

Plasmonic Nanostructures for SERRS Multiplexed Identification of Tumor-Associated Antigens

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Multiplexing allows the simultaneous investigation of different properties of cells and, therefore, it will become an invaluable tool for cell phenotyping and for in-vitro and in-vivo tumour diagnosis. Cell imaging is usually performed with fluorescent probes, using both organic fluorophores and semiconductor quantum dots.^[1,2] However, fluorescence suffers from limitations like photo-bleaching, in particular for organic molecules, and large emission spectral widths, even if spectral widths can be reduced in the case of quantum dots.^[3] Furthermore, biological samples show autofluorescence which reduces the dynamic range of the measurements. Metal nanoparticles show interesting properties in particular when they are used for producing the surface-enhanced Raman scattering (SERS) effect, which does not suffer bleaching and is characterized by vibrational bands very suitable for multiplexing measurements because they show very small bandwidths compared to fluorescence.^[4] Several tens of different signals could be in principle obtained with a single spectrum.^[5] Enhancement of the Raman signal, which has a very low scattering cross section with respect to fluorescence, can be achieved with metal nanostructures showing the so called “hot spots”, appearing in all those nanoparticle arrangements in which the local electromagnetic field can be increased by several orders of magnitude, making possible enhancements of the Raman signal up to 10 to 14 orders of magnitude.^[6] In this case single molecule detection becomes possible.

Hot spots with metal nanoparticles can be obtained with non spherical particles, but a simple and efficacious method is that of aggregation of nanoparticles. In fact, among nanoparticles, where the distances can be of the order of 1 nm or less, very high enhancements can be obtained by exciting their localized surface plasmons.^[7] Further enhancements can be obtained with the so called SERRS (surface-enhanced resonance Raman scattering), in which also the molecules linked to the surface of the metal nanostructures can be excited in resonance or preresonance.^[8] In this case one exploits the resonance effect without the problem of eliciting a fluorescence signal from the molecules, which disturbs Raman scattering, because fluorescence is usually quenched with respect to the Raman signal when a molecule resides in a hot spot.^[4,5]

SERS or SERRS were recently shown to be efficient also for in vivo multiplexing measurements, however no specific antigen targeting was reported.^[9–11] Very recently it was shown that these techniques can be associated to MRI and photoacoustic techniques for the imaging of a brain tumour.^[12] SERS was also used for in vivo recognition of one cell-associated antigen, the EGFR.^[13] However, tumor cells often show a characteristic phenotype due to the presence of more than one TAA (tumor associated antigen) and, therefore, the recognition of their expression pattern represents a crucial step in the diagnostic process.

Recognition of two antigens, using SERS, has been described for breast tumor cells in the identification of CD44 and CD24 markers^[14] or in prostatic cancer tissue^[15] for the expression of PSA and CK18 antigens, but improvement of the nanomaterials employed can be envisaged since in the first case a two step process was necessary and in the other an autofluorescence background analysis was needed due to the excitation in the green spectral region.

Prostate cancer is one the most frequent tumours in the western countries and we show herein how to prepare two new efficient SERRS gold nanostructures functionalized with antibodies to recognize very efficiently and simultaneously two TAAs, overexpressed in prostatic cancer cells, namely PSMA (prostate specific membrane antigen) and PSCA (prostate stem cell antigen) using one step process and excitation in the NIR spectral region to avoid autofluorescence.

The ability of detecting antigens on cancer cells can be very useful for the timely identification of tumour cells, the staging of the tumour and, based on its genetic and phenotypic

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features, its recurrence and metastatic potential. Multiplexing determination of such antigens with a single and simple measurement would make tumour cell identification and characterization easier, opening the way to diagnostic procedures leading to earlier and more precise diagnosis and, eventually, to more personalized therapies. PSMA and PSCA are prostate cancer antigens and their presence makes possible an evaluation of cell differentiation and the identification of a metastatic, recurrence-prone, drug-resistant phenotype when both antigens are present on the same cell.^[16–18]

We herein show how nanostructure aggregates lead to a very good multiplexing detection of PSMA and PSCA antigens on LNCaP and PC3 wild type or transfected cell lines expressing one of the antigens under investigation, both or none. We use gold nanoparticles, which are known to be biologically inactive when they are not coated with any stabilizing molecule like thiols, surfactants or citrate.^[19] For this reason we have set up a method based on laser ablation in solution (LASiS) for obtaining gold nanoparticles not coated with any molecule.^[20,21] The stability of the colloidal solution derives from the surface charges that the particles show after the laser ablation synthesis and therefore we do not need coating molecules serving as stabilizers. These nanoparticles can be very easily functionalized without recourse to ligand exchange reactions or extensive purification from any stabilizing molecules, thereby greatly simplifying the procedures for the preparation of the nanostructures.

Our strategy for the preparation of the nanostructures is that of linking, on aggregated gold nanoparticles, an antibody for a specific antigen and a SERRS reporter, i.e. a molecule with a strong SERRS enhancement effect, so that the binding between the antibody on the nanostructure and an antigen is revealed by the signal of the SERRS reporter present on the same nanostructure. Furthermore the nanoaggregate is coated with polyethyleneglycol polymers to avoid or minimize its non specific interactions with cells and to improve the selectivity of antigen recognition by the antibody. In this way we obtain multiplexing by mixing different nanostructures recognizing different antigens, which are detected with a single measurement by the spectra of the SERRS reporters associated to them. Antibodies and SERRS reporters are linked independently on the nanostructures so that a large number of combinations are possible.

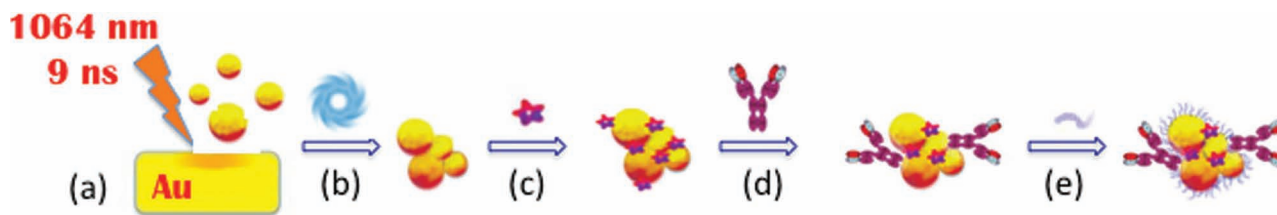
Our nanostructures, allow a very efficient and selective targeting, and SERRS spectra can be easily recorded with low intensity laser excitation of about 1 mW in the near infrared. We also show that our multiplexing measurements allow to

recognize, already at a qualitative level, namely without the need of a quantitative analysis, cells expressing PSMA or PSCA or both antigens.

The most important steps of the preparation of the nanostructures are reported in **Scheme 1**.

The synthesis of the gold nanoparticles (AuNP) is obtained with LASiS. A gold target, in bidistilled water, is ablated using 9 ns pulses of a Nd:YAG laser at 1064 nm (details of all the preparations and measurements are reported in Supporting information). Fluences of 10 J cm^{-2} allow a fast production of 10–20 mL of deeply colored solution (2–5 nM) of AuNP with average diameters of about 20 nm.^[20,21] The colloidal solution is usually stable for weeks without any stabilizing molecule. For obtaining particles, which are stable for months, a solution of NaCl (10^{-5} M) is used instead of pure water. It is known that Cl^- ions help in opening Au-O-Au bonds on the surface of the nanoparticles to produce Au-O^- charges which stabilize, by coulombic interactions, the colloidal solution.^[22] It was found that only less than 10% of the surface is occupied by these charged groups.^[23] ζ -potential measurements show for the synthesized nanoparticles values of more than -30 mV , which guarantees the stability of nanoparticles in solution. The toxicity of the nanoparticles is assessed using an “in vitro” assay (XTT assay) and we find no reduction of cell viability, as expected for gold nanoparticles without molecules covering their surface.

AuNP are centrifuged at 30 000 g for ten minutes and resuspended in water for three times to obtain aggregated nanoparticles, which are needed to obtain nanostructures with a sufficient number of hot spots for a strong SERS effect. Extent of aggregation is controlled by the charges on the nanoparticles. We verified that a low density of charges produces an insoluble pellet at the tube bottom after centrifugation. Examples of TEM images reported in **Figure 1a** show that the aggregated nanostructures have dimensions of the order of 100 nm with the presence of about 10–15 nanoparticles for each aggregate. More images are reported in Figure S1 (see Supporting Information). However, we controlled the aggregation using the UV-Vis-NIR spectra, which, as known, show pronounced extinctions with larger shifts to the NIR for aggregates with larger dimensions. In Figures 1. b1 and 1.c1 the spectra of the particles aggregated by centrifugation are shown as black lines. They show a pronounced extinction maximum at about 600–750 nm, which is important for exciting the localized Plasmon resonance of the nanoparticles in the NIR.



Scheme 1. Steps for the preparation of the nanoaggregates: (a) synthesis of gold nanoparticles by LASiS (laser ablation synthesis in solution); (b) aggregation of the nanoparticles by centrifugation; (c) linking of a thiolated reporter (TR-SH or MG-SH) to the nanoaggregate; (d) linking of an antibody (D2B or anti-PSCA) to the nanoaggregate; (e) covering of the nanoaggregate with PEG-SH.

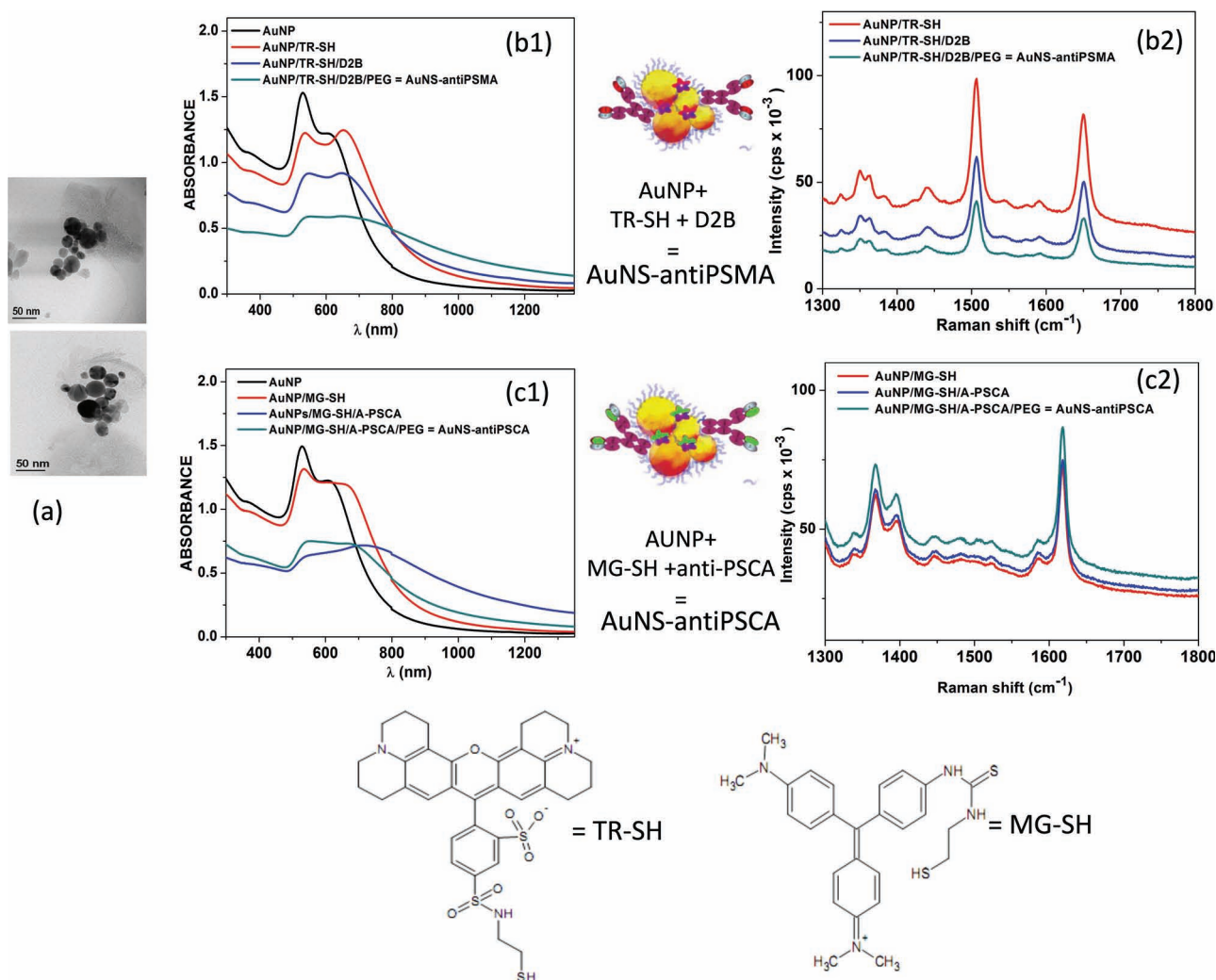


Figure 1. Extinction and SERRS spectra of gold nanostructures. (a) TEM measurements of aggregated nanoparticles obtained by LASiS; (b1) evolution of the extinction spectra of the aggregated gold nanoparticles during the preparation steps of AuNS-antiPSMA obtained with the TR-SH SERRS reporter and D2B antibody; (b2) evolution of the SERRS spectra of AuNS-antiPSMA nanostructure; (c1) evolution of the extinction spectra of the aggregated gold nanoparticles during the preparation steps of AuNS-antiPSCA obtained with the MG-SH SERRS reporter and anti-PSCA antibody; (c2) evolution of the SERRS spectra of AuNS-antiPSCA nanostructure.

We use two SERRS reporters, each linked to aggregated nanoparticles together with either one of two selected antibodies, one specific for PSMA (we call it D2B) and one selective for PSCA antigens (we call it anti-PSCA). We linked Texas Red functionalized with a thiol group (TR-SH) and D2B antibody for PSMA targeting on a first batch of aggregated nanoparticles and Malachite green functionalized with a thiol group (MG-SH) and an anti-PSCA antibody for PSCA targeting on a second batch of aggregated nanoparticles.

A SERRS reporter is linked to the aggregated nanoparticles by simply mixing a colloidal solution of nanoparticles with that of the SERRS reporter. The thiol group drives a fast binding of the molecules to AuNP. The nanoparticles are then purified from the unbound molecules by three centrifugation and dispersion steps in bidistilled water. The successive step in the preparation is the linking of the antibody to the nanostructures. By reacting the ammine groups of the lysine side chains with 2-iminothiolane we are able to functionalize the

antibodies with one thiol group, on average. After purification of the functionalized antibody by centrifugal ultrafiltration, the solution is mixed with aggregated AuNP already functionalized with a SERRS reporter. The colloidal solution is then purified by centrifugation and dispersed in PBS. In a final step of the synthesis, the functionalized nanostructures are allowed to react with polyethylene glycol chains (5000 MW, 0.5 mg/mL) functionalized with a thiol. In this way all the free surfaces of the nanostructures are coated with a layer of a protecting polymer, which helps in avoiding or minimizing non specific interactions with cells, without hindering the good targeting properties of the antibodies. After purification by centrifugation of the resulting nanomaterials, unspecific interactions of the nanostructures with biomolecules are further prevented by mixing with a solution of BSA (0.1 mg/mL). In the following paragraphs we refer to the nanostructures with TR-SH and D2B for targeting PSMA as “AuNS-antiPSMA” and to those with MG-SH and anti-PSCA for targeting PSCA as “AuNS-antiPSCA”.

In Figure 1.b1 and 1.c1 are illustrated the UV-Vis spectra and the SERRS spectra recorded at the different steps of the preparations of the two types of nanostructures. It can be observed that the aggregation of the nanoparticles, evident from the band at about 600–800 nm in Figures b1 and c1, can change during the different phases of the preparation, however only a small extension of the extinction to the near infrared spectral region, which reveal larger aggregations, can be appreciated. In any case a good extinction of the final nanostructures can be observed at about 633 nm where we excite the localized surface plasmon resonance to record the SERRS spectra. SERRS spectra, which show further enhancement with respect to SERS spectra, are expected^[8] because we use two reporters which are in resonance or preresonance with the laser excitation at 633 nm as one can appreciate in Figure S2 (see Supporting Information). The SERRS signal during the various preparation steps is reported in Figures 1. b2 and 1.c2, for the two types of nanostructures, and intense signals are evident also after the final step. This shows that the reporter molecules are present and stable on the aggregates hot spots during the different steps. It should be noticed that, without metallic nanoparticles, one would have registered only a strong fluorescence signal.

To set up the most appropriate cellular and antigenic context to carry out our investigation and to verify the targeting properties of the two types of nanostructures (i.e. AuNS-antiPSMA and AuNS-antiPSCA) we incubated both of them together with living cells expressing PSMA antigen only (LNCaP wild type cells), PSCA antigen only (PC3 PSCA-transfected cells), none of the antigens under study (PC3 wild type cells) and both PSMA/PSCA antigen (LNCaP PSCA-transfected cells). To obtain cells expressing the desired type of antigen, cells (i.e. LNCaP and PC3) were transfected with the PSCA antigen by recombinant DNA techniques obtaining the transfectants described above expressing both antigens (i.e. LNCaP-PSCA) or PSCA only (i.e. PC3-PSCA). Cell were transfected with PSCA cDNA to obtain cells expressing both antigens (i.e. LNCaP-PSCA) or PSCA only (i.e. PC3-PSCA).

The uptake and internalization of the nanoparticles by living cells are verified by producing resonance scattering images. **Figure 2** shows the confocal image of LNCaP cells just fixed before the analysis after incubation of the living cells with AuNS-antiPSMA for 3 h at 37 °C. The Au nanostructures can be identified as red spots, whereas the membrane and the nuclei are associated with green and blue colors (false colors), respectively, obtained with fluorescent molecules (see Supporting information).

Figure 3 illustrates the intensity of the SERRS signals obtained from single cells after incubation with a solution containing both AuNS-antiPSMA and AuNS-antiPSCA for three hours followed by washings with PBS and fixation.

Figure 3a clearly shows that the signals of TR-SH are observed in the cells expressing PSMA (LNCaP (PSMA+, PSCA-)), because TR-SH is associated to the antibody D2B, specifically recognizing PSMA. In Figure 3b very strong signals of MG-SH are recorded in cells expressing PSCA (PC3-PSCA (PSMA-, PSCA+)), because MG-SH is associated instead to the antibody anti-PSCA specifically recognizing PSCA. In Figure 3c only negligible signals are found in PC3

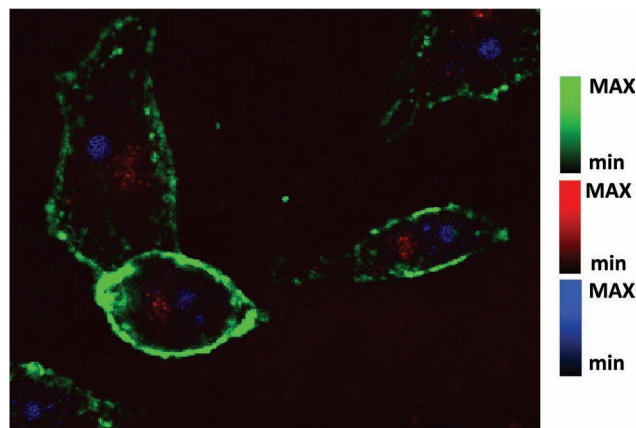


Figure 2. Confocal image of LNCaP cells expressing PSMA showing the uptake of the nanostructures within the cells. Living cells were incubated for 3 h at 37 °C and then fixed before the analysis. Gold nanoparticles are observed with resonance scattering at 633 nm and are visualized as red spots. Fluorescences are reported in green and blue for membranes and nuclei, respectively. Nanoparticles are included within cytoplasm but not within nuclei.

(PSMA-, PSCA-) cells because these cells do not express PSMA nor PSCA antigens. In Figure 3d is reported an example of the registered spectrum of an individual LNCaP cell (cell number 5) to illustrate that in the spectra it becomes very easy to recognize also a very low intensity signal, like that of the MG-SH present in this case (see Figure 3a).

One can qualitatively recognize cell expressing PSMA or PSCA antigens, or none of them, by recording the spectra of some tens of cells only. The different levels of SERRS signals measured at the single cell level are likely due to the heterogeneous expression of the target antigen by cells and/or to different internalization kinetics. Only in very few cases some lower non specific signals are observed and they are likely due to non specific binding of the antibodies.

Based on these results it can be concluded that, although both nanostructures are pre-mixed together and then added to cells, only one type of SERRS signal dominates the spectra or that very low signals are observed in the case of PC3. This shows that the antibodies maintain their efficient targeting properties and the very strong SERRS signals show that the nanostructures are stable.

The advantageous multiplexing properties of the prepared nanostructures can be appreciated in **Figure 4a**. In this case we incubated living LNCaP-PSCA transfected cells, which express both antigens, with the two types of nanostructures. SERRS spectra clearly show the presence of both TR-SH and MG-SH signals on the same cell and for all the cells, with dominating signal of one or the other SERRS reporter. This shows that different cells can show different expression of the two types of antigens, but the results are clearly different from those obtained analyzing cells expressing only one antigen or none. In Figure 4b is also reported the spectrum recorded for the cell number 15 showing clear SERRS signals of the two SERRS reporters. This result shows that multiplexing can be easily obtained, the measurements are simple and the spectra are directly interpreted.

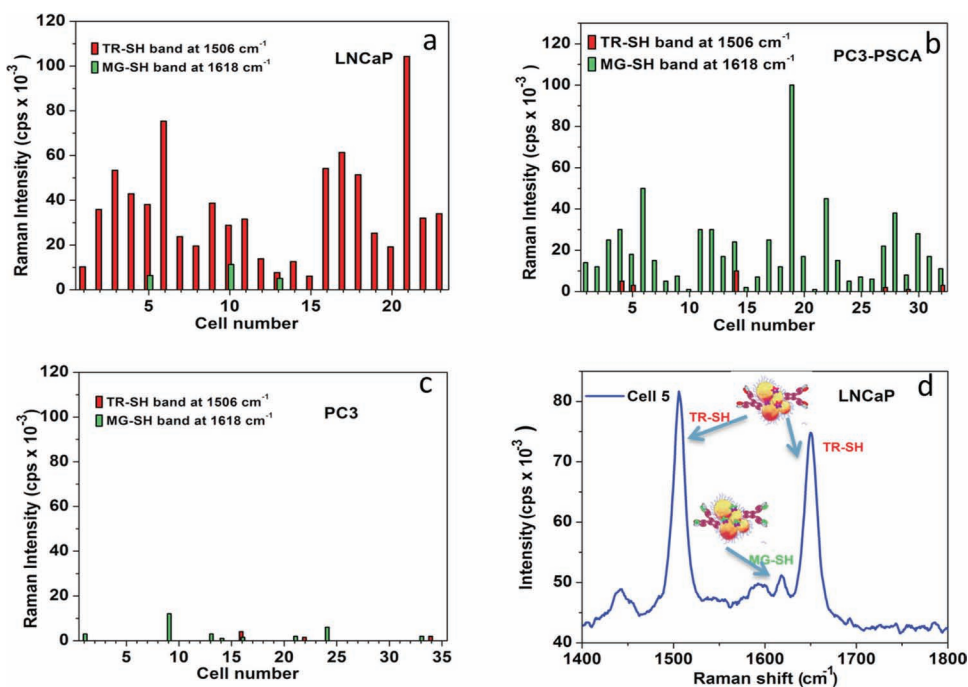


Figure 3. Intensity of the SERS spectra at 1506 cm⁻¹ (band of TR-SH) or at 1618 cm⁻¹ (band of MG-SH) of treated cells expressing PSMA or PSCA. Living cells were treated for 3 h at 37 °C, washed and fixed before the analysis. (a) Spectra for LNCaP cells expressing only PSMA. One can see the TR-SH SERS spectrum as expected; (b) spectra for PC3-PSCA cells expressing only PSCA. One can see the MG-SH SERS spectrum as expected; (c) spectra for PC3 wild type cells not expressing PSMA or PSCA. Only minor signals are recorded, as expected; (d) example of a recorded spectrum. This is the spectrum of LNCaP cell #5 in which the signal of TR-SH is observed but a small signal for MG-SH is also detected.

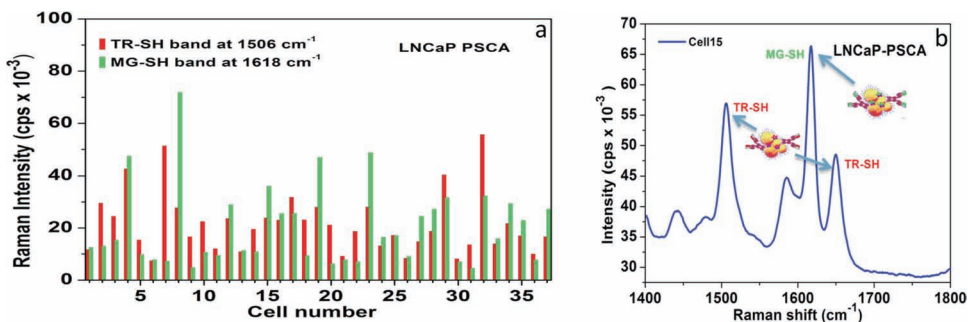


Figure 4. Intensity of the SERS spectra at 1506 cm⁻¹ (band of TR-SH) or at 1618 cm⁻¹ (band of MG-SH) of treated cells expressing PSMA or PSCA. Living cells were treated for 3 h at 37 °C with both AuNS-antiPSMA and AuNS-antiPSCA, then washed and fixed before the analysis. (a) Intensity of SERS spectra for LNCaP-PSCA transfected. One clearly observes spectra of both TR-SH and MG-SH for every cell, as expected. (b) Example of spectrum of LNCaP-PSCA: Cell #15 in which SERS signals of the two SERS reporters are clearly visible.

Comparison of Figure 3a-c and Figure 4 clearly shows that one can qualitatively distinguish expression of the cell antigens, which, as recalled, is important for staging of prostate tumour cells.^[16-18]

In conclusion, in the present report we have shown that the cell expression of different Tumour Associated Antigens can be investigated with a single measurement in which the characteristic vibrational bands of a SERS reporter associated on a gold nanostructure to a specific antibody, can be very easily recognized. The methodology employed for

the preparation of the nanostructures is of fundamental importance and the nanoparticles obtained by LASiS allow a simple and very efficacious synthesis.

The multiplexed detection of antigens is a powerful tool for the detection and identification of cell phenotypes. Clearly, the possibility of identifying also more than two Tumour Associated Antigens with only one SERS spectrum for the same cell, can be obtained by combining more SERS reporters to more antibodies linked independently on gold nanostructures and this may expand enormously the diagnostic potential in the clinics.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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- [1] J. A. Barreto, W. O'Malley, M. Kubeil, B. Graham, H. Stephan, L. Spiccia, *Adv. Mater.* **2011**, 23, H18.
- [2] J. V. Jokerst, S. S. Gambhir, *Acc. Chem. Res.* **2011**, 44, 1050.
- [3] X. Michalet, F. F. Pinaud, L. A. Bentolila, J. M. Tsay, S. Doose, J. J. Li, G. Sundaresan, A. M. Wu, S. S. Gambhir, S. Weiss, *Science* **2005**, 307, 538.
- [4] (Eds: D. Grahama, R. Goodacres) *Chem. Soc. Rev.* **2008**, 37, 875.
- [5] S. Schlücker, *ChemPhysChem* **2009**, 10, 1344.
- [6] S. Nie, S. R. Emory, *Science* **1997**, 275, 1102.
- [7] J. P. Camden, J. A. Dieringer, Y. Wang, D. J. Masiello, L. D. Marks, G. C. Schatz, R. P. Van Duyne, *J. Am. Chem. Soc.* **2008**, 130, 12616.
- [8] V. Amendola, M. Meneghetti, *Ad. Funct. Mat.* **2012**, 22, 353.
- [9] S. Keren, C. Zavaleta, Z. Cheng, A. de la Zerda, O. Gheysens, S. S. Gambhir, *PNAS* **2008**, 105, 5844.
- [10] G. von Maltzahn, A. Centrone, J.-H. Park, R. Ramanathan, M. J. Sailor, T. A. Hatton, S. N. Bhatia, *Adv. Mater.* **2009**, 21, 3175.
- [11] C. L. Zavaleta, B. R. Smith, I. Walton, W. Doering, G. Davis, B. Shojaei, M. J. Natan, S. S. Gambhir, *PNAS* **2006**, 103, 13511.
- [12] M. F. Kircher, A. de la Zerda, J. V. Jokerst, C. L. Zavaleta, P. J. Kempen, E. Mittra, K. Pitter, R. Huang, C. Campos, F. Habte, R. Sinclair, C. W. Brennan, I. K. Mellinghoff, E. C. Holland, S. S. Gambhir, *Nat. Med.* **2012**, 18, 829.
- [13] X. Qian, X.-H. Peng, D. O. Ansari, Q. Y.-Goe, G. Z. Chen, D. M. Shin, L. Yang, A. N. Young, M. D. Wang, S. Nie, *Nat. Biotechnol.* **2008**, 26, 83.
- [14] K. Lee, V. P. Drachev, J. Irudayaraj, *ACS Nano* **2011**, 5, 2109.
- [15] B. R. Lutz, C. E. Dentinger, L. N. Nguyen, L. Sun, J. Zhang, A. N. Allen, S. Chan, B. S. Knudsen, *ACS Nano* **2008**, 2, 2306.
- [16] R. M. Taylor, V. Severns, D. C. Brown, M. Bisoffi, L. O. Sillerud, *The Prostate* **2012**, 72, 523.
- [17] Y. Li, P. J. Cozzi, P. J. Russell, *Medicinal Res. Rev.* **2010**, 30, 67.
- [18] A. C. Cunha, B. Weigle, A. Kiessling, M. Bachmann, E. P. Rieber, *Cancer Letters* **2006**, 236, 229.
- [19] E. Boisselier, D. Astruc, *Chem. Soc. Rev.* **2009**, 38, 1759.
- [20] V. Amendola, M. Meneghetti, *J. Mater. Chem.* **2007**, 17, 4705.
- [21] V. Amendola, M. Meneghetti, *J. Phys. Chem. C* **2009**, 113, 4277.
- [22] J.-P. Sylvestre, S. Poulin, A. V. Kabashin, E. Sacher, M. Meunier, J. H. T. Luong, *J. Phys. Chem. B* **2004**, 108, 16864.
- [23] H. Muto, K. Yamada, K. Miyajima, F. Mafune, *J. Phys. Chem. C* **2007**, 111, 17221.
- [24] M. Colombatti, S. Grasso, A. Porzia, G. Fracasso, M. T. Scupoli, S. Cingarlini, O. Poffe, H. Y. Naim, M. Heine, G. Tridente, F. Mainiero, D. Ramarli, *PLOS One* **2009**, 4, e4608.

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