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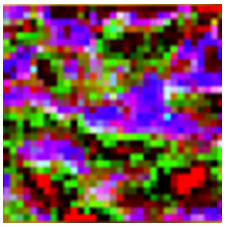
AFM-Raman

TERS Characterization of phospholipid bilayers and detection of nanoparticles



Application Note

TERS-lipids AFM22

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Abstract : This application note reports on TERS characterization of binary phospholipid bilayer systems deposited onto a gold coated coverslip. Such samples are used as model samples for directly and chemo-selectively visualizing the distribution of a lipid constituent at the nanometer scale. In addition, nanomaterials have been introduced to mimic the interactions between nanoparticles (NPs) and cellular membranes and better understand their toxicity on human health. The sensitivity of TERS for the detection and identification of NPs in phospholipid bilayers is demonstrated down to femtomolar concentration with a spatial resolution down to 7 nm. A specific liquid cell has also been developed to permit TERS measurements in solution and results of graphene oxide containing samples are presented.

Keywords : TERS, Raman spectroscopy, phospholipid bilayers, nanoparticles, nanomaterials risk assessment, nanomaterials toxicity, cells, membranes, graphene.

Context and issues

Nanomaterials (NMs) have gained prominence in technological advancements due to their tunable physicochemical properties, electrical and thermal conductivity, catalytic activity, light absorption, etc., with enhanced performance over their bulk counterparts. Despite their unique advantages and applications in both domestic and industrial sectors, the unpredictability of how nanoparticles behave at the nanoscale presents a double-edged sword and has raised the issue, as with any new material, of possible human health impacts. Due to the growth in the production of NMs and their increasing use in industrial applications, issues relating to their potential toxicity are inevitable. When nanoparticles are released into the environment, the cell membrane represents an initial interaction site for eukaryotic cells. Phospholipid bilayers which are the major constituents of membranes act as a barrier of selective permeability and carry out other specific roles in the cell. Studying the interactions between nanoparticles and cellular membranes requires a molecular chemical probe with nanometer resolution capability.

Potential/ Input from technique

Raman spectroscopy is a non-destructive and non-labelling technique which provides detailed information about chemical structure as it detects characteristic molecular vibration frequencies. But Raman microscopy fails to image nano-objects such as nanoparticles or lipid domains at the nanometer scale due to the optical diffraction limit. Tip-enhanced Raman spectroscopy (TERS) which provides nanoscale chemical mapping represents a promising tool for directly and chemo-selectively visualizing the distribution of a lipid constituent at the nanometer scale in bilayers and hence the presence and arrangement of nanoparticles inside the bilayers.

Starting point, what is known?

While TERS has become a tool of choice to characterize 2D materials[1-2] (e.g. graphene and transition metal dichalcogenides) and nanoparticles[3], it has been also successfully used to study biological samples including cells[4], viruses[5], proteins[6] and biomembranes[7]. A recent publication[8] from the group of Zenobi demonstrates TERS capability to probe the molecular organization of supported dipalmitoylphosphatidylcholine (DPPC) monolayers on Au(111) and highlights the correlation of topography and nanoresolved Raman response. In this application note, we report on TERS measurements carried out on binary (DPPC/Cholesterol and 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE)) phospholipid bilayer systems (Fig.1). Such systems are closer models to biomembranes compared to monolayers of one lipid

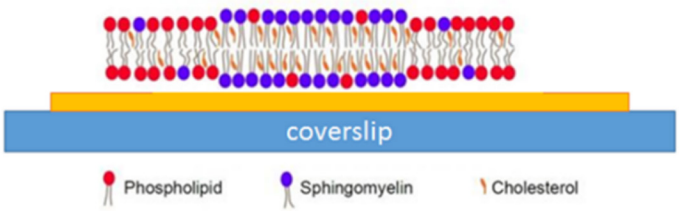

Figure 1 : Schematic diagram of supported bilayers on gold coated glass coverslip.

molecule. As a first step such samples were characterized by TERS. Then they are injected with nanoparticles to test their potential interaction and impact on molecular arrangement of membranes lipids. Measurements were carried out in air first and then in water, the ultimate goal being to be able to characterize nanoparticles within a lipid bilayer in water to closely mimic behavior of the transport nanomaterials across the membranes of eukaryotic cells.

Description of sample and measurement

The bilayer models were prepared using the Langmuir trough technique, with lipids deposited onto a gold coated coverslip at pressures between 25 and 30 mN/m.

TERS measurements were performed with either an XploRA Nano system or a LabRAM HR Nano system (both HORIBA Scientific, France) both combining a state-of-the art scanning probe microscope (OmegaScope) with a true confocal Raman micro-spectrometer. The laser excitation (red: 638 nm (XploRA) or 633 nm (LabRAM), 90 µW, *p*-polarized) is incident on the sample at an angle of 60° with respect to the sample plane. The laser light was focused using a 100× objective (NA=0.7) mounted on a piezo scanner for precise positioning and focusing of the Raman laser spot at the apex of the probe tip. The TERS probes were cantilever-based gold or silver coated AFM-TERS tips (OMNI TERS-SNC-Au/Ag, App Nano for HORIBA Scientific). Silver tips were used for air measurements whereas gold tips were used for liquid measurements as silver suffers from greater oxidation in water.

TERS maps were recorded together with topography scans using the patented Spec-Top™ mode: for each pixel a Raman spectrum is acquired with tip in direct contact with the surface with a typical interaction force of 2-10 nN. In between two pixels of the map, the sample moves in semi-contact mode to preserve the sharpness and plasmonic enhancement of the tip.

Cholesterols play key roles in controlling molecular fluidity in a biological membrane. A bilayer made of a mixture of DPPC and cholesterol is a more realistic membrane model than a homogeneous phospholipid bilayer. Cholesterol contains a short, thermally flexible, hydrocarbon tail with a rigid hydrophobic ring structure that is attached to a small, hydroxyl group that acts as a hydrophilic head. Due to this smaller hydrophilic head and smaller hydrophobic tail cholesterol always fits in between the lipids in a bilayer.

Since cholesterol and DPPC have close Raman signatures a sample was prepared with deuterated DPPC ($DPPC_{d62}$ with a deuterated chain) and non deuterated cholesterol (ratio of 3DPPC for 1 chol). It then becomes simple to distinguish C-D bonds vibrations from DPPC from C-H stretching modes from cholesterol (C-D vibrations occur at lower wavenumbers than C-H).

Fig. 2 shows several recorded TERS images (750 ms/pixel; 33 nm/pixel) overlaid on the AFM image. The **blue** and

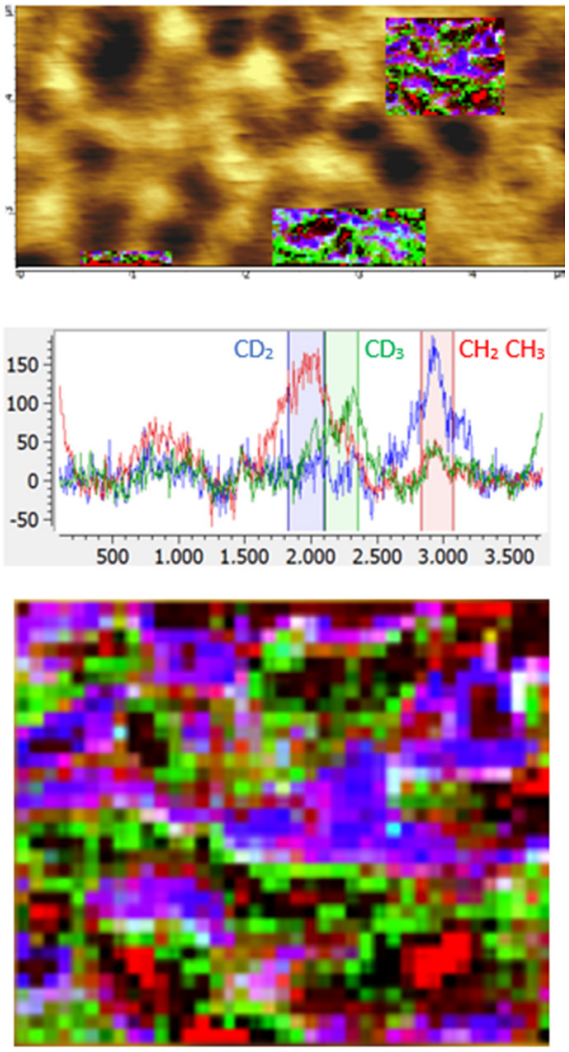

Figure 2: (a) TERS maps of deuterated DPPC /Chol bilayers overlaid on AFM topographic image. (b) Raman spectra defining cholesterol CH_2CH_3 band (red), and deuterated DPPC CD_2 band (blue), and CD_3 band (green). (c) Zoom of top right TERS map.

green bands between 2000-2500 cm^{-1} are associated with the CD_2-CD_3 stretching of DPPC while the 3000 cm^{-1} bands correspond mainly to cholesterol (**red** on map). It should be noted that the blue regions reveal areas where CD_2 bonds are preferentially excited while in green domains CD_3 bonds are more active. This is consistent with different orientation of the bonds, possibly related to the fluidity of the bilayers, since Raman signal intensity depends on the alignment of the laser polarization with the bonds, i.e. when they are parallel the signal is highest. Additionally, the formation of small (≈20-100 nm) nano-domains, enriched in cholesterol and localized preferentially in areas where the CD_2 bonds are excited is observed. These images clearly demonstrate the sensitivity of TERS to detect lipid bilayers.

Next lipid bilayers have been exposed to graphene oxide (GO) nanoparticles to explore interactions between them. As biosensor and drug delivery system candidates, the behavior of graphene materials in contact with cell membranes needs to be understood.

A 50 µl droplet of a picomolar suspension of GO in water was deposited onto a 3:1 ratio DPPC Cholesterol bilayer. This volume and concentration were selected to ensure a monolayer coverage of about 8.5%. The sample was left in air for 2 hours and then analyzed: Fig. 3a shows three TERS maps on top of the topographic AFM image acquired with a laser power of 90 µW and acquisition time of 800 µs. Three spectra from single pixels of the TERS maps at locations shown in Fig. 3b are depicted in Fig. 3c. While the topography is similar to the non-GO exposed sample and does not evidence the presence of GO, the occurrence in pixel spectra of the D and G bands from GO and CH stretching bands from Chol and DPPC indicates that GO is able to cross the phospholipid membrane in 2 hours and is no longer lying on top of the lipid bilayers. As shown in Fig. 3c, signature band are associated with a color for GO, Chol, DPPC/Chol which are G band (green), 1800 cm^{-1} shoulder (red) and 2900 cm^{-1} CH_3CH_2 (blue), respectively. Brown (mix of red and green) nano-areas indicate the presence of GO in cholesterol enriched phases while cyan (mixture of blue and green) nano-areas indicate the presence of GO in DPPC enriched phases. In a higher resolution map (not shown here) spectra from points distant by less than 7 nm exhibit D and G bands intensity variation of more than a factor of 5. Such a local GO concentration gradient clearly supports the fact that TERS, a non-labelled technique, can achieve a spatial resolution of only 7 nm.

After recording each TERS map, a scan of the tip is also acquired. The absence of any Raman peak in G-band spectral region confirms that there was no carbonaceous contamination on the tip and the laser power used is low enough to avoid burning the sample.

TERS is a suitable technique to detect the presence of femtomolar GO inside bilayers.

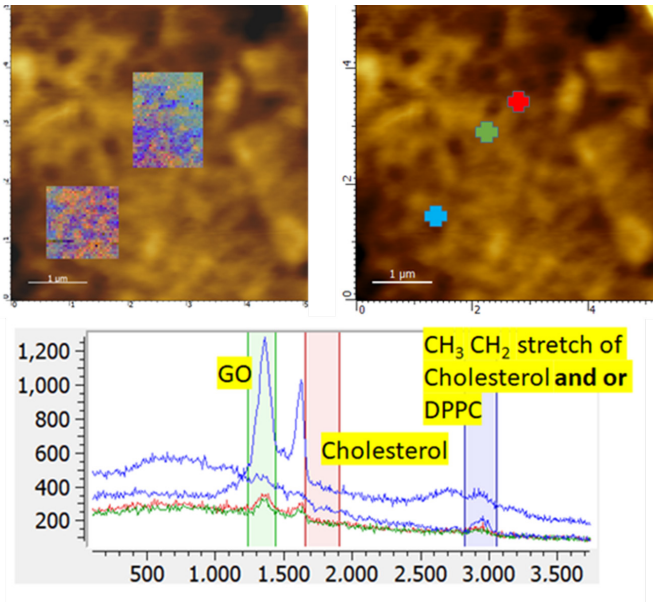

Figure 3: (a) TERS maps of DPPC /Chol bilayers and graphene oxide overlaid on AFM topographic image. (b) topographic AFM scan of same area locating three cursors from which Raman spectra are extracted and displayed in (c).

Finally, some preliminary data were obtained on bilayers in pure water. Pure water approaches the physiological environment of membrane cells since the assembly and stability of synthetic phospholipids bilayers is primarily driven by the hydrophobicity of the amphiphilic molecules. TERS in liquid is quite challenging in terms of laser-tip alignment and signal collection. Several hardware and procedure adjustments have been developed and details can be found in the "AFM-TERS measurements in a liquid environment with side illumination/collection" technical note in which CNT TERS mapping is reported.

A sample of a DMPE bilayer injected with a 50 µl droplet of a picomolar suspension of GO was prepared. 15 min after GO injection, the phase AFM scan in Fig. 4a shows the presence of phospholipids micro-domains but no GO sheets can be observed. A TERS map of the same area was obtained with an acquisition time of 2 s, a laser power of 180 µW and a pixel size of 31 nm. The TERS map overlaying the intensities of D band (red) and G band (blue) of GO as well as the CH stretching band (green) of DMPE exhibits some small islands –yellow colored – of 100 nm size enriched in GO.

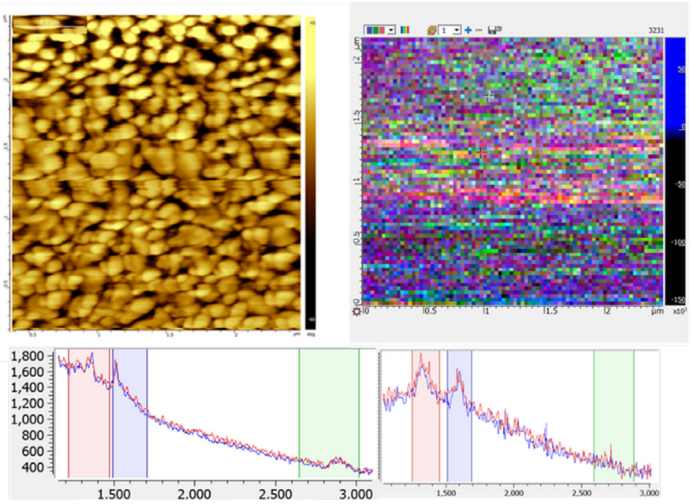

Figure 4: (a) Phase AFM map of a DMPE bilayer injected with a 50 µl droplet of a picomolar suspension of GO. The image was obtained in water in semi-contact mode at 1 Hz using a TERS tip. (b) TERS map acquired on the (a) area with an acquisition time of 2 s, a laser power of 180 µW and a pixel size of 31 nm. The TERS map overlays the intensity of D band (red) and G band (blue) of GO as well as the CH stretching band (green) of DMPE. (c) and (d) Near-field Raman average spectra (16 pixels) indicating the range of the three cursors of the TERS map.

The D and G bands are clearly observed as well as the CH_2-CH_3 stretch band in some areas. Near-field Raman average spectra (16 pixels) defining the range of three cursors of the TERS map are given in Fig. 4a and Fig. 4d.

Conclusion and perspectives

This application note shows how TERS chemically images the molecular arrangement of phospholipids bilayers with a spatial resolution never previously obtained of 7 nm in air without using any labelling technique.

The TERS microscope can visualize the phase separation in model membranes produced using the Langmuir trough method. This study also shows that injected graphene oxide particles can penetrate the bilayers within 3 hours of being deposited onto the artificial membranes in air.
A liquid cell has been specifically developed to carry out TERS measurements in water and a TERS map of DMPE bilayer exposed to GO evidences its penetration. These results demonstrates that TERS is an ideal tool for direct observation of molecular interaction mechanisms of cell membranes with nanomaterials.

Acknowlegdments

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