First Nano-Infrared Spectroscopy and Imaging Measurements of Single Phospholipid Bilayers.

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Abstract

Scattering-mode Scanning Near-Field Optical Microscopy (sSNOM) allows one to obtain absorption spectra in the mid-IR region for samples as small as 20 nm in size. This configuration has made it possible to measure FTIR spectra of the protein complement of membranes. (Amenabar 2013) We now show that mid-IR sSNOM has the sensitivity required to measure spectra of phospholipids in individual bilayers in the spectral range 800 cm^{-1} – 1400 cm^{-1}. We have observed the main absorption bands of the dipalmitoylphosphatidylcholine headgroups in this spectral region above noise level. We have also mapped the phosphate absorption band at 1070 cm^{-1} simultaneously with the AFM topography. We have shown that we could achieve sufficient contrast to discriminate between single and multiple phospholipid bilayers and other structures, such as liposomes. This work opens the way to further research that uses nano-IR spectroscopy to describe the biochemistry of cell membranes and model systems.

Introduction

Membranes are defining structures of cells. Their role extends beyond basic compartmentalization, and includes overall organization of proteins in space and time, energy storage, control of trafficking and homeostatic regulation, intracellular and intercellular signal transduction, recognition, and adhesion.

Despite intense interest, many issues in the structure and function of membranes are still unaddressed or have not reached a consensus. This is partly due to the challenges intrinsic to the study of individual membranes. In its simplest description, a cellular membrane consists of a bilayer of phospholipids and embedded proteins, which is a few nanometers in thickness and displays in-plane organization on the scale of the nanometer. The structure puts a severe constraint to spectroscopic studies of individual membranes because of its demands on both the sensitivity and the spatial resolution of the measurement.

Over the last few decades, infrared (IR) absorption spectroscopy has provided a major contribution to the understanding of the molecular properties of biological membranes and their model systems, such as phospholipid bilayers and multibilayers. (Tamm 1997, Tatulian 2003) Most measurements

have been carried out on samples of macroscopic dimensions, such as multibilayer stacks. IR microscopy configurations that rely on far-field optical geometries lack spatial resolution, because of the limits set by diffraction, and sensitivity, because of the low angle of incidence. The use of a grazing incidence geometry has allowed the study of extended individual bilayers and even single leaflets, including both lipid and protein components. However, grazing incidence measurements do not provide the spatial resolution necessary to study in-plane membrane organization.

The introduction of scattering Scanning Near-Field Optical Microscopy (sSNOM) has provided the sensitivity and spatial resolution to study minute amounts of biological material on the scale of 20 nm - 100 nm. (Huth 2012)

The capability to provide IR absorption spectra of single bilayers of the purple membranes from *H. salinarum* has been demonstrated. (Amenabar 2013) However, these studies were limited to the IR absorption of peptide bonds in patches of bacteriorhodopsin proteins and did not report any spectral contributions from membrane lipids. In particular, measurement of phospholipid headgroups in a bilayer membrane is expected to be a major challenge because of their lower concentration within the measurement volume.

In this work we demonstrate the possibility of measuring the IR absorption of phospholipid headgroups in single bilayers by using sSNOM. We use single and multiple bilayers of phospholipids deposited on a silicon surface as a sample. The system is a simplified model for biological membranes. Despite its compositional simplicity, it allows us to challenge the sensitivity and spatial resolution of sSNOM in spectromicroscopy and imaging experiments.

Experimental

DPPC bilayer stacks were formed on the surface of silicon wafers by deposition of a dispersion of single unilamellar vesicles (SUV).

sSNOM measurements were performed using a NeaSNOM microscope operating in tapping mode.

Results and Discussion

We deposited single bilayers and multibilayer stacks of DPPC by allowing liposomes to adhere and unfold on the surface of a silicon wafer. The resulting sample, as seen in AFM height maps recorded at room temperature, displays patches of single and multibilayers separated by areas of exposed silicon surface (Figure 1). Each bilayer is approximately 5 nm thick, as expected for DPPC bilayers below the transition temperature (41 °C). Multibilayers display a terraced structure that exposes steps corresponding to one, two, three or more bilayers. Unopened liposomes can be observed on top of the bilayers.

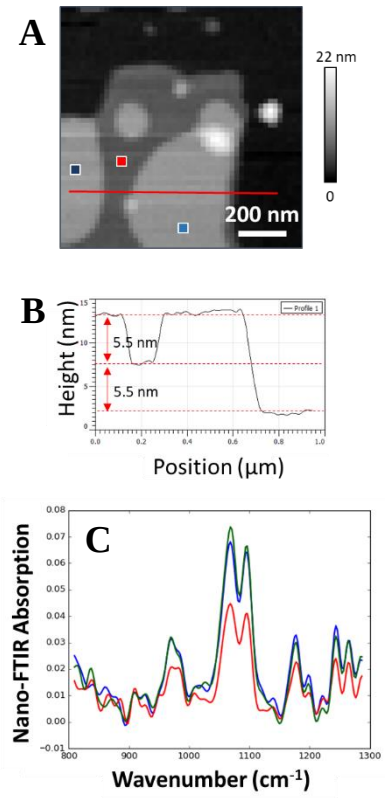

Figure 1. *sSNOM measurements of bilayer stacks of DPPC on a silicon substrate.* ***A.*** *AFM topography image of a region of the sample.* ***B.*** *Height profile along the red line in panel A.* ***C.*** *sSNOM spectra measured at the positions marked in panel A. Spectra are color coded with position markers. Red, single bilayer; Blue and Green, double bilayers.*

We use the AFM height map to survey the sample and select positions for the sSNOM spectral measurement, corresponding to different thickness values. Figure 1 shows the IR spectra measured with laser power emission in the 800 cm^{-1} - 1400 cm^{-1} range. Measurements were performed in three different positions, corresponding to single and double bilayers. The spectra show several bands above noise level. Band intensity and position are consistent with the spectra of macroscopic samples of DPPC recorded using the FTIR technique. The bands in this spectral region are assigned to vibrational modes of phospholipid headgroups and to bending modes of the methylene groups of the alkyl chains.

Comparison of the spectra measured at the three positions shows that the intensity of the bands from the double bilayer is about 50% to 100% higher than that of the bands from the single bilayer. This is expected for bilayers with 5 nm thickness, which are much smaller than the probing depth of sSNOM.

We performed sSNOM imaging of multibilayer patches by mapping the intensity of the sSNOM signal over the surface of the sample at 1070 cm^{-1}. Figure 2 shows maps of AFM topography, reflectivity, and sSNOM optical phase at 1070 cm^{-1}.

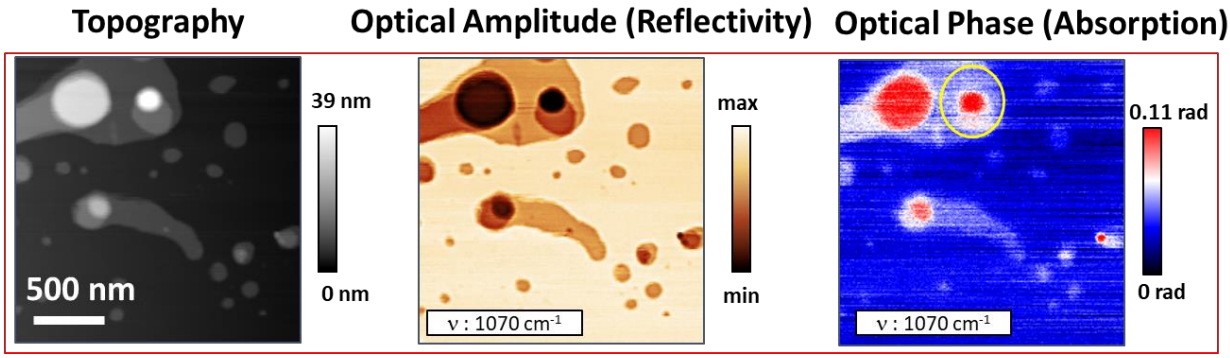

***Figure 2**. IR maps of multibilayers of DPPC recorded using the sSNOM signal at 1070 cm^{-1}.*

The peak at 1070 cm^{-1} corresponds to the absorption maximum for the stretching mode of the C-O-P phosphate ester bond and its spatial distribution tracks that of phospholipid headgroups. Figure 2 shows that in the optical phase map at 1070 cm^{-1} the contrast is large and it increases with the number of stacked bilayers in a given position, as expected from the thickness dependence of the spectra (Figure 1).

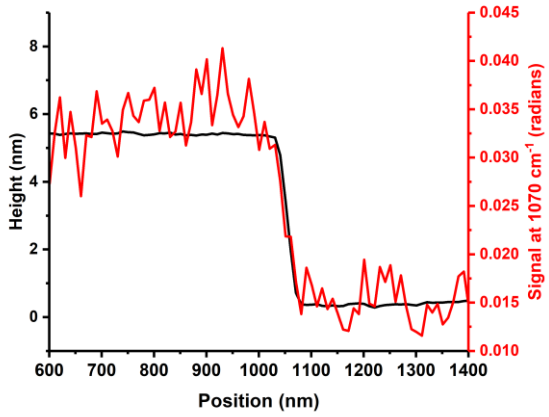

Figure 3. Line profile along the edge of a bilayer patch. Black trace: AFM topography profile. Red trace: sSNOM profile at 1070 cm^{-1}.

Figure 3 compares the AFM topography line profile at the edge of a patch with the corresponding phase profile at 1070 cm^{-1}, showing that the two track closely. The edge plot can be used to provide

an upper limit estimate for resolution of 30 nm or better, much smaller than is allowed by diffraction.

Conclusions

We have reported the first IR spectra of a single phospholipid bilayer using a near-field IR technique. We have investigated the spectral region between 800 cm^{-1} and 1400 cm^{-1}, which includes bands from both the phospholipid headgroups and the acyl chains. We have observed the same bands reported from the spectra of macroscopic samples, with a pattern of intensity that is consistent with the optics and polarization of light in the sSNOM experiment. The spatial resolution of the measurements is better than 30 nm, consistent with the dimensions of the tip. Overall, the quality of the spectra and the anisotropy displayed by some absorption bands from headgroups and alkyl chains indicate that the DPPC bilayers are highly ordered and oriented, which is in agreement with our current understanding of the structure of phospholipid bilayers.

The portion of the bilayer probed by the sSNOM experiment corresponds to about 500 nm^2. The footprint of a DPPC molecule in the bilayer plane is approximately 0.5 nm^2. Therefore, the signal for a single bilayer arises from about 2000 (1000 per leaflet) DPPC molecules. Despite the small size of the sample, the quality of the spectra obtained is high. That opens the way to detailed molecular studies of phospholipid bilayers, their interaction with biological molecules and, eventually, of the biological membranes for which they are models. The future challenge will be the development of sample holders allowing the performance of sSNOM experiments on samples of biological relevance with controlled environmental conditions, such as bilayers in contact with an aqueous phase.

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