to a flow-cell with a Tris-buffer solution (pH 8.9, 10 mM Tris, 100 mM NaCl) with 5 mM $CaCl_2$ and then analyzed by SFG.

The details of our broad-band SFG system are described elsewhere.[17,18] It consisted of a titanium:sapphire oscillator (MaiTai) and a regenerative amplifier (SpiteFire PRO), pumped by a Nd:YLF laser (EMPower) that generated a 2.2 mJ laser pulse at 800 nm with a 120 fs duration and a repetition rate of 1 kHz. Half of the output was used to pump an optical parametric amplifier system (TOPAS) to generate IR pulses that are tunable between 2.5 and 10 μm with a spectral width of approximately 300 cm^{-1}. The remaining output from the amplifier was sent to a home-made spectral shaper to generate a narrow-band pulse (circa 10 cm^{-1}) at 800 nm for improving the spectral resolution. A tunable visible pulse output from the UV to near IR region (250–1000 nm) with a typical spectral width of approximately 20 cm^{-1} was also possible using a narrow-band OPA (TOPAS white-NB). In this experiment, the visible pulse was fixed at 800 nm whilst the central wavelength of the IR pulse was fixed at 3.5 μm for the C–H stretching region (2800–3050 cm^{-1}) for L/D-DPPC and at 4.5 μm for the C–D stretching region (2000–2300 cm^{-1}) for $[D]_{75}$-L-DPPC. All SFG spectra were recorded with polarization combination of *p*-SFG, *p*-visible and *p*-IR and normalized by an SFG spectrum from a gold thin-film that was evaporated on the flat surface of the CaF_2 prism. One SFG spectrum was acquired over 1 minute, which allowed enough time resolution to follow the hydrolysis process. All experiments were carried out at room temperature (23 °C).

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[1] D. E. Vance, J. E. Vance, *Biochemistry of Lipids, Lipoproteins and Membranes*, Elsevier, Amsterdam, **2008**.
[2] a) D. L. Scott, S. P. White, Z. Otwinowski, W. Yuan, M. H. Gelb, P. B. Sigler, *Science* **1990**, *250*, 1541; b) J. E. Burke, E. A. Dennis, *J. Lipid Res.* **2009**, *50*, S237; c) K. Wagner, G. Brezesinski, *Curr. Opin. Colloid Interface Sci.* **2008**, *13*, 47.
[3] a) L. M. Van Deenen, G. H. De Haas, *Biochim. Biophys. Acta* **1963**, *70*, 538; b) M. K. Jain, M. R. Egmond, H. M. Verheij, R. Apitz-Castro, R. Dijkman, G. H. De Haas, *Biochim. Biophys. Acta Biomembr.* **1982**, *688*, 341.
[4] a) H. M. Verheij, A. J. Slotboom, G. H. De Haas, *Rev. Physiol. Biochem. Pharmacol.* **1981**, *91*, 91; b) G. C. Upreti, M. K. Jain, *J. Membr. Biol.* **1980**, *55*, 113; c) B. Z. Yu, F. Ghomashchi, Y. Cajal, R. R. Annand, O. G. Berg, M. H. Gelb, M. K. Jain, *Biochemistry* **1997**, *36*, 3870; d) F. Ghomashchi, Y. Lin, M. S. Hixon, B. Z. Yu, R. Annand, M. K. Jain, M. H. Gelb, *Biochemistry* **1998**, *37*, 6697; e) O. G. Berg, M. H. Gelb, M.-D. Tsai, M. K. Jain, *Chem. Rev.* **2001**, *101*, 2613.
[5] W. R. Burack, Q. Yuan, R. L. Biltonen, *Biochemistry* **1993**, *32*, 583.
[6] U. Dahmen-Levison, G. Brezesinski, H. Mohwald, *Thin Solid Films* **1998**, *327–329*, 616.
[7] a) D. W. Grainger, A. Reichert, H. Ringsdorf, C. Salesse, *FEBS Lett.* **1989**, *252*, 73; b) D. W. Grainger, A. Reichert, H. Ringsdorf, C. Salesse, *Biochim. Biophys. Acta Biomembr.* **1990**, *1023*, 365; c) K. M. Maloney, M. Grandbois, D. W. Grainger, C. Salesse, K. A. Lewis, M. F. Roberts, *Biochim. Biophys. Acta Biomembr.* **1995**, *1235*, 395; d) K. M. Maloney, D. W. Grainger, *Chem. Phys. Lipids* **1993**, *65*, 31.
[8] a) H. P. Vacklin, F. Tiberg, G. Fragneto, R. K. Thomas, *Biochemistry* **2005**, *44*, 2811; b) H. P. Wacklin, F. Tiberg, G. Fragneto, R. K. Thomas, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 1036.
[9] H. Speijer, P. L. Giesen, R. F. Zwaal, C. E. Hack, W. T. Hermens, *Biophys. J.* **1996**, *70*, 2239.
[10] a) J. Li, Z. Chen, X. Wang, G. Brezesinski, H. Mohwald, *Angew. Chem.* **2000**, *112*, 3187; *Angew. Chem. Int. Ed.* **2000**, *39*, 3059; b) X. H. Zhai, J. B. Li, G. Brezesinski, Q. He, H. Mohwald, L. H. Lai, Y. Liu, L. Liu, Y. Gao, *ChemBioChem* **2003**, *4*, 299.
[11] a) M. Grandbois, H. Clausen-Schaumann, H. Gaub, *Biophys. J.* **1998**, *74*, 2398; b) L. K. Nielsen, J. Risbo, T. H. Callisen, T.

Bjornholm, *Biochim. Biophys. Acta Biomembr.* **1999**, *1420*, 266; c) L. K. Nielsen, K. Balashev, T. H. Callisen, T. Bjornholm, *Biophys. J.* **2002**, *83*, 2617; d) P. Moraille, A. Badia, *J. Am. Chem. Soc.* **2005**, *127*, 6546; e) K. Balashev, N. J. DiNardo, T. H. Callisen, A. Svendsen, T. Bjornholm, *Biochim. Biophys. Acta Biomembr.* **2007**, *1768*, 90.

[12] a) A. Gericke, H. Huhnerfuss, *Chem. Phys. Lipids* **1994**, *74*, 205; b) M. Grandbois, B. Desbat, C. Salesse, *Biophys. Chem.* **2000**, *88*, 127; c) U. Dahmen-Levison, G. Brezesinski, H. Mohwald, J. Jakob, P. Nuhn, *Angew. Chem.* **2000**, *112*, 2889; *Angew. Chem. Int. Ed.* **2000**, *39*, 2775; d) S. A. Tatulian, *Biophys. J.* **2001**, *80*, 789; e) S. A. Tatulian, *Biochemistry* **2003**, *42*, 11898; f) X. L. Wang, S. P. Zheng, Q. He, G. Brezesinski, H. Mohwald, J. B. Li, *Langmuir* **2005**, *21*, 1051; g) J. Boucher, E. Trudel, M. Methot, P. Desmeules, C. Salesse, *Colloids Surf. B* **2007**, *58*, 73; h) D. P. Cherney, G. A. Myers, R. A. Horton, J. M. Harris, *Anal. Chem.* **2006**, *78*, 6928.

[13] a) Y. R. Shen, *The Principles of Nonlinear Optics*, Wiley, New York, **1984**; b) C. D. Bain, *J. Chem. Soc. Faraday Trans.* **1995**, *91*, 1281; c) G. L. Richmond, *Chem. Rev.* **2002**, *102*, 2693; d) Z. Chen, Y. R. Shen, G. A. Somorjai, *Annu. Rev. Phys. Chem.* **2002**, *53*, 437; e) S. Ye, M. Osawa, *Chem. Lett.* **2009**, *38*, 386.

[14] a) J. Liu, J. C. Conboy, *J. Am. Chem. Soc.* **2004**, *126*, 8376; b) J. Liu, J. C. Conboy, *J. Am. Chem. Soc.* **2004**, *126*, 8894; c) J. Liu, J. Conboy, *Biophys. J.* **2005**, *89*, 2522.

[15] a) X. Chen, H. Tang, M. A. Even, J. Wang, G. N. Tew, Z. Chen, *J. Am. Chem. Soc.* **2006**, *128*, 2711; b) X. Chen, J. Wang, A. P. Boughton, C. B. Kristalyn, Z. Chen, *J. Am. Chem. Soc.* **2007**, *129*, 1420.

[16] a) S. Roke, W. Roeterdink, J. Wijnhoven, A. Petukhov, A. Kleyn, M. Bonn, *Phys. Rev. Lett.* **2003**, *91*, 258302; b) X. Chen, L. B. Sagle, P. S. Cremer, *J. Am. Chem. Soc.* **2007**, *129*, 15104; c) E. Tyrode, M. W. Rutland, C. D. Bain, *J. Am. Chem. Soc.* **2008**, *130*, 17434; d) G. Ma, H. C. Allen, *Photochem. Photobiol.* **2006**, *82*, 1517.

[17] a) S. Ye, H. Noda, S. Morita, K. Uosaki, M. Osawa, *Langmuir* **2003**, *19*, 2238; b) S. Ye, H. Noda, T. Nishida, S. Morita, M. Osawa, *Langmuir* **2004**, *20*, 357.

[18] a) T. Nishida, M. Johnson, J. Holman, M. Osawa, P. B. Davies, S. Ye, *Phys. Rev. Lett.* **2006**, *96*, 077402; b) J. Holman, P. B. Davies, T. Nishida, S. Ye, D. J. Neivandt, *J. Phys. Chem. B* **2005**, *109*, 18723.

[19] We have optimized the alignment of our laser system in the C–H stretching region, which leads to a weaker SFG signals in the C–D stretching frequency region because of less overlap of the visible and IR beams.

[20] T. Inoue, S. Yanagihara, Y. Misono, M. Suzuki, *Chem. Phys. Lipids* **2001**, *109*, 117.

