Research Article

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Plasmonic Nanocavities-based Aperiodic crystal for Protein-Protein Recognition SERS sensors

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Abstract: The revelation of protein-protein interactions is one of the main preoccupations in the field of proteomics. Nanoplasmonics has emerged as an attractive surface-based technique because of its ability to sense protein binding under physiological conditions in a label-free manner. Here, we present a detailed experimental study of the use of aperiodic photonic nanocavities for plasmonic Surface Enhanced Raman Scattering (SERS) protein detection and recognition. The plasmonic crystal is designed on a 2D Thue-Morse array configuration. The SERS nanosensor is coated with a proper self-assembled monolayer to covalently bind Bovine Serum Albumin that is a well known model to study biological (specifically, protein) systems. The performance of the nanosensor is assessed by recording a new Raman (SERS) peak in the fingerprint region and by a giant enhancement of the SERS signal intensity, both reported and discussed.

Keywords: Photonic Crystals; Plasmonics; Sensors; Nanofabrication; Nanolithography; Raman; Surface Enhanced Raman Scattering (SERS); Localized Surface Plasmonic Resonance (LSPR); Metamaterials

1 Introduction

Within the field of proteomics one of the main target is to study the function of proteins and in particular how they interact mutually and with other biological entities. A major part of processes in biological pathways, rely on various protein-ligand and protein-protein interactions. In order to extract such information label-free surface-sensitive methods, such as the quartz crystal microbalance [1], electrical impedance [2], and surface plasmon resonance [3], have increased in popularity. One key value of these methods stems from the fact that with one of two interacting partners bound on a sensor surface, binding reactions can be transduced to a detectable signal via changes in the mechanical, electrical, and/or optical properties of such devices. A major challenge for protein analytical tools is that the transducer concept must be sensitive enough to detect low coverage of adsorbed proteins.

On the other hand, plasmonics is a rapidly growing field with strong impact in Science and Technology. In this context, the use of sensors is increasing in particular in applications for bio-sensing [4–10], whereas high sensitivity and miniaturization are strongly required. Plasmonic nanostructures are useful to obtain novel nanodevices that use the shift of the spectral properties of plasmons as transducer element of a chemical/biological signal. Surface Enhanced Raman Scattering (SERS) is a well-known plasmonic spectroscopic technique that allows to reveal the vibrational modes (stretching, bending) of specific chemical groups of molecules in touch with a SERS substrate [11–19]. In order to obtain a high efficient and specific SERS bio-detection, it is necessary: a) to well design specific nano-structured substrates with appropriated geometry and sizes; b) to appropriately choose chemicals that can specifically interact with a target biomolecule. For this reason the substrates are functionalized by self-assembling mono-layers (SAM) of specific substances that can in turn chemically interact with the target molecules. Concerning the design, we have recently reported on plasmonic nanocavities (NCs) based on photonic crystals for efficient plasmonic sensors [20]. It has been well-documented that SERS results from two effects,
electromagnetic field enhancement caused by localized surface plasmon resonances (LSPR) associated with metallic nanostructures and chemical enhancement caused by resonance Raman-like interaction between the metallic nanostructure and the adsorbate. Large SERS signals are expected when both the frequency of the incident laser and the scattered Raman electromagnetic field approach the resonance frequency of LSPR [14]. On the other hand, the spectral properties of the plasmonic resonance depend on the characteristic size of the pattern and they can be tuned by modifying the lattice parameters.

In the present work, we report on the use of NCs-based on an aperiodic crystal for protein-protein recognition by SERS spectroscopy. The peculiar characteristics of the plasmonic interaction in aperiodic crystals make them suitable to improve both efficiency and reproducibility of SERS sensors. In particular, using the Electron Beam Lithography (EBL) process we fabricated square NCs crystals based on a 2D-Thue-Morse (ThMo) sequence. Optical and plasmonic behaviors of the ThMo patterns have been widely reported in literature from both numerical and experimental point of views. They make such geometry an attractive candidate for the realization of high-performance nanosensors [21–28].

Regarding the functionalization, we decided to investigate the possibility to attach proteins on our substrate. The aim of this work is to open new possibilities to study protein-protein interaction and recognition [29–32] (e.g. antigen-antibody, phage-bacteria, actine-myosin reactions, et cetera), by the use of a label free high sensitive technology, such as SERS spectroscopy. For this purpose, we bind Bovine Serum Albumin (BSA) - a very known model for protein system studies - on the SERS sensors. The plasmonic surface (SERS substrate) is previously functionalized by a SAM of 4-amino-thio-phenol (4ATP). BSA is subsequently attached to the SAM of 4ATP by diazotization and coupling reactions.

2 Experimental

2.1 Materials

4-amino-thiophenol (4-ATP), Bovine Serum Albumin (BSA), Sodium Nitrite (NaNO₂), Acetic Acid (CH₃COOH), Isopropyl Alcohol (IPA), Ethanol (EtOH), Methoxy Benzene (Anisole), Indium Tin Oxide (ITO)-coated glasses (BK7) are purchased from Sigma Aldrich. The co-polymer methyl-styrene/chloro-methyl acrylate (ZEP) solution in Anisole (ZEP520A) is purchased from ZEONREX® Electronic Chemicals. In this document, by ZEP is indicated a solution of 11% of a copolymer (composed of methyl-styrene and chloro-methyl acrylate) and 89% of anisole (solvent).

2.2 Methods

ZEP solution preparation

The ZEP520A solution used in this work is obtained by a further dilution of ~47% (w/w) of ZEP520A in Anisole, in order to obtain a final concentration of the copolymer in Anisole as ~5.17 % (w/w).

EBL fabrication and morphological characterization

We used a Raith 150 EBL system to fabricate a Au square NCs based on 10th order ThMo array with a side size of the square $d = 185$ nm and minimum interparticle distances $a = 80$ nm (see inset in Fig. 1a). In the fabrication, a layer of ZEP with a thick of 100 nm is deposited by spin-coating technique on a 15 nm conductive ITO coated glass substrate, baked at 170° for 5 min and exposed to the 15.8 pA electron beam current with an area dose of 24 $\mu$C/cm². NCs are generated in ZEP layer after development by immersing the structure for 90 s in MIBK (Methyl isobutyl ketone), then 60 s in a solution (1:3) of MIBK: IPA followed by IPA rinse. Finally, the Au NCs arrays are created by evaporating 2 nm of Cr and 50 nm of Au films onto the ZEP surface. The e-beam evaporator is a SISTEC CL-400C. The nanostucture fabricated is characterized by a) Scanning Electron Microscopy (SEM – Raith 150), as illustrated in Fig. 1a and b) Atomic Force Microscopy (AFM - NT-MDT NTEGRA Spectra), as reported in Fig. 1b. Both size of the square nanocavities and minimum interpaticle distance appear regular and with a high resolution on the whole pattern.

Substrate functionalization by 4-amino-thiophenol (4-ATP): SAM formation

A SAM of 4-ATP on the gold substrate is simply obtained by depositing a solution of 4-ATP (100 $\mu$M in Ethanol) for 12 hours (overnight) at room temperature. In these experimental conditions the adsorption of the molecule on the gold surface is guaranteed, due to the presence of -SH group attached to an aromatic ring. After 12 h, the sample is washed many times with H₂O dd and EtOH and is dried under N₂ flux.
Figure 1: 2-layers square NCs in ThMo geometry: a) Scanning Electron Microscopy (SEM) images; b) Atomic Force Microscopy (AFM) topography measurement. Inset of Figure 1.a): magnification of the ThMo unit (a = 80 nm; d = 185 nm).

4ATP-SAM functionalization by BSA

BSA is subsequently attached to the SAM of 4ATP by diazotization and coupling reactions. In order to do that, micrograins of NaNO₂ are deposited in touch with the SERS substrate (200 × 200 µm²), to completely cover it. 300 µl of CH₃COOH are deposited droplet by droplet on the NaNO₂, and the production of nitrous acid is observed (HNO₂ in gaseous phase). When the production of HNO₂ goes to disappear, 300 µl of a solution of 3 mg / ml of BSA is deposited on the SERS surface and is left overnight. After 12 hours the sample is cleaned by repetitively washing the sample with H₂O dd and IPA.

Set-up for SERS

SERS measurements are realized by using the QE ProRaman system (Ocean Optics), configured for a linear polarized laser source with wavelength λ = 785 nm (power = 12 mW), a grating of 1200 lines / mm and an input slit of 50 µm. The spectra are collected in the range between 600–1800 cm⁻¹, by using 10 s of acquisition time and a 50X (N.A. = 0.75) microscope objective. The Raman system is coupled with an upright microscope Olympus BX51.

A Scheme for the Optical Set-up for SERS is reported in Ref. [17]. The Enhancement Factor (EF) was evaluated as in the previous work.

Numerical Simulation

We investigate the near-field of NCs-arrays performing numerical calculation using the Finite Difference in Time Domain (FDTD) method. We consider a ThMo 4-order lattice with the same geometry and size used for the fabrication. The structure is stimulated using a laser wavelength λ = 785 nm, linear polarized in the plane of the NCs parallel to the x axis (Fig. 3). Refractive indices used for the materials are n_air = 1, n_glass = 1.51, n_ITO = 1.78, n_ZEP = 1.55, while the Au is modeled using the Drude parameters. During the calculations, we set a spatial grid with a step size of 5 nm in each direction and a time step (in ct units) of 10⁻³ µm. Perfectly Matched Layer (PML) boundary conditions on the all direction are used. The Poynting vector intensity is calculated 15 nm above the plane of the NCs to avoid stair-stepped approximation error.

3 Results and Discussion

The fabrication process that we propose is based on a classic nanolithography method with the only noteworthy exception consisting in the omission of the final lift-off step (see Figure 2). Thus, we obtain a NCs-based quasi-crystal substantially made by a thin layer of gold (~50 nm thickness) deposited on a thin layer of ZEP (~100 nm thickness) that, in turn, is placed on a conductive and transparent substrate (ITO-glass). Furthermore, the quasi-crystal that we propose is based on a ThMo geometry, by using short inter-particles (inter-cavities) distance a = 80 nm, and NCs dimensions of d = 185 nm (see Experimental section). Thue-Morse pattern are particularly promising for plasmonic applications as demonstrated in literature where these patterns are used as SERS substrates (in a pillar configuration) showing a high enhancement factor [21].

Figure 2: Schematic sketch of the functionalization of a SERS substrate: a) NCs-based Thue-Morse SERS substrate (TMS); b) SAM of 4-ATP-functionlized TMS; c) SAM-4ATP reaction with BSA: simplified scheme for the diazotization reactions between 4-ATP and nitrous acid (HNO₂) formed by reaction of CH₃COOH and NaNO₂ and subsequent coupling with BSA; d) Chemical reactions describing the functionalization of the square-NCs with BSA.

Firstly, as it is evident from SEM and AFM topography measurements (Figure 1a and 1b, respectively), the fabrication process that we propose offers very high quality
and highly reproducible structures. This is not a trivial result if considering that the structure is constituted by soft matter and metal, and when considering that the lift-off process in nanolithography is a well assessed and standardized procedure, designed to obtain a different goal: gold nanopillars (and not NCs) configuration. The two layers (gold and ZEP) structure offers a first strong advantage that consists in a significant filtration of the fluorescence present in the SERS signal due to the glass substrate. Thus, one of the problems commonly encountered in fabrications of SERS substrates - namely, the choice for the correct fluorescent-free conductive and transparent substrate - in the case of NCs-based crystal can be overpassed. The choice of the ThMo array parameters (side size of the nanocavity and inter-cavities distance) is based on the results of FDTD simulations (see Figure 3). By simulation, the near-field is much more intense in proximity of the sides of the square NCs with the same direction of the linear polarization of the light used to excite the pattern and it decreases rapidly moving away from them. However, even in areas far from the NCs the field intensity doesn’t reach a negligible background value. Generating non-negligible near-fields in more region of the sensor is an important peculiar advantage that aperiodic patterns can provide with respect to their periodic counterparts. In fact, differently respect to the case of periodic patterns, where the field is substantially concentrate in proximity of the nanoelements, for aperiodic patterns the near-field in region far from the nanoelements can significantly contribute to the interaction with the analyte and so to the amplification of the output signal. We ascribe this last important feature to the peculiar multipolar interactions (long-range coupling) characteristic for aperiodic patterns. As a matter of fact, this characteristic improves the light-analyte interaction, so enhancing the sensitivity of the system, and in particular aperiodic nanostructures allow near-field distribution more uniform and with higher intensity making these types of patterns promising to develop sensing devices with high reproducibility and high output signals.

By accurately observing the above mentioned disposal of the hot spots (Figure 3), the highest intensity is into the NCs regions. The plasmonic near field distribution in NCs-based crystal should be reasonably comparable to that derived by a more classical holes-array configuration. Due to lack of gold in the holes, the holes-array configuration furnishes a filling factor less than 1, reducing the plasmonic active area useful for molecular sensing. The NCs-based crystal we propose is done by a real continuous metallic layer that increases the area available for the functionalization and makes the plasmonic field active for sensing also in the cavities. SERS enhancement factor, on the basis of the results obtained and the parameters used for measurements, is $3.8 \times 10^6$.

As mentioned in the introduction, our goal is the functionalization of NCs structure by BSA. This protein represents here a model to investigate the possibility to functionalize the NCs nanosensor for future studies in protein-protein recognition by plasmonic analysis. Usually, in order to attach on a gold substrate a molecule via covalent bonds or via chemo-sorption is required in its structure the presence of sulphidric groups (–SH) or disulphidric groups (–S–S–), respectively. In a protein, the amino-acid Cystein (Cys) contains a –SH group that could guarantee a strong link on a gold surface. However, BSA is a protein containing 580 amino-acids residues of which only the 6% is made by Cys residues. Thus, we have previously functionalized the nanosensor by 4ATP. This molecule is easily adsorbed and covalently attached on a gold substrate to form a SAM (see Experimental Section). This is due to the presence of -SH linked to an aromatic ring in its structure, as reported in the Figure 2b. At this stage, all the above mentioned advantages related to the particular two layer configuration proposed become evident when looking at the graph of Figure 4, that reports SERS measurements after 4ATP SAM formation. The SERS signal is very well resolved and all the peaks in the fingerprint region are clearly evidenced, since the fluorescence doesn’t affect the readability of the signal. The characteristic SERS peaks in the fingerprint region for 4ATP are those related to its sub-
stituted aromatic structure (–S–C– stretching, at 1078 cm$^{-1}$) and to the aromatic ring chain vibrations (–C–C– bending, at 1585 cm$^{-1}$) and are clearly evident in Figure 4. The characteristic peaks are also reported in the Table 1, where in the 1st and 2nd columns a comparison between Raman and SERS peaks for 4ATP is also detailed. Even if a thin layer of soft-matter (~100 nm of ZEP-layer) is present in the structure, the high intensity and quality of the signal confirm on the possibility to functionalize the NCs-based surfaces by SAM of thio-phenol derivatives. In the inset we also report on plasmonic measurements of the bare plasmonic quasi-crystal and of the functionalized one using UV-Vis extinction spectroscopy technique [33]. Once prepared a SAM of 4ATP we have operated a further functionalization of the NCs-platform by covalently attaching the BSA on it.

![Figure 4: Raman intensity (counts) vs wavelength (cm$^{-1}$) of a SAM of 4ATP on NCs-based SERS substrates. In the inset the extinction spectrum of the bare plasmonic quasi-crystal (red curve) and of the functionalized one (black curve) is reported.](image)

Theoretically, BSA can be simply deposited on the 4ATP-SAM and probably a huge number of weak hydrogen bonds can be sufficient to link the BSA on the SAM of 4ATP. However, reasoning in perspective, the device that we propose should be suitable for many different proteins different from BSA for which is desirable a sure and well identified link to the substrate.

For this reason, we have opted for the covalent attachment of BSA on the 4ATP-SAM by diazotization and coupling reactions: a) generating nitrous acid in proximity of the plasmonic surface; b) by coupling reaction of diazonium ion with specific amino-acidic residues of the BSA (reasonably Histidine (His) or Tyrosine (Tyr)). Diazonium ion on 4-ATP is generated by reaction in presence of NaNO$_2$ and CH$_3$COOH. In acidic conditions, 4ATP originates the ammonium-thiophenol (see Figure 2d) that, in turn, reacts with HNO$_2$ developed in the system by CH$_3$COOH and NaNO$_2$. The reaction is conducted under an optical microscope. It should be performed strictly in touch with the SERS surface, in order to completely involve the SAM of 4ATP. This procedure is necessary since the immersion of the sample in an acidic solution could bring to undesired lift-off effects such as the degradation of the NCs-based surface. Thus, we have operated on the surface of the sample by depositing a few micrograms of NaNO$_2$ (<100 µg on ~200 × 200 µm$^2$) to cover the SAM of 4ATP. The acidic conditions are guaranteed at nanoscale by depositing on the SERS surface micro-droplets of CH$_3$COOH (it is reasonable to assume that the acid is active on the structures at nanoscale by promoting the desired formation of the ammonium ion on 4ATP). In the same reaction, the production of HNO$_2$ it is optically monitored by observing HNO$_2$-bubbles production on the sample surface (HNO$_2$ in gaseous phase at RT). When the production of bubbles is exhausted, an aqueous solution of 3 mg of BSA/ml is deposited on the sample overnight. The aqueous solution of BSA, from one side allows the homogenously deposition of the BSA on the 4ATP-functionalized sample, while for the other side allows the complete neutralization of the nitrous acid production. Finally, the substrate is washed by water and IPA many times, before to be spectroscopically analyzed by SERS. We underline that the reaction doesn’t originate easily sub-products, since the 4-ATP molecule is covalently attached on the gold substrate, thus thiol function is protected. The SERS signal obtained after BSA functionalization is reported in Figure 5. The coupling is easily detected, since characteristic stable and strong Raman intensity peaks are present in the fingerprint region. Firstly, two new results have to be highlighted: a) the noteworthy peak shift of ~12 cm$^{-1}$ towards lower wavenumbers from 1585 cm$^{-1}$ (as recorded for the SAM of 4ATP) to 1573 cm$^{-1}$ (as recorded for the BSA-4ATP) associated to the 4ATP aromatic ring vibrations; b) the weaker shift of ~2 cm$^{-1}$, from the peak at 1078 cm$^{-1}$ (SAM-4ATP) to 1076 cm$^{-1}$ (BSA-4ATP), thus towards higher wavenumbers, that has an opposed direction with respect to the previous one. Both the results strongly suggest a novel real arrangement of the molecule under analysis after diazotization-coupling reaction; the first result (a) clearly indicates a variation of the nature of the substituent on the aromatic ring. This variation is strikingly confirmed by the appearance of a strong Raman peak at 1328 cm$^{-1}$, assigned to the vibrational stretching of the diazo-group (–N=N–) when inserted in a macro-molecular structure. Accordingly, the band at 1328 cm$^{-1}$ together with the other Raman bands
characteristic of the BSA-4ATP system exhibits a great enhancement of the fingerprint Raman signal, with vibrational features well distinguishable and stable in time. It is observed that the SERS intensity increases from around 1350 counts to 8050 counts for the peak at 1328 cm$^{-1}$. It is reasonable to assume that the Raman peak at 1328 cm$^{-1}$ is related to the 4ATP-N=N-BSA molecule, in which the 4ATP molecule is linked to the BSA via diazo-group (see Figure 2d) [34, 35].

4 Conclusions

This work has been directed toward investigating NCs-sensors based on aperiodic lattices functionalized by a SAM of 4ATP to chemically bind BSA. A comparison of the SERS response before and after protein immobilization is reported and discussed. A great enhancement of the whole spectrum and an additional band at 1328 cm$^{-1}$ is observed due to the presence of the BSA. Our results open up new interesting opportunities towards the use of a new class of SERS sensor for detection and study events at nanoscale between proteins and the functionalized surfaces.

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All the experimental results reported demonstrate on the possibility to chemically operate on NCs-sensors by reactions that involve proteins, salts and acids in gaseous and liquid forms. Thus, the protein-functionalized NCs-sensors are in perspective a useful platform to detect and study protein-protein recognition at nano-scale.

Table 1: Selected Raman and SERS bands of 4-ATP, 4-ATP-NCs, and BSA-4-ATP-NCs with their vibrational assignments.

<table>
<thead>
<tr>
<th>Vibrational assignment</th>
<th>4-ATP Raman (cm$^{-1}$)</th>
<th>4-ATP-NCs SERS (cm$^{-1}$)</th>
<th>BSA - 4-ATP –NCs SERS (via Diazocoupling) (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC str + NH$_2$ rock</td>
<td>1086s$^b$</td>
<td>1078</td>
<td>1076s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1142</td>
<td>1141m$^b$</td>
</tr>
<tr>
<td>CH bend</td>
<td>1174w$^b$</td>
<td>1173</td>
<td>1179w</td>
</tr>
<tr>
<td>CN bend</td>
<td>1206vw$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH str</td>
<td>1288w$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NN str</td>
<td></td>
<td></td>
<td>1328s</td>
</tr>
<tr>
<td>CC str + CH +rock + NH$_2$ rock</td>
<td>1388</td>
<td>1390w</td>
<td></td>
</tr>
<tr>
<td>CC str + NH$_2$ rock</td>
<td></td>
<td>1435</td>
<td>1437m</td>
</tr>
<tr>
<td>CC str + CH bend</td>
<td>1491w$^b$</td>
<td>1486</td>
<td></td>
</tr>
<tr>
<td>CC str + NH$_2$ bend</td>
<td>1590s$^b$</td>
<td>1585</td>
<td>1573s</td>
</tr>
<tr>
<td>SH str</td>
<td>2555</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Ref. [31]
$^b$ Intensity: s (strong); m (medium); w (weak); vw (very weak)

References


