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Nanostructured Gold Surfaces Coated By Silica-Based Layers as SPR and MS Imaging Platform

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Abstract **-**Current developments in the field of bioanalytical tools for research and diagnostic purposes tend to propose innovative and multifunctional solutions for studies of biomolecular interactions. Main requirements are expressed not only by sensitive and multiplex detection of binding phenomena. Also reliable identification of interactants is of big interest and opens up to many possible applications. In this scope, integration of surface plasmon resonance (SPR) assays with mass spectrometry methods provides high potential of analytical power. In this work we present development of silica/gold nanostructured layers as a versatile platform enabling analysis by both SPR and MS in imaging mode. Nanostructured gold substrates were prepared upon colloidal lithography and plasma-assisted processes whereas nanometric silica coating was obtained through sol-gel protocol from tetraethyl orthosilicate (TEOS) as precursor. Such surfaces were furthermore modified by devoted chemistries in order to allow addressing of biomolecules. We have therefore reported on analysis of interaction between immobilized model peptides and related target proteins by SPR imaging technique. Eventually silica films were modified by organic matrix molecules and provided detection of model analytes by MS imaging approach which has been demonstrated in this study.

Keywords**:** gold nanostructures, surface plasmon resonance, MS imaging, silica layers, FLAG peptide

1. Introduction

In the field of biosensing technologies, combination of several techniques has become a highly valuable approach aiming to fulfil requirements that are of great importance for many research and diagnostic applications (Turner, 2000). Developments are made in order to ensure high sensitivity of biorecognition, high-throughput and label-free analysis as well as integration of investigation levels such as detection of interactions followed by identification of interactants. These are also the motivations of the present work where we report on a biosensing platform able to perform in detection of biomolecular interactions by surface plasmon resonance (SPR) along with identification of bound analytes by mass spectrometry.

Here described nanostructured surface is based on optically thin gold layer containing bi-dimensionally distributed nano-holes and decorated by few-nm silica film. The array of nano-antennas embedded in gold substrate has a property to support a combination of delocalized and localized SPR modes that can be explored in a dedicated optical set-up of SPR imaging (Bottazzi et al., 2014). Thus, further surface functionalization may enable to address molecular probes and ensure characterization of biomolecular interactions in a label-free and real-time manner.

On the other hand, mass spectrometry measurements can provide qualitative and quantitative chemical data regarding material immobilized on analysed samples. MS analysis may be performed directly on nanostructured substrates and has been reported in the literature as surface-assisted laser

desorption/ionization (Silina and Volmer, 2013; Kawasaki et al., 2012). Here proposed gold/silica nanostructures are assayed in analogous approach towards detection of several molecular probes. SiO_2 film overlaying gold substrate is modified by organic matrix molecules in order to tune up the ionization potential and increase system sensibility for detection of bigger molecules such as peptides.

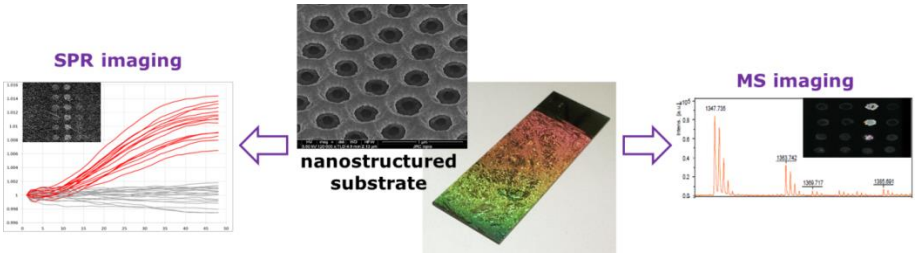


Fig. 1. Schematic representation of the nanostructured platform for SPR and MS imaging

2. Sample Preparation And Characterization

Gold nanostructured samples were obtained upon relatively low-cost fabrication process based on colloidal lithography as previously described by Giudicatti et al. (2010). Briefly, clean glass substrate (microscopic slide) was firstly spin-coated by 150-nm layer of polymethyl methacrylate (PMMA) and subsequently covered by colloidal monolayer of 500-nm polystyrene beads using Langmuir-Blodgett trough. Sample was then submitted to etching in oxygen plasma conditions (25sccm, 20mTorr, 142W) resulting in generation of nano-mask lattice with particular geometrical parameters. In the following step, manufactured array of nano-features was uniformly coated by 150-nm gold film generated by sputtering in plasma enhanced physical vapour deposition (PE-PVD) process. Eventually, the colloidal mask was removed from coating by ultrasonication bath and followed by O_2 plasma post-etching process aiming to remove residues of PMMA. The resulting nanomanufactured substrate was characterized by SEM unveiling nanostructured gold film perforated by 200-nm diameter nano-holes bi-dimensionally distributed with a lattice constant of 500 nm. Plasmonic responsiveness of the produced nano-features was strongly dependent on their geometrical parameters and could be easily assayed by simple reflectance measurements (Giudicatti et al., 2010). Nanostructured surfaces displayed clear decrease of the reflectance signal at around 900nm which was consistent to localised SPR mode as depicted by Fig.2.

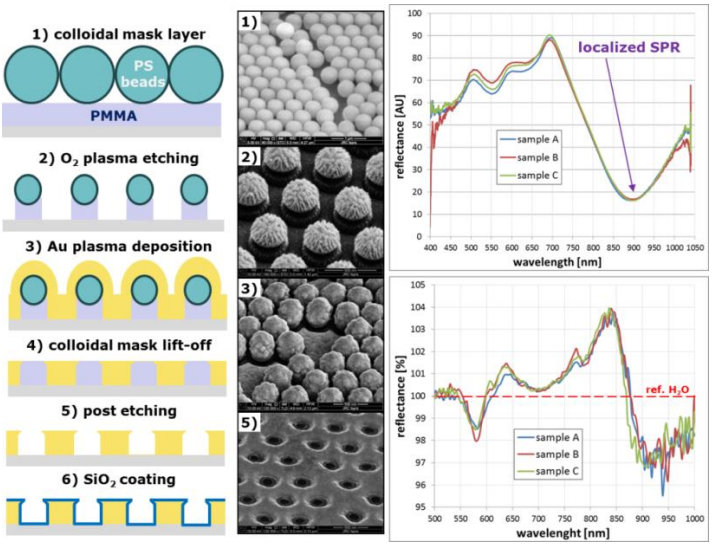
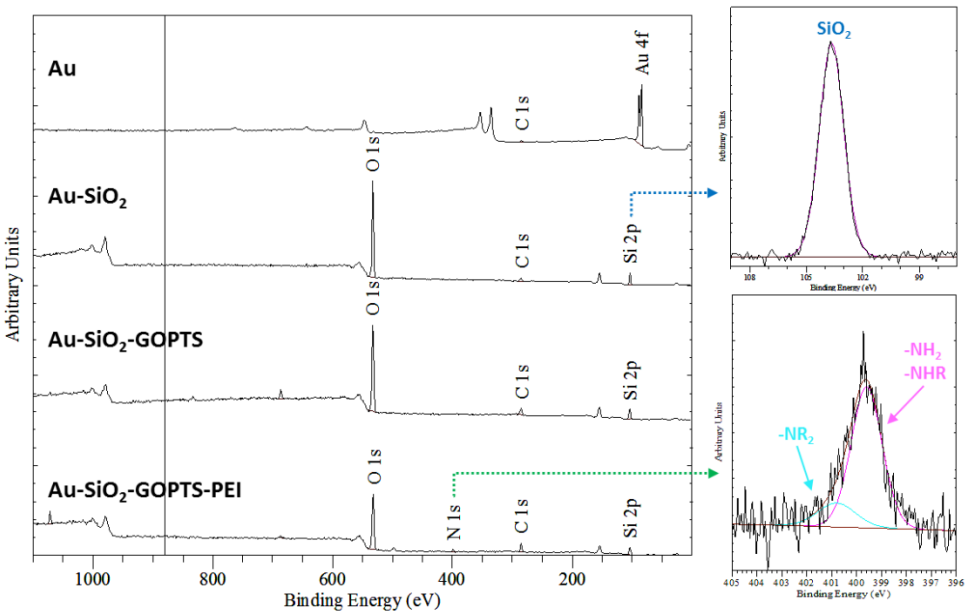


Fig. 2. Nanofabrication scheme along with SEM images at different steps of preparation (left). Spectral characteristics of the produced nanostructured surfaces measured in water (up right). Plasmonic response upon local change of the refractive index by incubation with 2% glycerol (bottom right).

Nanostructured gold surface was coated by ca.5-nm layer of silica generated through sol-gel synthesis protocol. Tetraethyl orthosilicate (TEOS) was used as precursor for hydrolysis and condensation condensation of silica substrate carried out in mild acidic conditions (pH 4.5-4.8). Upon maturation, sol-gel solution was diluted to 1-2% in ethanol and subsequently applied to generate silica layer of desired thickness by dip coating technique. XPS measurements (*AXIS Ultra*, *Kratos*, UK) evidenced the presence of Si2p signal attributable to silicon oxide of SiO_2 type confirming the high chemical quality of the coating. Moreover, the disappearance of the Au signal after silica deposition proved the film to be composed of uniform $(-O-Si-O-)_n$ lattice (*cf.* Fig.3). As shown by Floris et al. (2014) relatively low thickness of generated films enabled to maintain substrate plasmonic properties and further sensing.



SAMPLE	Au 4f	Si 2p	O 1s	C 1s	N 1s	C-C	C-O (C-N)
Au	65.83±1.30	-	-	34.17±1.30	-	100	0
Au-SiO_2	-	20.17±0.70	72.23±0.82	7.61±0.79	-	69.60±0.85	22.80±0.85
Au-SiO_2-GOPTS-PEI	-	16.15±0.74	66.28±1.33	11.26±1.03	-	62.50±1.33	37.50±1.33
Au-SiO_2-GOPTS-PEI	-	16.33±0.41	55.95±1.10	19.41±0.95	2.76±0.19	50.85±0.63	49.16±0.63

Fig. 3. Characterization of surface coating by XPS. Comparative table depicts elemental composition (in %) at each step of sample modification.

For the sake of mass spectrometry analysis, nanostructured gold surfaces were coated by silica layers modified with organic matrix molecules. Two MALDI matrices were chosen: CHCA (α-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid) that were incorporated into TEOS-based coating (Lin and Chen, 2002). Indeed, matrix molecules at 10000-20000ppm level were added to sol-gel preparation and therefore entrapped during condensation and silica polymerization step. Dip coating protocol was applied as described above, resulting in gold nanostructures coated by thin SiO_2 film chemically modified by DHB and CHCA moieties. The obtained substrate yielded in increased ionization efficiency for MS and was compatible with SPRi analysis.

3. Results And Discussion

The nanomanufactured samples were investigated by SPR imaging in terms of monitoring of biorecognition events with biomolecular probes immobilized on the surface. Protocol based on silane chemistry allowed further functionalization of silica-coated gold nanostructures for SPRi assays. Namely the aforementioned substrate was firstly activated by epoxysilane – 2% solution of (3-glycidyloxypropyl)-trimethoxysilane (GOPTS) in ethanol. Branched polyethyleneimine (PEI) was subsequently coupled over

the layer of epoxy moieties. For this purpose, surface was incubated with 1-5mM solution of the polymer in 50mM borate buffer (pH 8.0). Here proposed surface chemistry enabled to benefit from the silica layer and transform it into amine-rich coating. Aminosilanes could be applied alternatively but this solution was less preferred due to susceptibility to form multi-layered coatings (Zhu et al., 2012). All steps of chemical modifications were characterized in parallel by XPS measurements as reported by Fig.3. Spectral data evidenced proper distribution of binding energy peaks (Si2p, O1s, C1s) attesting related functionalization steps. In particular, the high-resolution N1s spectrum unveiled a detailed composition of PEI polymer containing mainly primary and secondary amines available for subsequent attachment of biomolecular probes.

For the need of the experiments hereafter described, a set of peptides was eventually addressed on the PEI-coated nanostructured chips. Amines were therefore activated by disuccinimidyl carbonate (DSC), a short homobifunctional cross-linker of NH_2 groups (Morpurgo et al., 1999). Using an automated piezo-dispenser, the following peptides were microarrayed onto the surface (buffer: 50mM borate, pH 9.0): FLAG [DYKDDDDK], FLAGC [DYKDDDDKC], R-BIOT [RAFK*YPFIK] and S-BIOT [SLLTEVETPIRNEWGSRSNDSSDK*] where K* stands for lysine conjugated with biotin moiety. The resulting peptide array was composed of several dozens of ca. 250µm diameter individual spots arranged with 500-µm pitch. Biochip was subsequently incubated with 20mM ethanolamine and blocked by Tween 0.05% (in PBS) prior to SPR imaging tests.

Nanostructured gold/silica surfaces decorated by devoted surface chemistry and peptide arrays were assayed by SPRi instrument described by Floris et al. (2014). Briefly, biochip surface was illuminated by 850-nm LED source and reflected light was registered by CMOS camera over few mm^2 with lateral resolution of around 10µm. Such optical set-up and more precisely SPR modes displayed by the nanostructures provided specific detection of biomolecular interactions expressed by relative variations of the refractive index at the surface. The biochip slide was loaded into microfluidic system and equilibrated with PBS buffer (pH 7.2) at a flow rate of 20µl/min. Subsequent incubations with protein targets (anti-FLAG mouse Ab and avidin) resulted in positive probe-specific signals and no significant unspecific binding was observed (*cf.* Fig.5). Imaging feature enabled to evidence patterns of immobilized peptides. Registered responses of up to 3-4 RU reflected compatibility of silica-based substrate for SPR detection which sensitivity was comparable to other chemistries based on gold SAMs (data not shown). Real-time monitoring of interactions allowed accessing of the kinetic data and drove to conclusions on differences of affinity among investigated peptides. Indeed, in our case FLAGC sequence demonstrated lower immuno-recognition level than its standard FLAG variant. Also S-BIOT turned out to bind stronger to avidin as compared to R-BIOT - most probably because of possible steric effects. Here reported experiments attest of multiplexing potential of SPR imaging platform that can be furthermore easily integrated with other technologies such as IMS.

Performances of the gold/silica nanostructured substrate embedding matrix molecules were eventually assessed by complementary label-free technique of MS imaging (Heeren et al., 2009). As reported in literature, sensitivity of detection in laser desorption/ionisation (LDI) approach is driven by the efficiency of nanostructured surfaces to produce ions, in particular from macromolecules, and is strongly dependent on chemical, physical and geometrical properties of the surfaces (Kuo et al., 2014; Silina et al., 2014). In this study, both CHCA and DHB modified samples were assayed on high-resolution FT-ICR mass spectrometer equipped with 355 nm laser source of 91µJ power (for 1kHz). To assess LDI potential of the aforementioned nanostructured samples, we chose a set of model compounds with wide mass range (260 Da – 1348 Da) and different physicochemical properties. Tested molecules were denoted as follows: SM1 – propranolol, S1 – 25-hydroxyvitamin D, S2 –17-hydroxy-progesteron, SM2 – olanzapine, peptide P1 [GRAFRGAG] and peptide P2 [RPKPQQFFGLM]. Solutions were prepared in water without addition of proton source (*e.g.* trifluoroacetic acid) which would promote formation of $[M+H]^+$ cations. Therefore we assessed the ionization potential of the surface itself. The assayed substances were manually spotted onto slides within the concentration range of 100µM to 10nM (*cf.* Fig.5) and dried. Samples were then submitted to laser irradiation with a lateral resolution of 100µm and the acquired MS data were evaluated in imaging mode enabled by devoted software.

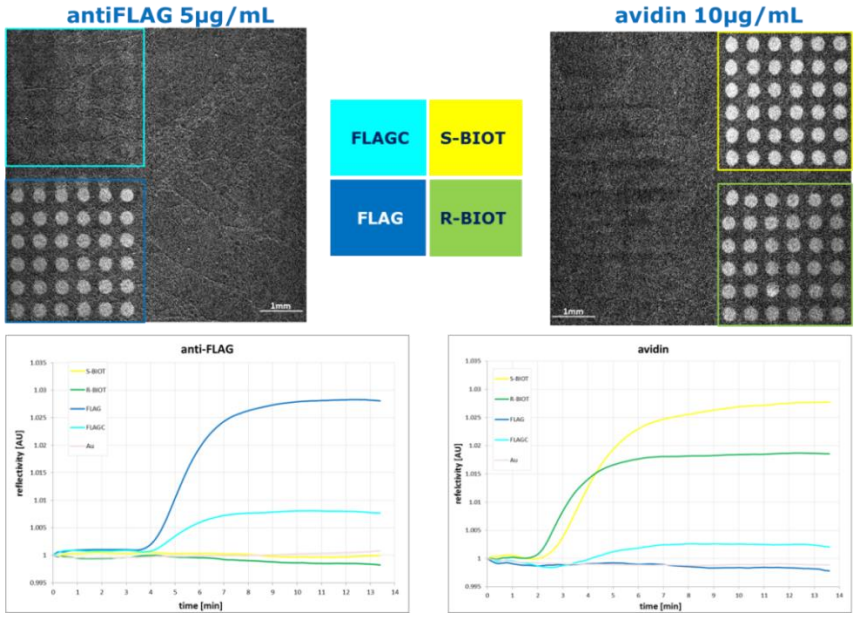


Fig. 4. Differential SPR images and kinetics data obtained upon loading of anti-FLAG Ab and avidin onto nanoplasmonic gold/silica/PEI surface functionalized with model peptide microarrays (spots of around 250-µm diameter). Signals are averaged for each type of probe peptide.

$[M+H]^+$ signals were readily registered for all assayed molecular targets. MS analysis resulted in identification of studied molecules with limit of detection down to 1pmol/µL. Mass spectra evidenced insignificant background noise in low mass range which allowed to detect small molecules. Gold clusters potentially released from substrate were not observed even at relatively high level of laser power. Tests performed on a flat gold substrate, instead of nanostructured one, modified with analogous coating yielded in significantly lower level of ionization (data not shown). Thus the presence of gold nano-features contributed to efficient absorption of administered laser energy. Moreover, the overlaying silica film was considered to ensure good thermal insulation, therefore allowing better energy confinement and facilitating desorption-ionisation events (Duan et al., 2010). This MS imaging tests demonstrated potential of the nanostructured platform to provide not only identification data but also information on localization of various molecules on the dedicated surface. The functionalizable substrate offers a possibility of selective MS analysis by generating affinity surfaces (immunoaffinity in particular) and combined with other label-free techniques such as SPR can open up to advanced biosensing applications.

Probe molecule	nanoAu SiO_2-DHB	nanoAu SiO_2-CHCA
SM1 (M.W. 259.34)	++	++
S1 (M.W. 412.65)	+	+
S2 (M.W. 330.46)	++	++
SM2 (M.W. 398.62)	++	++
P1 (M.W. 1036.10)	++	+
P2 (M.W. 1347.63)	+++	++

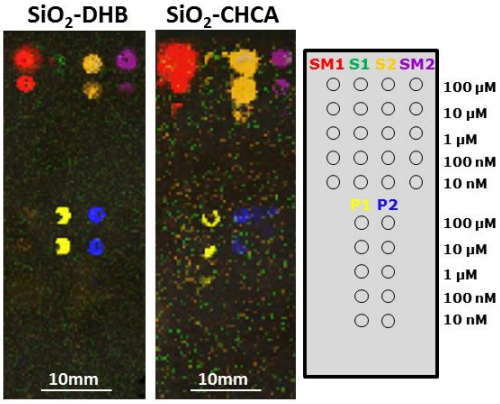


Fig.5. Identification of different molecular targets by MS imaging performed on nanostructured slides coated with silica layer embedding DHB or CHCA matrices. Comparative table represents the following limits of detection: 100pmol/µl (+), 10pmol/µL (++) and 1pmol/µL (+++). MS data were evaluated by Quantinetix™ imaging software.

4. Conclusion

In this work we report on nanostructured gold/silica substrate compatible with both SPR and MS imaging techniques. Biorecognition of various targets can be assessed on such versatile support by combination of these two high-throughput and label-free technologies. Here presented results consist of proof-of-concept approach that can open up to many biosensing investigations. Apart from multiplexity potential, imaging aspect brings additional spatial information which can be precious for identification of interactions in such applications like toxicity screening or epitope mapping.

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