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**Second harmonic generation to monitor the interactions of the antimicrobial
mycosubtilin with membrane-mimicking interfacial monolayers**

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Abstract

Mycosubtilin is a strong antimicrobial agent belonging to the iturinic lipopeptide family which contains a single tyrosine residue. Its cell target has been shown to be the cytoplasmic membrane. This tyrosine residue has been previously shown to be essential for the biological activity of mycosubtilin. Since we have previously demonstrated that tyrosine, an aromatic amino acid, can be used as an endogenous probe for the frequency doubling process, the presence of a tyrosine residue in mycosubtilin allowed us to investigate the interactions of mycosubtilin with biomimetic lipid monolayers at the air-water interface by Second Harmonic Generation (SHG). Mycosubtilin was added underneath dipalmitoylphosphatidylcholine (DPPC) or cholesterol monolayers at the air-water interface and significant increases in the surface pressure were observed in both cases. This observation demonstrates that mycosubtilin interacts with these biomimetic membranes. A light polarization resolved analysis of the SHG signals recovered for these two systems was then performed and confirmed that those interactions between the tyrosine residue in mycosubtilin and the membranes could be monitored by SHG. Furthermore, the differences exhibited by the nonlinear optical measurements for different membranes showed that these interactions depend on the nature of the biomimetic membrane present at the air-water interface.

Key words. mycosubtilin, biomimetic membranes, second harmonic generation, iturin, antimicrobial peptide

1. Introduction

Second Harmonic Generation (SHG) is the nonlinear optical process whereby two photons at a fundamental frequency are converted into one photon at the harmonic frequency. SHG has been shown to be an ideal tool to investigate molecular organization at the nanometer scale owing to its cancellation in centrosymmetric media like liquids within the electric dipole approximation. Hence, SHG has been applied in the past for the characterization of many different systems at liquid interfaces [1, 2]. Besides, recent studies aiming at extending its use to proteins and peptides in the absence of exogenous molecular probes have been recently performed. In particular, they demonstrated that aromatic amino acids like tryptophan (Trp) and tyrosine (Tyr) can be used as endogenous probes of proteins and peptides. The first hyperpolarizability of Trp and Tyr, namely their cross-section for the SHG process, and that of a short tripeptide containing two lysines and one Trp, were indeed determined by Hyper Rayleigh Scattering (HRS), an incoherent SHG process, in the bulk of an aqueous phase [3]. These measurements therefore showed that it is possible to devise experiments targeting Trp and Tyr residues in short synthetic peptides using SHG at the air-water interface [4, 5]. These results confirmed the previous works reported for Trp derivative at interfaces and extended them to the case of Tyr [6, 7]. As a result, through a detailed analysis of the nonlinear optical response, in particular when selecting the polarization state of the incoming and outgoing fields, a molecular picture of the interface can be retrieved, a powerful tool to investigate the interaction of Trp- and Tyr-rich peptides with phospholipids [2, 8].

In the present work, we perform SHG experiments probing at the air-water interface the presence of a biologically-active peptide containing a single Tyr residue. Indeed, mycosubtilin is constituted by the heptapeptide L-Asn→D-Tyr→D-Asn→L-Gln→L-Pro→D-Ser→L-Asn associated with a β -amino fatty acid residue [9]. Mycosubtilin is a strong antimicrobial cyclolipopeptide produced by *Bacillus subtilis* strains and involved in the biological control of plant diseases [10-11]. Its cell target has been shown to be the cytoplasmic membrane [12]. To model the interaction between the lipopeptide and the biological membranes, its interfacial properties and its adsorption to various lipid monolayers at the air-water interface have been extensively studied and it has been shown that cholesterol is a preferential partner of mycosubtilin when it interacts with biomimetic membranes [13-15]. We therefore investigated the

adsorption of mycosubtilin to interfacial monolayers constituted by DPPC or cholesterol by simultaneously measuring the SHG signal and the surface pressure by standard tensiometry.

2. Materials and methods

2.1. Chemicals.

Cholesterol (Chol) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), purchased from Sigma Chemical (St. Louis, MO), were dissolved in hexane-ethanol (9/1, v/v). Mycosubtilin, prepared as described previously [16], was dissolved in dimethylsulfoxide.

2.2. Film formation and surface pressure measurements.

Adsorption experiments were performed at 18°C using a small Teflon dish (4 cm diameter, 12 mL volume) and a pure water subphase (resistivity of 18.2 MΩ•cm). This low temperature was required for the optical experiments to prevent evaporation during the course of the measurements. The lipid monolayers were prepared by deposition of the DPPC or Chol solutions at the air-water interface, thereby setting an initial surface pressure Π_i of 10 mN/m. After stabilization of the lipid monolayer, mycosubtilin was injected into the subphase at a final concentration of 1.1 μM. To avoid fluctuations in the SHG optical measurements, the subphase was stirred only during the first minutes after the lipopeptide injection.

2.3. Second Harmonic Generation measurements

The SHG setup was based on a femtosecond Ti-sapphire oscillator laser source providing pulses with duration of about 70 fs at a repetition rate of 80 MHz (Spectra-Physics) [17, 18]. After passing through a low-pass filter to remove any unwanted harmonic light generated prior to the interface, the fundamental beam set to a wavelength of 800 nm and an averaged power of about 1 W was focused by a lens with a 10 cm focal length onto the air-water interface. The incidence angle was set at a value of 70° corresponding to an optimum incidence angle for the SHG intensity in reflection. The SH light was collected by a lens with a 10 cm focal length and separated from its fundamental counterpart by a high-pass filter. The SH light was detected with a water-cooled back-illuminated CCD camera (Andor) placed after a spectrometer (Jobin-Yvon). The fundamental input beam was linearly polarized and the input polarization angle γ was selected

with a rotating half-wave plate. The angle $\gamma = 0$ corresponds to a p-polarized fundamental beam and $\gamma = \pi/2$ to an s-polarized fundamental beam. An analyzer, placed in front of the spectrometer, was used to separate the S- and P-polarized SH intensities. In all the kinetic experiments, we used a p-in and P-out polarization configuration.

3. Results and discussion

3.1. Interaction of mycosubtilin with the DPPC monolayer

The interactions between mycosubtilin and the phospholipids were analyzed using DPPC as a model phospholipid. An initial surface pressure Π_i of 10 mN/m was chosen for the DPPC monolayer since it has been previously shown that mycosubtilin induces a significant increase in the surface pressure $\Delta\Pi$ for this initial pressure Π_i [14]. Before the mycosubtilin injection, the SHG signal of the DPPC monolayer at 10 mN/m was about one and a half times higher than that obtained for the neat air-water interface. After the mycosubtilin injection underneath the DPPC monolayer, the adsorption kinetics of the lipopeptide to the phospholipid monolayer was followed through the simultaneous measurements of the SHG signal and the surface pressure. The kinetics of the mycosubtilin-induced changes in the SHG signal and the surface pressure are shown in Fig. 1. The surface pressure increases gradually and then reaches a plateau corresponding to a surface pressure change $\Delta\Pi$ of about 15 mN/m. This indicates that mycosubtilin adsorbs to the interface. The SHG signal increased faster than the surface pressure until a plateau was also reached. To get further information on the molecular organization and/or orientation of the species present at the air-water interface, SHG light polarization curves were recorded for the DPPC monolayer in the absence and in the presence of mycosubtilin, see Fig. 2. The graph obtained for the pure DPPC monolayer at 10 mN/m, see Fig. 2A, is very similar to that obtained for the neat air-water interface (data not shown). The P-polarized curve has two maxima while the S-polarized one has four maxima. The maximum intensity of the P-polarized curve is about three times higher than that measured for the S-polarized one. The 45°-polarized curve has two maxima with intermediate intensity. The latter curve is required besides the P- and S-polarized ones to lift a phase ambiguity on the susceptibility tensor elements, see below. Light polarization curves of the DPPC monolayer were also recorded after complete adsorption of the lipopeptide (*i.e.* when the surface pressure reaches the plateau). The P-polarized curve still exhibits two maxima, as it was

observed with the neat air-water interface and the pure DPPC monolayer. However, the presence of mycosubtilin in the DPPC monolayer decreases significantly the intensity of the maxima of the S-polarized curve, see Fig. 2B. Thus, the ratio of the intensity maxima of the P- and S- polarized curves reaches about 10 while it was only about 3.5 in the case of pure DPPC monolayer. As a consequence, a smaller shift of the 45°-polarized curve is observed as compared to the shift in the absence of mycosubtilin. These data therefore confirm that the interaction of mycosubtilin with the phospholipid membrane is indeed monitored. In order to quantify this interaction, the P-polarized and S-polarized plots can be analyzed using the standard form of the SH intensity in the electric dipole approximation as a function of the input polarization angle γ [17]:

$$I_s(\gamma) \propto |a_1 \chi_{xxz} \sin(2\gamma)|^2 \quad (1a)$$

$$I_p(\gamma) \propto |(a_2 \chi_{xxz} + a_3 \chi_{zxx} + a_4 \chi_{zzz}) \cos^2 \gamma + a_5 \chi_{zxx} \sin^2 \gamma|^2 \quad (1b)$$

where the a_i ($i=1-5$) are five constants depending on the geometrical configuration and the optical indices of water and air at 800 nm and 400 nm. The quantities χ_{zzz} , χ_{zxx} and χ_{xxz} are the three non vanishing and independent quadratic susceptibility tensor components associated with an isotropic and achiral interface in the electric dipole approximation. Using Eqs.(1) to fit the experimental data, we obtain the values for the ratios χ_{zxx} / χ_{zzz} and χ_{xxz} / χ_{zzz} . These values are given in Table 1. For the pure DPPC monolayer, the results are similar to those obtained in the case of a neat air/water interface, as reported previously [19]. These ratios however dramatically drop in the presence of mycosubtilin demonstrating the occurrence of interactions between the peptide and the lipid membrane. The signature of this interaction is a reinforcement of the out-of-plane contribution, namely the χ_{zzz} element, of the non linear optical response.

3.2. Interaction of mycosubtilin with cholesterol monolayer

The interactions between mycosubtilin and sterols were analyzed using cholesterol as a model sterol. In a first step, we measured the SHG signal of a pure Chol monolayer at a surface pressure of 10 mN/m. The SHG intensity was about three times higher than the SHG signal of the neat air-water interface and about

two times higher than that of a pure DPPC monolayer at a surface pressure of 10 mN/m. This can be related to the high packing of the Chol monolayer. After mycosubtilin injection, we simultaneously measured the SHG signal and the surface pressure, see Fig. 3. A weak increase of the SHG intensity was observed immediately after the lipopeptide injection, while the surface pressure increase occurred later. This latter increase was also more significant, with a value $\Delta\Pi$ of about 35 mN/m. Such a time difference between the SHG increase and the surface pressure was also observed above with the DPPC monolayer. Furthermore, while the mycosubtilin-induced increase of the Chol monolayer SHG signal was weak, $\Delta\text{SHG} \sim 0.7$ a.u., as compared to its initial value of ~ 3 a.u., this increase is similar to that observed with the DPPC monolayer, $\Delta\text{SHG} \sim 0.6$ a.u.

The light polarization curves of the Chol monolayer in the absence and in the presence of mycosubtilin were then recorded, see Fig. 4. Without taking into account the different intensities, the light polarization curves of the pure Chol monolayer, see Fig. 4A, were very similar to those of the neat air-water interface (data not shown). The light polarization curves of the mixed Chol-mycosubtilin monolayer were then recorded after complete adsorption of the lipopeptide, see Fig. 4B. These curves are radically different from those of the pure Chol monolayer. This noteworthy difference between the light polarization curves of the Chol-mycosubtilin and pure Chol monolayers is observed in the practically complete disappearance of the S-polarized curve and the appearance of two weak peaks in the P-polarized curve at the fundamental polarization angles 90° and 270° .

To test whether these changes are the signature of the interaction of the lipopeptide with cholesterol, mycosubtilin was injected under the Chol monolayer at surface pressures of 3 mN/m and 30 mN/m. Light polarization curves of the Chol-mycosubtilin monolayers were then recorded after complete adsorption of the lipopeptide, *i.e.* when the surface pressure change $\Delta\Pi$ was stabilized at ~ 25 mN/m for the surface pressure Π_i of 3 mN/m or stabilized at ~ 10 mN/m for the surface pressure Π_i of 30 mN/m. In both cases, the P-polarized curves exhibited the two weak peaks at the fundamental polarization angles 90° and 270° besides the two maxima obtained at 0 and 180° (data not shown). In both cases, the complete disappearance of the S-polarized curve was observed. Using Eqs. (1), the elements of the susceptibility tensor were

determined in the case of a pure cholesterol monolayer and a mixed mycosubtilin-cholesterol monolayer, see Table 1. For the pure cholesterol monolayer, the extracted value for the χ_{zxx} / χ_{zzz} ratio is similar to that obtained in the case of a DPPC monolayer whereas a greater value is obtained for the χ_{xxz} / χ_{zzz} ratio. As for the DPPC monolayer, the presence of mycosubtilin induced a dramatic drop of the two elements χ_{zxx} and χ_{xxz} . The χ_{xxz} element vanishes as expected for a vanishing S-polarized intensity curve. Similarly, the absolute value of the ratio χ_{zxx} / χ_{zzz} also decreased as compared to the value obtained in the case of the pure cholesterol monolayer. However, this time the decrease is accompanied by a relative phase change. The latter change is associated with the response of the Tyr residue.

In conclusion, we have demonstrated that, while the SHG signals from the DPPC and Chol monolayers have not the same intensity, both give similar SHG light polarization curves. This similarity was not preserved when mycosubtilin interacted with the DPPC and the cholesterol monolayers. The mycosubtilin-induced changes in the SHG light polarization curves are however more pronounced in the case of a Chol monolayer as compared to the case of the DPPC monolayer. Hence, SHG is demonstrated to be a powerful tool to investigate Tyr containing peptides, similarly to Trp containing peptides. It is also sensitive enough to monitor peptides containing a single Tyr residue. Further work will be carried on to extract the molecular parameters from these results and conclude further on these interactions.

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Figure Captions

Fig. 1 Combined tensiometry (dash line) and SHG (solid line) intensity time dependence during the mycosubtilin adsorption for a DPPC monolayer at the air-water interface. The SHG intensity is normalized to the neat air-water interface. Time zero corresponds to the lipopeptide injection under the DPPC monolayer at a surface pressure of 10 mN/m. The final concentration of mycosubtilin in the pure water subphase was 1.1 μM

Fig. 2 SHG intensity as a function of the input polarization angle for a pure DPPC (a) and a mixed DPPC-mycosubtilin (b) monolayer. The SHG intensity is normalized to the neat air-water interface. P-polarized SHG intensity corresponds to ($\bullet$), S-polarized SHG intensity to ($\blacktriangle$) and 45°-polarized to ($\blacksquare$)

Fig. 3 Combined tensiometry (dash line) and SHG (solid line) intensity time dependence during the mycosubtilin adsorption for a Chol monolayer at the air-water interface. The SHG intensity is normalized to the neat air-water interface. Time zero corresponds to the lipopeptide injection under the Chol monolayer at a surface pressure 10 mN/m. The final concentration of mycosubtilin in the pure water subphase was 1.1 μM

Fig. 4 SHG signal intensity as a function of the input polarization angle for a pure Chol (a) and a mixed Chol-mycosubtilin (b) monolayers. The SHG intensity is normalized to the neat air-water interface. P-polarized SHG intensity corresponds to ($\bullet$), S-polarized SHG intensity to ($\blacktriangle$) and 45°-polarized to ($\blacksquare$)

Table 1 Values obtained for the χ_{xxz} / χ_{zzz} and χ_{zxx} / χ_{zzz} ratios from the adjustment to Eqs.(1).

	<i>Neat air/water interface^a</i>	<i>Pure DPPC monolayer</i>	<i>Mixed MS- DPPC Monolayer</i>	<i>Pure Cholesterol monolayer</i>	<i>Mixed MS- cholesterol monolayer</i>
χ_{xxz} / χ_{zzz}	0.38	0.32	0.19	0.32	0.00
χ_{zxx} / χ_{zzz}	0.08	0.10	0.02	0.21	-0.11

MS, mycosubtilin. ^a, taken from ref.[19]

line figure 1

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line figure 3

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