TITLE:

Atomic Force Microscopy on a multiplexed SPRi chip: a rigorous methodology for a deep qualification of extracellular vesicles subsets

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SUMMARY:

We propose a new generation of multiparametric analytical platform with increased throughput for the characterization of extracellular vesicles (EVs) subsets. Our method is based on a combination of multiplexed biosensing method with metrological, morphomechanical analysis by AFM, coupled with Raman spectrometer, to qualify vesicular targets trapped on a microarray biochip.

ABSTRACT:

Extracellular vesicles (EVs) are membrane-derived tiny vesicles, produced by all cells, that range from 50 to several hundred of nanometers in diameter, and used as means of intercellular communication. They are emerging as promising diagnostic and therapeutic tools for a variety of diseases. There are two main biogenesis processes used by cells to produce EV, giving EVs presenting differences in size, composition and content. Due to their high complexity - in size, composition and cell origin -, their characterization requires a combination of analytical techniques. Our project consists in developing a new generation of multiparametric analytical platform with increased throughput for the characterization of subpopulations of extracellular vesicles (EVs). To reach this goal, we start from the nanobioanalytical platform (NBA) established in the group, which allows an original investigation of EVs based on a combination of multiplexed biosensing method with metrological and morphomechanical analysis by AFM of vesicular targets trapped on a microarray biochip. We aim to complete this EVs investigation with a phenotypic and molecular analysis by Tip Enhanced Raman spectroscopy (TERS). The objective is to propose a multimodal and easy to use analytical solution to reach a deep qualification of EVs subsets in biological fluids with clinical prospects.

INTRODUCTION:

The infatuation in EVs research, combined with the challenges this field faces, has resulted in the development and implementation of a large variety of approaches and techniques for quantifying or characterizing these vesicles. None of these techniques, on their own, however, gives all the information allowing to characterize EVs subsets. The inherent heterogeneity of EVs, the diversity

of their biochemical & physical properties, prevents to get global analyses that are reliable and reproducible, especially for EVs contained in a mixture (crude sample). Detection and characterization methods EVs are therefore needed, individually and generally used systematically, in complement of the other methods, faster, but not selective [1].

High-resolution imaging, by transmission electron microscopy (TEM and cryoTEM) or atomic force microscopy (AFM), allows to determine the morphology and metrology of EVs with a nanometric resolution [2-7]. However, the main limitation of the use of electron microscopy for biological objects, such as EVs, reside in the need for a vacuum to carry out the exploration, which requires fixation and dehydrating the sample. Such preparation makes it difficult to transpose from the structures observed to the in solution EVs morphology. In order to avoid this dehydration of the sample, the technique of cryoTEM is the most widely used for EV characterization [8]. It is widely used for determine the ultrastructure of EVs. Immunolabelling of vesicles by biofunctionalized gold nanoparticles also make it possible to identify specific sub-populations of EVs and discriminate against them from others particles present in a complex biological sample. However, the low number of EVs analyzed by electronic microscopy, often makes difficult a characterization that is representative of a complex and heterogeneous sample.

To reveal this size heterogeneity, minimal information initiative for studies of extracellular vesicles of ISEV international community suggests analyzing a sufficient number of images of wide field, accompanied by more surface images reduced, revealing with high resolution EVs Individual [9].

Atomic force microscopy (AFM) occurs as an alternative to optical approaches and electronic diffraction techniques for the study of EVs. This technique uses a sharp tip hold by a flexible cantilever that scans the sample deposited on one support, line by line, adjusting the distance between the tip and the elements present, thanks to a feedback loop. It makes it possible to characterize the topography of the sample and to collect morphomechanical information [10-13]. The EVs can be scanned by AFM, either after being deposited on an atomically flat substrate, or after have been captured on a specific substrate functionalized by antibodies, peptides or aptamers, to characterize the various subpopulations [13-14]. By its ability to quantify and to simultaneously probe the structure, the biomechanics and the biomolecular content of EVs within complex biological samples, without the need for pre-treatment, labelling, or dehydration, the AFM is now increasingly used to characterize EVs in a fine and multiparametric way, under physiological conditions of temperature and medium.

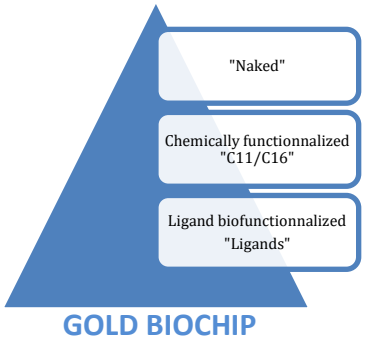
We propose here a methodology using a central gold biochip, with its capability to be (bio)chemically functionalized in a multiplexed format. This substrate consists in the corner stone of a powerful analytical platform combining biodetection of EVs subsets by surface plasmon resonance and atomic force microscopy metrology and morphomechanical characterization. This paper will show readers that combination of powerful technics assisted by a highly rigorous methodology in substrate preparation and data acquisition, makes the EVs analysis deep, resolve and robust.

PROTOCOL:

The principle of our approach is to prepare a gold substrate, to adsorb/graft or capture EVs subtypes, for their characterization in terms of phenotypes, size and morphology. This substrate can indeed present 3 types of interfaces, of growing complexity : naked, chemically functionalized or ligand microarrays.

Before describing the different steps of the protocol, here is presented schematically in figure 1 our NBA approach, coupling SPRi, AFM and Spectroscopy.

[Here Figure 1]



The central gold biochip constitutes the heart of our platform since all the label-free characterization technics are realized on it. Function of the need in EVs characterization (either global/total EVs or EVs subsets) and function of the limitation/demand of the methods used, we propose to work on 3 types of gold biochip surfaces : either naked, or chemically functionalized or biochemically (antibody, ligand, receptor, …) biofunctionnalized gold surface.

All the details/procedure is given below, but we propose here to present for which reasons and applications 3 types of biochip are generated:

Naked biochip: it enables the simple adsorption of EVs on gold : it is possible to play with the buffer used and to realize this adsorption either in a passive way (incubation then rinsing steps), or under flow (in SPRi). Moreover, this adsorption in passive way can be realized either on the whole chip (as a macroarray), or localized in microarrays thanks to a micropipette spotter. The “under flow procedure”, allows to follow the kinetics and the level of EVs adsorption. This approach on naked gold substrate is engaged when the chemical layer interface may interfere with the analytical method (i.e. for Raman spectroscopy in our case).

Chemically functionnalized biochip: the chemically functionalized biochip is used to create a dense “carpet” of EVs covalently bound on the gold surface, when the objective is to have a global view of the EVs sample. Indeed, in this case, the gold is functionalized by a thiolate mixture, and part of the thiolates are chemically activated to establish covalent binding with targets. Again, this strategy can be realized either in a passive way (incubation then rinsing steps / either in “macroarray” or in multiple microarrays thanks to a micropipette spotter), or under flow (in SPRi) to follow the kinetics and level of EVs grafting on the gold surface.

Ligand biofunctionnalized biochip: the chemically functionalized biochip is here also chemically activated but to covalently graft different ligands (antibodies, receptors, …), in order to capture

by affinity and selectively different EVs subsets coexisting in the biological sample.

1. Gold substrate preparation

The three types of surface are produced on Gold chip: 1) naked surface, 2) chemically functionalized C11/C16, and 3) biofunctionalized (ligands grafted on C11C16 layer). We will call them “naked”, “C11C16”, and “ligands” respectively from now onwards.

Gold Substrate preparation: The gold biochips were manufactured in-house in the clean room (at the “MIMENTO” Technology Center, Besançon, France). The home-made biochips are composed of glass slides (SF11) with the coating of chromium (2nm Cr) and gold (48nm Au). The coating was done by Physical Vapor Deposition (PVD) with the help of Plassys DC magnetron sputtering. The length of the biochip was 28 mm, width 12.5 mm, and thickness 0.5mm [15].

Chemical functionalization: the naked chips were functionalized by incubating overnight in the mixture of mercapto-1-undecanol (11-MUOH : “C11”) & mercapto-1-hexadecanoic acid (16-MHA “C16”) 90%/10% by mole respectively reaching 1 mM in absolute ethanol, under agitation at room temperature. This step will form a stable Self Assembled Monolayer (SAM), which is useful in grafting of ligands. The biochips were cleaned with absolute ethanol and ultrapure water, then subsequently dried and stored under clean-room conditions.

Activation and EVs or ligand grafting on chemically functionalized biochip : the biochip was cleaned with ultra-pure water and then to activate C16 carboxylic groups, it was incubated in the mixture of 200 mmol/L EDC and 50 mmol/L Sulfo-NHS for at least 30minutes before the experiments.

To immobilize EVs or ligands on the chip, after EDC-NHS activation, we then go either, in passive way to get a macroarray (through one single drop) or a microarrays format (thanks to a micropipette spotter), or under flow, to graft on the whole surface the EVs or ligands. This constitutes our EVs or “ligands'' modified chips.

[Here Figure 2]

For ligand grafting, the molecules were diluted at 200µg/ml, in their optimal pH solution. The optimum pH for grafting antibodies was determined previously by pre-concentration experiments done on SPR-Biacore 3000 instrument from GE Healthcare/now Cytiva life sciences. It should also be noted that the grafting conditions will change with clones of antibodies that we are using, it is therefore recommended to determine the conditions before moving to SPRi experiments.

After activation of the chip, 300 nl of EVs/ligand solution was added by using spotter as shown in the figure 2.

Note: A piece of paper submerged in water should be kept in the both left and right wells to avoid the evaporation of droplets. This step is important to maintain the EVs/ligands in the optimum condition for their stability and functionality.

After spotting, the biochip was kept under a sonic bath (frequency 37 kHz, and power 30%) for 30 min incubation.
The biochip was washed from top with ultra-pure water and then was placed on a prism, which is of the same refractivity index (RI) as the biochip.
While adjusting the biochip on the top of the prism, a droplet (~ 2.3 µl) of oil was used which has the same RI as that of the prism to create an uniform thin layer between the biochip and the prism. This step was done to have a continuous medium of the same RI in the optical path.

Note: It is important to avoid incorporating any bubble in the oil layer at this step, as it will change the optical properties in the path and will hinder further analysis.

2. Surface Plasmon Resonance imaging

SPRi Plex II from Horiba Scientific was used for this experiment. The biochip was mounted on the SPRi-plex system. The flow rate of the buffer was kept at 50 µl/min.

Note: In case of any bubbles, increase the flow rate up to 500-1000 µl/min; also inject Octyl Glucoside (OG) 40 mM frequently to remove them as soon as possible.

Conditioning of the gold biochip: Afterwards CCD image acquisition was performed to define the Region of Interest (ROI) as well as the ligand families (when multiplexed biochips is used).
The plasmon curves were obtained. In the case of a passivating step (thanks to albumin) performed inside the apparatus, we recommend selecting a working angle to get the optimum sensitivity towards the surface, thus establishing a quality control of the surface reactivity.

Note: this passivation step is important when the chip is prepared for affinity/capture biodetection, in order to reduce non-specific interaction between the sample and biochip surface.

The kinetic monitoring was chosen. The RSA (200 µg/ml, prepared in Acetate buffer pH 4.5) was injected at 50 µl/min for 4 min, which was followed by the injection of Ethanolamine (1M) at 20 µl/min for 10 min, to deactivate the carboxylic groups still present and reactive on the surface. The biochip was then washed by injecting OG 40mM at 50 µl/min for 4min.

Sample Injection: the Plasmon curves were redefined after passivation and the working angle was chosen according to the ligand this time. In kinetic monitoring, we reduced the flow rate to

20 µl/min and waited for the baseline to be stable. The sample was injected at a concentration of choice generally for 10 min, the kinetics of interaction is followed and the reflectivity variation was measured at the end of injection.
The different samples that were injected will be described in the results section.

After sample injection : there are 2 ways to finish the SPRi experiment :
1st way "unfixed/in liquid": we take out the biochip from the SPRi apparatus, maintaining a liquid drop on it and go for further AFM characterization of the surface in liquid
2nd way "fixed": glutaraldehyde (0.5%) diluted in water was injected at 20 µl/min for 10 min to fix the objects captured on the biochip. Then water is injected to rinse the surface and the biochip is taken out and washed very gently with distilled water and then air dried to be further analyzed under AFM.

3. Atomic Force Microscopy (AFM)

Bruker-JPK NanoWizard® 3 Bioscience AFM was used for AFM characterization. Contact mode was used to scan the biochip, in air. Quantitative Imaging mode is used to scan the biochip in liquid conditions. To identify the position of the respective microspots on the biochip, a glass slide with a mask was used. The biochip was then aligned on the top of this mask as shown in the below picture.

[Here Figure 3]

Positionning of the tip: Using the CCD camera on top of the AFM was used to localize the cantilever on the correct spot that we want to scan. We used triangular shaped cantilever with 200 µm length, 28 µm width, and a spring constant of 0.08 N/m. The laser was then aligned on the top of the cantilever, at a position to have an optimum response in the feedback control mechanism.

Scanning: Once engaged and in contact on the biochip surface, the AFM acquisition started, in contact or in quantitative imaging mode, from 3 to 5 big area (typically 10 x 10 µm²) to small area (1 x 1 µm²). The aim is a) to get an AFM characterization that is representative of the whole mm² spot, and 2) to visualize enough EVs and at a good resolution to have a robust analysis (a minimum of 300 EVs counted and analyzed for each condition), to make metrology and morphology measurements.
These AFM images were then further processed with JPK Data processing software, and treated thanks to Gwyddion or Mountains SPIP ones.
AFM image treatment:
The AFM images were treated with JPK Data processing software first, the height channel was selected. A polynomial fit was chosen to be subtracted from each line to obtain straightened scan

lines. These images were then further analyzed by Gwyddion SPM data processing software to obtain the size (height, diameter and calculated effective diameter) and number of EVs. The height threshold was selected on gold grains, to eliminate the roughness of the surface. Usually our rough gold substrate (RMS around 3 nm) and the presence of the chemical and the ligand layers bring us to choose a threshold at 8.5 nm.

The result of this AFM characterization consists in a) counting the EVs number present on each AFM image of 3x3µm² and for the 10 images collected on each spot), and b) in measuring their size (measured diameter & measured height giving a calculated effective diameter). To reach that, we generate graphs showing the size (measured height, measured diameter and calculated effective diameter) of EVs, with each particles counted represented by a dot.
An approach of estimating the effective diameter based on the calculation of the effective volume of the object is adopted as reported in the literature [3]. The "effective diameter" was calculated in two steps. In Gwyddion software, with the grain extraction module, the grains were marked by a height threshold value. Then several properties of the marked grains such as height, diameter were extracted. In the second step, with these values, the volume of the grains was estimated. Then an "effective diameter" equivalent to a sphere of the same volume was derived.

[Here Figure 4]

Thus, at the end of our characterization, the NBA platform enables to correlate the biodetection signal, and then the phenotyping, to a number and the size of EVs subsets.

REPRESENTATIVE RESULTS:

Determination of the optimum pH conditions for ligand grafting

The different ligands used to prepare the biochips are tested function of the pH and their availability to interact with the thiolates chemical layer. For that, the ligands are diluted in acetate buffer at different pH and injected on the biochip chemically functionalized with C11C16 layer. The solutions are injected randomly on the surface, and a detergent (octylglucoside at 40mM) is injected after each ligand injection to recover the baseline. This "pre-concentration" test allows to determine the optimal for each ligand grafting. In the example presented in the figure 5, pH6 was selected as the better pH for this ligand grafting.

[Here Figure 5]

SPRi CCD images

The SPRI CCD images registered on biochip (naked, chemically functionalized or presenting microarrays) once inserted in the SPRi machine, are presented in figure 4. In the case of microarrays presenting biochip, the image is taken after albumin passivation of the surface. Duplicate or triplicate of spots are systematically realized on the chip, and a negative control is automatically also present on the chip. The negative control consists in a irrelevant antibody most of the time.
In SPRi, when the biochip is used without spotting - for adsorption on naked gold or for grafting

directly EVs on chemical functionalized biochip -, ROI are chosen arbitrarily in the sensing area. As an example in figure 6C are represented ROI chosen on a naked gold chip before injection of EVs.

[Here Figure 6]

Thanks to this SPRi CCD image, we have then the possibility to ignore certain spots if such mentioned problems appeared during the grafting. The SPRi CCD image allows also to estimate the homogeneity of the grafting inside the spot, the reproducibility of grafting between the different spots of a same ligand, and finally to ensure that EVs biodetection will be proceed on equivalent arrays in terms of surface density.

SPRi results

When ROI are selected, the baseline is stable, then the sample can be injected. The figure 7 shows different results, obtained on a multiplexed biochip revealing a high signal-to-noise ratio, for certain immunoarrays (the reflectivity signal being xx compared to the response on negative control that is xx). In figure 7B is presented the result in EVs adsorption, obtained after injection of EVs sample on a naked gold chip.

[Here Figure 7]

AFM characterization

After SPRi experiments and EVs loading on biochip (either by adsorption, grafting or affinity capture), AFM is engaged, following the methodology to scan firstly big scans (10x10µm²) then further (around 10 at least) smaller ones (few µm²). Figure 8 shows example of large and small scale AFM images of EVs on biochips.

[Here Figure 8]

Gwyddion analysis

Gwyddion software was used to process the data for each vesicles visualized on every batches of AFM images. Measured height and Diameter of each EVs were determined, from which the effective diameter was obtained.

In the software we have first to select “data treatment”, then “Grains”, then “Label with threshold”, then the threshold value has to be adjusted. We determined that 8.5nm was a good one. Then the grains have to be ”filtered”, and the “number of grains” appears. In “grain distribution” the height volume and diameter of grains have to be selected. Thus, a table presenting 3 columns with Height, Volume and Diameter values for all the grains detected at this threshold, per image, is obtained, in txt format.

FIGURE AND TABLE LEGENDS:

Figure 1 : the NanoBioAnalytical (NBA) Platform combining Surface Plasmon Resonance imaging (SPRi) (A), Atomic Force Microscopy (AFM) (C) and infrared/Raman (nano)spectroscopy, all engaged on the same substrate, a multiplexed gold chip (B).

Figure 2: Gold biochip (left), micropipette spotter (middle) and the biochip after spotting with ligands droplets of 300 nl each (right).

Figure 3: Biochip characterization by AFM. After SPRi experiment, the chip is either fixed and dried or maintained in liquid for AFM characterization. (A) The glass slide machined (with 2 perpendicular positioning wedges, indicated with a "w" on the picture) and presenting a mask fitting with the localization of the biochip 16 microarrays. By light exposure and transparency, it enables, once installed for the AFM characterization, to place the AFM tip on the desired spot to characterize. (B) The biochip installed on the "mask" slide and under a drop of buffer, to scan in liquid conditions. (C) SPRi image of the 16 microarrays. (D) One microarray imaged by optical microscopy after immunocapture of biofunctionnalized calibration nanoparticles of 920nm in diameter. The white squares indicate the sampling of the different areas scanned by AFM into each spots of interest to make the AFM characterization robust.

Figure 4: Results generated by the AFM characterization of EVs on a biochip. (A) Metrology of EVs on one spot (antiCD41 immunoarray), determined with a threshold of 8.5nm and after injection of EVs sample at 10e8/ml. From the top to the bottom are presented measured particles diameter, effective calculated diameter and height. (B) Histogram generated from the data in (A), showing the distribution of EVs in effective diameter. Results obtained in air (in red: sample fixed and dried) and in liquid (in blue : unfixed).

Figure 5: Pre-concentration tests led in Biacore 3000 SPR apparatus to determine the optimal pH for ligand grafting. The sensorgram present the level of interaction function of time of one ligand injected randomly (in pH) at the same concentration and during 2 min on the surface. OG is the detergent allowing to recover the baseline between each injection.

Figure 6: SPRi CCD image of the biochip. (A & B) Multiplexed biochip, after albumin passivation. In (A) a chip with no default ; in (B) some defaults that can appear on the chip : fusion of spots (i), weak grafting (ii) or dusts or "contaminants" (iii). The ROI, in color in the spots (one color per ligand family), are choosen avoiding those "contaminants". When spots fusionned, we notify it and the spots are either ignored or named as "mixture of ligands 1 & 2". (C) Naked gold chip without microarrays, for experiment consisting in adsorption of EVs on gold. The blue arrow indicates the flow direction. This chip does not present spots, and ROI are chosen to register the reflectivity signal from the line 1 (L1, red circles) to the line 4 (L4, purple circles) during the sample injection.

Figure 7: SPRi experiments of EVs injection on biochip. (A) Capture experiment on multiplexed biochip, showing reflectivity signal on different ligands. Here the signal-to-noise ratio for the different ligand is really good (and especially on antiCD41 spots) since the response on the negative control is negligible. (B) Adsorption experiment of EVs on naked biochip. Sensorgram presenting the reflectivity of the chip after the EVs sample injection (4), and after conditioning

the chip with 2 flushes of buffer (1 & 2) and OG cleaning (3). On this biochip there is no negative control, but the reflectivity signal (its kinetics, its stability after injection) is high, meaning that those EVs are able to adsorb and stay on gold chip.

Figure 8: AFM characterization of EVs on biochip (here images obtained in contact mode on dried sample). (A) One example of large scanned area to have a representative view of the mm² ROI on chip. This result was obtained after covalent grafting of EVs on chemically modified surface. (B) Another example of large scanned area obtained after capture of EVs on immunoarrays. (C) A closer view of the objects to get high resolution and enabling the metrology (in height & diameter) of EVs. (D) A closer view of the biochip given the image in (A), with a 3D tilted image. (E) A zoom on one large EVs adsorbed on naked gold biochip, on a tilted 3D image. Z scale is 30 nm and 20 nm for A, B and C images respectively.

DISCUSSION:

The NBA platform, combining biodetection of EVs on multiplexed gold substrate and AFM characterization, enables a deep qualification of nanovesicles present in complex biological fluids. Indeed, thanks to the selectivity of the biochip, crude samples can be injected, and the vesicle contribution to be highlighted.

Our approach consists then to characterize EVs adsorbed/grafted or captured by affinity, on a rough gold substrate. AFM, that is conventionally used on atomically flat substrate, shows here all its performance on rough gold chip, presenting different sort of interfaces and being micro/nanostructured.

Also, the multiplexed biochip presenting several spots to scan (until 16 up to now), we have been developing our NBA approach on fixed and dried sample due to the time needed for a deep & highly resolved AFM investigation of each spot. Different tests realized in the lab showed that this procedure (fixation, drying and imaging in air) does not impact significantly the metrology of EVs.

There are two main critical steps in our approach: a compromise to select the chip sensitivity for all the different grafted ligand, and the EVs dissociation from the substrate at the end after injection.

Indeed, we use a SPRi machine working with one single angle of resonance, meaning that we have to select the better compromise in plasmon response on all the ligands spots ; for some ligands the detection will be really sensitive (close to the angle), for others less sensitive if the fixed angle value is farer than the others.

The second critical step is the dissociation of EVs from the substrate, after injection. If the dissociation is too high (because EVs present, for example, only few specific proteins at their surface), we won't be able to scan them afterwards. These situations are really rare, but when it happens, we worked at different flow rate and injection duration, to work around the problem.

The limitation of our method could rely in the representability of the AFM observations compared to the mm² area of EVs interaction on the biochip. For that we have absolutely to scan different areas on the spot, from big to small ones and at different places inside the spot. We

even try to scan in the ROI area, for the AFM characterization to be as much as possible correlated to SPRi response.

This coupled & label-free approach, assisted by a rigorous methodology in the preparation of the biochip and in the AFM acquisition mode, constitutes a method of choice to characterize EVs in sample even complex and crude. Thus, no preanalytical steps on the sample, no labelling or exalting procedure are needed in our method, while EVs subsets are deeply characterized. These different elements place our NBA platform as a very relevant method for deep and resolved analysis of EVs directly in crude sample.

Our recent developments consists in improving the sensitivity in biodetection and the discrimination between EVs subsets coexisting in a sample, that potentially co-interact on the same arrays. To increase the sensitivity, we are switching currently from the SPRiPlex instrument to a XelPleX one, that enables to select several angles for EVs detection on the different spots. To improve the discrimination of EVs subsets, we are facing different challenges : a) getting the infrared & raman signature of each EVs subsets on biochip, b) increasing the number of spots realized on the biochip (from 16 to 100) and c) increasing the throughput of analysis thanks to high-speed AFM. Moreover, HS AFM will then allow also to characterize EVs subsets, rapidly, and in liquid condition.

ACKNOWLEDGMENTS:
The work was supported by the region Bourgogne Franche Comté and the EUR EIPHI graduate school (NOVICE project, 2021-2024). Part of this work was done in RENATECH clean room facilities.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

[1] W Boireau, C Elie-Caille, Extracellular vesicles: Definition, isolation and characterization Medecine Sciences: M/S, 2021, 37 (12), 1092-1100

[2] Brisson AR, Tan S, Linares R, et al. Extracellular vesicles from activated platelets: a semiquantitative cryo-electron microscopy and immuno-gold labeling study. Platelets (2017), 28(3) : 263-71.

[3] Yuana Y, Oosterkamp TH, Bahatyrova S, et al. Atomic force microscopy: A novel approach to the detection of nanosized blood microparticles. Journal of Thrombosis and Haemostasis. (2010), 8(2):315-323. doi:10.1111/j.1538-7836.2009.03654.x

[4] Sebaihi N, de Boeck B, Yuana Y, Nieuwland R, Pétry J. Dimensional characterization of extracellular vesicles using atomic force microscopy. Measurement Science and Technology. (2017);28(3):034006. doi:10.1088/1361-6501/28/3/034006

[5] Beekman P, Enciso-Martinez A, Rho HS, et al. Immuno-capture of extracellular vesicles for individual multi-modal characterization using AFM, SEM and Raman spectroscopy. Lab on a Chip. (2019);19(15):2526-2536. doi:10.1039/c9lc00081j

[6] Malenica M, Vukomanovi´c M, Kurtjak M, et al. Perspectives of Microscopy Methods for Morphology Characterisation of Extracellular Vesicles from Human Biofluids. Biomedicines (2021), 9(6) : 603.

[7] Verweij, FJ; Balaj, L, ... Van Niel, G. The power of imaging to understand extracellular vesicle biology in vivo, (2021) NATURE METHODS 18 (9), pp.1013-1026

[8] Théry C, …Boireau W, … ELIE-CAILLE C., … and Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines, Journal of Extracellular Vesicles (2018) VOL. 8, 1535750

[9] Obeid S., Sung, P-S., Le Roy B., Chou M-L., Shieh S-L., ELIE-CAILLE C., Burnouf T., Boireau W. NanoBioAnalytical characterization of extracellular vesicles in 75-nm nanofiltered human plasma for transfusion: a tool to improve transfusion safety. Nanomedicine: Nanotechnology, Biology, and Medicine (2019) pii: S1549-9634(19)30061-9.

[10] Obeid S, Ceroi A, Mourey G, Saas P., Elie-Caille C., Boireau W. Development of a NanoBioAnalytical platform for «on-chip» qualification and quantification of platelet-derived microparticles. Biosens Bioelectron. (2017), 93:250-259

[11] Ridolfi, A; Brucale, M; (...); Valle, F, AFM-Based High-Throughput Nanomechanical Screening of Single Extracellular Vesicles (2020), Analytical Chemistry, 92 (15), pp.10274-10282.

[12] Vorselen, D; van Dommelen, SM; (...); Roos, WH, The fluid membrane determines mechanics of erythrocyte extracellular vesicles and is softened in hereditary spherocytosis (2018), Nature Communications, 9, 4960, DOI 10.1038/s41467-018-07445-x

[13] Hardij J, Cecchet F, Berquand A, et al. Characterisation of tissue factor bearing extracellular vesicles with AFM : comparison of air-tapping-mode AFM and liquid Peak Force AFM. J Extracell Vesicles (2013) ; 2 : 21045.

[14] Jorgensen M, Bæk R, Pedersen S, et al. Extracellular Vesicle (EV) Array: microarray capturing of exosomes and other extracellular vesicles for multiplexed phenotyping. J Extracell Vesicles (2013) ; 2 : 20920

[15] Remy-Martin F, el Osta M, Lucchi G, et al. Surface plasmon resonance imaging in arrays coupled with mass spectrometry (SUPRA-MS): Proof of concept of on-chip characterization of a potential breast cancer marker in human plasma. Analytical and Bioanalytical Chemistry

508 (2012);404(2):423-432. doi:10.1007/s00216-012-6130-4
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Figure 1

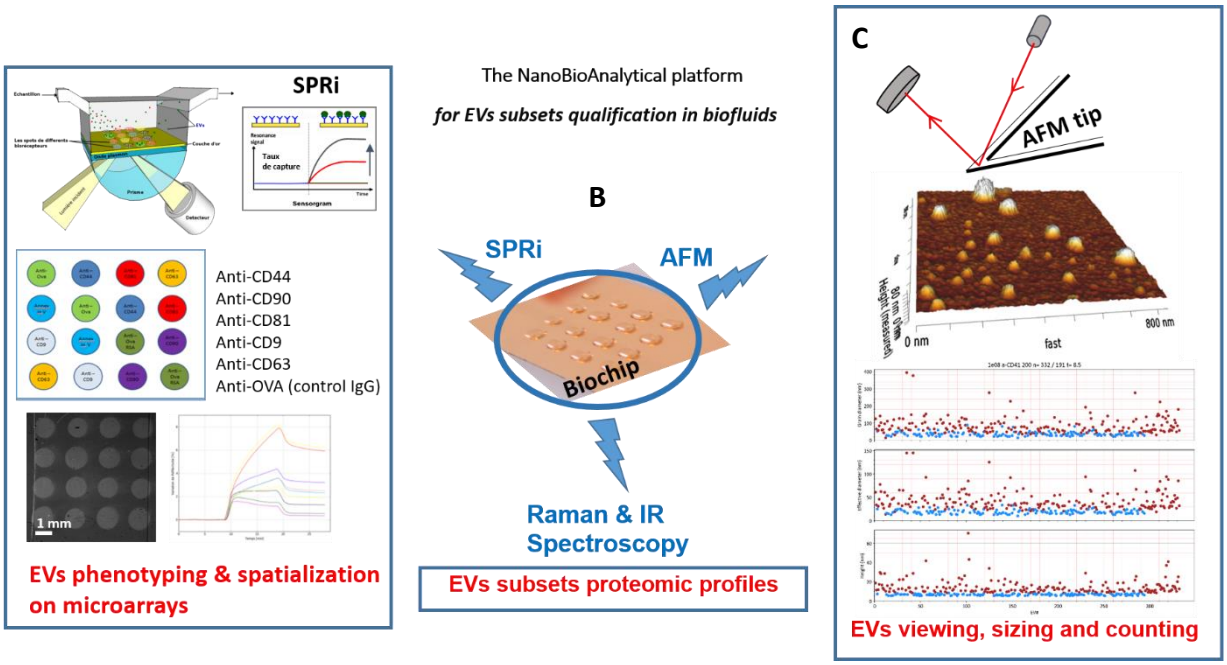

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Figure 2

Figure 2: Gold biochip (left), micropipette spotter (middle) and the biochip after spotting with ligands droplets of 300 nl each (right).

Figure 3

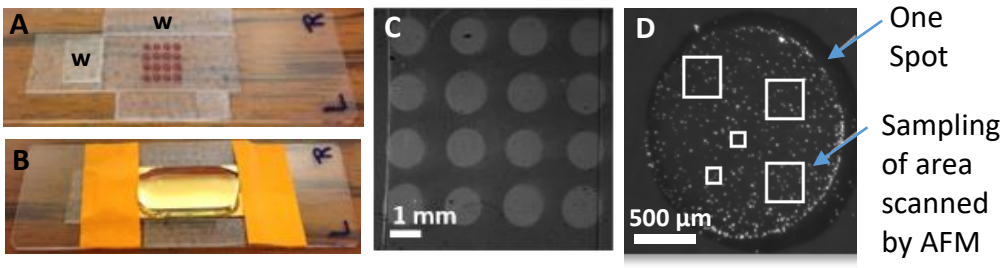

Figure 3: Biochip characterization by AFM. After SPRi experiment, the chip is either fixed and dried or maintained in liquid for AFM characterization. (A) The glass slide machined (with 2 perpendicular positioning wedges, indicated with a "w" on the picture) and presenting a mask fitting with the localization of the biochip 16 microarrays. By light exposure and transparency, it enables, once installed for the AFM characterization, to place the AFM tip on the desired spot to characterize. (B) The biochip installed on the "mask" slide and under a drop of buffer, to scan in liquid conditions. (C) SPRi image of the 16 microarrays. (D) One microarray imaged by optical microscopy after immunocapture of biofunctionnalized calibration nanoparticles of 920nm in diameter. The white squares indicate the sampling of the different areas scanned by AFM into each spots of interest to make the AFM characterization robust.

Figure 4

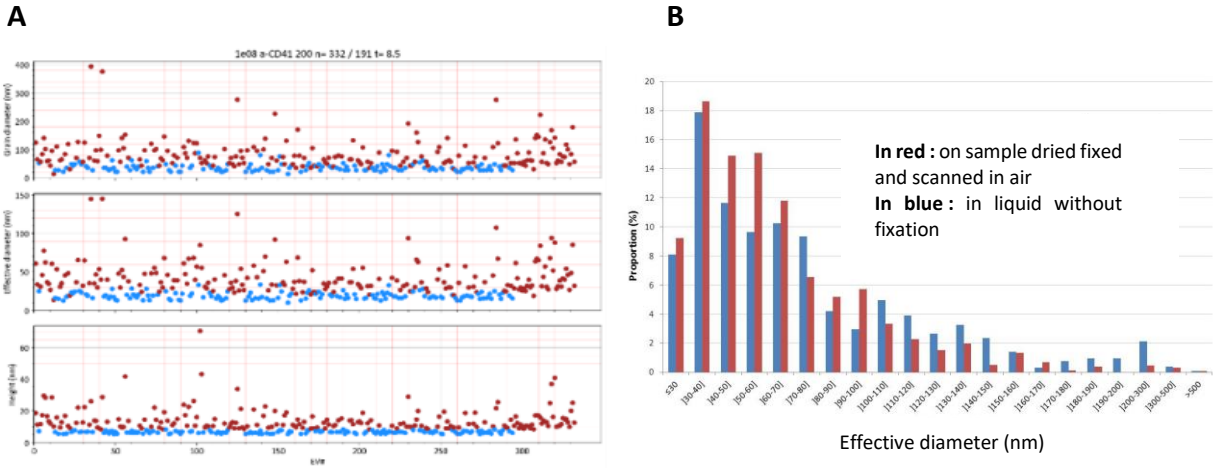

Figure 4: Results generated by the AFM characterization of EVs on a biochip. (A) Metrology of EVs on one spot (antiCD41 immunoarray), determined with a threshold of 8.5nm and after injection of EVs sample at 10e8/ml. From the top to the bottom are presented measured particles diameter, effective calculated diameter and height. (B) Histogram generated from the data in (A), showing the distribution of EVs in effective diameter. Results obtained in air (in red: sample fixed and dried) and in liquid (in blue : unfixed).

Figure 5

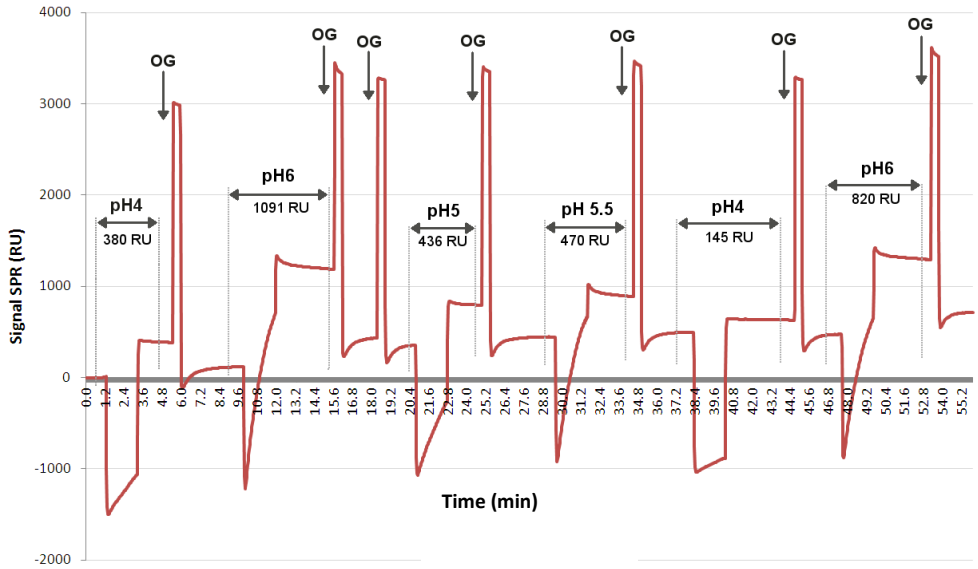

Figure 5 : Pre-concentration tests led in Biacore 3000 SPR apparatus to determine the optimal pH for ligand grafting. The sensorgram present the level of interaction function of time of one ligand injected randomly (in pH) at the same concentration and during 2 min on the surface. OG is the detergent allowing to recover the baseline between each injection.

Figure 6

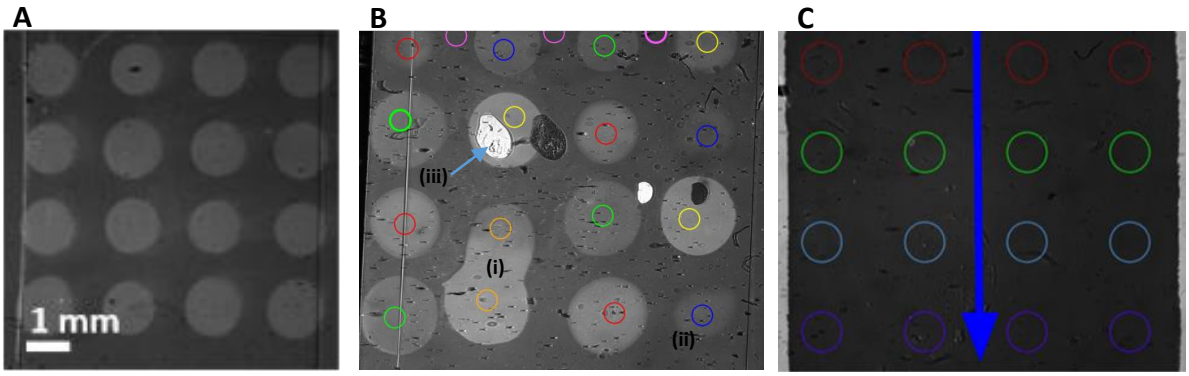

Figure 6 : SPRi CCD image of the biochip. (A & B) Multiplexed biochip, after albumin passivation. In (A) a chip with no default ; in (B) some defaults that can appear on the chip : fusion of spots (i), weak grafting (ii) or dusts or “contaminants” (iii). The ROI, in color in the spots (one color per ligand family), are choosen avoiding those “contaminants”. When spots fusionned, we notify it and the spots are either ignored or named as “mixture of ligands 1 & 2”. (C) Naked gold chip without microarrays, for experiment consisting in adsorption of EVs on gold. The blue arrow indicates the flow direction. This chip does not present spots, and ROI are chosen to register the reflectivity signal from the line 1 (L1, red circles) to the line 4 (L4, purple circles) during the sample injection.

Figure 7

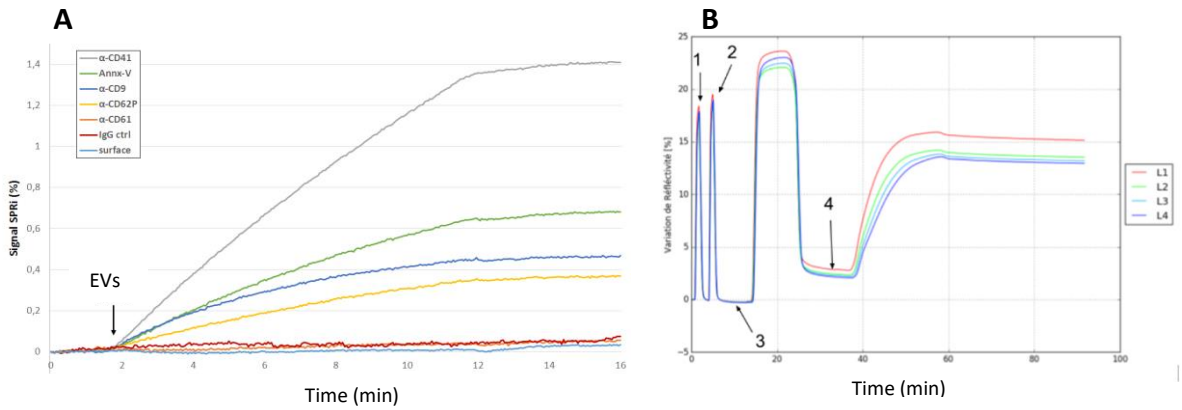

Figure 7: SPRi experiments of EVs injection on biochip. (A) Capture experiment on multiplexed biochip, showing reflectivity signal on different ligands. Here the signal-to-noise ratio for the different ligand is really good (and especially on antiCD41 spots) since the response on the negative control is negligible. (B) Adsorption experiment of EVs on naked biochip. Sensorgram presenting the reflectivity of the chip after the EVs sample injection (4), and after conditioning the chip with 2 flushes of buffer (1 & 2) and OG cleaning (3). On this biochip there is no negative control, but the reflectivity signal (its kinetics, its stability after injection) is high, meaning that those EVs are able to adsorb and stay on gold chip.

Figure 8

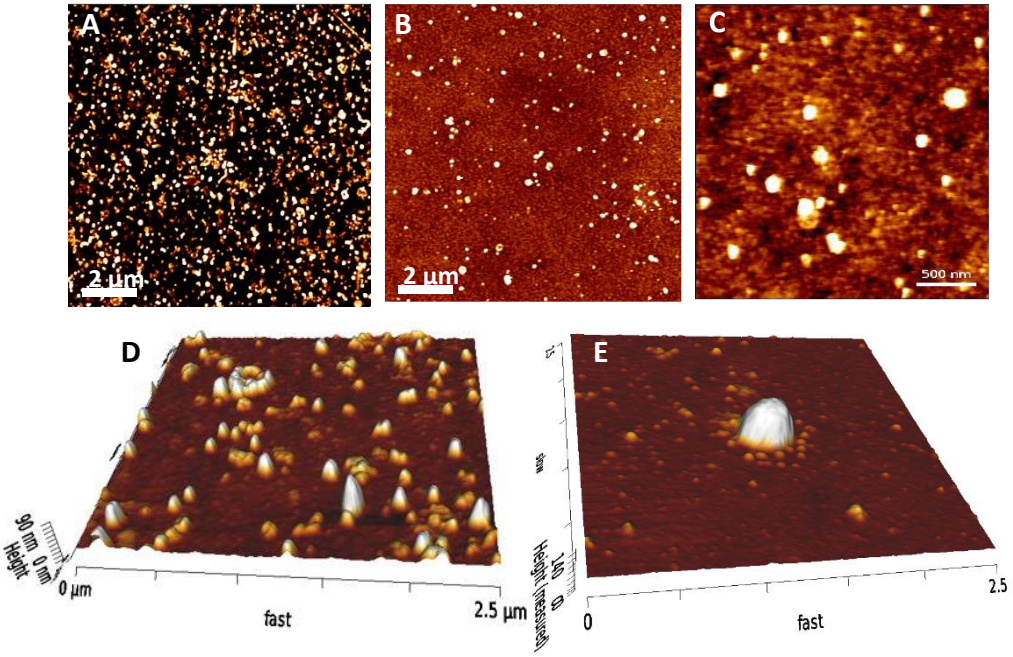

Figure 8: AFM characterization of EVs on biochip (here images obtained in contact mode on dried sample). (A) One example of large scanned area to have a representative view of the mm² ROI on chip. This result was obtained after covalent grafting of EVs on chemically modified surface. (B) Another example of large scanned area obtained after capture of EVs on immunoarrays. (C) A closer view of the objects to get high resolution and enabling the metrology (in height & diameter) of EVs. (D) A closer view of the biochip given the image in (A), with a 3D tilted image. (E) A zoom on one large EVs adsorbed on naked gold biochip, on a tilted 3D image. Z scale is 30 nm and 20 nm for A, B and C images respectively.