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A new micro minimally invasive biopsy tool for molecular analysis

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***Abstract*-In this paper, we present a new micro minimally invasive biopsy tool adapted to proteomic mass spectrometry analysis. The concept is born from a multidisciplinary collaboration in the field of proteomics, cancer research and microtechnology.**
In mixing different skills, we have developed and manufactured a miniaturized biopsy tool using microtechnology techniques in order to minimize tissue damage. Dedicated chemically functionalized zones were added to the device in order to increase capture yield, and specificity during tissue apposition. Fields of application range are from cancer research to neurodegenerative diseases study.

I. INTRODUCTION

Today, clinical diagnostic and therapeutic monitoring techniques are evolving toward miniaturization.
Moreover, molecular profiling has been validated as an important complement to classical anatomopathology providing a more precise diagnosis and therapeutical response prediction.
However for relevant biomarkers discovery, biopsies are needed which can be difficult to obtained in particular for brain tissue.
Existing techniques and biopsy tools are all based on sampling pieces of tissue, with potential clinical side effects depending on the biopsy location. In the brain, some highly functional areas such as substancia nigra for example, can not even be investigated without deleterious effects.
Furthermore, in pathologies such as brain tumors, the peripheral or even surrounding healthy tissue can contain very relevant biomarkers predicting tumor recurrence and treatment efficiency. However, performing biopsies on healthy brain tissue using classical techniques is unthinkable and remain uninvestigated.
We present in this paper a miniaturized new minimally invasive tool particularly adapted to human brain tissue investigation providing a dramatic decrease in side effects. This new patented concept is called "affixing" and consists in simply pressing the sampling tool in contact with the tissue to be analyzed. During contact, the tissue remains intact while only infinitesimal quantity of biological molecules are gathered on the "imprint" tool, enough to be directly analyzed by laser desorption mass spectrometry.
Furthermore, specific chemical functionalization on the affixing surface allows direct analysis of proteins and peptides without the need of standard extraction procedure.

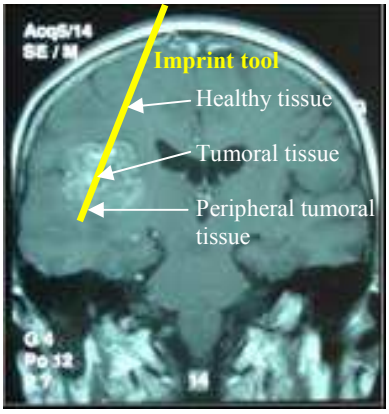

Fig. 1. Principle of axial cartography in a brain tumor

This affixing tool has several advantages: reduce sampling size, specific surface affinities for biomolecules (i.e. proteins) as well as the possibility of having a direct interface with laser desorption MS.

- ✓ The miniaturized imprint tool has a double surgical interest; reduced size and to the opportunity to have a sample with an axial cartography.

Imprinting tools are manufactured using microtechnology techniques, which provide high miniaturization therefore reducing collateral damages during surgery and opening the possibility of reaching delicate brain areas.
While imprinting a tumor, an axial cartography can be recovered (Fig.1) providing simultaneously on the same tool, an imprint of healthy, tumoral and peripheral tumoral tissues. Spatial analysis of the proteomic expression becomes possible.

- ✓ The imprint tool can be directly interfaced with the laser desorption mass spectrometry analyzer.

This aspect presents two advantages: on one hand, the experiments are simplified and on the other hand, loss of proteins and peptides are avoided for the usual sample preparation steps.
Simplification of the overall analytical process enhances reproducibility because of fewer manipulations steps, thus becoming less "operator dependent". Furthermore, this detection performed directly on the imprinted tool avoids damaging or even destroying some proteins or peptides, and saves precious time in particular during surgery.

Finally this tool is more sensitive because these preparation steps are avoided and because of its huge surface enabling the recovery of more proteins and especially the discovery of new proteins / biomarkers.

II. TECHNICAL ACHIEVEMENTS

Using microtechnology methods for manufacturing the silicon tool can provide high developed microstructured surfaces in order to compensate size reduction by increasing protein adsorption. Moreover, protein adsorption is somewhat selective since the microstructured surface has been chemically functionalized with different chemical affinity properties in order to preferentially capture certain class of proteins.
The imprint tool can be used directly as the laser target in a laser desorption mass spectrometer such as MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time Of Flight). The efficiency of the laser desorption/ionization on functionalized oxidized silicon has been demonstrated.

Microtechnologic manufacturing

Each tool is designed individually, as a chip in the microelectronic industry, and manufactured collectively on 100mm silicon wafers (Fig.5).
The manufacturing consists mainly in standard photolithography and Deep Reactive Ion Etching (DRIE) process running in the LETI MEMS clean rooms.
In order to obtain high developed surfaces, octagonal micro pillars were designed. They have 80µm diameter, 240µm pitch and are 50µm high (Fig. 3 and 4). These pillars represent a 50% surface increase compared to a smooth surface. This geometry has been optimized considering on one hand overall surface increase due to the pillars and on the other hand sufficient pillar spacing for correct matrix and protein crystallization very important for the laser desorption step.

A controlled thermal oxidation is carried out at the end of the process to cover the silicon with 500nm of silicon dioxide for chemical functionalization.
The tools are then diced, measuring each 300µm x 600µm x 2mm (Fig.6) and are ready for the chemical treatment.

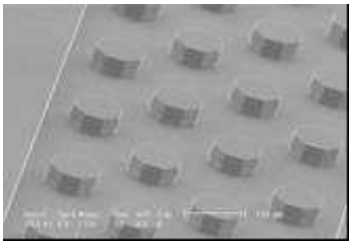

Fig. 3. Surface with micro pillars

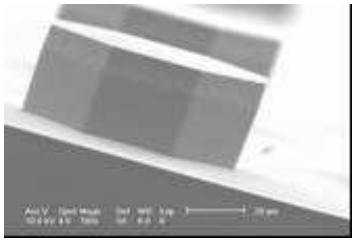

Fig.4. Detail of one micro pillar

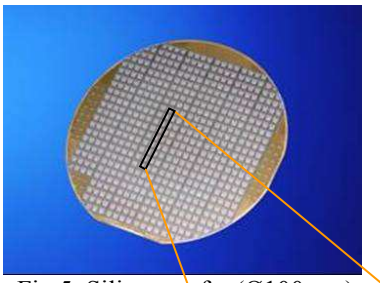

Fig.5. Silicon wafer (Ø100mm)

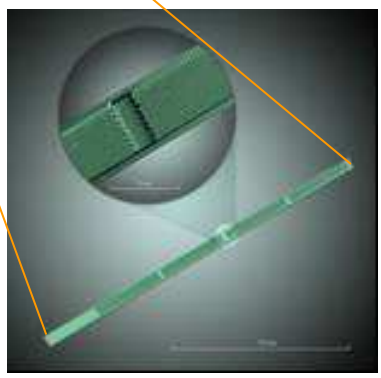

Fig.6. Individual chip

Chemical functionalization

The chemical functionalization process described bellow is used for efficient surface grafting of the tool.
First, the silicon oxide layer (500nm) was hydrated for 2 hours with a solution of NaOH 2,5N/Ethanol 40%/60% in volume and rinsed successively in water and ethanol under ultrasonic stirring in order to have hydrophilic silica surface (contact angle < 10°).
The grafting reaction is performed under an inert atmosphere thermo-regulated at 0 °C for 24h with a 10^{-2} M solution of silane dissolved in dry trichloroethylene. The tool is then successively rinsed with trichloroethylene, ethanol, and chloroform under ultrasonic stirring to remove the excess of silane and the physisorbed organosilane molecules.
Four main proprietary chemistries were developed: a hydrophobic surface directly obtained after grafting the silane and a hydrophilic, cationic and anionic surface obtained by performing one post-silanisation step.
Chemical surface modification is composed of a molecular monolayer, which allows for much more reproducible results compared to multilayer treatment such as polymer coatings. This reproducibility in capturing proteins is a very important parameter for biomarkers analysis.

Chemical functionalization and protein adsorption of the surface are illustrated on Fig.2.

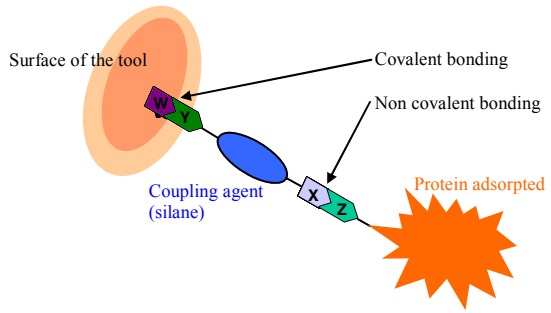

Fig. 2. Principle of the surface chemical functionalization and proteins adsorption

Experimental method

Experiments are carried out following three critical steps on brain tissue; first, validations on healthy mice, second, pre clinical "ex vivo" validations on human brain tumors obtained extemporaneously during surgery, and finally, human "in vivo" validations once the toxicology studies of our tool are performed.

Experimental set up is the following: The imprint tool is placed in contact ("affix") for about 30s on the tissue. After a washing step to remove unspecific biomolecules, a drop of sinapinic acid matrix (SPA) was added to the tool surface and let dried for laser desorption analysis. The imprinted tool is directly analyzed in the laser desorption mass spectrometer (SELDI-TOF ProteinChip® System PCS 4000, Ciphergen®). All "affixing" experiments were performed by the Inserm team.

Tool efficiency is assessed by the number and the intensity of peaks detected by MS.

III. Results

First, protein adsorption efficiency of chemically modified silicon chips was compared to classical surgical stainless steal.

Fig. 7 shows that peaks on the imprinted tool, especially for low mass proteins/peptides (<12000Da), are stronger, much more numerous and well-defined for smooth hydrophobic and anionic surfaces coated silicon (spectrums 3 and 4), as compared to two kind of surgical stainless steal (spectrums 1 and 2) used for biopsies.

Fig. 8 and Fig. 9, compare the mass spectra obtained on a micro pillar surface structuration to a smooth surface for respectively a hydrophobic and an anionic surface. Peaks are once again much more numerous, intense and well-defined for microstructured surfaces than on a smooth surface.

This clearly shows the importance of chemical functionalization and microstructuration on the protein adsorption yield, thus drastically enhancing the sensitivity and specificity of the MS analysis.

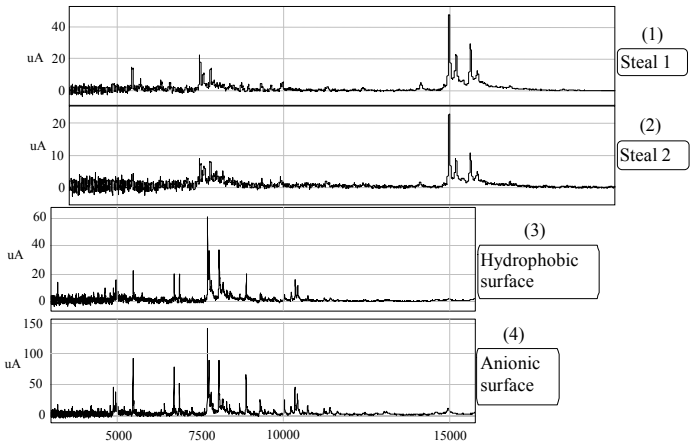

Fig.7. Mass spectrums for comparison of our tool to classical steal

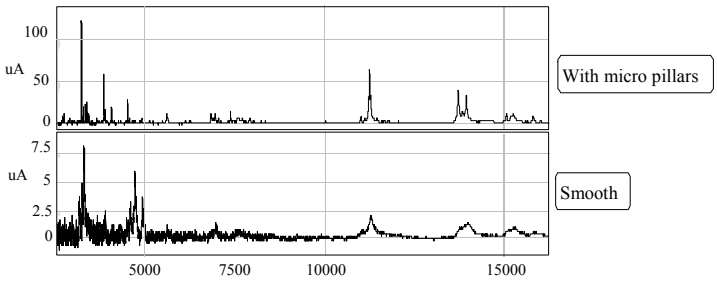

Fig.8. Mass spectrums for hydrophobic surfaces

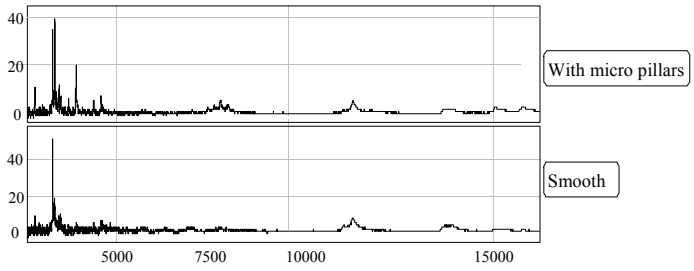

Fig.9. Mass spectrums for anionic surfaces

Complete analysis of all the "affixing" spectrums obtained on human glioma shows a huge difference in the number of detected peaks, with 32 peaks obtained on a microstructure surface versus 12 on a smooth surface for a hydrophobic surface.

IV. Conclusion

The proof of concept for the "affixing" principle has been demonstrated in the cancer research field, particularly for brain tumor. The imprinting tool has been validated extemporaneously "ex-vivo" on human cancerous brain tissues. This work validates an innovating diagnostic approach using molecular profiling obtained by direct fingerprint of human pathological tissue. These promising results comfort us in performing an "in vivo" validation on patients as soon as the toxicology results are obtained.

The developed tool can be used for other kind of cancers, such as lung or breast cancers. It can become an important mean targeted at diagnosis and treatment monitoring for cancer but also much wider applications.

The opportunity of molecular fingerprinting without tissue removal could have scientific interest in neurodegenerative pathologies investigations such as Parkinson or Alzheimer diseases. With this kind of tool, deep brain regions can be reached without damage enabling the discovery and the validation of new relevant biomarkers and providing useful understanding of diseases development. For example, on Parkinson patients during electrode implantation surgery, our tool could be used to make a biological "imprint" of regions involved in brain degeneration. This would provide biological data from fresh tissue which was impossible to obtain for such diseases so far.

The major advantages of this new tool are the lack of damage to surrounding tissues during the imprint and the optimized surface enabling greater sensitivity and specificity.

Acknowledgments

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References

[1] *Apparatus and methods for obtaining a molecular fingerprint*. Patent submission n° PCT/IB 2006/000064 January 16th, 2006. Priority US n° 60/643 592 January 14th, 2005
Depositor: INSERM, Inventors: A. Bouamrani, D. Ratel, A-L Benabid, J.-P. Issartel, F. Berger.

[2] *Dispositif de prélèvement moléculaire par contact*. EN 0550303, 02/02/2005.
Depositor: CEA, Inventors: P. Caillat, M.L. Cosnier, F. Martin.

[3] *Nouveaux composés silanes et leur utilisation pour fonctionnaliser des supports solides et immobiliser sur ces supports des molécules biologiques.* EN 0650360, 01/02/2006.
Depositor: CEA, Université de Montpellier II, Inventors: M. Granier, G. Lanneau, F. Martin.

[4] *Nouveaux composés silanes et leur utilisation pour fonctionnaliser des supports solides et immobiliser sur ces supports des molécules biologiques.* EN 0650361, 01/02/2006.
Depositor: CEA, Inventor: F. Martin.