Oligonucleotides detection by SERS analysis for biodiagnostic applications

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Surface-Enhanced Raman Scattering (SERS) spectroscopy plays an important role in materials science, biophysics, medical diagnostics, and molecular biology for high-sensitive label-free detection.
In particular, SERS have been widely applied to biological fields for the detection of different biomolecules such as peptides [1] whole proteins, DNA, and even cells [2].
Detection of micro-RNAs (miRNAs), small, non-coding, 18 to 24-nucleotide single-stranded sequences, is of great relevance in gene regulation affecting essential processes such as cell proliferation, cell death, tumor genesis, and mammalian cell development.
Herein, we present a protocol aimed to the immobilization of thiolated cDNA oligonucleotides (~22 bp) on plasmonic metal-dielectric nanostructures consisting of silver nanoparticles (Ag NPs) sticked on porous silicon substrates (pSi). These sensing platforms were successfully tested as efficient SERS substrates for the detection of other biomolecules so that they could be considered promising tools for miRNA sequences hybridization with the respective immobilized complementary cDNA probes.

Experimental: synthesis of Ag NPs sticked to the pSi surface

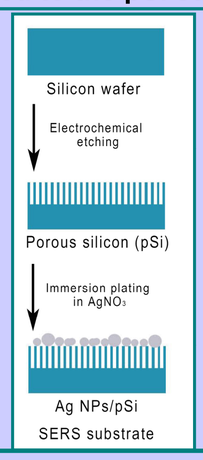
Boron-doped silicon wafer (34 mΩ/cm)

Anodization process in HF based solution (20:20:60 HF:H_2O:CH_3CH_2OH) and current density of 100 mA/cm^2

Porous Si with 80 % of porositiy

$SiH+H_2O+2AgNO_3 \rightarrow SiOH+2Ag+2HNO_3$
Impregnation of pSi in $AgNO_3$ (Redox reaction), rinsing in HCl 1 mM 10 sec

Ag NPs/ pSi substrates



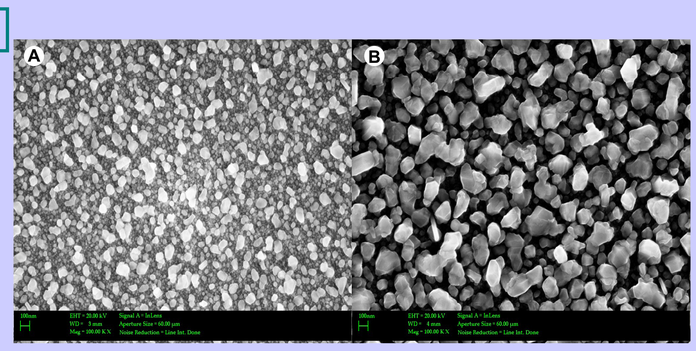


Fig.1 Ag NPs substrates obtained by immersion plating in 10 mM $AgNO_3$ solution: T=50 °C, Dipping time= 30 sec (A) 60 sec (B)

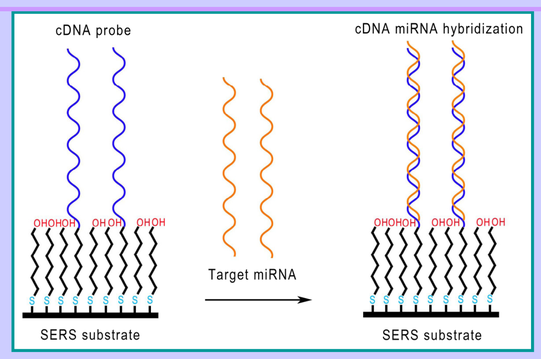
Substrates obtained by these conditions (Fig.1B) allow to match the localized surface plasmon resonance with the excitation source (Ar-Kr laser at 514.5 nm).

The optical response in terms of plasmonic resonance is strictly related to the substrates morphology. The resulting densely packed silver crystallites give extremely high surface enhancement of the Raman signal [3, 4].

Parameters affecting Ag particles density and average size
- concentration of $AgNO_3$ in the aqueous solution
- dipping time
- process temperature

Biological Assay designed and optimized for solid SERS substrates

The aim of our protocol is the immobilization of thiolated cDNA oligonucleotides (~22bp), (5'[C6-SH]ACCCAGTAGCCAGATGTAGCT3') by chemical bonding between the thiol group (-SH) and the Ag nanoparticles, resulting in a self-assembled monolayer (SAM).



The SAM formation has been optimized by co-immobilization of the cDNA probes with specific spacer/blocking agents. This functionalization procedure is fundamental to avoid the unspecific physical adsorption of the charged oligonucleotides to the Ag surface and to promote the successful hybridization between the cDNA probes and the complementary miRNA sequences (5'[Cy5]AGCUACAUCUGGCUACUGGGU3').

Sample pretreatment	• DTT reduction of thiolated cDNA (SS to SH) • 95 C for 2 min (cDNA and miRNA uncoiling) then rapid cooling
Temperature of incubation	• Room Temperature (RT) for cDNA immobilization • 65 C for cDNA-miRNA hybridization
Buffer	• **DNA**: PBS-tween pH 7.2, PBS-NaCl 1M pH 7.2, TE-tween pH 7.5 (10 mM Tris, 1 mM EDTA, 0.05% tween 20), TE 1 M-NaCl pH 7.5 • **RNA:** SSC (saline sodium citrate) 1X pH 7.0, SSC 4X-SDS 0.1% pH 7.0
Time of incubation	• Over Night (O/N.) • 1 hour
Blocking agents/spacers (simultaneously incubated with probes)	• Alkanethiols (terminating -CH_3, -COOH, -OH, -EG) • Cysteine • BSA

Tuning of working condition starting from Microarray protocols

Optimization on Ag/Si flat substrates by means of ELISA test with biotinilated oligo-nucleotides and HRP-streptavidin to support SERS analysis

Selected protocol for SERS analysis

1. Ag/pSi substrates pre-wet in TE-tween, pH 7.5 (5 min);
2. cDNA probes reduction in DTT and pre-treatment at 95 C for 2 min to promote oligo uncoiling, followed by rapid cooling (30 sec in ice bath) [5];
3. Simultaneous O/N incubation (co-immobilization) at room temperature of cDNA-SH probes 15 µM in TE-tween with blocking agents/spacers [6];
4. Wash (3x5 min) in TE-tween 0.5 X, pH 7.5 to remove unspecific binding;
5. Ag/pSi substrates pre-wet in the SSC buffer, pH 7.5 (5 min);
6. miRNA pre-treatment at 95 C for 2 min to promote oligo uncoiling, followed by rapid cooling (30 sec in ice bath) [5];
7. Incubation of miRNA sequences (15 µM in SSC 1X) at 65 C for 5 min;
8. Cool down at room temperature (30 min);
9. Wash (3x5 min) in SSC 1X pH 7.5 to remove unspecific binding;
10. Rinse in ddH_2O (5 min) before SERS analysis;

SEM characterization: compatibility of our substrates with the experimental parameters of the biological protocol

Preliminary tests without bio-species

Buffer

Temperature of incubation

Time of incubation

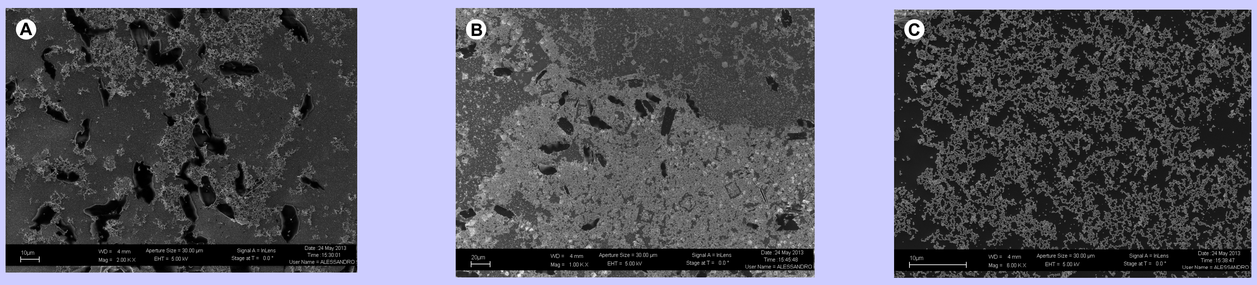
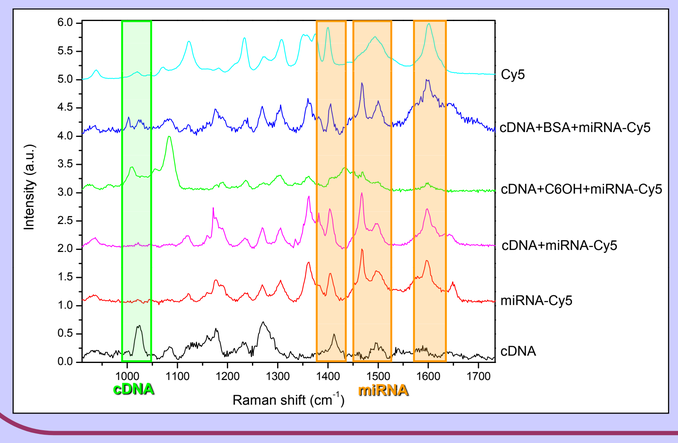


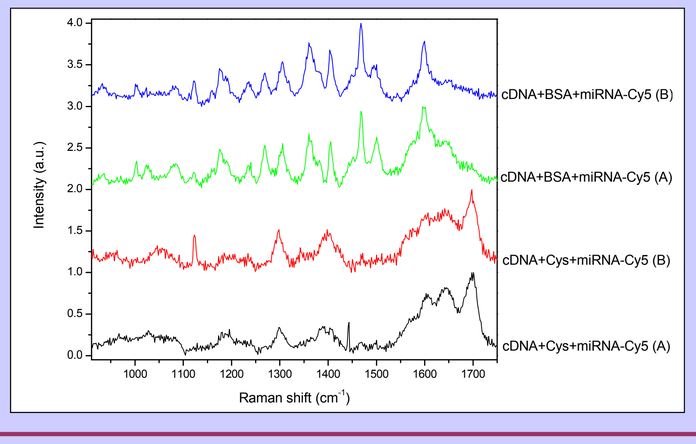
Fig. 2 Ag NPs substrates were incubated O/N in the TE -tween buffer, pH 7.5 at room temperature (A); substrates were then incubated O/N in the SSC buffer pH 7.2 at 65 °C (B) and finally rinsed in ddH_2O to remove salts (C).

SERS analysis

Preliminary detection of cDNA-miRNA hybridization on AgNPs/pSi



Reproducibility test on different AgNPs/pSi treated with the same protocol



Conclusions

- SERS-active substrates were synthesized on mesoporous silicon by immersion plating to obtain Ag NPs/pSi sensing surfaces suitable for cDNA immobilization;
- A biological protocol to study the interaction between cDNA probes and complementary miRNA sequences was developed on Ag NPs/pSi substrates;
- By means of blocking agents/spacers and cDNA co-immobilization promising results have been obtained during preliminary analysis, although further investigations are required;

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