Biochemical Characterization of Cell Electropermeabilization using the Raman Effect

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Abstract

The main consequence of the interaction between cells and pulsed electric fields is the destabilization of the plasma membrane leading to its permeabilization (allowing, for example, an uptake of external molecules by the biological cells). We detail here a highly sensitive method, coupling Coherent Anti-stokes Raman Scattering (CARS) microscopy and Spontaneous Raman Spectroscopy (SRS), to characterize the biochemical consequence of membranes electropermeabilization.

1.Introduction

Electropermeabilization characterizes the permeabilization of the plasma membrane due to a high voltage (several MV/m) and short pulsed electric field. The parameters of the electric pulses can be tuned to obtain reversible to irreversible permeabilization. Electrochemotherapy [1] is one of the medical applications of the interaction between tumor cells and a specific electromagnetic field that results in the increase of the efficiency of anticancer drug (bleomycin) by a factor between 1000 and 10000. Cell electropermeabilization has been known for decades. However, the underlying mechanisms of the bioelectromagnetic interaction between pulsed electric field and cells supporting cell electropermeabilization are still not fully understood [2]. One hypothesis explaining the membrane destabilization is the presence of chemically altered membrane phospholipids after the electrical excitation [3]. Spontaneous Raman Spectroscopy (SRS) is a powerful tool to probe the chemical bounds of samples [4]. Comparing the vibrational spectra of biological sample before and after pulsed electric field can provide critical bandwidths to characterize electropermeabilization. Coherent Anti-stokes Raman Scattering (CARS) microscopy acquires images of a sample at a specific vibrational frequency. Based on the critical bandwidths previously determined with Raman spectroscopy, it is possible to follow the evolution of the biochemical composition of the membrane during a pulsed electric field. The study is performed on two study systems: Giant Unilamellar Vesicles (GUV) of phospholipids which are a simple mimic of the cell membrane and DC-3F cells (chinese hamster lung fibroblast cell) which are a classical cell line used to study electropermeabilization.

2.Materials and Methods

2.1 Electroformation of giant unilamellar vesicles and lipid extraction

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, AL). DOPC was dissolved in chloroform at a mass concentration of 5mg/mL. The standard solution was stored at -20°C.

The vesicles were prepared at 6°C using an electroformation protocol [5]. 30µL of the lipid solution was deposited on the conducting side of two glass slides coated win indium tin oxide (Sigma, Saint Louis, MO). The

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chamber was filled with a sucrose solution (240mM sucrose, 1mM NaCl, 1mM KH_2PO_4/K_2HPO_4). The slides were connected to a function/arbitrary waveform generator (HP Agilent 33120A, Santa Clara, CA) and sinusoidal voltage of 25mV peak to peak and 8Hz was applied. The voltage was increased by 100 mV steps every 5 minutes, up to a value of 1225mV and maintained under these conditions overnight. Finally, a square-wave AC field of the same amplitude was applied at 4Hz for one hour in order to detach the GUVs from the slides.

The lipids were extracted from the vesicles solution with a methanol-chloroform solution (volumic ratio 2:1). This solution was added, in a volumic ratio 5:1, to the vesicles. The low phase of the solution was kept and evaporated under vacuum with nitrogen flow during 1 hour. The lipids were suspended in 50µL of methanol-chloroform and stored at -20°C. The theoretical concentration of the lipids in the final solution is 2.4mg/mL, supposing that the efficiency of the electroformation and the extraction were 100%.

2.2 Cell culture

The DC-3F cells were grown in MEM - Minimum Essential Medium (GIBCO Laboratories, Cergy-Pontoise, France, 31095) with addition of 10% fetal bovine serum (GIBCO Laboratories, Cergy-Pontoise, France, 10091-148) and supplemented by antibiotics. The cells were maintained in a humidified atmosphere at 37˚C and 5% CO_2.

2.3 Nanosecond Pulse Generator

A commercially available generator (FID GmbH, Model FPG 10-ISM 10, Burbach, Germany) with an output impedance of 50 Ohm (50 Ω) was used to treat the GUV and the cells. It generates trapezoidal monopolar pulses of 10ns half width. The magnitude goes from 3kV to 8kV. The rising edge, fixed by the semiconductors commutation lasts between 0.4 and 0.6ns. The falling edge lasts between 1 and 1.5ns.

2.4 Spontaneous Raman Spectroscopy

SRS experimental setup was designed to probe the vibrational footprint of biological samples. Nanosecond pulsed neodymium-doped yttrium aluminum garnet (Nd-YAG) laser (Light Solution, Mountain View, California, USA) excites a crystal doubler to get a laser beam at 532nm and 160mW. The laser beam was focused with an objective (Zeiss, Plan-APOCHROMAT 10x/0.45, Oberkochen, Germany) in order to maximize the excitation and the collection of the Raman signal. To eliminate the non-useful signal, the desexcitation beam is filtered with two interference filters (Semrock, LP-03-332-RE-25D RazorEdge, Lake Forest, USA) and two notch filters (Schott, OG550, Mainz, Germany). The signal was focused on an optical fiber connected to the spectrometer (Princeton Instruments, Acton Insight 400B, Massachusetts, USA). The software Winspec/32 v2.6.11.0 (Princeton Instruments) remoted the spectrometer. Baseline and spikes were removed by data process with python using Spyder 2.1.12.

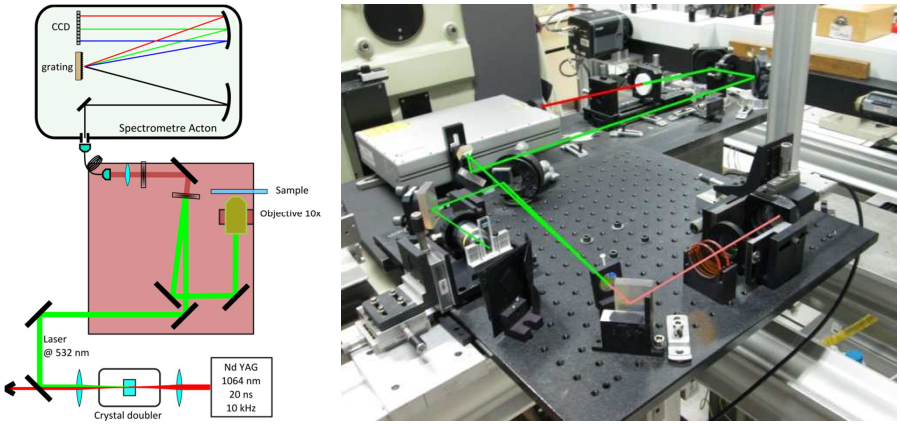


Figure 1: SRS experimental setup

2.5 Coherent Anti-stokes Raman Scattering microscopy

At UMR8203, a unique and homemade device combines nanosecond pulse generator and wide-field CARS microscopy allowing to acquire images of living cells with a high time resolution (3ns). Silve *et al.* [6] have detailed the experimental setup of this wide-field CARS microscope. The coherent excitation maximizes the signal from the biological sample which can be then detected in one laser shot.

3.Results

In the literature [7-8], the critical bandwidths to characterize lipid oxidation are associated to 1263 cm^{-1} (symmetric rock in *cis* double bond δ(=C-H)), 1292cm^{-1} (in-phase CH_2 twist), 1433 cm^{-1} (scissoring mode of CH_2), 1646 cm^{-1} (*cis* double bound stretching υ(C=C)) and 2800 cm^{-1} - 3000 cm^{-1} (CH stretch of CH_2 and CH_3).

In order to maximize the Raman signature of the DOPC, the Raman signature of the substrate had to be as low as possible in the explored bandwidth. The comparison of different substrates (Figure 2) shows that microscope micro slide and suprasil substrates are the best to reduce as much as possible the background signal.

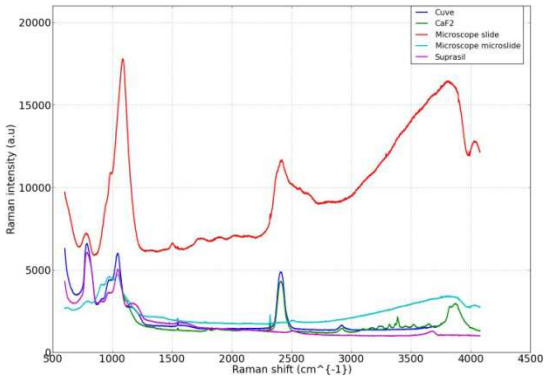
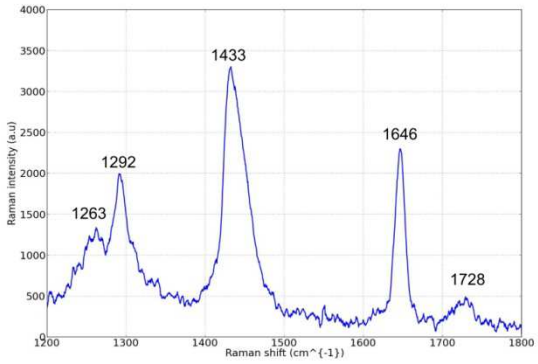


Figure 2: Comparison of Raman signature of different substrates at 160mW output laser power and 1second integration time. Blue: Cuvette (Hellma Analytics, 100-QS, Mülheim, Germany), Green: CaF2, Red: Microscope slide, Clear blue: Microscope micro slide, Purple: Suprasil

In order to increase the output power of the laser without burning the sample, a homemade hermetic chamber was designed. This chamber was composed of two microscope micro slides where the sample stands in between. Several drops of the sample were deposited on the first microscope micro slide. The solvent were evaporated at room temperature during two minutes. One drop of water milli-Q was deposited on the dry sample and the chamber was closed by the second microscope micro slide. To avoid the water to evaporate, the edge of the second microscope micro slide was glued to the first microscope micro slide with varnish. Several drops around 5µL of DOPC in standard buffer (chloroform) at 5mg/mL were deposited on the substrate.

Each time, the spectrum of the chamber and of the sample inside the chamber were acquired under the same conditions (power laser and integration time). The difference between those two spectra, calculated with python, reports the Raman signature of the sample alone. The Raman spectrum of the dry DOPC was acquired with an output power laser of 160mW for 30s (Figure 3).

a)



b)

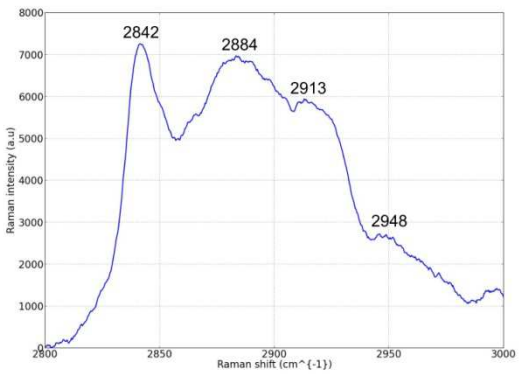


Figure 3: Spectra of the dry DOPC from a standard solution at 160mW power laser and 30 seconds integration time. a) 1600 cm^{-1} bandwidth, b) 2800 cm^{-1} bandwidth

With the same protocol, the spectrum of the dry lipid extracted from a GUV solution was acquired in the hermetic chamber (Figure 4).

a) b)

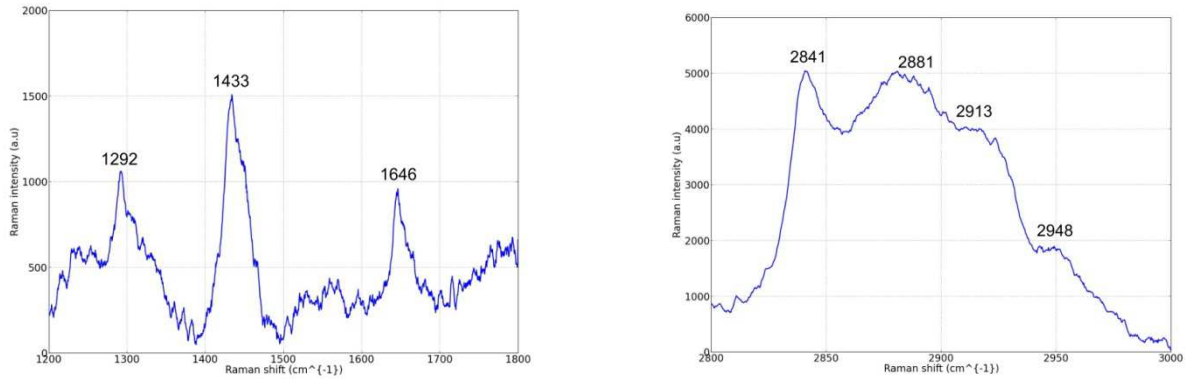


Figure 4: Spectra of lipid extract from DOPC GUV solution at 160mW power laser and 30 seconds integration time. a) 1600 cm^{-1} bandwidth, b) 2800 cm^{-1} bandwidth

The Raman signature of a standard lipid and a lipid extract from a vesicles sample shows that the critical bandwidths of the lipid are slightly modified by the electroformation and the extraction steps. The bands are wider in the GUV configuration than in the standard solution. The general magnitude of the Raman intensity indicates that the concentration in lipid was lower in the GUV sample than in the standard solution, indicating a loss of lipid during the electroformation and extraction steps.

4.Conclusion and perspectives

We succeed to design an experimental setup sensitive enough to detect the Raman signature from lipid extract from GUV in solution. We are now able to acquire the spectrum of the GUV after the application of pulsed electric fields. By comparing the Raman signature of pulsed vesicles and control vesicles, we will be able to link the results with a potential chemical alteration of the phospholipids. The same protocol will be done with DC-3F cell line. The CARS microscope will be a powerful tool to have spatial information on the underlying mechanisms.

5. Aknowledgments

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