



PROJECT FINAL REPORT

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1. Final publishable summary report

1.1 executive summary

The main goal of our proposal was to develop a novel optical nanobiosensor based on extraordinary vibrational signal enhancement of the proteins to be detected. To reach vibrational signal enhancement, we exploited the optical properties of specially designed metallic nanoparticles, which should act as nanoantenna and the associated field enhancement to obtain a direct detection of proteins bound to the nanoparticle.

Thus, our sensor reached high sensitivity provided by the established large enhancement of vibration signals due to the resonant excitation of the nanoantenna device used as substrates. The aim was to detect only a few proteins with concentration much lower than 1pM and finally to reach detection threshold such as femtomole or lower. High molecular selectivity has been reached with the functionalisation of the nanoantenna. Such functionalisation selectively favours the immobilisation of the protein to be detected at the vicinity of the nanoparticle surface, providing the best enhancement and then the detection of the targeted protein. Our nanobiosensor includes two main components: the nanoantenna device, which corresponds to our sensor transducer and the functionalisation, which corresponds to its bioreceptor. Then, each functionalised nanoantenna device used as vibrational signal enhanced system can be considered as an individual and specific nanosensor of proteins. As a consequence, our nanobiosensor integrated in a vibrational spectroscope allows the detection and the analysis of the enhanced vibrational signal from the targeted proteins and thus corresponds to a diagnosis instrument.

Our nanobiosensor has been validated on the detection of proteins on body fluids. These proteins have been chosen since they have been identified as specific biomarkers of common pathologies. This validation was applied to improve their detection (better sensitivity, decrease of the detection threshold) and open the way to the early diagnosis.

1.2 summary description of project context and objectives

Despite some recent advances, the detection of traces of biological species in medical diagnosis remains a challenge at the European level. Biological sensors able to detect specific biological species need further progress, not only new technical solutions but also applications of new ideas and more sensitive methods. Indeed, the detection of biomarkers specific to some diseases for example can be of real importance for medical diagnosis. A large number of disease biomarkers are proteins and their presences in body fluids (blood, plasma, saliva...) are considered as indicators of the presence of the diseases. Thus, their detection is a key point in order to enable physicians to provide quickly the right therapy and their detection in low concentration could enable the early diagnosis of serious diseases.

Low-cost label free detection devices with a very high sensitivity and selectivity will help remove that bottleneck if used on a wide scale. Due to their complexity, the design of such devices requires an interdisciplinary collaboration as well as complementary expertises between research groups that cannot be found at a single laboratory level.

It is in this context that the Nanoantenna consortium made of twelve partners combining physicians, biologists, chemists and physicists was created. Taking profit of the most recent advances in nanotechnologies, surface engineering and biotechnologies, the Nanoantenna project was a European multidisciplinary project aiming at developing a highly sensitive and specific nanobiosensor based on extraordinary vibrational signal enhancement of molecules dedicated to the *in vitro* proteins detection and disease (cancer, cardiovascular or infectious diseases) diagnosis.

The project was based on a three-step principle as follows:

Nanoantennas

High sensitivity was reached with metallic nanoparticles, used as nanoantennas. When excited by an incident light, they have the ability to enhance locally the vibration signal of molecules deposited on the nanoparticle surface. For one resonantly excited nanoparticle, this enhancement enables the observation of a very few quantity of proteins.

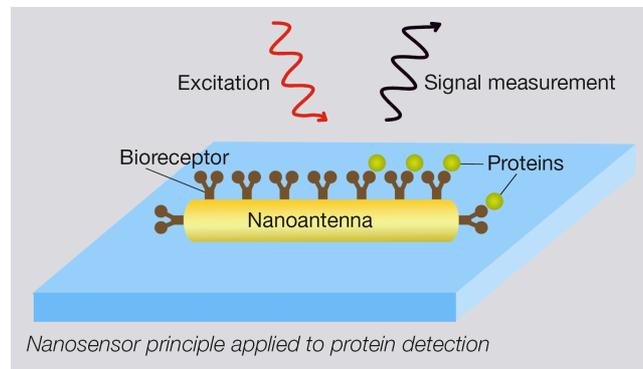
Bioreceptors

High molecular selectivity was reached with the functionalisation of the nanoantenna with specific bioreceptors. Such functionalisation selectively caught the protein to be detected at the vicinity of the nanoparticle surface, providing the best enhancement and then the detection of the targeted protein.

Nanobiosensor

Each functionalised nanoantenna device was an individual and specific nanobiosensor of proteins. As a consequence, the nanobiosensor allows the detection and the analysis of the targeted proteins and thus can be integrated in a diagnosis process.

The following figure gives an illustrative explanation of the sensor principle:



In order to develop our sensor system, our approach was divided into three main components:

- The molecular target to be detected by the nanobiosensor: proteins in our case.
- The transducer: the nanoantenna device
- The bioreceptor: the functionalisation layer to capture the targeted proteins at the nanoantenna surface

These three components were developed and optimised during the project. Thus one of the objectives was to determine the best components to be integrated in the nanobiosensor. After integration of all the components in a single system, the objectives were to determine the characteristics (sensitivity, selectivity, reproducibility...) of our nanobiosensor and to validate it for the *in vitro* detection of proteins in body fluids.

Our project was then divided in 5 scientific workpackages:

- *WP1, Biosensor: Protein spectral signature*
- *WP2, Transducer optimisation: Optical properties of nanoantennas*
- *WP3, Bioreceptor optimisation: Biological functionalisation of the nanoantennas*
- *WP4, Nanobiosensor integration and Diagnosis instrument development*
- *WP5, Validation of the nanobiosensor*

1.3 description of the main S&T results/foregrounds

Since the scientific results are directly related to the WP progresses, they will be presented WP per WP in relation with the WP objectives.

2.2.1 WP1: Biosensor: spectral signature

The main objective of this WP was to acquire Raman and IR spectra of specific proteins, to assign the spectral features to molecular structures and to correlate spectral markers with the presence of the targeted proteins. Thus, we have determined the spectral fingerprint of the proteins targeted in the project enabling a direct label-free detection and molecular identification of the proteins, It has provided spectral markers and allowed us to discriminate between proteins with similar structures.

As a consequence, we have determined all the spectral signature of the targeted proteins and we have been able to provide a database of the reference spectra to be detected by the nanobiosensor as well in SERS and SEIRS.

For the detection of the proteins, the following specifications have been chosen: excitation wavelengths for SERS (785 and 660 nm) and Infrared wavelengths to be used for SEIRS (between 5,88 and 6,7 μm). For the spectral signature, it was determined to focus on the following spectral range: 1200-1700 cm^{-1} and more specifically on the Amid I bands located between 1500 and 1700 cm^{-1} since it was found that they were the most intense in Infrared and Raman spectra for all proteins.

Significant results

- Determination of the spectral signature of all the targeted proteins of the project
- Supply of a spectral database including the reference spectra of the proteins to be detected.

2.2.2 WP2: Transducer optimisation: Optical properties of nanoantennas

This WP has provided the physical know-how on nanoantennas. We have been able to provide a quantitative characterization the optical properties (plasmon resonances and field enhancement) of individual and of coupled nanoantennas on a wide spectral range (UV-visible-IR) and at the various scales (microscopic and nanoscopic). We have determined the influence of the morphological/geometrical parameters: size and geometry, coupling parameters (far field and near field coupling) on the optical properties of nanoantennas, and on the spectral enhancement factor (basic principle of the transducer function). Some theoretical models and electromagnetic numerical calculations of the optical properties of nanoantennas and of the coupling mechanisms in nanoantenna arrays for optimal performance were developed.

Electron-beam lithography has turned out to be the best way for reproducible production of nanoantenna arrays with a smooth nanoantenna surface morphology. The produced nanoantennas showed spectral properties and resonant field enhancement in accordance to the calculations. The tuning parameters for application to surface-enhanced infrared spectroscopy (SEIRS) of the targeted molecules of this project (in the amide band's range) were clarified. Also in the visible range significant resonance enhancement was proven. An almost inert substrate was chosen according to the application needs in SEIRS and SERS.

The results have indicated sufficiently high near-field enhancement to reach the objective of the project. We concentrated on the problem of optimization of field enhancement in narrow gaps at the proper frequency for SEIRS and SERS. We showed that coupling of narrow gaps in electric field direction increases field enhancement considerably but shifts the resonance frequency. For SERS polarization parallel and perpendicular was investigated. In SERS optimization was much more challenging than for SEIRS for several reasons: Enhancement should be high for both excitation and scattered wavelengths. The excitation wavelength cannot be chosen freely; the available laser techniques were considered as well as the possible contributions of resonance effects that depend on the excitation wavelength. Short wavelengths were less useful because of fluorescence and damping of plasmons. The horizontal resonance mode may not yield the biggest enhancement in SERS but it is

advantageous for the application in the project that it has a rather stable frequency position. Our article on a combined SEIRS-SERS study (SERS with the horizontal mode) was published in ACS Nano 2013.

The experiments and calculations for the determination of the optical properties of coupled nanoantennas by the various partners were done. Dimers and chains of nanoantennas were investigated. It turned out, that dimers are preferred because small errors in the production of a narrow gap may not disturb the function of the whole array. A paper on the coupling in the IR was published in Optics Express and one is in preparation for the visible range.

New methods have been implemented. The situation of an antenna on a supporting substrate was simulated with Finite Different Time Domain (FDTD) calculations of the optical and infrared response. Independent calculations predicted resonance spectra very close to those that were measured. The emphasis on both the development of the tools for optimization and the inverse problem resolution and on the full 3D Finite Element Model was considered. It was dedicated to the description of the nanoantenna geometry in detail and its surrounding in a realistic manner, in order to determine the best shape and the best size of the nanoantennas for the highest SERS signal to increase the biosensor sensitivity.

Significant results

Several significant results are achieved:

- High quality nanoantenna arrays with SEIRS and SERS activity were made, including nm-sized gaps between tip ends.
- Infrared near-field enhancement at a hot spot was directly measured with scattering SNOM.
- The tuning of antenna resonances via the geometry (length, height, width) of individual antennas was clarified.
- Theoretical calculations were done in accordance to experiments. So, the tuning behaviour was understood.
- Since many years, theory has predicted a distinct spectral shift between the near- and far-field optical responses of plasmonic antennas. We combined near-field optical microscopy and far-field spectroscopy of individual infrared-resonant nanoantennas to verify experimentally this spectral shift for the first time experimentally. Numerical calculations corroborate our experimental results. Furthermore, we have studied numerically the implications of this spectral shift in SEIRS, showing that it has to be considered in order to optimize the molecular spectral absorption contrast in plasmonic (bio)-sensing devices.

2.2.3 WP3: Bioreceptor optimisation: Biological functionalisation of the nanoantennas

The main objective of WP3 was to control and optimise the nanoantenna device functionalisation for biological species. The nanoparticle surface has to be functionalised to change its chemical reactivity and to become specific to the targeted proteins. The adequate functionalisation of the metallic nanoparticle was determined to increase the selectivity of the nanobiosensor. The affinity of the resulting biomimetic bioreceptors to specific biomarkers was assayed to select the best procedure for immobilization of biorecognition elements on nanoantennas.

EBNA-1

The designed EBNA-1 aptamer was tested to detect the protein using quartz crystal microbalance (QCM) method. The detection system is based on an EBNA-1 binding to the aptamer immobilized on the Au-surface of a QCM electrode. The reported biosensor was successfully used to quantify EBNA-1 concentration in buffer solution. It was demonstrated

that the detection method was sensitive and selective against EBNA-1 and able to detect 50 ng/mL of the protein while working in label-free mode. However, it is highly desirable to decrease the detection limit as low as possible. Therefore we have also attempted here a signal amplification using a biocatalytic precipitation of a soluble substrate to an insoluble product on the electrode surface. This signal amplification system allowed for a 100 times lower detection limit of 0.5 ng/mL.

MnSOD

The aptamers for MnSOD were developed by SELEX process. For this process DNA library was prepared and mixed with the target protein. For separation of unbound and bound DNA we used 5 % Native PAGE. The band of protein-DNA was cut and extracted from the gel. DNA was amplified by PCR and dsDNA separated to ssDNA by Streptavidin dynabeads®. All the steps were repeated again. After 9 SELEX rounds the obtained family of ssDNA has been cloned and sequenced. Having the results of this sequencing we were able to synthesize DNA aptamers with affinity for MnSOD.

To determine the surface coverage of gold with Fab fragments, QCM was chosen. Immobilization of MnSOD was performed. QCM electrode was cleaned with Piranha solution (sulphuric acid: 30 % hydrogen peroxide, 1:1). Chemical cross-linker dithiobis(succinimidyl propionate) (Sigma-Aldrich) in dimethylsulfoxide, 4 mg/ ml, was injected into the flow cell containing the QCM electrode. Washing was performed with dimethylsulfoxide and next with water and HEPES 20 mM, pH 7, MnSOD, 100 µg/ml was applied. Again HEPES was utilised in the washing step. Next the buffer was changed to binding buffer (100mM NaCl, 20mM Tris-HCl pH 7.6, 2 mM MgCl, 5 mM KCl, 1 mM CaCl six and nine SELEX round), 100 nM, was injected into the flow cell and washed with binding buffer. The control experiment was performed with DNA library before SELEX rounds.

Thus, we obtained the family of ssDNA's with some affinity for MnSOD (9 SELEX round (up)). This family is being sequenced through expression in E.Coli. Having the results of this sequencing we have been able to synthesize DNA aptamers with high affinity for MnSOD.

Significant results

Several significant results are achieved:

- The synthesis of several bioreceptors to be integrated in the nanobiosensor: Fab fragments, aptamers for MnSOD and EBNA-1 proteins.
- The detection of proteins using such bioreceptor with high affinity and low detection limit.
- A functionalisation protocol was provided to the final users and used during the validation workshop.

2.2.4 WP 4: Nanobiosensor integration and diagnosis instrument development

The scientific activities of WP 4 concerned the integration of the different components developed in the previous WPs with an ad-hoc spectrometer, aimed at setting up a biomolecular sensor with optimised characteristics. More specifically, the activities included:

1. Determination of the relevance of the technological solutions adopted for the nanoantenna production and functionalization in view of the nanobiosensor integration.
2. Development of a diagnosis instrument prototype.
3. Integration of the instrument with the nanoantenna chip and determination of the nanosensor characteristics.

The nanobiosensor integration has been accomplished. Electron-beam lithography (EBL) combined with thermal, electron evaporation and lift-off turned out to be the best suited technologies for reproducible production of nanoantenna arrays. Scaling up of EBL, an

intrinsically serial technique, to mass production is possible with electron beam direct writing using state of the art machines reaching a writing speed of 100MHz up. Such machines can reach a rate of about 100 nanoantenna chips per day, i.e. 30K chips per year. If a higher throughput is needed nano imprinting lithography, an intrinsically parallel technique, could be a good candidate, allowing for the fabrication of nanoantenna arrays. EBL would be needed, in this case, only for the fabrication of the nanostructured master.

The best nanoantenna parameters for nanosensor production have been determined. Gold has been preferred to other plasmonic materials (e.g. silver, aluminum) because of its time stability allowing further functionalization steps even after months from the production date. On each chip two planar arrays of nanoantennas ($100 \times 100 \mu\text{m}^2$) are lithographed allowing for reproducibility tests.

For SERS and SEIRS the most efficient nanostructure geometries were determined and tested. The functionalization protocols determined in the WP3 for MnSOD and EBNA have been validated for the nanobiosensor operation in this WP and in the WP5.

The spectroscopic development has been accomplished. A specific spectroscopic prototype has been developed for protein detection using the nanoantenna-based nanobiosensor. This development has been done using a commercial system adapted to take into account the main constraints due to the use of the nanobiosensor and its characteristics.

The nanobiosensor has been simulated, tested and characterized for what concerns sensitivity (i.e. limit of detection), linearity, reproducibility and reliability for both SERS and SEIRS.

The SERS tests on the nanobiosensor have been started with simple probe molecules and the functionalized sensor has been validated on MnSOD, EBNA and GIPC1 within the activities of WP5.

The results can be summarized as follows:

- Theoretical simulations have shown that the effect of the functionalization layer (aptamer) on the nanoantennas can be neglected for what concerns the shift of the nanoantenna resonance induced by the binding of the molecules themselves. These results are confirmed by experiments on nanoantennas functionalized with aptamers.
- The optimal fabrication parameters for maximum SERS from nanoantennas have been determined via theoretical simulations for both operation conditions of the nanosensor in air and in liquid, and they agreed with the experiments.
- Experiments on nanoantennas have shown that the decay length of the enhanced fields (in the visible) is indeed larger than the dimension of the aptamer length, confirming that, once bound, the target protein still benefits of an enhanced excitation field even in presence of an aptamer functionalization layer.
- The SERS characterization of the nanosensor was done using a probe molecule.

Results are the following:

- At 660nm, the limit of detection is 10nM. At 785nm, the limit of detection is 100pM and can reach 1pM with specific nanoantenna geometries.
- In all experiments a SERS signal increase is observed Vs the concentration up to a concentration of 10 μ M.
- A statistical analysis was carried out for each excitation wavelength and nanoantenna geometry. A reproducibility of the average signal with a standard deviation of 30% was observed.
- The IR nanosensor characteristics (SEIRS) have been tested directly on proteins

- Proteins were analysed in dry conditions as an example of enhanced unspecific protein detection. Based on the detection of the enhanced Amide I and Amide II signals. The limit of detection was found to be 100nM.
- IR near-field characterization was done on the nanosensor functionalized with aptamers and with MnSOD absorbed using a scattering SNOM coupled with a quantum cascade laser resonant with the Amide I band of the protein. Results showed that with the adopted protocols either no protein has been absorbed or that the quantity was too small to be detected with infrared scattering SNOM. Measurements on gold flat films confirmed that the absorption of the protein occurs in an inhomogeneous way on the surface, it was not clear if this was due to clustering of the aptamer functionalization layer, or it was inherent to the protein capture.
- Integration of SERS and SEIRS measurements on the same nanoantenna chip was demonstrated using a probe molecule. Enhancement factors of 10^5 and 5×10^2 were found for SEIRS and SERS, respectively.

The main characteristic of the nanobiosensor is its ability to detect low concentration of molecules. It has been demonstrated that pM and nM concentration can be reached for respectively SERS and SEIRS. These detection thresholds are very promising and are competitive comparing to other detection techniques as ELISA. Moreover, these thresholds are reproducible as well as the detection signal. It means that the nanobiosensor is a reproducible one. Finally, the nanobiosensor can be used with both methods SERS and SEIRS.

Significant results

- A spectrometer prototype, portable, capable to carry out LSPR analysis and SERS has been developed.
- The nanobiosensor concept, i.e. enhanced molecular and protein detection using the nanoantenna + molecular layer + spectrometer assembly, has been demonstrated for what concerns both SERS and SEIRS.
- It has been proved that properly designed nanoantennas allow for simultaneous SERS and SEIRS molecular analysis.
- SERS detection limits of probe molecules have been reached in the pM range using nanoantenna.
- SEIRS detection limits of proteins (unspecific binding) have been reached in the nM range using nanoantenna.

2.2.5 WP5: Validation of the nanobiosensor

The aim of the workpackage 5 was to validate the nanobiosensor on direct applications, to compare it with the detection techniques already available and to explore its capabilities in real conditions for diagnosis on complex samples such as serum or saliva from patients.

MnSOD detection

The workpackage focused on the MnSOD protein. A large number of antioxidant systems are involved in the scavenging of ROS, including the superoxide dismutase (SOD) family of proteins. MnSOD exists in two forms: a precursor one with 222 amino-acids in which the mitochondrial targeting sequence is cleaved within the mitochondrial matrix, leading to a mitochondrial mature form and an active enzyme, a homotetramer with one manganese ion per subunit.

A commercial enzyme immunosorbent assay (ELISA) kit was used to detect the MnSOD. The plate used strips were coated with a mouse monoclonal antibody specific to human MnSOD.

The standard protein was a recombinant human MnSOD. The secondary antibody was a biotin labelled mouse anti-human MnSOD antibody, which was revealed by an avidin linked horseradish peroxidase. The substrate used was a TMB (tetramethylbenzidine) solution. The minimal detectable dose of human MnSOD in this kit was 78 pg/ml. There was no cross-reactivity with the other isoforms of SOD. The intra-assay and inter-assay precisions were less than 15%. In collaboration with the Hepatology department of the Jean Verdier Hospital in Bondy (France), 234 patients with dysmetabolic disorders, 292 alcoholic patients, among them 169 with cirrhosis, 57 with small HCC and 66 with large HCC were enrolled. The level of MnSOD was determined in the plasma of these patients by the use of ELISA. There is a statistically significant difference between all pathological groups (dysmetabolic disorders, cirrhosis and both HCC groups) as compared to the healthy control subjects. Furthermore, as compared to cirrhosis and to curative HCC groups, the level of MnSOD in patients with large HCC in which the treatment remains at this time palliative, was significantly higher, suggesting that seric MnSOD could be a prognostic biomarker but not diagnostic one. MnSOD was also detected in the saliva of patients with cardiovascular disease. The range of the detected level was similar at those obtained in the plasma. Some of the samples in which MnSOD level was detected by ELISA were used for the Nanobiosensor experiments. For the MnSOD detection, the nanoantenna were functionalised using the specific aptamer developed in the WP3. The investigation of biological media showed positive results. First experiments done with saliva and serum led to great success in MnSOD protein detection at very low concentrations. Specificity of the nanobiosensor was demonstrated. A calibration curve could be performed. Experiments for evaluating repetability (variability of the measurements obtained by one person while measuring the same item repeatedly) or reproducibility (variability of the measurement system caused by differences in operator behavior) were not large enough in order to validate these characteristics.

Anti-GIPC1 detection

The Nanoantenna surface was functionalized using the GIPC1 protein on the nanoantenna. We have not been able to get a clear SERS spectrum of the GIPC1 protein. It could be explained by the possibility that the proteins were on the top of the nanoantenna and not on the nanoantenna side, where electromagnetic enhancement is the most important. After incubation in a serum containing Anti-GIPC1, some new bands can be observed. However, these features are not so clear since they have a small intensity and it can not be completely excluded that they come from the spectrum noise. It means that further experiments are needed to effectively demonstrate that we could detect the Anti-GIPC1 in human serum using SERS.

Using SEIRS, when ramping up the concentration of GIPC1 we start to detect the amide I vibration at 10^{-4} M. At this concentration, however, we also noticed agglomerates on the surface, which have diameters of a few tens of micrometers. Blocking with mercaptohexanol (MHO) slightly changed the spectral response, but could not prevent BSA from binding to the surface. With MHO and BSA, both blocking the unspecific binding sites on the antenna, protein binding from human serum (diluted 1:10 in PBS) was detected. It should be pointed out here, that the amide I signal after incubation with body fluids (here serum, but same was true for saliva above) was clearly red shifted to about 1635 cm^{-1} . This indicated the binding of a different species and clearly demonstrated the ability of antenna enhanced IR spectroscopy to discriminate different analytes.

The investigation of biological media showed positive results with SERS. First experiments done with saliva and serum led to great success in MnSOD protein detection at very low

concentrations. In the case of Anti-GIPC1 detection, further experiments are necessary to assess the nanobiosensor efficiency.

SEIRS of adsorbed biomolecules could be demonstrated using body fluids. Unfortunately, such detection was not possible at low concentration. It has also been noticed that the unspecific binding can disturb the detection, which indicates the extremely high requirements for functionalization of the gold surface.

Thus the nanobiosensor has demonstrated its ability for the *in vitro* detection of proteins in body fluids but we still need to go one step further to demonstrate its efficiency for the disease diagnosis.

Significant results

- The detection of the targeted proteins in low concentration with the nanobiosensor
- The detection of MnSOD in body fluids (saliva and serum)
- The proof of concept of the nanobiosensor

1.4 potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

1.4.1 Potential Impacts

Impact on Science and Technology

The development of the nanobiosensor has needed intensive studies of its different components: optical properties of the nanoantenna, functionalisation and vibrational fingerprint of the targeted molecules. Thus, all the research studies related to these three components had an impact on each research field: physics, chemistry and biology. But one of the main impacts addressed the pluridisciplinary field since the integration of the nanobiosensor components has implied the convergence of several fields of expertise and then a strong interaction between the partners working at the interface of several disciplines.

Our project had also an impact on technologies since a new nanobiosensor technology has been developed and directly applied in the biomedical field. Its abilities to detect proteins in low concentration and in body fluids have been demonstrated. It gives evidence that the Nanoantenna concept is suitable to set up a new innovative nanobiosensor with unique characteristics based on the most recent advances in nanotechnologies and biotechnologies. Furthermore, the *in vitro* detection of biomarkers at low concentration paves the way to the early disease diagnosis. Thus, we have been able to demonstrate the relevance of our nanobiosensor and we have given a proof of concept of our sensor. For the diagnosis instrument, a spectroscopic instrument specifically designed as a diagnosis instrument prototype has been developed by the consortium. Thus, in the near future, we will be able to develop an actual nanobiosensor taking into account all the considerations of the clinical difficulties. This last point will be all the most favoured by the presence of the two industrial partners both in technological field and in medical field in the consortium and by the fact that these two partners want to continue the collaboration in this sensor development.

Impact on health and nanomedicine

The aim of the Nanomedicine, Nanotechnology for health, is to advance our understanding on how to more efficiently promote good health, to prevent and treat major diseases. This issue address ambitious and responsible researches, developments and innovations to strengthen the competitive scientific and industrial position of Europe in the area of Nanotechnologies and to improve the quality of life and the health care of its citizens.

One of the research priorities in Nanomedicine is the Nanotechnology-based Diagnostics to identify disease at the earliest stage possible, ideally at the level of a single molecule and it is important to promote the development of diagnostic tools of better sensitivity, specificity and reliability. Our project was in direct line with this issue since our final results opened the way to a new methodology to detect diseases, through nano- and biotechnologies, and had enable the reduction of the detection limits compared to the current methodologies. Thus, our project has provided a new technological tool to detect important biomarkers, enhancing reliability and accuracy of detection. Since we developed a fast and sensitive (picomolar range) nanobiosensor, we assume that our project could have an impact on detection of diseases and then on the early diagnosis and the therapy monitoring. This impact will be all the more important since the choosen applications concern notably the cancer detection (MnSOD and GIPC1 are biomarkers for respectively liver and breast cancers), which is one of the six priority diseases at the European level and which imposes a high socio-economical burden on society. Indeed, the cancer is currently the second leading cause of death in Europe, while it shows probably the highest clinical complexity. As a consequence, our nanobiosensor could have a wide influence in patient management, in improving patient's quality of life and in lowering mortality rates.

Furthermore, one of the most rapidly advancing fields in medical diagnostics is the point of care (POC). Since our nanobiosensor is simple to use and since we have developed a compact and transportable spectroscopic instrument to analyse the sensor, the nanoantenna system seems to be especially well suited for POC. Application of the nanoantenna system for POC tests would allow specific and rapid determination of a relevant analyte or a whole diagnostic panel in the doctors office or the emergency ward, places where regular assays for protein analytes such as ELISAs or latex enhanced assays cannot be performed.

Potential impact on other application fields

Many other fields could be concerned by such a nanosensor such as the environment monitoring (detection of pollutants in water for example), the food safety (detection of dioxin, distinguishing *Listeria monocytogenes* from other harmless *Listeria* species...) or even homeland security for the detection of biowarfare / bioterror agents (such as anthrax). Some new developments are in progress to propose new applications and the detection of new targets by using the nanoantenna concept.

Our nanosensor could also have some impacts on the other detection methods since it can also be seen as a first investigation method (easy using and fast detection method). It will be a complementary technique for first sample screening before using more costly or slower techniques such as mass spectrometry in chemistry or ELISA in biology, for result confirmation.

Impact on competitiveness and economy

The European Council in Stockholm in 2001 invited the European Commission to "examine measures required to utilise the full potential of biotechnology and strengthen the European biotechnology sector's competitiveness, in order to match leading competitors".

The Nanoantenna project has contributed to achieve this goal, since our final results gave the proof of concept that our nanobiosensor based on the Nanoantenna approach has the ability to detect *in vitro* some proteins in body fluids at low concentration. We have also showed the sensor added value compared to other detection methods and we gave evidence of the feasibility of the development of a simple nanobiosensor allowing a fast and direct detection of molecules such as proteins. With such a result, our nanobiosensor enhances and improves the molecular detection and our set-up is able to compete with other detection systems, since it is a low cost and highly sensitive sensor. The involvement of industrial partners (Horiba

Jobin Yvon and Technoclone), in the project was of real importance since it allowed an easy and natural technological transfer. This latter issue has been all the more favoured since HJY has developed a specific spectroscopic set-up for the nanobiosensor analyses and TC has produced some specific biological compounds for the biomedical diagnosis. Even if the results should be confirmed and that some further experiments are necessary to determine the exact ability of our nanobiosensor for the disease diagnosis. Our project has opened the way of some potential industrial exploitation.

The project had also some immediate effects on the industrial partners. Thus for HJY, the project has allowed to address within the consortium several issues. HJY acquired a deeper understanding of the requirements in biology and related fields, areas in which HJY takes part and in which HJY addresses already in its product strategy for today and even more for the future. HJY developed a dedicated compact Raman systems with increased sensitivity, increased spatial resolution and full automated “hands-off” operation, needed for non-specialists (medical and biological laboratories, forensic laboratories) and then expanded its area of expertise in the application of Raman spectroscopy and SERS with dedicated designs. For the industrial partner TC, the consortium has been able to determine the relevance of technological solutions available throughout the project concerning the development of our nanobiosensor and its applications to diagnosis. Thus, the involvement of the industrial partners has allowed the development of a new product.

At the moment, our nanobiosensor prototype is at the level 3 or 4 on the Technology Readiness Level. It needs some further developments to envisage its potential commercialisation. In this aim, the industrial partners want to continue their collaboration with the scientists of the consortium to go one step further. For example some new collaborative projects are in progress with HJY partners and some have been submitted with TC notably in the framework of the FP7 program. Even if such proposals were not successful, it has allowed defining some new directions for the results exploitation and for the nanobiosensor development.

Thus, we assume that this project had an impact on the industrial partners of the project and then on the economy and the competitiveness of our industrial partners.

Training impact

Nearly each partner has recruited one PhD student or a post-doctor in the framework of the project. They have worked directly and full time on the project. All these PhD students and post-doctors have participated to the consortium meeting. It was the occasion for them to present their own work and to be directly in contact. They have also collaborated all together in the same research field. We assume that this “Research community” have enhanced the collaboration between all the partners. This was all the more relevant since the PhD students and post-doctor involved in the project were fully integrated in this wide European project and in the research management with the consortium meetings and all the collaboration exchanges. Thus, in the way of the development of the European research space, the implication of the PhD students and post-doctors in such collaborative project had an impact on their research and their abilities to collaborate at the European level.

Furthermore, the project included several consortium meetings but also some workshops and one summer school. This has given the opportunity to give some lectures on specific fields important for the project in direction of the participants but more especially in direction of the students (PhD, master...) involved in the different partners’ laboratories. The PhD student got some training on their research field but also on other fields since the project was a multi-disciplinary one. They were able to get knowledge on physics, biology and chemistry. Moreover, to have a good understanding between the partners and the PhD students, some training times were organised for the PhD student and researchers in laboratories involved in

another field. For example, the PhD students in physics have spent one month training in a laboratory of biology to learn how can be used the proteins and how the biosensor can be validated. One training was also organised on the surface chemistry and the functionalisation protocol in order to integrate the different components of the nanobiosensor. In the opposite way, biologists have spend some time in a laboratory of physic to learn about the optical instruments was working and how to use the nanobiosensor. We hope that at the end, all the PhD students, the post-doctors and the researchers involved in the project got a strong formation in both biotechnologies and nanotechnologies.

1.4.2 Dissemination

Internal communication within the consortium.

In terms of communication and dissemination inside the consortium, a restricted area for project members serving as an exchange platform has been put in place to allow the transmission of information in the consortium and to have access to all the documents produce by the project (deliverables, minutes, agenda, publications...). Eight meetings have also been organised (each 6 months) to gather all the partner members and to talk about the scientific aspects and to present the results of the project. Moreover, four workshops with external invited speakers were organised within the project for the partners training. Thus several topics in relation with the project objectives and tasks were approached: nanostructure production, surface functionalisation, plasmonics, biosensors. Such meetings have developed a strong dynamic between the involved researchers giving them the possibility to communicate, exchange and disseminate their results with other researchers having different backgrounds. It has allowed so far for instance students, PhD, post-docs, to extend their knowledge by visiting other laboratories and by presenting their research results within the project community, providing a research space at the European level.

External communication outside the consortium.

In terms of communication and dissemination outside the consortium, in the project framework a complete range of dissemination tools have been put in place: a website presenting the project, a leaflet promoting the project, the organisation of workshops and conferences, participation to international and national congresses, publications...

Thus, to make known the results of the projects, project members regularly attend national and international conferences (175 communications has been given for the whole project duration) with large audience. In such presentation (talk or posters), the Nanoantenna project was acknowledged. Several articles have already been published in international journals (64 accepted and published papers for the whole project duration) and others should be submitted after the end of the project. A book related to the "Nanoantenna" concept and entitled "Nanoantenna: Plasmon-Enhanced Spectroscopies for Biotechnological Applications" has been published in the 24 January 2013 by Pan Stanford Publishing group. The editors of this book are Marc Lamy de la Chapelle and Annemarie Pucci, members of the project consortium.

Moreover, several events have been organised by the consortium and supported by the project: one summer school in April 2011 and three international congresses (workshop on Optical Biosensors, Nanobiophotonics and Diagnostics in November 2011, Electromagnetic and Light Scattering XIII in September 2011 and International Conference on Enhanced Spectroscopies in October 2012).

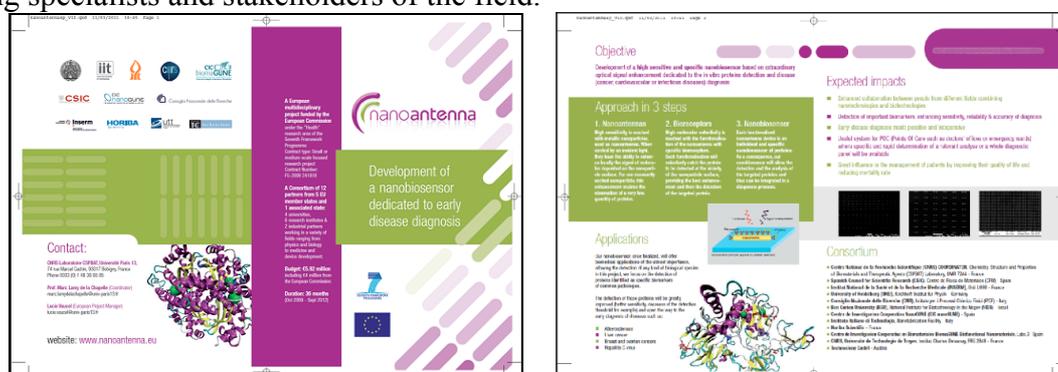
Project web Site

The website of the Nanoantenna project has been launched in November 2009: www.nanoantenna.eu. It includes a publicly accessible area and restricted area with an access limited to the consortium member. The public area provides information such as:

- Basic information: contacts, budget, duration, EU and 7th FP logos
- Main goal of the project
- Applications
- Impacts
- Publications related to “Nanoantenna”
- List of consortium partners.

Leaflet

A leaflet has been designed and printed; the definitive version was finalized on March, 11th 2011. 2000 leaflets have been ordered and have been given to the partners so that they can distribute them. It has guaranteed that information about the project was largely diffused among specialists and stakeholders of the field.



Nanoantenna leaflet

Networking and cluster participation

The project coordinator was involved in a cluster created on the EC initiative gathering several EC funded projects around one thematic: Targeted Nanopharmaceutics and Early Diagnostics for health. This gave the opportunity to exchange information and ideas about EU projects not only regarding their management, but also about their scientific findings. Project Management Team has been involved in this cluster taking part in meetings and events organized for the cluster to exchange and communicate on common issues related to the activities of the different projects.

A written presentation of Nanoantenna project was sent to the European Commission in February 2011 following a request from the EC DG Research which was preparing a compilation of information about EC funded projects belonging to the Targeted Nanopharmaceutics and Early Diagnostics for health Cluster.

Workshops and conferences organisation

Seven workshops were organised during the project duration.

Distinction is made between internal Workshops (where a few speakers are invited to make a presentation and exchange with Nanoantenna project members) and external Workshops (open to everybody who is willing to attend the event).

- Internal workshops

Five workshops were organised:

- *Workshop on micro and nanofabrication*, Genoa (Italy), 28 April 2010
- *Workshop on gold modification and aptamers*, San Sebastian (Spain), 4 October 2010

- *Workshop on Plasmon Bio Nanotechnologies*, Messina (Italy), 2 May 2012
- *Workshop on Biosensors*, Troyes (France), 23 October 2012
- *Validation Workshop*, in Beer-Sheva (Israel), 4-7 February 2012. During this workshop, all the different partners involved in the validation of the nanobiosensor were gathered in the Ben Gurion University in order to perform in the same place all the experiments needed to validate the sensor. The spectrometer prototype was sent to BGU for the week and for the experiments.

- **External workshops**

- *Summer School on Plasmonics, Functionalization and Biosensing* was organised in Heidelberg (Germany), April 25-29 2011. Audience: 85 participants
- *Workshop on Optical Biosensors, Nanobiophotonics and Diagnostics* was organised in the Dead Sea (Israel), November 5-9 2011. Audience: 65 participants. At this workshop, three specific sessions were devoted to the presentation of the results of the Nanoantenna project.

The consortium has also participated to the organisation of 2 international conferences, which have been supported by the project:

- *Electromagnetic and Light Scattering XIII* (ELS XIII), Taormina (Italy), September 26-30 2011, <http://elsxiii.unime.it/>, Audience: 100 participants.
- *International Conference on Enhanced Spectroscopies* (ICES), Porquerolles (France), October 3-5 2012, <http://www.im2np.fr/ICES2012/index.html>, Audience: 85 participants.

This latter conference was the first one organised specifically on the enhanced spectroscopies and was organised at the initiative of the Nanoantenna consortium.

For both conferences, the Nanoantenna logo was observable on the conference web site and on the conference booklet.

Conferences participations

122 communications (invited talks, talks or posters) have been given by project members in international conferences. 35 communications (invited talks, talks or posters) have been given by project members in national conferences. Presentations of the project and project results were done and the project and EC were acknowledged.

Publications

63 publications and 8 proceedings have already been published to present some results of the project. Some publications have been done in high-level journals with high impact factor such as Nature, Nature Photonics, Nano Letters, ACS Nano... Nearly 25% of all the publications have been done in journal with an impact factor higher than 10 and nearly 40 % of all the publications have been done in journal with an impact factor higher than 5. This indicates the high quality of the results obtained during the Nanoantenna project and its high impact on the scientific community.

Some are also in preparation since all the project results have not been disseminated.

Nanoantenna book

A book related to the “Nanoantenna” concept and entitled “Nanoantenna: Plasmon-Enhanced Spectroscopies for Biotechnological Applications ” has been published in the 24 January 2013. Marc Lamy de la Chapelle and Annemarie Pucci are the editors of this book. Consortium Nanoantenna members as well as external experts were invited to make a contribution to this book by writing a chapter. An editor note written by Marc Lamy de la Chapelle and Annemarie Pucci acknowledged the Nanoantenna project and the European Commission funding.

1.5 project public website

Project website: www.nanoantenna.eu

Nanoantenna logo



Nanoantenna project partners

- Chemistry, Structure and Properties of Biomaterials and Therapeutic Agents ([CSPBAT](#)) Laboratory, UMR 7244, Centre National de la Recherche Scientifique ([CNRS](#)) – France (COORDINATOR)
- Centro de Fisica de Materiales (CFM), Spanish Council for Scientific Research ([CSIC](#)) – Spain
- Unit [U698](#), Institut National de la Santé et de la Recherche Médicale ([INSERM](#)) – France
- Kirchhoff-Institut für Physik, [University of Heidelberg](#) (UHEI) – Germany
- Istituto per i Processi Chimico Fisici (IPCF), Consiglio Nazionale delle Ricerche ([CNR](#)) – Italy
- National Institute for Biotechnology in the Negev (NIBN), [Ben Gurion University](#) (BGU)- Israel
- Centro de Investigacion Cooperativa [NanoGUNE](#) (CIC nanoGUNE) – Spain
- Nanofabrication Facility, [Istituto Italiano di Tecnologia](#) – Italy
- Horiba Scientific – France, Emmanuel Froigneux
- Biofunctional Nanomaterials – Laboratory 3, Centro de Investigacion Cooperativa en Biomateriales [BiomaGUNE](#) – Spain
- Institut Charles Delaunay, FRE 2848, CNRS, [Université de Technologie de Troyes](#) – France
- [Technoclone](#) GmbH – Austria

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