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Fabrication of a microfluidic platform for investigating dynamic biochemical processes in living samples by FTIR microspectroscopy

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ABSTRACT

Here we present the optimization of fabrication steps for realizing an infrared–visible microfluidic chip to study single-living cell behaviour in physiological environment by synchrotron radiation FTIR microspectroscopy. We optimized subtractive and additive lithographic processes on CaF_2 substrate, employing X-ARP 3100/10 photoresist both as etching-mask and for the device fabrication. Using prototype microfabricated liquid cells 9 and 5 μm thick, we measured the response of small groups of THP1 monocytic cells to mechanical compression and chemical stimulation with fMLP using conventional IR globar source, aiming to evaluate biochemical rearrangements of leukocytes during the capillary circulation or recruitment processes. Stimulated monocytes have spectral features recognizable, differentiating them from unstimulated, especially affecting the spectral region 1280–1000 cm^{-1}, characteristic of nucleic acids and carbohydrates, and specific band ratios, such as proteins on lipids and methylene on methyl. Spectra variations have been correlated with biochemical events such as transcription, synthesis of new-proteins and variations in membrane fluidity.

1. Introduction

The establishment of reliable living single-cell analysis techniques is a crucial point for understanding the molecular bases of cell behaviour in response to external stimuli, either chemical or mechanical [1]. Microfluidic devices offer a unique platform for this purpose since they allow the integration of a variety of operation such as single-cell selection, positioning or lysis as well as separation and detection of cellular analytes [2]. Moreover, microfluidic devices are able to confine cells in compartments near their intrinsic volume, thus minimizing dilution effects and increasing detection sensitivity. From a detection standpoint, microchips have to be fabricated in materials transparent with respect to the probing technique. Visible and fluorescence microscopy were among the first investigation methods to be integrated in microfluidic platforms and they still are the most employed, taking advantage from the standardized fabrication protocols of suitable substrates such as glass, quartz or plastics.

We aim to expand cellular microanalysis in physiological environment to FTIR microspectroscopy (μ-FTIR), a label free, not damaging, powerful and versatile tool for biological sample investigation. This technique has not the selectivity of a single molecule analytical tool; nevertheless, by the analysis of specific absorption bands [3], it offers a fast snapshot of cellular response to specific stimuli in terms of variation of protein structure, membrane composition and order, nucleic acid conformation as well as differences in relative concentrations of major cellular macromolecules [4,5]. Despite its great potentialities, fabrication protocols for both infrared and visible (IR–VIS) transparent materials are not well-established and technical problems related to the strong water absorption in the Mid-IR regime has be taken in consideration.

Herein we present the optimization of fabrication steps for producing an IR–VIS transparent microfluidic chip, suitable for exploring biochemical changes in living-cells upon different stimulations. In particular we are interested in studying the cellular behaviour of individual leukocytes, in order to understand their internal rearrangements induced both by capillary circulation and/or chemoactivated extravasation through narrow endothelium interstices. Preliminary results on mechanical compression and chemical stimulation with fMLP (N-formyl-Met-Leu-Phe) of THP1 leukocytes in a

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prototype microfabricated liquid cell are reported and future developments of the research activity outlined.

2. Experimental

2.1. Fabrication of IR–VIS transparent devices

In order to investigate by μ-FTIR leukocytes' response to mechanical deformation during capillary circulation or induced by chemotaxis, we designed the device shown in Fig. 1a, where the narrower channels mimic capillaries and epithelium intercellular interstitials. The device was realized on calcium fluoride by photolithography with X-ARP 3100/10 (AllResist GMBH) and wet etching. First we carved larger measurements chambers in CaF_2 by wet etching, and then we defined the upper level of the device by patterning a second layer of photoresist.

2.1.1. X-ARP 3100/10 characterization

X-ARP 3100/10 is an experimental positive photoresist developed by AllResist GmbH. The resist was spun at thicknesses from 4 to 9 μm varying the spin rate from 6000 to 1000 rpm. Prebaking conditions were optimized in order to achieve the better aspect ratio of the structures and set at 105 °C for 2 min. Exposure doses were changed accordingly with resist thickness from 180 to 300 mJ/cm^2 (Karl-Suss MJB3). The development step was done in AR 200-26 developer for 90 s and the final rinse in DI water gave the release of the lithographed pattern (Fig. 2a).

2.1.2. Wet etching of calcium fluoride

For the wet etching process of CaF_2, a saturated solution of $NH_4Fe(SO_4)_2$ was employed, using a X-ARP 3100/10 pattern as mask. Due to the low chemical reactivity and the poor solubility of CaF_2 (0.0017 mg/100 mg in water at 20 °C, insoluble in most bases and acids, soluble in ammonia salts [6]), the etching at room temperature was very slow (100 nm/h) and of unsatisfactory quality. By increasing the temperature to 30 °C, enhancing both the reaction rate and the solubility of the products, we reached an etching rate of 500 nm/h and a better pattern transfer (fig. 2b). The whole etching process did not alter at all the IR transmittance of the substrate, even if no protective layer was added on the backside (Fig. 2c).

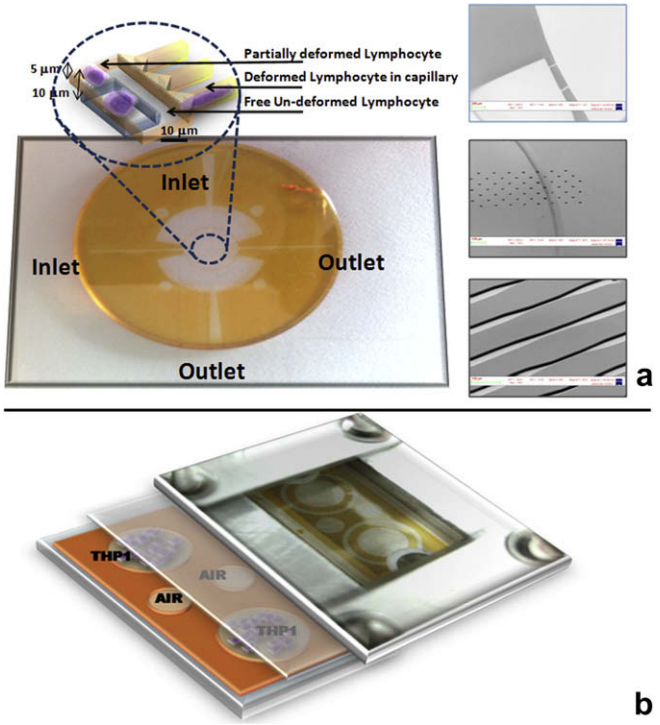

Fig. 1. The fabricated CaF_2 devices. (a) The final fluidic device. The large features, like inlet channels and reservoirs are 10 μm thick while small channels are 5 μm thick, 5–20 μm wide. In the insets: SEM images of the section variable channels realized by optical lithography and of the etched features. (b) Prototype liquid cell employed for the measurements with conventional IR source.

2.1.3. Fabrication of the prototype liquid cell

The prototype liquid cell we used for preliminary μ-FTIR measurements with conventional source, shown in Fig. 1b, was realized by photolithography of X-ARP 3100/10 spun on calcium fluoride. The device consists of two large wells for the confinement of the cells in liquid environment and two smaller holes for air background acquisition. Devices 5 and 9 μm thick have been realized and tested.

3. Measurements

3.1. Cell model

The human cell line THP1 (American Type Culture Collection, Rockville, Md.) [7], in-vitro established and displaying many monocytic characteristics, was employed. Cells 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), 100U/mL penicillin, 100 μg/mL streptomycin, in incubator 37 °C with 5% of CO_2.

RPMI 1640 was purchased from Biowhittaker. Synthetic fMLP (N-formyl-Met-Leu-Phe) was purchased from Sigma–Aldrich. FBS (Fetal Bovine Serum) was purchased from Hyclone.

Cell vitality in PBS buffer and NaCl 0.9% physiological solution, both suitable for FTIR studies, were determined by cytofluorimetric assays, and NaCl was chosen since it has no signals in the phosphate region of the IR spectrum.

3.2. Spectra acquisition

Experiments were carried out at the infrared beamline SISSI (Synchrotron Infrared Source for Spectroscopic and Imaging) at the Elettra Synchrotron Laboratory, Trieste, Italy [8] according to the following procedure. After being removed from the incubator, the cells were counted in a Bürker chamber and a vitality test with trypan blue was performed. The growth medium was removed by centrifugation (Eppendorf Microcentrifuge) at 1600 rpm for 5 min, and then substituted with NaCl 0.9% physiological solution; the procedure was repeated twice, ensuring the complete buffer exchange. The cells were then re-suspended in NaCl 0.9% physiological solution supplemented with glucose 5 μM. For the experiments, 1 μL of cell's suspension was dropped in the device and the chemical stimulation was induced by adding 1 μL of fMLP 1 μM.

FTIR transmission spectra were acquired using a Bruker Hyperion 3000 Vis–IR microscope mounting a mid-band HgCdTe detector, coupled with Bruker Vertex 70 interferometer. Both interferometer and microscope, sealed with an in-house designed box, were purged with nitrogen in order to reduce spectral contributions for environmental water vapour and carbon dioxide. Repeated spectra on THP1 cell groups were collected with globar source setting knife-edge apertures at 40 × 40 μm, using 15X Schwarzschild condenser and objective, co-adding 512 scans with a spectral resolution of 4 cm^{-1}, starting to collect data 30 min after the cells were dropped in the device. The delay time is imposed by the liquid cell assembling, the sample screening and the IR