REAL-TIME BIOCHEMICAL RESPONSE UPON CHEMICAL STIMULATION OF LIVING MONOCYTES INVESTIGATED BY FOURIER TRANSFORM INFRARED MICROSCOPY (µ-FTIR)

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ABSTRACT
This paper aims to present novel results on biomedical applications of FTIR microspectroscopy (µ-FTIR) in the field of real-time investigation of biochemical processes occurring within living cells under physiological conditions. During the past years, our group optimized fabrication strategies on IR-VIS transparent materials for the realization of new microfluidic devices. We further improved the experimental setup by fabricating a fully functional IR-VIS transparent microfluidic chip. This system allowed us to follow the response of circulating white-blood cells to the chemical stimuli induced by fMLP (Formyl-Methionyl-Leucyl-Phenylalanine) cytokine, simulating an inflammation process. Transcription phenomena, new protein synthesis and increased cell membrane fluidity were monitored within the first minutes of stimulation, demonstrating the applicability of µ-FTIR in studying living systems.

KEYWORDS: Living Cells, FTIR Microspectroscopy, Label-free detection, Fluidic Devices.

INTRODUCTION
FTIR microspectroscopy (µ-FTIR) is a label-free microanalytical technique that allows collecting spatially resolved chemical information of the measured samples by recording vibrational spectra, allowing the direct correlation between sample morphological features and its vibrational local pattern.

µ-FTIR of biological species, fixed or dried, such as plant and animal cells or tissues have been employed for many purposes: differentiation of microbial cells and strains [1], characterization of pathological states [2], discrimination of the effects of drugs or pollutants on cellular biochemical behavior [3] and for many other applications [4]. The exploitation of analytical capabilities of µ-FTIR in lab-on-a-chip devices for single cell analysis is mainly hindered by two factors i) the strong absorbance of water in the medium infrared region (MIR, 4000–400 cm⁻¹) and ii) the almost total experience for the microfabrication of the most common IR-compatible substrates.

In intensity, position and shape of cellular infrared bands in the MIR are sensitive to the cellular biochemistry: composition and state of order of lipids, cellular protein structure, nucleic acid conformation and relative concentration of cellular constituents. In the MIR fall also some liquid water bands, primarily the symmetric and asymmetric O-H stretching (3000-3600 cm⁻¹) and the H-O-H bending centered at ~1640 cm⁻¹. The later hides completely the Amide I band (1600-1700 cm⁻¹), diagnostic for the cellular protein structure. In order to disclose Amide I and recover fundamental biochemical information from hydrated sample’s spectra, the water contribution has to be subtracted, therefore avoiding the saturation of bending water band. Due to the quite high water extinction coefficient (ε = 20 L·mol⁻¹·cm⁻¹), pathlengths shorter than 9 µm has to be guaranteed and carefully controlled, imposing a microfabrication approach in realizing fluidic devices.

Infrared transparent materials have been available since many years in form of single or poly crystalline windows, with a thickness ranging from few microns to some millimeters. Table 1 summarizes the most interesting properties of commonly employed IR and VIS transparent substrates from a mechanical, thermal and chemical point of view.

<table>
<thead>
<tr>
<th>Material</th>
<th>IR transmission range [cm⁻¹]</th>
<th>Refractive index (at 3µm)</th>
<th>Young Modulus [GPa]</th>
<th>Density [g/cm³]</th>
<th>Thermal conductivity [W/mK]</th>
<th>Water solubility [g/100 g of water]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fused Silica</td>
<td>50,000-2500</td>
<td>1.41</td>
<td>72</td>
<td>2.20</td>
<td>1.4</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Diamond</td>
<td>45,450-2325</td>
<td>2.38</td>
<td>1050</td>
<td>3.515</td>
<td>&gt;1800</td>
<td>Insoluble</td>
</tr>
<tr>
<td></td>
<td>1665-285</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaF₂</td>
<td>66,666-1110</td>
<td>1.42</td>
<td>76</td>
<td>3.18</td>
<td>9.7</td>
<td>0.00151 (20°C)</td>
</tr>
<tr>
<td>BaF₂</td>
<td>50,000-770</td>
<td>1.46</td>
<td>53</td>
<td>4.89</td>
<td>11.72</td>
<td>0.12 (25°C)</td>
</tr>
</tbody>
</table>

The choice of a suitable material for fabricating microfluidic devices should take in consideration both thermal and mechanical properties of the substrate. After all, most of the fabrication processes involve thermal treatments and/or mechanical manufacturing, such as drilling or polishing, so too hard or brittle materials will result extremely difficult to handle. Another very important issue is the chemical stability of the window materials, since wet and/or dry chemical treatments are often employed for shaping 3D features.

Diamond and fused silica are quite inert in aqueous environment, nevertheless they are not transparent in the entire MIR [5]. Barium fluoride posses the better IR transparency, but it is too soluble in water, resulting cytotoxic. We found a good compromise in CaF₂, which have just one drawback: the lack of standard protocols for microfabrication.
The microfabrication strategies we developed hence will make available fluidic devices for the real-time monitoring of living cells with IRMS.

EXPERIMENTAL

The photolithographic and etching processes we developed and already published [6] were joined for fabricating a 3D fluidic device; in Figs. 1 an example of microfluidic chip pattern and assembly is shown. The fluidic device has two inlets, one for the cells and the other for the injection of chemicals, and two outlets, for fluidic control and waste collection. The internal-measurement chamber is divided in two different zones, one larger for cell allocation and one smaller for chemical perfusion, separated by a series of small channels 5 μm in width and 10 μm in length, 20 μm spaced. The cell chamber presents also two different thicknesses, 9 and 5 μm, the deeper, obtained by directly etching 4 μm in CaF₂. Two more wells are patterned, not connected to the fluidic part of the chip, to collect the air background during measurements. The device is sealed by thermal bonding with another CaF₂ drilled window, using a thin layer (100 nm) of SU-8 photoresist as adhesive promoter. The complete device is connected to external pumping system by an aluminum holder specifically designed (Figure 1a). The system is then thermalized by fitting it inside a copper-made water-circulating chamber (Figure 1c).

U937 monocyte cells (mean average diameter 8-10 μm) were cultured in RPMI medium (RPMI 1640: 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 10% FBS), with 100U/ml penicillin, 100 μg/ml streptomycin, in incubator at 37°C with 5% of CO₂. Before measurements, the culture medium was substituted by NaCl 0.9% physiological solution supplemented with glucose 5μM: this condition has been checked by cytofluorimetric measurements to not alter at all cell cycle and viability for the short periods of data acquisition [7]. The U937 cell suspension (1.000.000 cells/mL) was injected into the measurement chamber via a manual syringe. Cells were let to diffuse into the measurement chamber. Once identified an isolated cell (sampled area 10X10 μm), IR transmission spectra have been acquired using a Bruker Hyperion 3000 Vis-IR microscope mounting a mid-band HgCdTe detector, coupled to a Bruker Vertex 70 interferometer at the infrared beamline SISSI (Synchrotron Infrared Source for Spectroscopic and Imaging) at the Elettra Synchrotron Laboratory, Trieste, Italy [8]. The brightness advantage of Synchrotron Radiation (SR) has been exploited for measuring a single-cell. After the collection of repeated spectra on the un-stimulated monocyte for 24 minutes, fMLP was injected through the second inlet port and let to diffuse into the cell-measurement chamber. Immediately after the injection, the measurements were started again on the same cell, co-adding 512 scans per spectrum at 4 cm⁻¹ spectral resolution with a scanner velocity of 40 kHz, which roughly means one spectrum every 4 minutes.

RESULTS AND DISCUSSION

Using the fluidic device shown in Fig.1, we tested the response of U937 monocytic cell line to fMLP cytokine, simulating some steps of an inflammation process. fMLP binds to a specific G-coupled membrane receptor. The receptor activation triggers intracellular signal transduction pathways, resulting in the correct biological response, for instance, migration, phagocytosis, antibody-dependent cell mediated citotoxicity, degranulation, superoxide production, transcriptional activation, and actin reorganization [9].

Some minutes after the fMLP injection, an increase in the intensity of both symmetric and asymmetric bands of nucleic acids can be appreciated (see Fig. 2b). In particular the RNA contribution at 1245 cm⁻¹ [10] increases, along with the signal of Amide II, related to newly synthesized proteins [11]. Such trend indicates the activation of biochemical processes of transcription and synthesis within the cell upon fMLP stimulation. The appreciable change of methyl band could be ascribed to variations in cellular membrane composition, probably related to a superior cell motility upon activation.
Figure 2  a. A picture of the single U937 living cell measured aside the time evolution of spectra (1000-3000 cm⁻¹), during the time course of the chemical stimulation experiment with fMLP, highlighting the two spectral regions in which major variation have been noticed (insets b and c). In the two graphs are plotted the values of integral of asymmetric PO₂⁻ stretching of nucleic acids (massif 1250-1240 cm⁻¹) and of CH₃ asymmetric stretching band (massif 2975-2950 cm⁻¹) (respectively insets d and e), at 38 minutes from the starting of the measurement, and 8 minutes after the injection of fMLP, it is visible a sudden and contemporaneous increase in signals due to the response of the cell to the chemokine.

CONCLUSION
As far as we know, this is one of the first experiments demonstrating the suitability of µ-FTIR for the investigation of on-going eukaryotic cell biochemical processes.
The design flexibility offered by microfabrication allows expanding the range of µ-FTIR experiments to new fields of science. The first steps we moved gave quite promising results, and demonstrated the great potentialities of µ-FTIR in monitoring the biochemical behavior of living bio-systems usable with this new type of microfluidic devices for real time, label-free cell analysis, at single cell level by exploiting the high brilliance of SR sources.

REFERENCES

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