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Development of optical (T-LSPR) biosensors, based in nanoplasmonic thin films, for fast *Legionella pneumophila* detection in patients or environmental samples.

PhD thesis proposal

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Abstract

This project aims to develop Transmittance-Localized Surface Plasmon Resonance (T-LSPR) Biosensors for fast detection of Legionella pneumophila. The detection mechanism proposed relies on shifts of the LSPR band of Au nanoparticles, due to changes of the dielectric function of the surrounding medium when analyte molecules are adsorbed. Since LSPs can be excited by the passing-through light, the detection system can be relatively simplified. A palmtop detection system will be design and fabricated, consisting of a light source, the plasmonic thin film with LSPR bands, microfluidic channels and micropumps, light detectors and all the integrated microelectronics necessary to the system. To promote the selectivity of the nanoplasmonic films, they will be functionalized with biorecognition elements (antibodies or DNA). This type of biosensors will allow to detect the presence of the pathogens faster and with very low detection limits, when compared to the conventional detection methods.

State of the art

Legionella is a Gram-negative bacterium present in freshwater habitats and that can colonize man-made water system [1] and can be dispersed by aerosols generated by showers, faucets, cooling towers, etc. [2]. Legionella pneumophila is responsible for more than 90% of cases of Legionnaires' disease and outbreaks of community-acquired and nosocomial *L. pneumophila* infection have been recently described in Portugal. While *L. pneumophila* have at least 16 serogroups, the serogroup 1 is responsible for most of the American and European clinical cases [3].

The detection of this pathogen from clinical or environment samples is mainly performed by culture plate methods, which takes up to 10 days [3]. The detection of *L. pneumophila* infections can also be executed using the urinary antigen test due to its simplicity, speed and in-situ [4], however, this only detects the serogroup 1, producing a detection blind spot for the remaining serogroups [5].

Antibodies detection methods usually relies on the detection of pathogen' specific proteins and are used due to the low detection limit (103 CFU/mL). However, they are not appropriate for heat or chemically processed samples (e.g. water treatment stations). Furthermore, the Portuguese legal limit of *L. pneumophila* in the environment is 102 CFU/L (Portaria n°353-A/2013). Methods based on DNA stands as a viable alternative since their detection limit is already lower than protein-based methods [6].

Therefore, an alternative, highly sensitive and easy method with a short detection time is needed to improve patient's survival and prevent *L. pneumophila* outbreaks. (2030 Agenda - Objective 3.3.)

Localized Surface Plasmon Resonance (LSPR) has emerged as a capable technique in the field of label-free biosensing [7]. LSPR is associated with resonance of incident electromagnetic (EM) waves with collective oscillations at the interface of noble metal surfaces with a dielectric medium [8], leading to strong EM fields with an absorption band at a specific wavelength [9]. In LSPR sensing, these EM fields are sensitive to changes in the refractive index (RI) of the surrounding medium [10]. Due to the stronger EM field confinement, a smaller penetration of the evanescent

field into the dielectric is observed [11], translating in an increase of sensitivity for RI changes near the nanoparticles' surface [12,13]. These characteristics are suitable for the development of a diagnostic tool to quickly detect biological entities whose detection with lower concentrations is crucial.

LSPR absorption band can be excited by the transmitted EM radiation, described as Transmission-LSPR (T-LSPR) [14,15], which can simplify the detection system, opening the possibility of constructing a miniaturized device [16].

However, the development and production of reliable T-LSPR platforms requires reproducible and stable optical properties [17]. Magnetron sputtered nanocomposite thin films, with noble nanoparticles embedded in a dielectric matrix [18], such as those composed of Au [19,20] or Ag [21] dispersed in a dielectric matrix, have already showed potential as T-LSPR platforms. The LSPR absorption band can be tuned by changing several deposition parameters [22] and post-deposition annealing treatments [23]. The Au:TiO_2 system has been a subject of intense research in the last years in the investigation group, and "optimized" conditions for its preparation have been reached [24].

Other critical issue in T-LSPR biosensors is functionalization of the nanoparticles with biorecognition elements [25], such as antibodies [26], aptamers [27] or even DNA [28]. These molecules are used to functionalize the sensor , leading to the immobilization of the analyte molecules near the nanoparticles' surface [10].

Objectives

This project aims to develop a functional Transmittance-LSPR biosensor prototype able to detect the presence of *L. pneumophila*. To achieve this main goal, some objectives must be accomplished:

1) Optimization of nanoplasmonic thin films with tailored Transmittance-LSPR (T-LSPR) bands. This will involve several steps:
    a) Production of the thin films, composed of Au nanoparticles, embedded in a dielectric matrix (TiO_2) using reactive magnetron sputtering and post-deposition thermal annealing;
    b) Characterization of the thin films in terms of composition, structure and morphology, with special emphasis to the shape and size distributions of the Au NPs;
    c) Correlation between the nanostructure of the films and their LSPR absorption bands characteristics (width, peak position and intensity, etc.);
2) Immobilization of *L. pneomophila* specific biorecognition elements (DNA molecules) on the surface of the nanocomposite thin films:
    a) Design and selection of the biorecognition molecules to functionalize the surface of the thin film.
    b) Development of the protocol of immobilization with the selected molecules;
    c) Validation of the specific detection by testing different types of samples.

3) Preparation of the T-LSPR detection setup for liquid samples. This involves the design and fabrication of microfluidic platforms, micropumping studies and its adaptation to the system;
4) Design and production of the photodiodes and microelectronics for the detection system;
5) Construction of an integrated approach to T-LSPR system for the detection of L. pneumophila in clinical and environmental cases.

Tasks

1. Bibliographic review

Throughout work plan, the current state of scientific work produced on the relevant topics related with the work will be followed.

2. Optimization of Au:TiO_2 nanoplasmonic thin films with tailored Transmittance-LSPR (T-LSPR) bands.

2.1 Production of the thin films

Production of nanoplasmonic thin films, composed of gold nanoparticles embedded in a TiO_2 matrix. The thin films will be deposited by reactive magnetron sputtering of a pure Ti target, with different amounts of gold placed on its erosion zone, in a reactive atmosphere ($Ar+O_2$). The starting point of the films' depositions will be the work developed by the candidate [24].

Magnetron sputtering is associated to low-cost production, relative simplicity and versatility, and is considered environmentally friendly. Furthermore, it was shown in previous studies that the amount of gold used is minimum, being that a 32 mm^3 piece of gold can produce hundreds of sensing platforms.

After the deposition, the films will be thermal annealed to promote the necessary microstructural changes (nanoparticles' growth), so they can manifest localized plasmonic resonances (LSPR). Different annealing protocols will be used to optimize the nanoparticles' shape and size distributions inside the dielectric matrix.

2.2. Characterization of the films

This task will provide fundamental understanding of the physical and chemical properties of the films, namely in terms of composition, structure and microstructure. In this sense, the microstructure of the films in terms of structure (X-Ray diffraction), morphology, grain size, shape of the nanoparticles, size distribution, phase composition (TEM/SEM, etc.) and surface topography (AFM) will deserve emphasis since they will allow the tailoring of the overall films' responses.

The characterization of the films will be performed in national/international laboratories, by means of already established collaborations.

2.3. Characterization of the Transmittance-LSPR band

The optical response of the films will be studied using the transmittance spectra and the LSPR bands (T-LSPR) will be correlated with their microstructure, namely with the i) nanoparticles' size, shape and distribution, ii) phase composition and crystallinity, iii) surface and growth morphology. This will allow to tailor the LSPR band for biosensing.

3. Development of the T-LSPR-biosensor prototype for detection of pathogenic species

To detect specific analytes, the nanoplasmonic films must be functionalized with biorecognition elements. The functionalized nanocomposite thin films will be used to prepare LSPR-biosensor prototypes, which will be tested using the T-LSPR detection setup.

Thus, several DNA probes complementary *L. pneumophila* target sequence will be designed and tested. The single stranded probe DNA (pDNA) will have 19-mer to 21–mer, complementary to specific target genes/ intergenic sequences. To determine specificity, complementary and randomly sequenced DNA probes, will be purchased and tested. At least three pDNAs will be selected to be immobilized to the Au:TiO_2 thin film.

The selected probes will then be converted into peptide nucleic acids (PNAs) which are nucleic acid analogues known to bind very effectively and with high sequence specificity. A key factor in the success of the DNA-sensor is the choice of a proper immobilization approach, thus several protocols will be tested [29,30].

Hybridization reaction will be conducted by incubation of complementary and mismatch DNA sequences with the immobilized PNA probes and the reaction will be followed by LSPR band measurements. After these optimizations and selection of the best PNA probe, DNA from *L.pneumophila* and from other bacteria species (*E.coli*, *B.sublilis*, etc.) will also be tested for validation of the sensor.

4. Microfluidic design, production and micropumping

To miniaturize the detection system and adapt it to liquid samples, a microfluidic platform will be designed and constructed. It will be fabricated in polydimethylsiloxane (PDMS), which is a well-established polymer used in microfluidics, and, more importantly, it's optically transparent in the visible spectrum. The microfluidic channels can be produced by simple methods, without the use of cleanrooms, using SU-8 moulds produced by UV photolithography.

For optimizing the pumping flow rate inside the microchannels, it will be used a neMESYS Syringe Pump. At the final prototype and, for a portable device, the pumping system will be composed by programmable piezoelectric micropumps and micro-valves and automatized by a microcontroller.

5. Detection system and microelectronics design and production

To create a microsystem as small as possible, the fabrication of the photodetectors in CMOS technology features a small silicon area and allows their integration with the readout electronics in a single chip. Study the photodiodes junction depth and oxide layers inherent to the CMOS fabrication will be performed to obtain high quantum efficiency optical photodiodes at the required wavelengths. Protection rings for the different photodiodes will be studied to prevent signal interferences and improve their signal-to-noise ratio. Furthermore, high-selective optical bandpass filters could be used in the illumination light source for narrow each spectral band.

Readout electronics for photodiodes signals detection will be also designed, allowing the analog to digital conversion of the photodiode's signals, using a current-to-frequency converter.

All the simulations and layout of this task will be done using Cadence IC Tools. This CMOS will be fabricated in a silicon foundry.

6. Assembly of the portable T-LSPR system and proof-of-concept.

After both optical and microfluidic systems being tested, they will be integrated into a single platform specifically designed for this purpose.

A portable palmtop USB connectable T-LSPR biossensorial system will be constructed, integrating the light source (e.g. LEDs), the functionalized nanoplasmonic thin film with the microfluidic PDMS platform on top, the photodiode with integrated electronics and with the programmable microcontroller. The system will be enclosed in 3D printed box that will support the various components and keep them aligned.

T-LSPR-biosensor will be tested to determine if it can detect the presence of *L. pneumophila* in samples. After validation, the economic potential of the prototype will be analyzed.

7. Reports, publications and thesis.

Throughout the proposed work plan, a significant focus will be assigned to the attendance of international conferences and to the submission of high-quality research papers to recognized international peer-reviewed journals.

The last six months will be devoted to summarizing the results and write the Thesis, including critical evaluation and prospects for prototype improvement.

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