CONSIGLIO NAZIONALE DELLE RICERCHE ISTITUTO DI FISICA APPLICATA “NELLO CARRARA”

NANO probes for cancer theranostics and imaging by Molecular Beacon based on fluorescence and SERS

“Short-Term Mobility” 2013

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Scientific report on the research activity developed at the Fitzpatrick Institute for Photonics, belonging to the Duke University (Durham, North Carolina, USA) during the period 21 October – 9 November 2013

Summary

1. Introduction

Optical sensors have a large impact in the fields of life science research, drug discovery and medical diagnostics. The recent advances in nanotechnology and photonics have led to a new generation of nanotools, capable of probing even the single cell: it has already been demonstrated that nanobiosensors can detect biochemical targets and proteins inside living single cells[1]. The solid support chosen as substrate for the immobilization of the biorecognition element was a tip of an optical fibre tapered at nanoscale size, intended to be used in the future to probe directly inside the cytoplasm of living cells[2].

Particular interest for this STM-project has been focused on oligonucleotide probes capable to turn on or to modify their light emission upon the interaction with well-defined molecular targets, which are termed hairpin probes. In particular, the optical characterization of the oligonucleotide interactions has been measured with two different optical methods, based on fluorescence (performed at IFAC) and based on surface enhanced Raman spectroscopy – SERS (performed at Duke University).

The hairpin probes are single-stranded DNA molecules that possess a stem-and-loop structure. The loop portion of the molecule can form a double-stranded DNA in the presence of a complementary sequence. In this project, I will refer to the hairpin probe developed for measurements in fluorescence as a molecular beacon (MB); on the other hand, I will refer to the hairpin probe developed for measurements in SERS as a molecular sentinel (MS). The MB is labelled with a fluorophore and a quencher on the two ends of the stem. The fluorophore fluorescence is switched-on when the MB hybridizes to the target sequence. The MS probe consists of a DNA strand having a Raman label molecule at one end and a metal nanoparticle at the other end. The plasmonics nanoprobe uses again, as in the MB, a hairpin-like stem-loop structure to recognize target DNA sequences. The sensing principle of molecular sentinels, however, is quite different from that of molecular beacons. With MS systems, in the normal configuration (i.e., in the absence of target DNA), the DNA sequence forms a hairpin loop, which maintains the Raman label in close proximity of the metal nanoparticle designed to induce an intense SERS signal of the Raman label upon laser excitation. Upon hybridization of a complementary target DNA sequence to the nanoprobe hairpin loop, the Raman label molecule is physically separated from the metal nanoparticle, thus leads to a decreased SERS signal.

In particular, in this STM-project, the attention was focused on the RNA messenger (mRNA) for survivin[3], a protein highly expressed in most types of cancer. In this case the MB/MS could act not only as detector of the over-expression of the mRNA for the survivin (diagnosis) but also as the blocking agent of the synthesis of the protein itself (therapy).

As preliminary study, before entering living cells, the activated nanotips were tested in buffer solutions. The comparison between the two different optical set-up were performed and here reported.

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[1] T. Vo-Dinh, Y. Zhang, "Single-cell monitoring using fiberoptic nanosensors", Wiley Interdisciplinary Reviews: Nanomed. Nanobiotech. 3, 79-85 (2011).

[2] A. Barucci, S. Berneschi, F. Cosi, G. Nunzi Conti, S. Pelli, F. Quercioli, S. Soria and G.C. Righini, "Fiber optic nanoprobes for biological sensing", Proc. SPIE 8011, 80118X (2011).

[3] P.J. Santangelo, B. Nix, A. Tsourkas, G. Bao, “Dual FRET molecular beacons for mRNA detection in living cells”, Nucl. Acids Res. 32 (2004) e57.

2. The NANOtip fabrication

The nanotips used were produced both at Fitzpatrick Institute for Photonics, Duke University, and at the Institute of Applied Physics, CNR, using different methodologies.

2.1. CO_2 laser pulling

The nanotips were realized on Polymicro technologies INC fibres (200 μm external diameter). The laser pulling method, performed at Duke University, consists of local heating of an optical fibre using a laser and subsequently pulling the fiber apart. Fabrication of nanosensors requires techniques capable of making reproducible optical fibres with submicron-sized diameter core. The laser pulling process is a time-dependent heating effect where laser power, timing of pulling, velocity setting, and pulling force are important parameters that contribute to the taper shape and tip size. Since transmission efficiency is highly dependent on the taper shape, it is crucial to control the tip shape in the fabrication of high-quality nanoprobes. Figure 1 illustrates the experimental procedures for the fabrication of nanofibers using the micropipette puller (Sutter Instruments P-2000)[1].

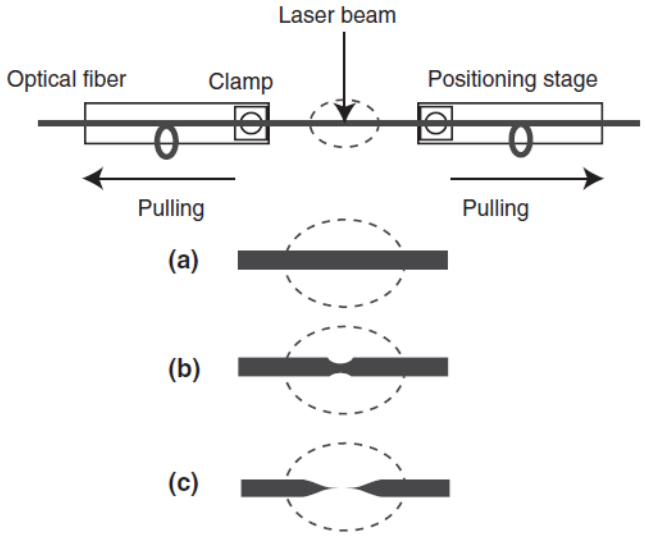

Figure 1. The fabrication of nanofibers through laser pulling. (a)–(c) The course of pull in time. In (c), a nanotip has been formed[1].

2.2. Chemical etching

The nanotips were fabricated by chemical etching (using the HF principle), at the Institute of Applied Physics, starting from 500 micron - diameter multimode optical fibre down to 100 nm at the tip. The complete procedure has been submitted for a patent owned by the CNR.

In Figure 2 a picture of the optical fibre nanotip realized with the chemical etching procedure is shown.

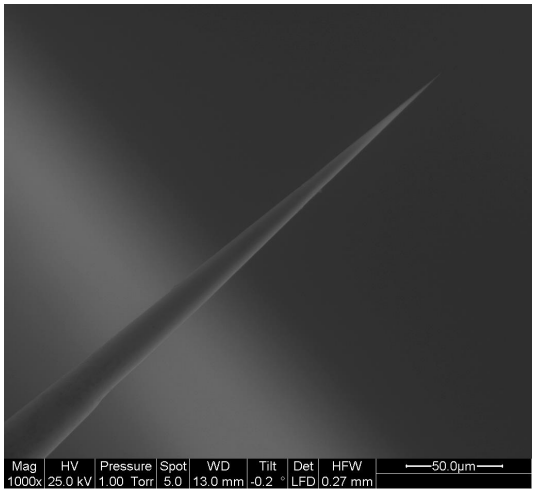

Figure 2. Picture of the optical fibre nanotip

3. The designed probe-sequences ad the chemical procedure applied on the NANOtips

The interest for this project was focused for the development of molecular beacon (MB) and molecular sentinel (MS) for the mRNA for survivin. In particular, once the sequence complementary to the mRNA has been defined, the two different probes were designed following the requirements for the two different applications.

3.1. The designed sequence for MS and the applied chemical protocol

In table 1 the sequences designed for the MS used on the SESRS-system are showed. In particular, the red part of the MS is the sequence complementary to the mRNA for survivin. The black parts are involved in the loop of the hairpin configuration. The SH-group was chosen for the probe immobilization on the silver nanoparticles used for SERS and the Cy5 was the marker for the Raman signal. Moreover, the table shows the target sequence that is specific for the MS and exactly complementary in the red part. The non-complementary sequence has a completely random sequence.

Survivin	MS	5'-SH-C6-CGACGGAGAAAGGGCTGCCACGTCG-Cy5-3'
Survivin	target	5'-CCCCTGCCTGGCAGCCCTTTCTCAAGGACC-3'
Non complementary		5'-GTGTAGGGATTATAGAGTCGCTTTC-3'

Tabel.1. Designed probe for the MS, the specific target and the non-complementary sequence

A scheme of the chemical protocol is here reported:

1. 15 min in piranha solution
2. 20 min in milliQ H_2O
3. Wash with EtOH

4. 2h silanization with APTES in EtOH 10% (@ room temperature). The tips were dipped for about 3 mm.
5. Wash the tips with EtOH, dipped gently
6. Wash the tips in milliQ H2O, dipped gently
7. Over night, tips in [Ag] about 0.1nM (the Ag nanoparticles are in H_2O stabilized with citrate)
8. Tips were washed in milliQ H_2O, dipped gently
9. The probe (MS) was heated for 1 minute in water bath at about 80°C, then left to go back at room temperature slowly (to optimize the hairpin formation)
10. Over night probe immobilization ([MS] 1μM, in 0.5 M NaCl and 10 mM SPB (sodium phosphate buffer: 0.26 g KH_2PO_4; 2.17 g Na_2HPO_4-$7H_20$; 8.71 g NaCl; 800 mL H_20; adjust the pH and bring volume to 1 L with H20) pH 8.0 buffer)
11. Wash the tips in 0.5 M NaCl and 10 mM SPB, pH 8.0 buffer, dipped gently
12. 1h surface passivation with mercaptohexanol [MCH] = 1mM in 0.5 M NaCl and 10 mM SPB, pH 8.0 buffer
13. Wash the tips in 0.5 M NaCl and 10 mM SPB, pH 8.0 buffer, dipped gently
14. Take the reference measurement for all the tips after MCH-passivation
15. 1h of reaction with the target sequence and the non-complementary sequence, the tips were tested for SERS signal observation.

3.2. The designed sequence for MB and the applied chemical protocol

In table 2 the sequences designed for the MB used on the fluorescence-system are showed. In particular, the red part of the MB is the sequence complementary to the mRNA for survivin.

Survivin MB 5'-(ATTO647N)CGACGGAGAAAGGGCTGCCACG**X**CG(BBQ)-3' **X**=C_6-dT Thio

Survivin target 5'-CCCCTGCCTGGCAGCCCTTTCTCAAGGACC-3'

Non complementary 5'-GTGTAGGGATTATAGAGTCGCTTTC-3'

Tabel.2. Designed probe for the MB, the specific target and the non-complementary sequence

The black parts are involved in the loop of the hairpin configuration. The SH- group was chosen for the probe immobilization on the silanized nanotips, via a cross-linker bonding (SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate). The two side-end markers were chosen as couple for the FRET, ATTO647N as the fluorophore and the BBQ as the quencher. Moreover, the table shows the target sequence that is specific for the MB and exactly complementary in the red part. The non-complementary sequence has a completely random sequence.

A scheme of the chemical protocol is here reported:

1. 15 min in piranha solution
2. 20 min in milliQ H_2O/5 min MeOH/5 min milliQ H_2O/5 min MeOH
3. 2h silanization with 5% APTMES in MeOH/H_2O (1:1) (@ room temperature). The tips were dipped for about 3 mm.

4. Wash the tips with MeOH/H_2O/MeOH, dipped gently
5. 1 hour @ 100°C in oven
6. 1 hour in [SPDP]=4mM in PBS, pH 7.4
7. Tips were washed in PBS and tris-buffer (10 mM tris-HCl, 10 mM $MgCl_2$, pH 8), dipped gently
8. The probe (MB) was heated for 5 minute in water bath at about 80°C, then left to go back at room temperature slowly (to optimize the hairpin formation)
9. **Over night** probe immobilization ([MB] 1μM, in tris-buffer @ 4°C)
10. Wash the tips in tris-buffer, dipped gently
11. Take the reference measurement for all the tips
12. 1h of reaction with the target sequence and the non-complementary sequence, the tips were tested for fluorescence signal observation.

4. The Optical set-up

Two different optical setups were used for the two different approaches: SERS-based setup, developed at Duke University and fluorescent-based setup, developed at IFAC-CNR

4.1. The SERS-based optical setup

The detection scheme of the SERS-based optical setup is shown in Figure 3.

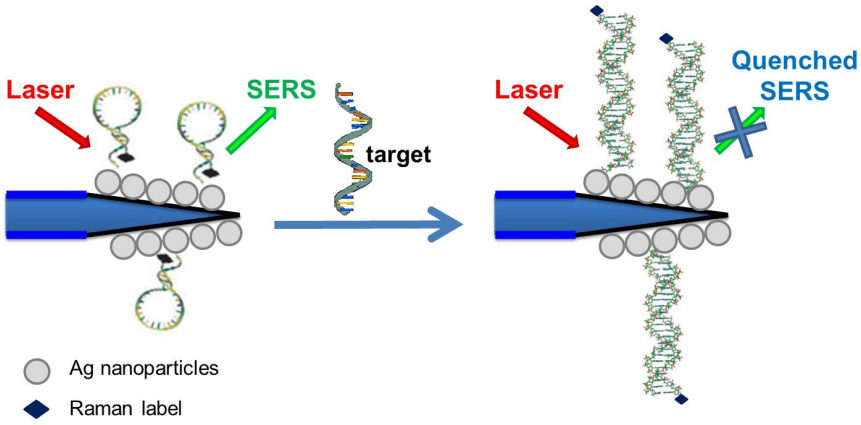

Figure 3. SERS-based optical setup

The complementary side ends of the MS hairpin hybridize into 6 basepair-stem sequence allowing formation of a stable hairpin structure at room temperature in the absence of a complementary DNA target. The 3'-end of MS hairpin probes was modified with Cy5 Raman label. Conjugation of MS probe onto the silver nanoparticles (Ag-NP) surface was achieved by using an alkyl thiol substituent at the 5'-end. In the absence of target sequence, the MS's stem loop is in the closed state. At this state, the Cy5 dye is in close proximity to the Ag-NP surface (<1 nm), inducing a strong SERS signal. When the complementary target DNA sequence is added, it hybridizes with the MS, forcing the stem loop to open. At this state, the Cy5 dye is physically separated from the Ag-NP surface. The opened stem loop results in a quenched SERS signal.
The laser excitation was at 635nm and the spectra collected were between 400 and 1800 nm.

4.2. The fluorescence-based optical setup

The fluorescence-based optical setup is shown in figure 4.

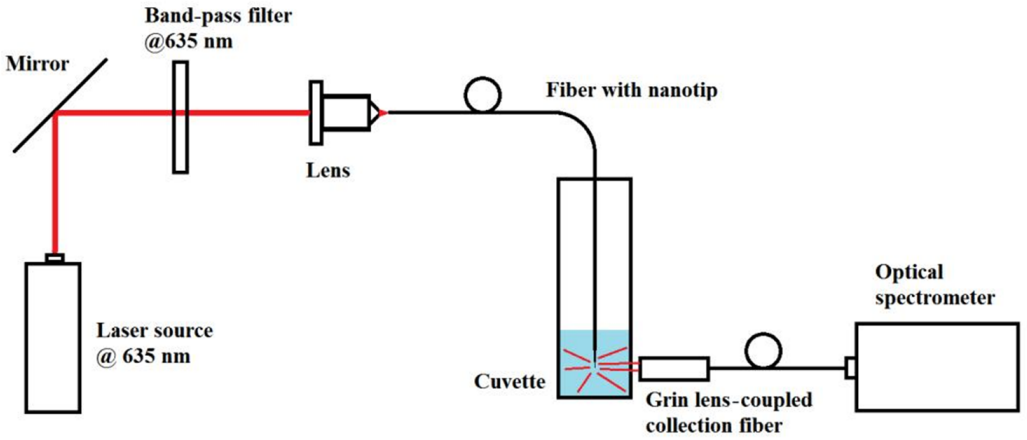

Figure 4. Fluorescence-based optical setup

The surface of the nanotip was internally excited by a laser diode emitting at 635 nm coupled to the nanotip fibre. The fluorescent signal was collected at 90° by a GRIN lens focused on the very tip area, coupled to a multimode optical fibre and sent to an Andor Shamrock 303 spectrograph.

5. Results and discussion

Preliminary results on the use of the two complementary methods are here reported. In particular, the MS and MB hairpin-probes suitable for mRNA for survivin (an anti-apoptotic protein involved in many different types of cancer) were designed and characterised. The response of the two sensing nanoprobes in the presence of the interaction with 1 μM specific sequence with the MS and MB immobilised on the nanotips are shown in figure 5 and 6, respectively, revealing the potentiality of the two optical approaches.

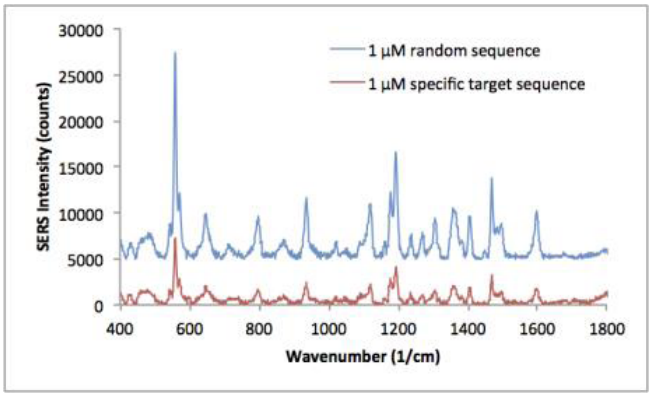

Figure 5. SERS spectra of the MS immobilized on the fiber NANOtip
after the reaction with 1 μM random sequence (blue) and 1 μM specific target sequence (red).

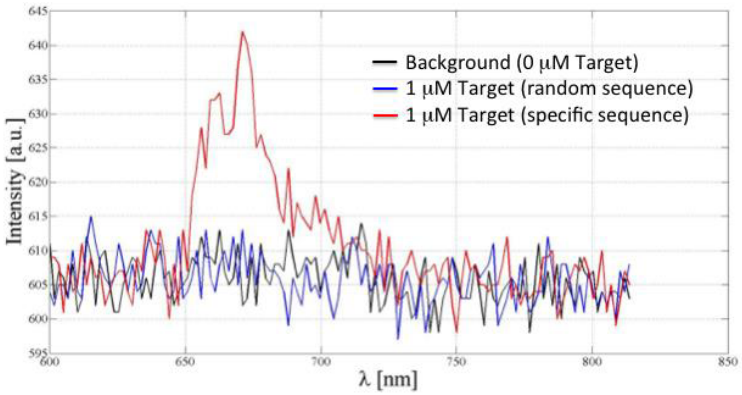

Figure 6. Fluorescence spectra of the MB immobilized on the fiber NANOtip, in buffer solution (black), after the reaction with 1 μM random sequence (blue) and 1 μM specific sequence (red), respectively.

6. Conclusions

The results expected by this Short Term Mobility program, were: the development and characterization of Molecular Beacons (MB) and Molecular Sentinel (MS) to be used in theranostics by means of sensing and imaging of tumor cells. The signal related to the interaction of the MB or MS to the target sequence, would have been measured by fluorescence and surface enhanced Raman spectroscopy (SERS). Further aim of the stay, would have been to consolidate the collaboration between the two institutes involved, between which a memorandum of understanding is already signed.

The main tasks were fully achieved with the design and characterization of the MS and MB hairpin-probes suitable for mRNA for surviving. Moreover, the two different setups based on SERS and on fluorescence were compared, revealing the potentiality of the two optical approaches; bringing to the submission of an abstract to an international conference (see Publications). The last, but not least, the collaboration with the prestigious Fitzpatrick Institute for Photonics, at Duke University, was consolidated.

Publications

The STM program, giving the possibility to consolidate the already existing collaboration between the two involved institute, gave also the possibility to already submit an abstract to the Conference: **EUROPT(R)ODE 2014**, 13-16 April, Athens, Greece (please, see the attached file to this report).