# **Folate-based Single Cell Screening**

## Using Surface Enhanced Raman Microimaging

C. Fasolato<sup>1,2</sup>, S. Giantulli<sup>3</sup>, I. Silvestri<sup>3</sup>, F. Mazzarda<sup>1</sup>, Y. Toumia<sup>4</sup>, F. Ripanti<sup>1</sup>, F. Mura<sup>5</sup>, F. Luongo<sup>1</sup>,

F. Costantini<sup>5</sup>, F. Bordi<sup>1,6</sup>, P. Postorino<sup>1</sup>, and F. Domenici<sup>\* 1,4</sup>

<sup>1</sup> Dipartimento di Fisica, Università Sapienza, P.le Aldo Moro 5, Rome, Italy

<sup>2</sup>Center for Life Nanoscience, Istituto Italiano di Tecnologia, V.le Regina Elena 291, Rome, Italy

<sup>3</sup> Dipartimento di Medicina Molecolare, Università Sapienza, P.le Aldo Moro 5, Rome, Italy

<sup>4</sup> Dipartimento di Scienze e Tecnologie Chimiche, Università di Roma Tor Vergata, Via della Ricerca

Scientifica, Rome, Italy

<sup>5</sup> Dipartimento di Chimica, Università Sapienza, P.le Aldo Moro 5, Rome, Italy

<sup>6</sup>CNR-ISC UOS Roma, Sapienza Università di Roma, P.le A. Moro 5, 00185 Roma, Italy

# **Supplementary Information**

### Section S1: Nanovector preparation: chemistry

A solution of folic acid (FA) (at the concentration of  $7.8 \times 10^{-3}$  M) was prepared in MilliQ water. To ensure that FA does not precipitate, because of saturation, the solution was stirred at each step. FA bears in its structure two carboxylic groups (-COOH), which constitute the reactive electrophilic centers involved in the nanovector biofunctionalization. Conjugation of FA is achieved via the activation of these groups by means of 2 cross-linker specific molecules, that react with the substrate subsequently: "EDC" (1-ethyl-3-[-dimethylaminopropyl] carbodiimidehydrochloride), i.e. a "coupling" reagent functional to the formation of a highly unstable intermediate (O-Acilisourea active ester) and "NHS" (N-hydroxy - succinimide), a different activator that reacts to form a less labile complex (activated ester succinate).

In order to ensure the activation of the carboxylic groups of FA, EDC and NHS were added to the folate solution with the ratios FA/EDC (1:1) and EDC/NHS (3:1) [s1]. The final solution was incubated for 30 minutes at room temperature ( $25^{\circ}$ C) to form the stable covalent adduct FA-NHS.

The terminal stage involves the reaction between the adduct and the 4-aminothiophenol (4ATP) modified nanoparticles (Nps). The FA is kept in excess to favor maximum functionalization of the Nps. It is important to stress that the reaction of covalent functionalization between FA and 4ATP does not occur with a stoichiometric ratio of 1:1 as a result of steric hindrance. The reaction was carried out at a temperature of 4°C for 12 hours. Then, the solution was dialyzed for 24 hours against MilliQ water in order to remove the residual FA. Meanwhile, the presence of FA in the dialysis water was monitored over time by means UV-Vis absorption spectroscopy.

As already explained, the reaction mechanism discussed above requires a first activation, of one of the carboxyl groups of FA, by the reactive portion of EDC (carbon-heteroatom double bond) (**Figure S1.1**).



### Figure S1.1: 1,2 nucleophilic addition.

This is a "1,2 nucleophilic addition" (i.e. a  $\pi$  bond is removed with formation of 2 new covalent bonds). The intermediate that is formed is an active "high-energy" ester. Then, the second activation of the carboxylic group via NHS (N-hydroxysuccinimide) takes place. This molecule is used in organic synthesis and in biochemical reactions, mostly assisted by a "coupling reagent" as EDC, as here chosen. The activated acids can react with amines to form, for example, amides, while a normal carboxylic acid reacting with an amine can form a salt compound only.

The second step implies that the intermediate 'O-acilisourea active ester' undergoes a SN2 substitution reaction (i.e. a reaction where a bond is broken and a new bond is formed simultaneously in the absence of the carbocationic intermediate), where the EDC is substituted with the NHS to form an ester of N-hydroxysuccinimide. Therefore, the carbonyl carbon of FA is now electrophilic enough to be attacked by a nucleophilic agent which, in our case, is the amino group of 4ATP (**Figure S1.2**).



Figure S1.2: First SN2 reaction.

The last step can be summarized in a second SN2 nucleophilic substitution, which, if occurring at slightly basic pH, allows the release of NHS by breaking a second ester bond and with the formation of an amide bond. The group R' of in the reaction obviously represents the 4ATP and the complex FA-4ATP-Np has been formed successfully (**Figure S1.3**).

$$R_{3}-C-O-N + R'-NH_{2} \xrightarrow{pH > 7} R_{3}-C-N-R' + HO-N$$

Figure S1.3: Second SN2 reaction.

A sketch of the two main steps of the Np functionalization reaction is shown in **Figure S1.4**. As can be noticed, the final structure of the nanovector there schematized is one of the possible binding configurations (being thought the most probable **[s2]**) involving the two carboxyl groups of folic acid. Both the configurations constitute an oriented binding, as the interacting part of the folate molecule, the heterocyclic part, is the farthest from the Np, being left externally with respect to the Np surface, ready for biointeractions.



**Figure S1.4**: Main steps of the gold nanoparticle functionalization: AuNp are firstly conjugated with the Raman reporter 4ATP (1), than conjugated to folic acid (2). The sketch of the final structure is a result of one of the possible oriented binding configurations involving the carboxilic moieties of folic acid. In such configurations the folate pteroyl group is kept exposed to water and free to bind to membrane receptors.

Section S2: Nanovector characterization

### 1) Stability of the nanovector size in water and other solutions

The high Z potential (-38 mV) of the folate nanovector prevents it to aggregate through electrostatic repulsion also in the cell physiological media as proved by light-scattering experiments in PBS and cell culture medium.

According to the hydrodynamic diameter distributions shown in **Figures S2.1** and **S2.2**, the slight variation in the diameter values confirms that the nanovector is stable in both the dispersions. The increase in the size dispersion noticeable in **Figure S2.2** can be related to the presence of protein aggregates and other particulate, as suggested by the size dispersion of the cell culture medium itself (green spectrum, **Figure S2.2**).



**Figure S2.1**: Size distribution by intensity of folate functionalized Nps in water (red) and in PBS (green).



**Figure S2.2**: Size distribution by intensity of cell culture medium (green), folate functionalized Nps in water (red) and folate functionalized Nps in cell culture medium (blue).

### 2) AFM- SERS coupled measurements

The nanovector diameter was characterized not only by DLS and SEM measurements (as showed in **Figure 2** of the manuscript), but also by AFM imaging.

Tapping-mode AFM images on the FA-4ATP-AuNp solid aggregates, previously measured by microRaman, were acquired in air and at room temperature using a Dimension Icon microscope, equipped with a Nanoscope V Controller (Bruker AXS, Germany), at CNIS Laboratories (Sapienza University). A cantilever with force constant of 40 N/m was driven at the scan rate of 0.5 Hz, for different scan sizes at 512x512 pixel resolution.

As can be noticed in **Figure S2.3**, the high resolution of AFM measurements allowed us to determine the precise diameter of the functionalized Nps, which is centered around 65 nm (with deviation of ~10%, according to the size variability of the pristine Nps ).



Figure S2.3: (A) AFM image of some isolated, folate functionalized gold nanoparticles.(B) Height profile of the nanoparticle marked in panel (A).

We were also able to correlate AFM and SERS measurements collected on the same clusters, as previously reported in **[s3]**. The result is shown in **Figure S2.3**, where the AFM image of a micrometric Np aggregate, obtained by dropcasting from the nanovector water solution, is compared with the SERS intensity map acquired on the same aggregate. As can be noticed, and as expected, there is a good correspondence between the presence of the Np cluster and the detection of SERS signal.



**Figure S2.4**: (A) Raman map on a cluster of folate-functionalized Nps, obtained by integrating 4ATP C-C stretching band (around 1580 cm<sup>-1</sup>) and (B) corresponding AFM measurement.

### Section S3: Quantification of available 4ATP and FA molecules on AuNp surface

The number of tethered FA molecules was deduced from the difference in available 4ATP before and after functionalization. The number of 4ATP molecules available on the AuNps surface before and after FA functionalization was estimated experimentally, by quantifying the available primary amine groups using Coomassie Brilliant Blue (CBB) dye standard addition method **[s4]**.

CBB solution at pH 2.2 was prepared in (H<sub>2</sub>O, MeOH, CH<sub>3</sub>COOH) with the proportions (80%, 15%, 5%), respectively. Typically, the Np suspension was sonicated for 2 minutes in a sonicator bath, then CBB was gradually added until saturation. At each addition step the suspension was incubated for 10 minutes at RT in the dark, then followed by centrifugation at 3900 rpm for 15 minutes. The absorbance of free CBB in the supernatant was measured by UV spectrophotometry at 610 nm using a double beam JASCO V-630 (Milan, Italy) UV–vis spectrophotometer with a 1 cm cell. One CBB molecule is able to bind one single primary amine site **[s4]**. Therefore, the amount of CBB bound to form the complex 4ATP-CBB is exclusively equivalent to the amount of 4ATP groups available on the NPs surface which allows as well an indirect quantification of FA attached on the Nps (**Figure S3.1**).



**Figure S3.1:** Titration of 4ATP primary amine groups available on the AuNps surface: supernatant absorbance vs. the added amount of CBB to the nanoparticles. The red and black lines correspond to the linear titration fit, before and after CBB saturation, of FA-4ATP-AuNps and 4ATP-AuNPs, respectively. The intersection point is the critical saturation value that determines the maximum amount of CBB bound.

The number of FA molecules/AuNp is calculated following the equation below:

$$N = \frac{(nc1 - nc2) \times N_A}{C}$$

Where: nc1, nc2 are the molar amounts of CBB bound to 4ATP-AuNps, and to FA-4ATP-AuNPs, carried out from the black and red fit curves of **Figure S3.1**, respectively;  $N_A$  is the Avogadro number; and C is the number of AuNps.

Accordingly, the mean number of 4ATP molecules on the AuNp surface has been determined as equal to  $12000 \pm 1000$ . The average number of tethered FA molecules was quantified as equal to  $1900 \pm 200$  molecules per nanovector.

Section S4: Raman and SERS assignment and data analysis

# 1) Assignment



**Figure S4.1**: Raman spectrum on a cell dried on Silicon substrate (black) is compared to the reference spectrum of the substrate (blue). In red, the frequency range of the most intense cell peaks is highlighted.



**Figure S4.2**: Raman spectrum on a cell dried on Silicon substrate (black) is compared to the reference spectrum of the substrate (blue) in the spectral region of the most intense cell peaks. The cell peaks assignment is reported.



Figure S4.3: SERS spectrum of the nanovector with attempted assignment [s5,s6].

#### 2) SERS data analysis

SERS spectra were acquired on functionalized 4ATP-Np and FA-4ATP-Np after inducing their selfassembly into disordered Np clusters on a clean glass slide (the procedure is reported in **[s3]**). Acquisition was performed using 100x objective, 1mW incident power and a 1800 lines/mm diffraction grating, which ensured a spectral resolution better than 1 cm<sup>-1</sup>. In order to calculate an average SERS signal we collected several spectra from different, 2  $\mu$ m spaced, points, typically over a 16x16  $\mu$ m<sup>2</sup> sample surface. We noticed that measurements from distinct points did not differ in spectral shape but basically by an overall intensity factor as expected from a self-assembled SERS substrate.

We focused our analysis on the peak at 1580 cm<sup>-1</sup>, which results to be composed of two distinct components, assigned to two different symmetries of C-C stretching of the benzene ring **[s6]**. It is known that a modification of the relative weight of these two components reflects a change in the charge distribution of the molecule driven by a mechanical stress or, more commonly, by the onset of charge transfer processes due to molecular modifications **[s6,s7]** (as in our case, because of covalent bonding).

Specifically, as shown in the simplified scheme of Figure S4.4, the modification of the primary aromatic amine in favor of the formation of an amide bond (see section S1) triggers the mesomeric delocalization of the amide nitrogen lone pair and hence the occurrence of multiple aromatic resonance structures **[s8]**.



**Figure S4.4**: The typical resonance structures of acetanilide are shown. These derive from the non-bonding valence electron pair of the aromatic amino substituent diverted to the adjacent carbonyl group of the amide bond, and provide in turn an electronic delocalization change along the "Raman active" aromatic C-C bonds.

In **Figure S4.4**, we show the comparison of the SERS spectra acquired on 4ATP-Nps and on FA-4ATP-Nps. Notice there is no substantial change in the characteristics of the C-S stretching peak (centered at 1079 cm<sup>-1</sup> in the 4ATP-Np case and at 1080 cm<sup>-1</sup> in the FA-4ATP-Np sample), while there is a neat modification in the C-C stretching band. Indeed, the center of mass of this spectral structure, calculated as the first momentum of the data distribution, moves from 1578 to 1573 cm<sup>-1</sup> upon folate functionalization. This is caused by the changes in the relative weight of the two spectral components of the band, which are highlighted in the figure with the red arrows.



**Figure S4.4**: SERS spectra of 4ATP-Nps (blue) and of FA-4ATP-Nps (red) in the spectral regions of the C-S stretching band (around 1080 cm<sup>-1</sup>) and of the C-C stretching (around 1580 cm<sup>-1</sup>). Experimental data are plotted as symbols, fitting is plotted as a solid line. In the right panel, the two fitted peaks composing the spectral structure are plotted as

dashed-dotted and dotted lines. Red arrows highlight the changes occurring upon folate functionalization.

## Section S5: SEM imaging

FESEM was employed to characterize the measured, treated cells (on their Silicon substrate). For most of the cell images here shown, the backscattering imaging mode was chosen (see **Figures S5.1, S5.2, S5.4**, **S5.5**). The backscattering imaging mode ensures a high contrast visibility for gold on the cell sample, being the scattering cross section of gold higher than the one of the other materials on the sample, as it is proportional to the third power of the elemental atomic number **[s9]**.



Figure S5.1: Reference image of the substrate, backscattering mode.



**Figure S5.2:** Treated cell culture on the substrate, backscattering mode. The red rectangle identifies the area of the subsequent zoomed-in image.



**Figure S5.3:** Cell surface in ordinary mode. Gold nanoparticles are visible on the cell membrane. In red, the area of the subsequent zoom is highlighted.



**Figure S5.4:** Cell surface at a higher magnification, backscattering mode. Gold nanoparticles are well visible on the cell membrane and below the membrane (these latter are indicated by the arrows). In red, the area of the subsequent zoom is highlighted.



**Figure S5.5:** Cell surface at a higher magnification, backscattering mode. Gold nanoparticles are well visible on the cell membrane and below the membrane.

SEM imaging confirms that the nanoparticles are located on the cell membrane. From **Figure S6.4**, it seems that some Nps are internalized inside the cell, as can be noticed by the halo which covers the bright Np image (see the red arrows in the **Figure S5.4**). We are confident that this effect is due to the presence of the membrane over the Np. Indeed, the power of the incident electron beam during the acquisition (15 keV), according with **[s8]**, exceeds the threshold of "transparency" of the cell membrane to electron beams.

### Section S6: SERS screening details

SERS screening was performed using 50x (0.50 n.a.) objective and a variable power between 2.5 and 10 mW focused on a laser spot of about 3  $\mu$ m diameter. The adopted procedure allowed us to acquire signal from the 50% of the cell surface approximately. Indeed, if a cell has an average diameter around 15-20  $\mu$ m, we performed measurements every 8  $\mu$ m. Considering the area of the laser spot, we can give an estimate of our covering of the cell surface.

The laser power was tuned in order to avoid the bleaching of the cells, which was common in the HeLa cell sample, due to the presence of a larger amount of Nps bound to the cell membrane.

The average signal acquired on the sample was then integrated in the spectral range of the SERS C-C peak (around 1580 cm<sup>-1</sup>) and in the region of the Silicon peak (around 950 cm<sup>-1</sup>). The normalization to the substrate peak allowed us to prepare a dataset of comparable SERS intensity (per cell) on which we performed our statistical analysis.

The parameter distributions were plotted in histograms with logarithmic binning and were fitted with a lognormal distribution, which proved to be the best function to reproduce the datasets **[s10]**.

### Section S7: Cell viability tests

We report herein the results of MTT assay on PC-3, HaCat and HeLa cell lines. All cell lines were treated with 1 pM FA-4ATP-Np solution; only HeLa cell line was also treated with 1 and 2 pM Np solution (see also the **Experimental** section of the manuscript for details). After incubation for 2 and 24 hours at each concentration, cell viability was evaluted with MTT.

In order to determine if the lower viability recorded was due to the presence of the nanovector or to the stress due to water addition, ultrapure water was added on HeLa cell line at different concentrations (2 hours incubation). MTT assays show the same decrease in viability on water-treated and on Np-treated wells, and proves that this effect is not correlated with the action of the Nps.

Consequently PC-3 and HaCat cell lines were treated with the highest concentration of ultrapure water (50 parts of ultrapure water in 50 parts of medium culture) for 2 and 24 hours and they did not show any decrease in viability.

## a) Absorbance and cell viability for HeLa line



# b) Absorbance and cell viability for PC-3 line



## c) Absorbance and cell viability for HaCaT line



**Figure S7.1:** HeLa (a), Pc3 (b) and HaCat cell lines (c) were treated with folate functionalized Np solution and ultrapure water for 2h and 24h. Cells viability was evaluted with Mtt assay. Columns and bars represent average and SD values for 3 indipendent experiments. All results were analyzed by ANOVA, and the significance was evaluated by the Tukey honestly significant difference (HSD) post hoc test. The level of significance was established at p < 0.05. Statistical analyses were performed using GraphPad Prism 5 Software.

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