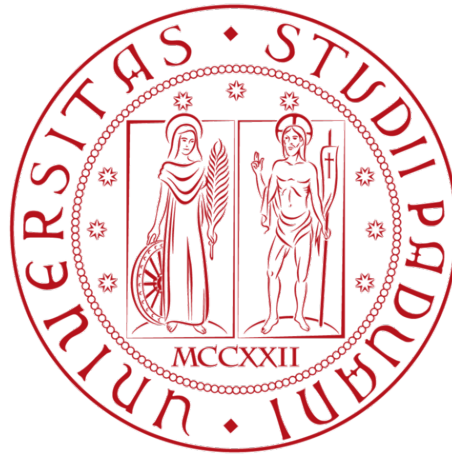




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TESI DI LAUREA MAGISTRALE IN FISICA

Plasmonic NanoHole Arrays for Label-free Biosensors

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*To my family,
those who supported me
and myself.*

*“All science is either physics or stamp collecting.”
Ernest Rutherford (1871 - 1937)*

*“Imagination is more important than knowledge.
Knowledge is limited. Imagination encircles the world.”
Albert Einstein (1879 - 1955)*

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Introduction

The remarkable and fascinating properties displayed by nanoscaled structures, such as optical, magnetic, mechanical and catalytic properties, have attracted in the last years growing attention in many research fields, from nanophotonics to data-storage [1] and telecommunications, reaching the medical, biological and healthcare fields [2]. Moreover, due to the countless possible applications they provide and to the versatility and adaptability they display, nanostructures have been largely employed for different purposes: biosensing, lasing, optical switches, optical filters, light sources, waveguides, microscopy, surface enhanced spectroscopy (SERS) [3], lithography, imaging and so on, driven by the need to work with smaller, faster and efficient devices.

The present work focuses on plasmonic properties of nanostructures to be used as optical biosensors. Plasmonics is a branch of nanophotonics. Nanophotonics studies the behaviour and the interaction of electromagnetic (EM) radiation, in the NIR-Vis-NUV range, with materials at the nanoscale. Plasmonics explores electromagnetic fields confinement in dimension on the order of or smaller than the wavelength (λ), going beyond the diffraction limit ($\lambda/2$) and in particular it investigates the interaction between electromagnetic radiation and conduction electrons at the interface with a metal or inside small metallic nanostructures. At this scale, noble-metals nanostructures display unexpected and outstanding optical properties, depending on their dimensions, morphology and composition.

In particular, this thesis work concerns nanostructured biosensors, which nowadays constitutes an intriguing option as device for diagnosis and monitoring of diseases, drug discovery, proteomics, environmental detection of biological agents. In fact, these devices perform ultrasensitive, real-time and label-free diagnosis which could allow to prevent or identify in time serious diseases, like Alzheimer, heart attack, pancreatic cancer and so on. Thus, it is important that these devices may guarantee the detection of low concentration (sub-pM) of small molecules like mRNA, proteins, antibodies, antigens, DNA chains, bacteria, etc. [4–6]. In this regard, nanostructured specific biosensors are based on the receptor-analyte principle, where the occurred binding reaction is then converted into an optical signal by a transducer, namely, the metallic nanostructure.

The physical phenomenon on which we focus on and which we exploit in our biosensors is the *Surface Plasmon Resonance* (SPR), resulting from the coupling of light with conduction electrons of the metallic structure, which can be divided in *Extended Surface*

Plasmon Resonance (E-SPR) and *Localized Surface Plasmon Resonance* (L-SPR). The latter involves the study and application of subwavelength nanoparticles, like *NanoPrism Array* (NPA) or *Semi-NanoShell Array* (SNSA) [7]. LSPR devices display higher local sensitivity and lower bulk sensitivity with respect to ESPR devices, like *NanoHole Array* (NHA), which take advantage of the excitation of *Surface Plasmon Polaritons* (SPPs) [8], which can be either propagating SPPs (PSPPs) or localized SPPs (LSPPs). SPPs are electromagnetic waves that travels at the interface between a metal and a dielectric. In its name are enclosed both a coherent oscillation of the surface conduction electrons inside the metal and the propagation of an EM wave inside the dielectric. Hence, the spectral position at which the resonant condition at which SPPs excitation occurs is strongly dependent on the dielectric medium at the interface. Thus, as the analyte binds to the receptor of the suitably functionalized NHA, a change in the SPR condition is revealed, providing the main advantage to the plasmonic device: label-free biosensing. Moreover, choosing an highly selective receptor leads to high-specificity. Finally, the reason why NHA have been chosen is due to the fascinating optical property they display: *Extraordinary Optical Transmission* (EOT), first studied by Ebbesen in 1998 [9], ascribable to the excitation of SPPs.

In this thesis work, NHA are designed, synthesized, characterized, functionalized and tested. A nanohole array is a thin metallic film of thickness $50 \div 100$ nm, patterned with a periodic array of holes, in our case, an hexagonal array of circular holes. Indeed, in this condition transmittance results to be greater than that of a single hole of area equal to the sum of all the single nanoholes area. The NHA fabrication method employed is the *NanoSphere Lithography* (NSL) [10], which represent a high throughput, cost effective and versatile technique. For transmittance measurements a simple experimental setup is needed, which is feasible of real-time measurements making it a suitable Lab-on-a-chip device [11]. Recalling the EOT property, measuring the transmittance spectrum of a NHA it is possible to observe a sharp intense peak occurring at a specific wavelength, i. e., EOT peak. Furthermore, the high sensitivity displayed by this devices together with the low *Limit of Detection* (LoD), namely, the minimum concentration detectable, make NHA a promising tool for biosensing essays.

The aim of this work is to study the physical features of NHA, optimizing the NSL process in order to conveniently tailor its optical properties, allowing a reproducible protocol for high throughput nanostructures fabrication and providing the best morphological and optical characteristic. NHA are finally tested, assessing their bulk and local sensitivities and LoD, by means of the Biotin-Streptavidin system, two proteins displaying high affinity, comparing experimental results with numerical simulations.

The work is arranged as follows:

Chapter 1 Theory of EM radiation-metallic nanostructure interaction.

Chapter 2 Theory of light transmission through apertures and EOT.

Chapter 3 Description of the NHA fabrication protocol: NSL.

Chapter 4 Basic principles of biosensing with nanostructures, functionalization protocol

and expected results from simulations.

Chapter 5 Optical and morphological characterization of the synthesized NHA.

Chapter 6 Results from the biosensing tests.

Chapter 1

Plasmonic properties of metallic nanostructures

Plasmonics is a branch of *nanophotonics* which investigates the interaction of electromagnetic (EM) radiation with metals and in particular its coupling with surface conduction electrons, leading to EM waves confinement in volumes of size of the order of or smaller than the wavelength at the surface of the metallic structures, leading to intriguing phenomena such as Extraordinary Optical Transmission through sub-wavelength nanoholes in a metallic thin film.

1.1 Interaction of EM radiation with metals

The interaction between EM and metals is in general thoroughly described by a classical treatment based on Maxwell equations. The quantum mechanical description is needed only when the size of the nanostructures is around or below 1 nm, which is not the case in the present study. This interaction shows a strong dependence on the frequency of the field and the electronic structure of the metal.

There are three ranges of interest to consider:

- Low frequency range: microwave and far-infrared;
- Visible range: from infrared (IR) frequencies up to ultraviolet (UV) frequencies;
- High frequency range: UV frequencies.

In the low frequency range only a negligible fraction of the impinging radiation penetrates to a depth, called *skin depth*, thus making it possible to use the perfect conductor approximation. In this regime metals displays high reflectivity, preventing EM waves propagation through the structure. This explains why metals are employed as cladding in waveguides and resonators.

In the IR-UV range field penetration and dissipation increase significantly, prohibiting to scale low frequencies photonic devices to the visible regime.

Finally, at UV frequencies metals allow the propagation of EM radiation acting as a dielectric. This behavior depends on the band structure of the metal: alkali metals exhibit ultraviolet transparency because of the free-electron-like response, whereas noble metals, such as gold or silver, show a strong absorption because of inter-band transitions.

These optical and dispersive properties are characterized by a *complex dielectric function* $\epsilon(\omega)$ which can be determined both empirically or theoretically through use of the *Drude model*. First of all, it is important to recall the macroscopic Maxwell equations in order to describe the electromagnetic response of metals:

$$\nabla \cdot \mathbf{D} = \rho_{ext} \quad (1.1a)$$

$$\nabla \cdot \mathbf{B} = 0 \quad (1.1b)$$

$$\nabla \times \mathbf{E} = -\frac{\partial \mathbf{B}}{\partial t} \quad (1.1c)$$

$$\nabla \times \mathbf{H} = \mathbf{J}_{ext} + \frac{\partial \mathbf{D}}{\partial t} \quad (1.1d)$$

These four equations gather the four macroscopic fields:

- $\mathbf{D}$: the dielectric displacement;
- $\mathbf{B}$: the magnetic induction or magnetic flux density;
- $\mathbf{E}$: the electric field;
- $\mathbf{H}$: the magnetic field;

where ρ_{ext} and $\mathbf{J}_{ext}$ are the external charge and the current densities, respectively. There are two further equations which links $\mathbf{D}$ with $\mathbf{E}$ and $\mathbf{H}$ with $\mathbf{B}$:

$$\mathbf{D} = \epsilon_0 \mathbf{E} + \mathbf{P} \quad (1.2a)$$

$$\mathbf{H} = \frac{1}{\mu_0} \mathbf{B} - \mathbf{M} \quad (1.2b)$$

which introduce the polarization $\mathbf{P}$ and the magnetization $\mathbf{M}$, where ϵ_0 and μ_0 are the electric permittivity and the magnetic permeability of vacuum, respectively. However, we will not take into account the magnetization term by considering only non magnetic media, limiting our discussion to electric polarization effects. $\mathbf{P}$ measures the electric dipole per unit volume inside the medium, thus it is related to the internal charge ρ_{int} , via $\nabla \cdot \mathbf{P} = -\rho_{int}$ which, by means of the charge conservation equation $\nabla \cdot \mathbf{J} = -\frac{\partial \rho_{int}}{\partial t}$, provides:

$$\mathbf{J} = \frac{\partial \mathbf{P}}{\partial t} \quad (1.3)$$

This relation, together with the request of a nonmagnetic, linear and isotropic material, leads to proportionality of $\mathbf{D}$ with $\mathbf{E}$ and $\mathbf{H}$ with $\mathbf{B}$:

$$\mathbf{D} = \varepsilon_0 \varepsilon \mathbf{E} \quad (1.4a)$$

$$\mathbf{B} = \mu_0 \mu \mathbf{H} \quad (1.4b)$$

where ε is the relative permittivity and μ is the relative permeability¹ of the medium. Introducing the conductivity σ we can write another constitutive linear relationship which links $\mathbf{J}$ to $\mathbf{E}$:

$$\mathbf{J} = \sigma \mathbf{E} \quad (1.5)$$

ε and σ are both used to describe electromagnetic phenomena and are also related to each other. Historically, the latter is preferred for low frequency phenomena, whereas the former is widely used at optical frequencies. Equations 1.4a and 1.5 are correct only if we are considering linear and dispersionless, both spatially and temporally, media. A more general non-local description of the fields can be obtained from the following equations:

$$\mathbf{D}(\mathbf{r}, t) = \varepsilon_0 \int dt' d\mathbf{r}' \varepsilon(\mathbf{r} - \mathbf{r}', t - t') \mathbf{E}(\mathbf{r}', t') \quad (1.6a)$$

$$\mathbf{J}(\mathbf{r}, t) = \int dt' d\mathbf{r}' \sigma(\mathbf{r} - \mathbf{r}', t - t') \mathbf{E}(\mathbf{r}', t') \quad (1.6b)$$

A more clear expression of 1.6 is possible by means of the Fourier transform in the reciprocal space $(\mathbf{K}, \omega)$, which turns the convolutions into multiplications:

$$\mathbf{D}(\mathbf{k}, \omega) = \varepsilon_0 \varepsilon(\mathbf{k}, \omega) \mathbf{E}(\mathbf{k}, \omega) \quad (1.7a)$$

$$\mathbf{J}(\mathbf{k}, \omega) = \sigma(\mathbf{k}, \omega) \mathbf{E}(\mathbf{k}, \omega). \quad (1.7b)$$

It is now possible to derive the relationship between ε (*dielectric function*, from now on) and σ via 1.2a, 1.3 and 1.7:

$$\varepsilon(\mathbf{k}, \omega) = 1 + \frac{i\sigma(\mathbf{k}, \omega)}{\varepsilon_0 \omega}. \quad (1.8)$$

This is the general form of the dielectric response. For our purposes, i. e., for studying plasmonics properties which involve frequencies from the IR to the UV, we can focus only on the dielectric function. We can furthermore simplify eq. 1.8 in the limit of *spatially local* response, which is valid as long as the wavelength λ of the EM radiation in the medium is larger than all characteristic dimensions like electrons mean free path or the unit cell size (*long-wavelength limit*). This condition still holds at UV frequencies being $\lambda \sim 10^1 \div 10^2 \text{ nm}$ and yields: $\varepsilon(\mathbf{K} = 0, \omega) = \varepsilon(\omega)$. Moreover, equation 1.8 gathers bounded free charges: the first describe polarization effects, the latter the contribution to current

¹ $\mu = 1$, due to the nonmagnetic medium hypothesis

flow. However, this distinction is marked at low frequencies, but at optical frequencies this difference becomes blurred.

The dielectric function is a complex function of the angular frequency ($\varepsilon(\omega) \in \mathbb{C}$, $\omega \in \mathbb{R}$) whose general form is $\varepsilon(\omega) = \varepsilon_1(\omega) + i\varepsilon_2(\omega)$. It can be empirically determined by means of reflectivity experiments, assessing the complex refractive index $\tilde{n}(\omega) = n(\omega) + i\kappa(\omega)$ of the material through $\tilde{n} = \sqrt{\varepsilon}$, or explicitly:

$$\varepsilon_1 = n^2 - \kappa^2 \quad (1.9a)$$

$$\varepsilon_2 = 2n\kappa \quad (1.9b)$$

$$n^2 = \frac{\varepsilon_1}{2} + \frac{1}{2}\sqrt{\varepsilon_1^2 + \varepsilon_2^2} \quad (1.9c)$$

$$\kappa = \frac{\varepsilon_2}{2n} \quad (1.9d)$$

where κ is the extinction coefficient which determines the optical absorption of EM waves inside the material through Beer's law:

$$I(x) = I_0 e^{-\alpha x} \quad (1.10a)$$

$$\alpha(\omega) = \frac{2\kappa(\omega)\omega}{c} \quad (1.10b)$$

where I_0 is the intensity of the input beam, α is the absorption coefficient linked to κ .

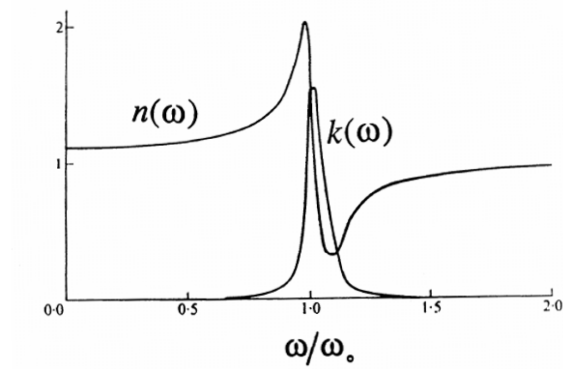


Figure 1.1: Refractive index, $n(\omega)$, and extinction coefficient, $\kappa(\omega)$, trend at the resonance.

A simple model which displays how to estimate $\varepsilon(\omega)$ is the Drude model of free electron gas.

1.2 Ideal metal description: *the Drude model*

The Drude model is basically a *plasma model*, which states that a gas of free electrons is free to move in a constant field due to the positive ions. Hence, it assumes that:

- electrons are delocalized from the lattice ions and withstand to the *equipartition theorem*;

- long-range *electron-ions* and *electron-electron* interactions are neglected;
- the only possible collisions are instantaneous with average time τ called *relaxation time*.

The validity of this model is limited in the case of noble metals because of the inter-band transitions that occur at visible frequencies, but one simply includes band structures features into the effective mass of the electron. If an external field is applied, the electrons oscillate with a motion damped by collision occurring with a characteristic frequency $\gamma = 1/\tau \sim 10^{14} \text{ s}^{-1} = 100 \text{ THz}$ at room temperature.

The starting point of the model is the equation of motion for an electron under an harmonic external electric field $\mathbf{E} = \mathbf{E}_0 e^{-i\omega t}$:

$$m\ddot{\mathbf{x}} + m\gamma\dot{\mathbf{x}} = -e\mathbf{E}. \quad (1.11)$$

which leads to a particular solution $\mathbf{x}(t) = \mathbf{x}_0 e^{-i\omega t}$, where $\mathbf{x}_0$ is the complex amplitude which incorporates the phase shifts between the driving field and the medium response via:

$$\mathbf{x}(t) = \frac{e}{m(\omega^2 + i\gamma\omega)} \mathbf{E}(t) \quad (1.12)$$

We can calculate the macroscopic polarization explicitly:

$$\mathbf{P} = -ne\mathbf{x} = -\frac{ne^2}{m(\omega^2 + i\gamma\omega)} \mathbf{E} \quad (1.13)$$

and the dielectric displacement through 1.2a:

$$\mathbf{D} = \epsilon_0 \left(1 - \frac{\omega_p^2}{\omega^2 + i\gamma\omega} \right) \mathbf{E} \quad (1.14)$$

where we introduced the *plasma frequency* of the free electron gas $\omega_p = \sqrt{\frac{ne^2}{\epsilon_0 m}}$. This yields the equation for the dielectric function:

$$\epsilon(\omega) = 1 - \frac{\omega_p^2}{\omega^2 + i\gamma\omega} = \left(1 - \frac{\omega_p^2}{\omega^2 + \gamma^2} \right) + i \left(\frac{\omega_p^2 \gamma}{\omega(\omega^2 + \gamma^2)} \right) = \epsilon_1(\omega) + i\epsilon_2(\omega). \quad (1.15)$$

We now inspect the behavior of metals with respect to the plasma frequency. At frequencies $\omega < \omega_p$ metals retain their metallic character and the Drude model holds. When frequencies approach ω_p and the term $\omega\tau \gg 1$ (high frequency approximation), an undamped free electron plasma is obtained, whose dielectric function is purely real as:

$$\epsilon(\omega) \sim \epsilon_1(\omega) = 1 - \frac{\omega_p^2}{\omega^2} \quad (1.16)$$

As previously mentioned, in this frequency range the model is no longer valid for noble metals because of the interband transitions, which entail an absorption effect and therefore an increase in ϵ_2 . At higher frequencies $\omega \gg \omega_p$, $\epsilon(\omega) \rightarrow 1$ and up to this point this model

holds for ideal metals, whereas for real metals and especially for noble metals it needs an extension in the $\omega > \omega_p$ region, because the response is dominated by the free s electrons, while the filled d band, close to the Fermi surface, provides a strong polarization. This problem is solved phenomenologically by adding the ion cores residual polarization term $\mathbf{P}_\infty = \varepsilon_0(\varepsilon_\infty - 1)\mathbf{E}$ to 1.2a, turning $\mathbf{P}$ into solely free electron polarization term. From 1.15 it yields:

$$\epsilon(\omega) = \left(\varepsilon_\infty - \frac{\omega_p^2}{\omega^2 + \gamma^2} \right) + i \left(\frac{\omega_p^2 \gamma}{\omega(\omega^2 + \gamma^2)} \right) \quad (1.17)$$

where $1 \leq \varepsilon_\infty \leq 10$.

Noble metals This model is valid for noble metals until interband transitions occur, as one can see in figure 1.2 where experimental data for both imaginary and real part of $\epsilon(\omega)$ from Johnson and Christy [12] are plotted against the theoretical dielectric function from Drude model. There is a good agreement for energies below the interband transition threshold: 2 eV (600 nm) for copper and gold and 4 eV (300 nm) for silver. In particular, in the case of gold this model does not hold already passing from NIR to Vis.

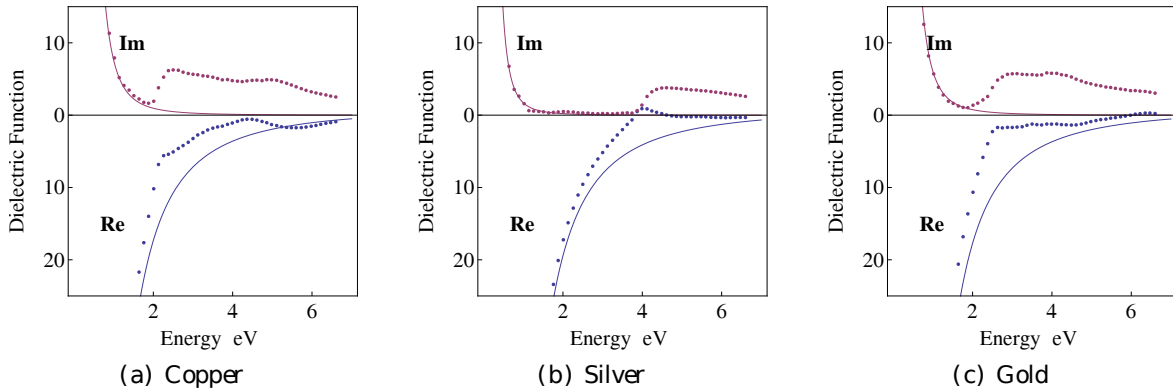


Figure 1.2: Comparison between Drude model dielectric function (*solid line*) and experimental data from Johnson and Christy [12] (*dotted line*).

1.3 Real metal description: *the Lorentz-Drude model*

As we have seen previously, the Drude model shows some discrepancies if we consider noble metals, due to the interband transitions excited by photons that efficiently promotes the bounded d electrons below the Fermi surface to higher bands. The equation of motion for bounded electrons is:

$$m\ddot{\mathbf{x}} + m\gamma\dot{\mathbf{x}} + m\omega_0^2\mathbf{x} = -e\mathbf{E}. \quad (1.18)$$

which describes interband transitions with a classical interpretation assigning a resonance frequency ω_0 to the d electrons. By solving a set of these equations it is possible to model

the noble metals dielectric function considering the polarization contributions from each one of them in the form of a Lorentz oscillator [13], giving:

$$\epsilon(\omega) = \epsilon_\infty - \frac{\omega_p^2}{\omega^2 + \imath\gamma\omega} + \sum_{i=1}^N \frac{A_i}{\omega_i^2 - \omega^2 - \imath\gamma_i\omega} \quad (1.19)$$

where N is the number of the interband transitions considered, thus the last term represents N Lorentz-oscillator terms, in which ω_i represents the resonant frequency of the i -th interband transition, while ω is the radiation frequency.

If we consider for instance 4 interband transitions, we obtain the L_4 model. Thus, renaming the coefficients in eq. 1.19, we obtain:

$$\epsilon(\omega) = \epsilon_\infty - \frac{\imath\sigma}{\epsilon_0\omega} + \sum_{i=1}^4 \frac{C_i}{\omega_i^2 + \imath A_i\omega + B_i} \quad (1.20)$$

These coefficients have been calculated by Nordlander and Hao [14] for silver and gold and are displayed in table 1.1:

	$Au(\epsilon_\infty = 1.1156, \sigma/\epsilon_0 = 1355.01s^{-1})$			$Ag(\epsilon_\infty = 1.4783, \sigma/\epsilon_0 = 3157.56s^{-1})$		
	$A_i[\text{eV}]$	$B_i[\text{eV}^2]$	$C_i[\text{eV}^2]$	$A_i[\text{eV}]$	$B_i[\text{eV}^2]$	$C_i[\text{eV}^2]$
$i = 1$	$-8.577 \cdot 10^4$	$-1.156 \cdot 10^4$	$5.557 \cdot 10^7$	$-1.160 \cdot 10^5$	-3050	$3.634 \cdot 10^8$
$i = 2$	-2.875	0.0	$2.079 \cdot 10^3$	-4.252	-0.8385	112.2
$i = 3$	-997.6	-3090	$6.921 \cdot 10^5$	-0.4960	-13.85	1.815
$i = 4$	-1.630	-4.409	26.15	-2.118	-10.23	14.31

Table 1.1: Table of the L_4 model coefficients (eq. 1.20) calculated by Nordlander and Hao [14] for Au and Ag.

Figure 1.3 shows the goodness of the L_4 analytical model dielectric function $\epsilon(\omega)$, which suitably fits the experimental data, thus representing a valuable tool for numerical calculations, thanks to its computationally simple formula.

1.4 Plasma oscillations: *Plasmons*

1.4.1 Volume Plasmons

We have already introduced the plasma frequency ω_p in eq. 1.14 highlighting that when $\omega > \omega_p$, $\epsilon(\omega)$ is approximately real, thus making the metal act like a dielectric. From Maxwell equations, setting $\mathbf{J}_{\text{ext}} = 0$, it is possible to obtain a dispersion relation for propagating waves in the metal:

$$\omega^2 = \omega_p^2 + k^2 c^2 \quad (1.21)$$

This equation outlines a longitudinal quanta oscillation of the electrons, called *volume plasmons*, with respect to the fixed ions with the characteristic plasma frequency, allowing

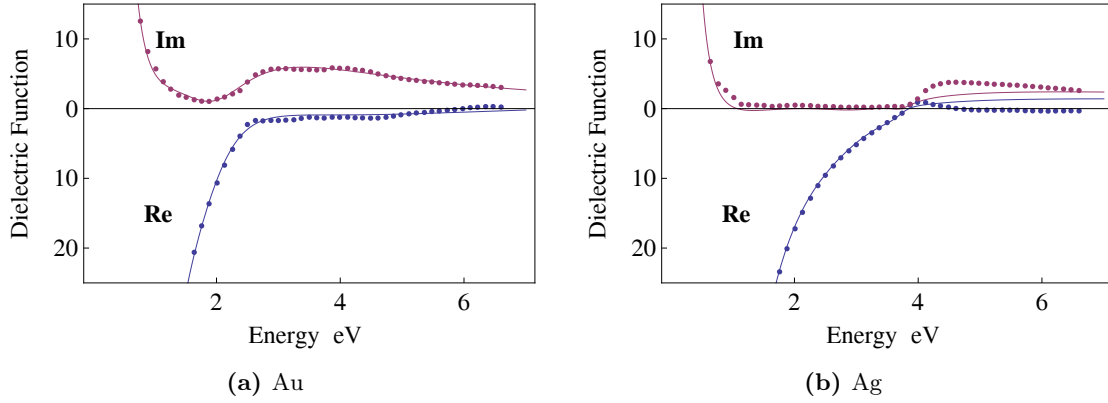


Figure 1.3: Comparison between the dielectric function of the $L4$ model and the experimental data from Johnson and Christy for Au and Ag.

only transverse EM waves to propagate through the metal. Because of this inability of volume plasmons, linked to their longitudinal nature, to couple with transverse EM waves, it is evident that the only way to excite a volume plasmon is by particle impact.

1.4.2 Surface Plasmons Polaritons

The most relevant phenomenon for the present work, regarding plasmon oscillations, is the *Surface Plasmon* (SP). This kind of oscillation occurs at the interface between a dielectric and a metal, i. e., at the interface between a material with a negative dielectric function and a positive permittivity medium. When it involves propagating surface oscillation, it is called *Surface Plasmon Polariton* (SPP), contrarily, in the case of confined surface oscillation in three-dimensional nanoparticles, we speak of *Localized Surface Plasmons* (LSP), which will be briefly discussed further on, in section 1.4.3.

First of all, SPPs are named that way because they involve charge motion in a metal (Surface Plasmon) and EM waves in a dielectric (Polariton). Thus, SPPs are longitudinal EM excitations arising from the coupling of the external field and the electron plasma. This oscillations are evanescently confined in the perpendicular direction on a sub-wavelength scale, because $\lambda_{SPPs} \leq \lambda_{EM}$, involving a high local field intensity which leads to EM field enhancement.

We now sketch out a mathematical description of the SPPs phenomenon. We first define the geometrical features and properties of the system (fig. 1.4):

- single, flat interface ($z = 0$) between a dielectric and the metal;
- dielectric: ($z > 0$), non-absorbing, $0 < \varepsilon_d(\omega) \in \mathbb{R}$;
- metal: ($z < 0$) $Re[\varepsilon_m] < 0$.

Starting from Maxwell equations it is possible to obtain the *D'Alembert equation* for the electric field:

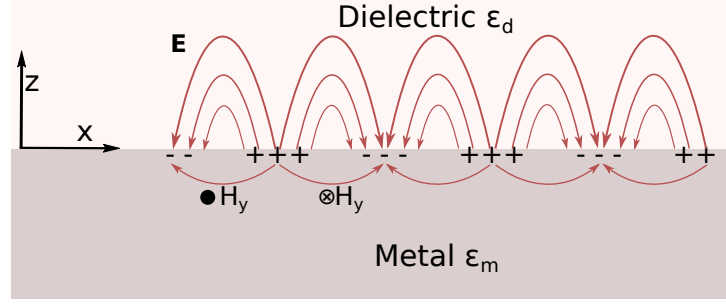


Figure 1.4: Geometry scheme of the dielectric-metal system and SPP propagation at interface. Electric and magnetic fields for the p-polarized SPP are also described.

$$\nabla^2 \mathbf{E} - \frac{\varepsilon}{c^2} \frac{\partial^2 \mathbf{E}}{\partial t^2} = 0, \quad (1.22)$$

assuming the dielectric function to be constant over distances on the order of optical wavelengths. If we suppose the electric field to have an harmonic time dependence $\mathbf{E}(\mathbf{r}, t) = \mathbf{E}(\mathbf{r})e^{-i\omega t}$, it yields the *Helmholtz equation*:

$$\nabla^2 \mathbf{E} + k_0^2 \varepsilon \mathbf{E} = 0, \quad (1.23)$$

where $k_0 = \frac{\omega}{c}$ is the wave vector of the propagating wave in vacuum. For the specific system geometry we choose, we have traveling waves along the x -direction and $\varepsilon(\omega)$ depending on the z -direction. Hence, we are dealing with propagating waves of the form $\mathbf{E}(x, y, z) = \mathbf{E}(z)e^{i\beta x}$, where we have defined β as the *propagation constant*, i. e., the component of the wave vector in the propagation direction. We find then:

$$\frac{\partial^2 \mathbf{E}(z)}{\partial z^2} + (k_0^2 \varepsilon - \beta^2) \mathbf{E}(z) = 0. \quad (1.24)$$

which reminds eq. 1.23.

An analogous equation holds for the field $\mathbf{H}$. To solve these two equations, boundary conditions at the interface must be set.

If we define the scattering plane as the plane in which both the incident and reflected wavevectors lay, we obtain two different sets of solutions, depending on the polarization of the incident radiation:

- transverse magnetic (TM), in which the magnetic field $\mathbf{H}$ is perpendicular to the scattering plane;
- transverse electric (TE), in which the magnetic field $\mathbf{E}$ is perpendicular to the scattering plane;

It is simple to show that SPPs only occurs for TM polarization, making $\mathbf{E}$ to propagate with β along x , no propagation along y and an evanescent component along z . We therefore find a dispersion relation for the TM modes which holds either for absorbing or non-absorbing metals:

$$\beta = k_0 \sqrt{\frac{\varepsilon_m \varepsilon_d}{\varepsilon_m + \varepsilon_d}} \quad (1.25)$$

If we consider an *ideal conductor metal*, which displays no attenuation ($\varepsilon_m(\omega) \in \mathbb{R}$), at the interface with a dielectric, whenever the condition $Re[\varepsilon_m]Re[\varepsilon_d] < 0$ is satisfied, i. e., whenever the two media display opposite signs of the real part of the dielectric permittivities (always in the case of a conductor and an insulator, see the conditions introduced above), a dispersion relation can be found.

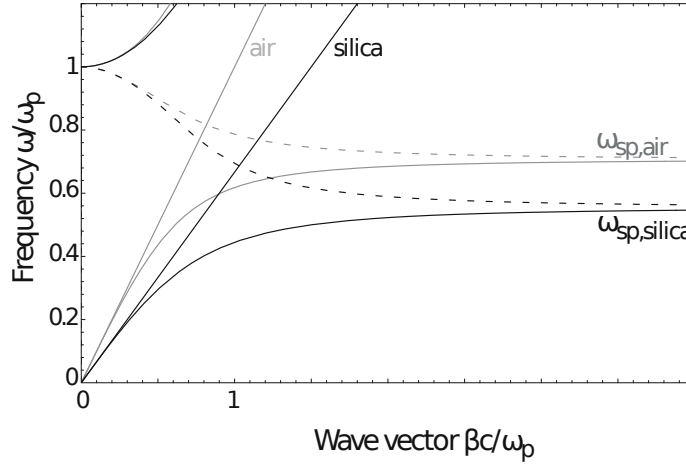


Figure 1.5: Dispersion relation of SPPs at air/metal (grey) and silica/metal (black) interfaces for ideal metals. Solid curves represent $Re[\beta]$, dashed curves $Im[\beta]$ and straight lines are the light lines.

Figure 1.5 shows the dispersion relations of two configurations, air/metal ($\varepsilon_{air} = 1$) and silica/metal ($\varepsilon_{Si} = 2.25$) interfaces, where both the real (continuous curves) and imaginary (broken curves) part of the wave vector β are plotted. The SPP modes curves lie on the right of the respective light lines $\omega = kc/n$ (straight lines), because of their bound nature, displaying a frequency gap region with the radiative modes in which β is purely imaginary, thus prohibiting propagation. For large values of k , the SPP curve tends to the *surface plasmon frequency* ω_{sp} , which is a finite value obtained by substituting 1.16 into 1.25:

$$\omega_{sp} = \frac{\omega_p}{\sqrt{1 + \varepsilon_d}} \quad (1.26)$$

As $\omega \rightarrow \omega_{sp}$, $k \rightarrow \infty$, thus $v_g = \frac{\partial \omega}{\partial k} \rightarrow 0$, which means that the oscillation mode acquires a static character: it becomes a *surface plasmon*.

For *real conductor metal* (fig. 1.6) both the metal dielectric function ε_m and the SPP propagation constant β are complex. In particular, $Im[\varepsilon_m] \neq 0$ because conduction excitations of conduction electrons suffer both from free-electron and interband damping. Hence, traveling SPPs display an attenuation length $L = 2Im[\beta]^{-1} \sim 10 \div 100 \mu\text{m}$ (in the visible regime) and ω_{sp} is reached at a finite wave vector value, providing a lower bound both to the oscillation wavelength λ_{sp} and to the perpendicular confinement at the interface (fig. 1.7).

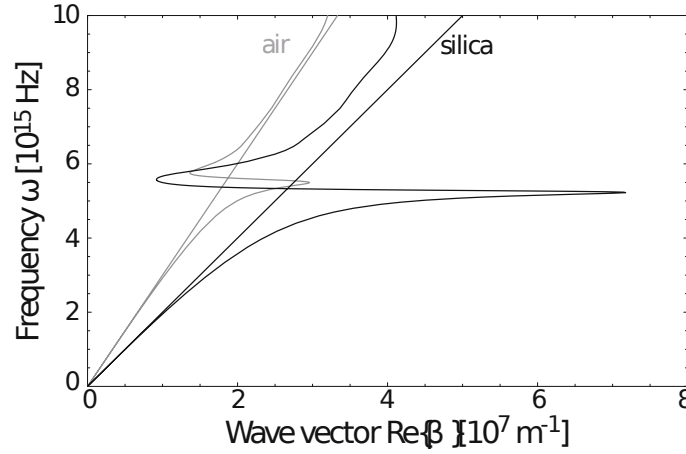


Figure 1.6: Dispersion relation of SPPs at air/silver (grey) and silica/silver (black) interfaces for real metals.

From figures 1.5 and 1.6 one can notice that the propagation constant β is always greater than the wave vector k in the dielectric, leading to evanescent decay in both side of the interface. Moreover, the SPPs dispersion curve lies to the right of the light line, which means that no SPPs excitation at the interface is possible through direct EM radiation interaction. But two way to couple radiation with SPPs are possible: *prism coupling* and *grating coupling*, as discussed in next section 1.5.

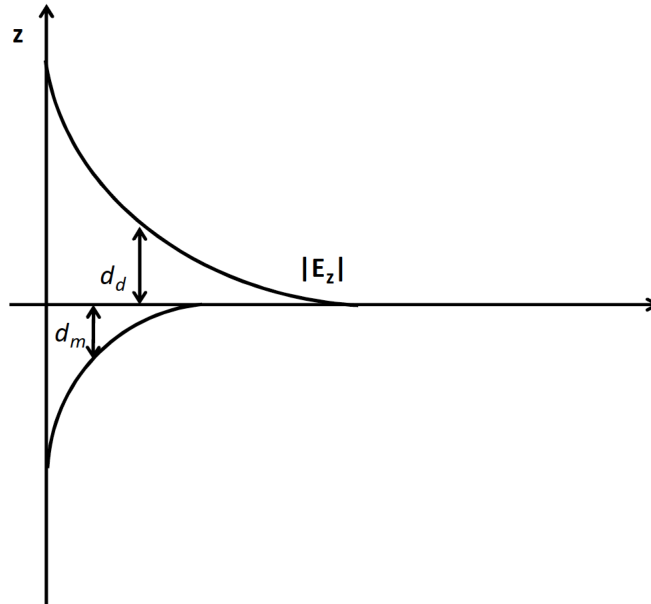


Figure 1.7: Exponential decay of the SPP field at the interface

1.4.3 Localized Surface Plasmons

We have introduced before the existence of another type of surface plasmons: *Localized Surface Plasmon* (LSP). This kind of oscillations does not need any coupling mechanism for they can be excited directly with an EM radiation, due to the restoring force acting on the electrons driven by the EM radiation field, linked to the shape and size of the particles. In fact, LSP are non-propagating oscillations and occur whenever the excitation is three-dimensionally confined into nanostructures such as sub-wavelength nanoparticles. This phenomenon leads to an enhancement of the field both inside the nanoparticle and outside the nanoparticle close to its surface, at a resonance frequency which depends both on the shape and size of the metal nanoparticles.

The theory mostly refers to the so called "Mie theory", published by Gustav Mie in 1908 [15], and is analytically developed for spherical and ellipsoidal particles. It treats the spherical particles as non-interacting scattering centers, by means of the expansion in multipole partial waves. The first approximation is the *dipole approximation*, where the radius R of the nanoparticles is assumed to be small compared to the wavelength of the radiation λ and the interparticle distance d . Thus, the problem is reduced to a non interacting dipole system. We can now make a distinction between the near-field regime and the far-field regime: if $d \ll \lambda$ (near-field), the interaction scales with d^{-3} displaying a strong field in the space between adjacent particles; if $d \gg \lambda$, (far-field) the interaction is dominated by the d^{-1} term.

1.5 SPPs-light coupling

We already outlined that to excite SPP a phase-matching condition is needed, due to the fact that the propagation constant β is always greater than the component of the radiation wave vector k_x along the interface. Hence, few coupling techniques are here shown.

1.5.1 Prism coupling

A way to obtain the phase-matching with SPPs is to make use of a three-layer system, where a thin meta film is the middle layer between two dielectric environment with different dielectric function. For simplicity, let the latter be air ($\varepsilon_1 = 1$) and a prism ($\varepsilon_2 > 1$). A light beam which experiences reflection at the interface between the prism and the metal will have an in-plane momentum $k_x = k\sqrt{\varepsilon_2} \sin \theta$ sufficient to excite SPPs at the air/metal interface. In figure 1.8 it is possible to see that the result of this configuration is to "lower" the air light line, obtaining an intersection with the air/metal curve, i. e., a coupling. This coupling mechanism has two possible configurations, Kretschmann configuration and Otto configuration, and is also known as attenuated total internal reflection (ATR), because the excitation of the SPPs is detected as a minimum in the reflected light in the prism, as it remains confined at the interface. Kretschmann geometry consists in evaporating a thin

metal film on one side of the prism, whereas in the Otto configuration, there's a gap of air between the prism and the metal layer, as can be seen in figure 1.9.

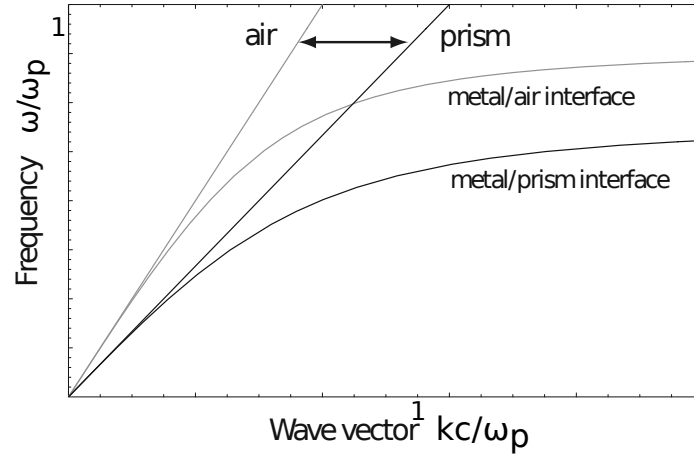


Figure 1.8: Dispersion relation of SPPs at metal/air (grey) and metal/prism (black) interfaces and air and prism light lines.

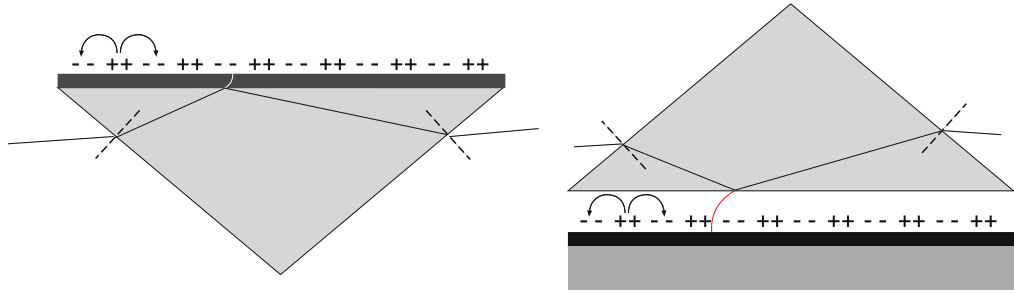


Figure 1.9: Kretschmann coupling configuration (left) and Otto coupling configuration (right).

1.5.2 Grating coupling

Another way to excite SPPs is by "modifying" directly the metallic film, patterning the surface with a lattice of grooves or holes. The missing momentum will then be provided to that of the EM radiation by the reciprocal lattice vectors. For simplicity, if we consider a one-dimensional lattice of period a , the phase-matching equation is straightforward:

$$\beta = k \sin \theta + \frac{2\pi}{a}j \quad (1.27)$$

where $k \sin \theta = k_x$ is the in-plane component of the light momentum and $\frac{2\pi}{a}j$ is a reciprocal wave vector of the lattice ($j \in \mathbb{Z}$). A simple scheme of this configuration is shown in figure 1.10.

Eq. 1.25 still holds whenever the corrugations are slight (depth ~ 10 nm). The occurred coupling can be detected, as in the case of prism configuration, as a minimum in reflection,

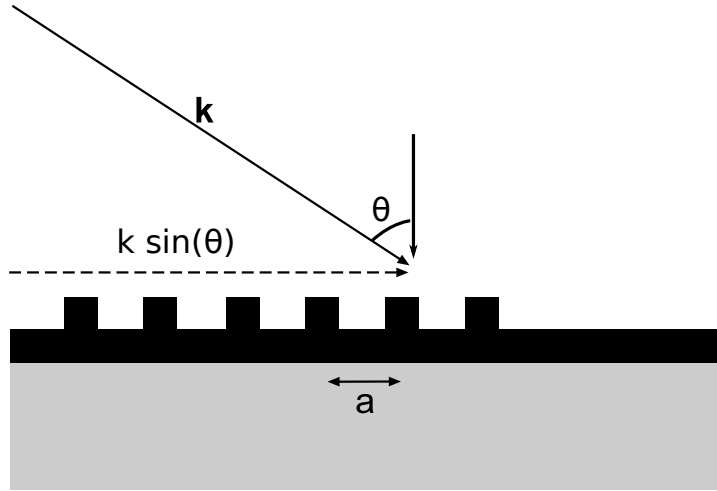


Figure 1.10: Grating coupling configuration. The missing momentum $k \sin \theta$ is shown.

but with grating it is possible to obtain also the reverse process: traveling SPPs can couple to light and radiate if they meet up a grating along the surface.

If the corrugations are enough deep, they became a strong perturbation at the interface, giving rise to localized modes and in turns eq. 1.25 is no more valid. In particular, in the case of an ordered array of holes through a metal film, it is possible to observe a peculiar optical property: the *extraordinary optical transmission* (EOT), which is used as the main optical transduction signal for the plasmonic biosensors in the present work. The basics of EOT will be discussed in the following chapter.

Chapter 2

Light propagation through sub-wavelength holes

One peculiar optical property related to the physics of nanoholes array (NHA) is the transmission of light through apertures beyond the diffraction limit and, in particular, the *Extraordinary Optical Transmission*. This phenomenon was first observed by Ebbesen [9] in 1998. Before studying this peculiar phenomenon, early transmission theories and, in our case, transmission of light through sub-wavelength circular holes will be briefly discussed.

2.1 Early Theories

2.1.1 Huygens-Fersnel principle and Kirchhoff's scalar diffraction theory

The *Huygens-Fersnel principle* (late 17th century) describes the transmission through holes of radius $r \gg \lambda_0$, where λ_0 is the wavelength of the incident radiation. In this case, the transmission coefficient T , equal to the ratio of the total transmitted intensity to the incident intensity on the holes area, is close to 1. This principle is a useful, simple tool to describe diffraction effects and can be seen as an approximation of the *Kirchhoff scalar diffraction theory* (second half of the 19th century), in which an opaque, perfectly black screen and a scalar wave equation is considered.

However, this theory is valid only in the case $r \gg \lambda_0$ and becomes useless when we consider the E- and H- field vector waves, as Bouwkamp pointed out [16]:

"If the scalar Kirchhoff formula is applied to each of the rectangular components of the electric and magnetic vectors, then the six wave functions so obtained do not in general satisfy Maxwell's equations."

The problem arises because we are trying to solve a vectorial problem (as the light field) by using a scalar formalism.

We now need a theory in the case of small (sub-wavelength) apertures, able to satisfy Maxwell's equations.

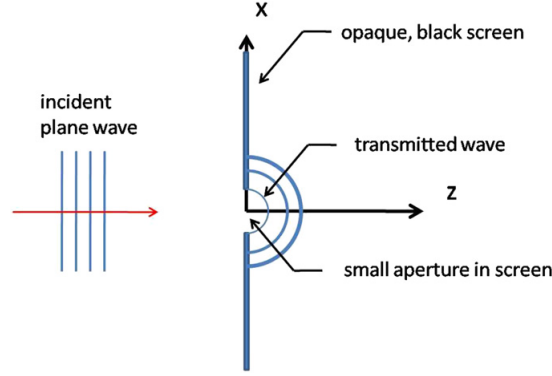


Figure 2.1: Kirchhoff diffraction theory scheme.

2.1.2 Bethe-Bouwkamp theory¹

The first attempt to make a theoretical insight in light transmission through a circular sub-wavelength hole in a thin perfectly conductor screen at normal incidence was developed by Bethe (1942) [17]. In this case, there is no propagation of light through the hole, but only transmission by tunneling. The hypothesis ‘*perfectly conducting*’ screen means that the metal film has infinite conductivity, no skin depth (EM radiation does not penetrate the screen) and TE modes are discarded. Bethe found that the transmission coefficient, normalized to the hole area, can be written as:

$$T = \frac{64}{27\pi^2} (k_0 r)^4 \propto \left(\frac{r}{\lambda_0}\right)^4 \quad (2.1)$$

where r is the hole radius, $k_0 = \frac{2\pi}{\lambda_0}$ is the incident radiation wave vector. Thus, we find that T falls off as λ^{-4} (Fig. 2.2a), predicting much less light transmission than Kirchhoff scalar diffraction theory, where T falls off as λ^{-2} . Moreover, in Bethe’s theory a strong drop in the transmission is expected as λ becomes larger than r and, if a real depth of the hole is taken into account, T becomes exponentially attenuated (Fig. 2.2b), due to the diffraction limit, $\lambda > 4r = \lambda_c$, beyond which light transmission becomes a tunneling process (where, λ_c is the cutoff wavelength). Notice that the cutoff condition might occur at longer wavelength when finite conductivity is considered.

Even though Bethe’s theory seems to provide a reasonably solid model, experimental results display some discrepancies, like the resonant-like behaviour of the transmission spectrum [6, 18] in figure 2.3:

which is linked to the discovery of the previously mentioned phenomenon, EOT. This behaviour seems to find an explanation in the excitation of SP modes, as will be discussed below.

¹The contribution of Bouwkamp to Bethe’s work consists in corroborating the validity of his theory in the far-field and proposing a correct near-field equation. [16]

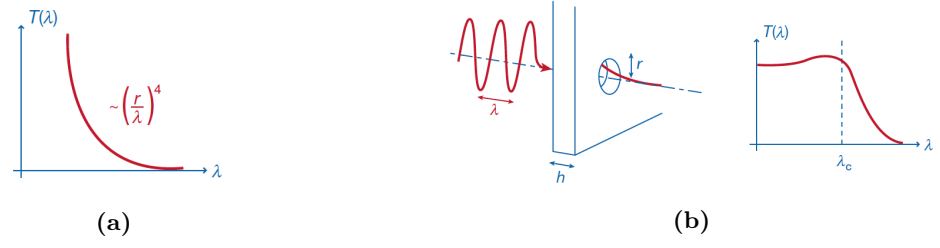


Figure 2.2: Transmission spectrum of visible light through a sub-wavelength hole in Bethe's approximation (*left*). Cylindrical waveguide of depth h and transmission spectrum with the exponentially decreasing tail (*right*).

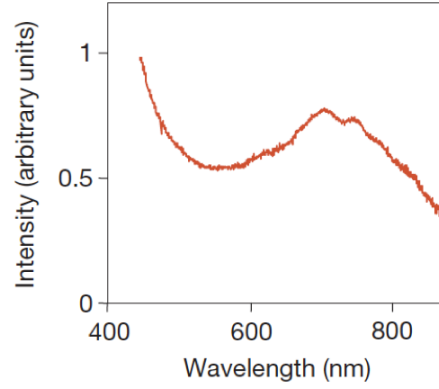


Figure 2.3: Transmission spectrum of a circular hole. Notice the resonant-like peak superimposed on a smooth background. [6, 18]

2.2 Extraordinary Optical Transmission

We introduced before that some discrepancies arise between Bethe-Bouwkamp model and experimental results, i. e., transmission enhancement. This phenomenon is linked to the presence of some type of resonance and according to the most accepted theory [6], it is ascribed to SP resonances. In fact, Bethe-Bouwkamp model does not consider the possibility of exciting propagating and evanescent modes at the edges of the hole.

2.2.1 NanoHole Array

In 1998, Ebbesen et al. [9] discovered that a metal film pierced with an ordered array of sub-wavelength holes (NHA) display an enhancement in the transmission, compared to theoretical previsions. In his experiment Ebbesen used a squared array (900 nm of pitch) of circular nanoholes (150 nm of diameter) milled in a thin silver film (200 nm thick). Fig. 2.4a shows the transmission spectrum at normal incidence, while fig. 2.4b displays the dispersion relation extracted from the energy of the transmission peaks at different angles. The term '*extraordinary*' means that the transmission efficiency exceeds 1, or alternatively, that the light transmitted by the NHA is more than that of a single hole, whose area corresponds to the sum of the nanoholes area. As we said previously, the coupling of light with a grating structure excites SPPs that travel through the holes

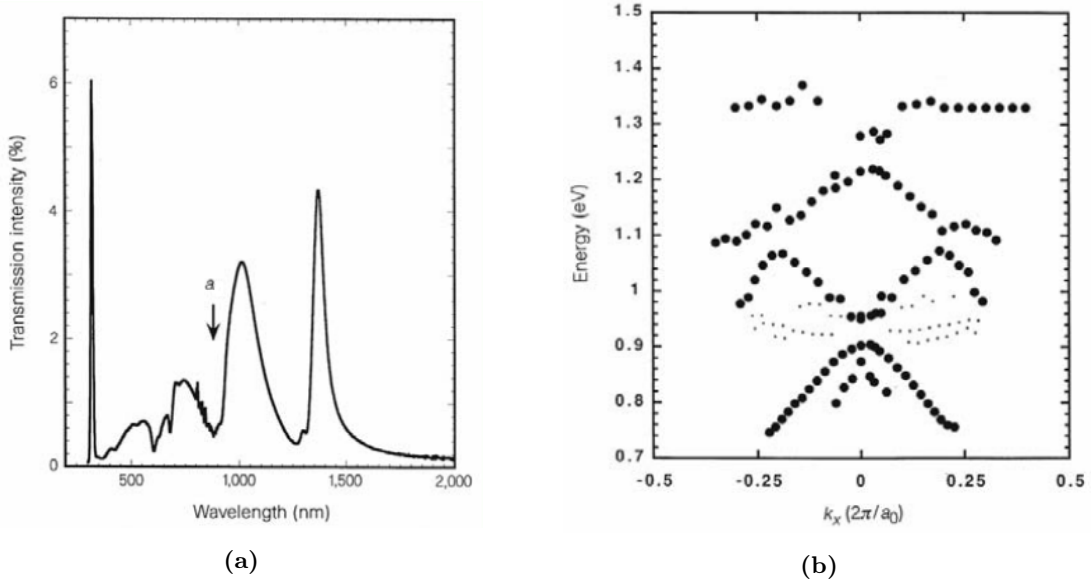


Figure 2.4: Transmission spectrum at normal incidence of a 200 nm thick silver film with a square array of circular holes of diameter 150 nm and 900 nm of pitch (*left*). Dispersion relation of SPPs at different angles of incidence (*right*).

edges reaching the other side, thus, coupling with light and re-radiating in the far-field. Moreover, the fundamental difference between smooth and periodical structured metal films is that, by illuminating the latter even at normal incidence, a SPP can be excited on both surfaces (under certain conditions). The missing wave vector between the in-plane light wave vector and the SP wave vector is provided by diffraction on the periodic surface.

If we consider a square lattice of holes with periodicity a , from eq. 1.27 we obtain:

$$\beta = k \sin \theta + mG_x + nG_y = k \sin \theta + (m + n) \frac{2\pi}{a} \quad (2.2)$$

where k is the wave vector of the incident radiation, G_x and G_y are basis wave vector of the reciprocal lattice with n and m being Miller integer indexes defining the scattering order and the SPP propagation direction. To obtain enhanced transmission peaks we need a resonance condition to be satisfied and therefore SPPs waves, called *SPP-Bloch waves*, which are standing waves consistent with Bloch's theorem [19, 20]. A simple relation provides the matching condition to give rise to this resonance at normal incidence:

$$\lambda_{SPP} = \frac{a}{\sqrt{m^2 + n^2}} \sqrt{\frac{\epsilon_m \epsilon_d}{\epsilon_m + \epsilon_d}} \quad (2.3)$$

taking advantage of eq. 1.25 and assuming a flat interface between the metal and the dielectric. This approximation leads to a slight underestimation of the wavelength of the resonance peaks than those observed experimentally.

In our case, the lattice of interest is an hexagonal hole array (Sec. 3.1.2). Let a be the periodicity, a similar relation can be found:

$$\lambda_{SPP} = \frac{a}{\sqrt{\frac{4}{3}(m^2 + mn + n^2)}} \sqrt{\frac{\epsilon_m \epsilon_d}{\epsilon_m + \epsilon_d}} \quad (2.4)$$

The limit of this approximation resides in the fact that a NHA does not constitute a small perturbation at the interface, thus β relation for SPPs needs to be more accurate. Another source of inaccuracy is linked to the semi-infinite dimension of the single interface, while we work with two finite interfaces which can be in contact with different dielectrics, thus providing two different spectra depending on which side of the NHA light impinges on.

Hence, when eq. 2.4 is satisfied, EOT phenomenon occurs. Speaking in terms of the normalized transmission T_{norm} , i. e., the ratio between the transmission coefficient of the nanohole array, T_{NHA} , and the sum of the ordinary transmission of each single hole, T_h :

$$T_{norm} = \frac{T_{NHA}}{\sum_j T_h} \quad (2.5)$$

as we already mentiond, EOT means that $T_{norm} > 1$, which means that the transmitted light is more than the one which impinges on each single aperture. This happens because, thanks to SPPs, not only the portion of the light beam that illuminates the holes area is involved in the transmission process, but also the one that impinges on the metal interstices. An interference phenomenon, as a result of a Bragg scattering process analogous to that of crystalline atomic lattices, occurs and the spectral selectivity of the EOT matches with Bragg resonances whose orders are identified by the (m, n) integers [21].

If we go back to figure 2.4a, we notice that several peaks are observed, but two of them rise at a wavelength greater than both the hole diameter and the array periodicity. This suggests that EOT is not caused by ordinary light tunneling through the holes, but reasonably by light funneling by means of SPPs propagating within the holes walls.

Geometrical parameters

An important feature of NHA resides in the simplicity of the spectral properties tuning by changing different geometrical features, whose nanofabrication will be shown in next chapter

Periodicity a From the coupling equations 2.3 and 2.3, it can be easily seen the proportionality of the peak position to the periodicity of the array. Adjusting this parameter it is possible to shift the EOT peak in the desired spectral region.

Hole diameter d This parameter controls the process in which light transmission occurs: for d large enough, propagation modes are allowed and the transmission is modulated by resonances waveguide modes. Since we are working with sub-wavelength apertures, $d \leq \lambda/2$, only tunneling modes are allowed. Typical diameters treated in this work are below ~ 330 nm for which the resonance occurs at $\lambda > 900$ nm. Therefore, our transmission spectra are linked to SPPs and their funneling through the holes.

Thickness t This is a critical parameter, because if t is smaller than the skin depth, $t < \delta$, the excited SPPs at the front interface might couple and produce SPPs at the rear interface, if the dielectric environment is the same on both sides. However, for the

wavelengths range we are considering (UV-Vis-IR), the skin depth results much smaller than the film thickness, $\delta \sim 5 \div 10$ nm, while $t \sim 100$ nm. Hence, only SPPs funneling through the holes governs the transmission process.

Hole shape The shape of the holes determines the coupling efficiency depending on the polarization of light. Higher intensity in the transmission spectra has been observed for square holes rather than for circular holes. The advantage of employing circular holes is due to its symmetry, making the response similar for different incident light polarizations.

2.2.2 Fano resonance

In the last part of the previous paragraph we mentioned the interference process which takes place when SPPs are excited, i. e., at the SP resonance. Resonance peaks usually display a Lorentzian or Breit-Wigner (BW) line-shape and arise from the interference of two counter waves in the same scattering channel. The EOT peak instead shows an asymmetric line profile due to wave interference of different channels. This type of resonances are called *Fano resonances* and have been first described by Ugo Fano in 1935 [22] (his study concerned atomic physics). In our case, the "different interfering channels" interpretation involve a resonant continuum state (CS) with a non-resonant discrete state (DS) (fig. 2.5) [23].

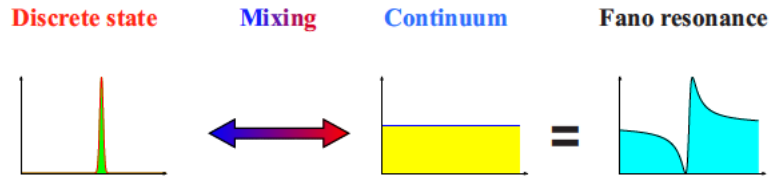


Figure 2.5: Interference of a discrete state with a continuum state resulting in a Fano-type resonance. [23]

The former is represented by the SP resonance, while the latter by the Bragg-diffraction channel of the nanohole array structure. Another interpretation is the interference between a propagative mode of the incident radiation and the evanescent mode of surface plasmons. An intuitive analytical discussion to understand this type of resonances is provided below.

Classical analogy of Fano Resonances

We already know that a system under an external excitation, for a certain frequency, called *resonance frequency* or *natural frequency*, may display a resonance, thought as an enhancement of the response of the system. A simple way to introduce resonances is by means of the harmonic oscillator under a periodic driving force. When the frequency of the driving force is equal to the eigenfrequency of the oscillator, the latter displays a enhancement in the amplitude of its displacement reaching its maximum value. On the other hand, many systems may display the opposite phenomenon, suppressing their

response at a certain matching condition: *antiresonance*. In this case a simple example is the two weakly coupled harmonic oscillators in which one is driven by a periodic force (see figure 2.6a). This system displays two resonances slightly shifted with respect to the eigenfrequencies of the two free oscillators: $\omega_- = \omega_1 + \Delta\omega_2$ and $\omega_+ = \omega_2 + \Delta\omega_2$. In particular, the forced oscillator displays the usual enhancement in the amplitude close to its natural frequency, at ω_- , and an unusual sharp peak at ω_+ . The former resonance shows the usual *Lorentzian* or BW profile, while the latter has an asymmetric profile with a vanishing amplitude coinciding with ω_2 preceding the peak at ω_+ (fig.2.6b and fig.2.6c). This is due to the destructive interference resulting from the action of the driving force and of the second oscillator on the first one. This destructive interference is one of the basic properties of the Fano resonance, which is unique with respect to other resonances [23].

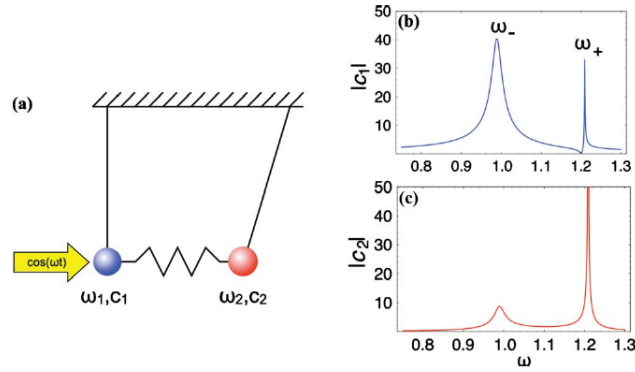


Figure 2.6: *a)* Two harmonic coupled oscillator, one of which is driven by a periodic force. *b)* Amplitude of the first oscillator as a function of the frequency. Symmetric and asymmetric resonance peaks and their respective ω_- and ω_+ eigenfrequencies are shown. *c)* Amplitude of the second oscillator as a function of the frequencies and the symmetric resonance peaks. [23]

Fano resonances in plasmonic nanostructures

We now present another simple theoretical model related to the present case of plasmonic nanostructures [24, 25]. As we said previously, the Fano resonance arises when an interference between CS and DS occurs and in our case the CS is represented by the plasmon resonance, PR, while DS by the Bragg-diffraction modes of the lattice. This interaction gives rise to a new mixed state that accounts for both interfering channels. The Fano function has the following form:

$$\sigma(\epsilon) = \frac{(\epsilon + q)^2}{\epsilon^2 + 1} \quad (2.6)$$

where q is a coupling factor that measures the asymmetry of the curve and is defined as the excitation probability ratio between DS and CS; ϵ is the reduced energy defined as: $\epsilon = 2(E - E_d)/\Gamma_d$, where E is the incident photon energy, E_d and Γ_d the energy and the width of the discrete state respectively. By tuning the q parameter we obtain three different cases 2.7:

- $q \rightarrow \infty$: Lorentzian or Breit-Wigner line-shape, the profile is determined by the transition through the DS being the probability of exciting the CS small;
- $q = 0$: a symmetric antiresonance arises, called Breit-Wigner dip;
- q is finite: an asymmetric lineshape is obtained

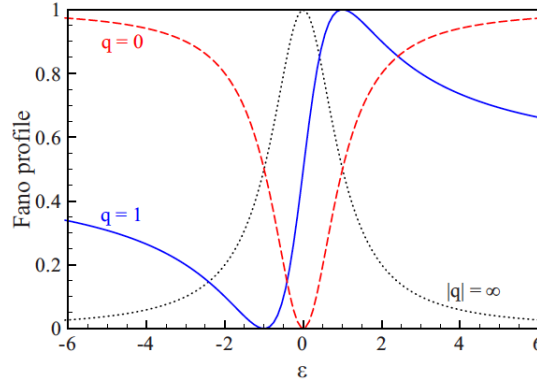


Figure 2.7: The three possible Fano profile as a function of the reduced energy with respect to the q value: the Lorentzian (or BW) for $q \rightarrow \infty$; the BW dip for $q = 0$; the Fano resonance shape for finite values of q (e. g., $q = 1$). [23]

It is interesting the fact that in eq. 2.6 appears no feature of the plasmon resonance, which will be included in the coupling to the CS. Let us assign to the DS the quantum state $|d\rangle$, $|c\rangle$ to the CS state and $|i\rangle$ to the incident state, while w , g and v are respectively the coupling factor of $|i\rangle \rightarrow |d\rangle$, $|i\rangle \rightarrow |c\rangle$ and $|d\rangle \rightarrow |c\rangle$, as shown in figure 2.8. Let $\mathcal{H}_0$

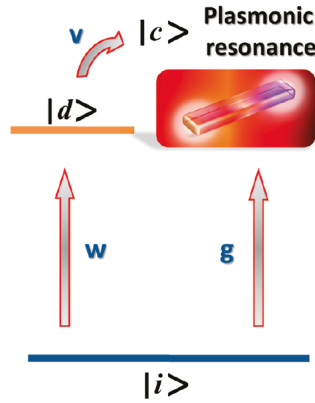


Figure 2.8: Scheme of the Fano interaction between $|i\rangle$, $|d\rangle$ and $|c\rangle$ and the respective coupling factors. [24]

be the free Hamiltonian and V a coupling Hamiltonian. Thus, $\mathcal{H}_0$ and V matrix elements are:

$$\langle d|V|d\rangle = E_d = 0 \quad (2.7a)$$

$$\langle c|V|c'\rangle = E\delta(E - E') \quad (2.7b)$$

$$\langle c|V|d\rangle = v\sqrt{\mathcal{L}(E)} \quad (2.7c)$$

where E_d has been set as the origin, while $|c\rangle$, $|c'\rangle$ and E , E' are different continuum states and their respective energies. The most important equation is 2.7c, which gives the coupling between DS and CS and is determined by the plasmonic line-shape $\mathcal{L}(E)$, which is a Lorentzian with energy position E_p and width Γ_p , and by the coupling factor v . Furthermore, all other matrix element of V are assumed to be vanishing.

Hence, the Hamiltonian of the system is $\mathcal{H} = \mathcal{H}_0 + V$, which leads to the eigenvalue problem: $\mathcal{H}|\Psi\rangle = E|\Psi\rangle$, where Ψ is the new mixed-state quasi-CS. The incident photon $|i\rangle$ is coupled to DS and CS by the Hamiltonian W :

$$\langle i|W|d\rangle = w \quad (2.8a)$$

$$\langle i|W|c\rangle = g\sqrt{\mathcal{L}(E)} \quad (2.8b)$$

By solving the eigenvalue problem and by calculating the probability that $|i\rangle$ might excite $|\Psi\rangle$, i. e., $|\langle i|W|\Psi\rangle|^2$, the Fano profile is obtained. By normalizing that probability to that of exciting the CS neglecting the DS, that is, the PR, 2.6 is readily obtained, with q and ϵ being explicit function for the PR:

$$\frac{|\langle i|W|\Psi\rangle|^2}{|\langle i|W|c\rangle|^2} = \frac{(\epsilon + q)^2}{\epsilon^2 + 1} \quad (2.9a)$$

$$q = \frac{vw/g}{\Gamma_d(E)/2} + \frac{E - E_p}{\Gamma_p/2} \quad (2.9b)$$

$$\epsilon = \frac{E}{\Gamma_d(E)/2} - \frac{E - E_p}{\Gamma_p/2} \quad (2.9c)$$

where $\Gamma_d(E) = 2\pi v^2 \mathcal{L}(E)$ is the DS energy width.

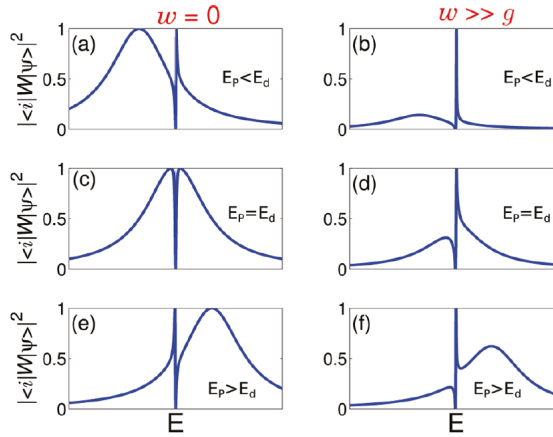


Figure 2.9: Fano resonances regimes, $w = 0$ (a, c, e) and $w \gg g$ (b, d, f), in the common case of $\Gamma_p \gg \Gamma_d$ ($\Gamma_p = 10\Gamma_d$), for different energy relations, $E_p < E_d$ (a, b), $E_p = E_d$ (c, d) and $E_p > E_d$ (e, f)

Because of the fact that now q and ϵ are no longer constants, but depend on the coupling factors and on the PR features, the Fano resonance shows a variety of asymmetric line-shapes depending on the physical system considered. Figure 2.9 shows the different profiles

of the Fano function in the most common case, that is, when $\Gamma_p \gg \Gamma_d$, accounting the possibility of a weak coupling with the DS, $w = 0$ (a, c, e), a strong coupling with the DS, $w \gg g$ (b, d, f), and also the possible energy situations: $E_p < E_d$ (a, b), $E_p = E_d$ (c, d) and $E_p > E_d$ (e, f). For our purposes, we won't enter further in the discussion of the mentioned possible situations, but we simply add that the EOT phenomenon of the NHA falls between the two cases: $w = 0$ and $w \gg g$. From eqs. 2.9 it is possible to calculate the minimum of the Fano resonance, which occurs for $q = -\epsilon$, corresponding to $E = -vw/g$, which does not depend on the PR, but only on the coupling factors.

It is important to add that, in addition to SPPs, the impinging radiation penetration inside the metal gives rise to strong localized surface plasmons on the rim of each hole, making it a *hot spot*. This hot spot results in an increase of the effective hole diameter together with the cutoff wavelength, assisting the funneling process of forbidden modes, eventually increasing the transmission. However, the main contribution to EOT is due to SPPs [18]

The Fano profile of the EOT spectrum explicitly shows that this phenomenon is highly frequency-selective and easily spectrally tunable (section 2.2.1), making it a useful tool for different practical applications, like in the present case of label-free optical biosensors.

Chapter 3

Synthesis of Nanohole Array

We already mentioned that one of the main practical feature of the nanohole array is the simple tunability of its geometrical parameters in order to adjust its spectral properties. The most widely used method is *lithography*, due to its simplicity in tuning the geometrical features by patterning a metal film through the use of a mask. In this chapter, the most common lithographic techniques of nanohole arrays (and nanostructures in general) will be illustrated. Then, an in-depth description of the technique employed in this work, i. e., *Nano Sphere Lithography*, will be presented.

Photolithography [26,27] This method involves light to transfer a geometrical pattern on a film or from a photomask to a light-sensitive layer called "photoresist" and then, by chemical etching, the pattern is transferred to the film, removing the photoresist. However this technique is not employed in nanostructure fabrication due to its diffraction-limited resolution: of the order of $\sim \lambda/2$ of the incident radiation. Thus, if deep UV light is employed, resolution reaches the order of 50 nm, but with X-ray lithography [28] this diffraction limit is overcome, reaching higher resolution, together with higher costs.

Electron Beam Lithography (EBL) This is one of the most used methods, since it can reach sub-10 nm resolution designs. It consists in a narrow focused beam of electrons of tens of keV. Hence, the surface is coated with an electron-sensitive material on which the pattern is directly drawn, without employing any mask. The limit of this technique resides in the high cost and low sample throughput which prohibit a sustainable high production.

Focused Ion Beam Lithography (FIB) [27] Similar to the EBL, focused ion beam lithography involves a narrow focused beam, in this case of ions, and does not need the use of a mask as well. Moreover, it does not need any resist material since the beam impinges directly on the surface. With respect to electrons, ions, which are heavier, display a smaller wavelength and thus, a negligible diffraction effect. Ebbesen used FIB in the experiment that lead him to the discovery of EOT (section 2.2.1, [9]). However, even this method provides a high resolution, down to 2-nm, but exhibit the same issue of the EBL.

Nano Sphere Lithography (NSL) Born as "natural lithography", by Deckman [29], this fabrication technique, which is the one used in this work, was renamed and perfected by Van Duyne [10] and consists in a multistep procedure that allows to easily control the size, shape and pitch of well-ordered nanostructure arrays with defect-free areas of the order of $\sim 10 \div 100 \mu\text{m}^2$. The first step is molding a *self-assembled monolayer* (SAM) mask ($\sim 1 \text{ cm}^2$) of monodisperse size of polystyrene nanospheres (NS) (diameter $D \sim 0,1 \div 1 \mu\text{m}$) on a substrate. This monolayer arrangement gives a hexagonally close-packed lattice of nanospheres.

Defects may arise from polydispersity in the sphere diameter or from vacancies and slip dislocations, caused by the assembling methods like spin-coating, drop-coating or another method which we took advantage of and which will be now discussed.

Then a metal film can be deposited through the mask both as it is (e. g., to obtain nanoprism arrays) or after an etching process, which shrinks the spheres without affecting the interparticle distance, i. e., the periodicity, thus obtaining a nanohole array (fig. 4.1).

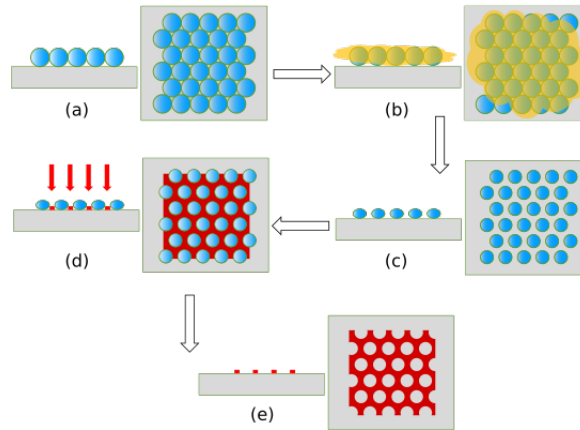


Figure 3.1: Scheme of the NSL fabrication process to obtain the NHA: (a) SAM, (b) RIE, (c) ried mask, notice that the NS are reduced in dimension, but the periodicity has not been affected, (d) sputtering of the metal layer and (e) NHA, after NS removal.

One of the reasons why we used the NSL, rather than the EBL and FIB, is because this method exhibits high throughput and cost-effective production.

3.1 Nanosphere lithography

The steps of NSL fabrication method will be discussed in more detail in this section. These steps consist in the cleaning of the substrate, the assembling of the mask, the shrinking of the PS, the metal deposition and finally the removal of the mask and its cleaning.

3.1.1 Cleaning of the substrate

The substrate involved in the NHA fabrication are monocrystalline silicon (Si) and Soda Lime Glass (SLG) with lateral size of couple of centimeters. Si wafer are employed as "monitoring" substrates, because they can be easily imaged with the SEM (scanning electron microscope), without charging. SLG displays a relatively smooth surface, making it a good choice in order to reduce mask imperfection. But the surface morphology is not the only parameter to influence the mask order. Also impurities on the substrate, such as dust or organic residuals, must be removed. To accomplish it, two cleaning processes are performed: the *acid piranha* and the *basic piranha* baths.

The acid piranha consists of a 3:1 solution of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). The substrates are immersed in the acid piranha at the temperature of 90 °C for about 1 hour. This solution is a strong oxidizing agent and it serves to remove organic impurities and make the surface highly hydrophilic. After this process, the substrates are thoroughly rinsed in Milli-Q water (18.2 M Ω -cm resistivity).

Then, the substrates are immersed in the basic piranha, a 3:1 solution of ammonium hydroxide (NH_4OH) and hydrogen peroxide. This bath, at the temperature of 90 °C, lasts about 20 minutes and it further improves the hydrophilicity of the surface.

3.1.2 The mask: self-assembling monolayer

The mask self-assembling is made by taking advantage of the work of Schatz [30]. A colloidal solution of polystyrene nanospheres (NSs), of nominal diameter $D = 522$ nm, in water and 2-propanol is first prepared. Then, a substrate is fixed to a T-shaped arm of a motorized dipper and exposed to the previous solution. The substrate is then dipped into Milli-Q water, letting the NSs float on the water surface where they form a compact monolayer, due to the meniscus between the alcoholic dispersion and the water and the evaporation of the former. The monolayer is then manually collected with another SLG or Si substrate. It is dipped through use of a tweezer and pulled out extremely slowly, by going backwards and upwards, to let the capillarity of the water and the hydrophilicity of the substrate to work properly in order to let the spheres adhere on the substrate surface. All these steps are shown in figure 3.2.

Hence, the spheres are left to stand, to allow water evaporation in order to dry and compact the mask into an hexagonal close-packed lattice, thanks to the capillarity forces (fig. 3.3).

Figure 5.4 shows different images of the NSs SAM on a substrate, by means of Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM).

3.1.3 Reactive Ion Etching

The next step in the NSL process is the reactive ion etching (RIE), a process that allows to shrink the PS nanospheres without affecting the lattice periodicity, providing the negative mold for the NHA. RIE erodes the nanospheres with a gradual and controlled

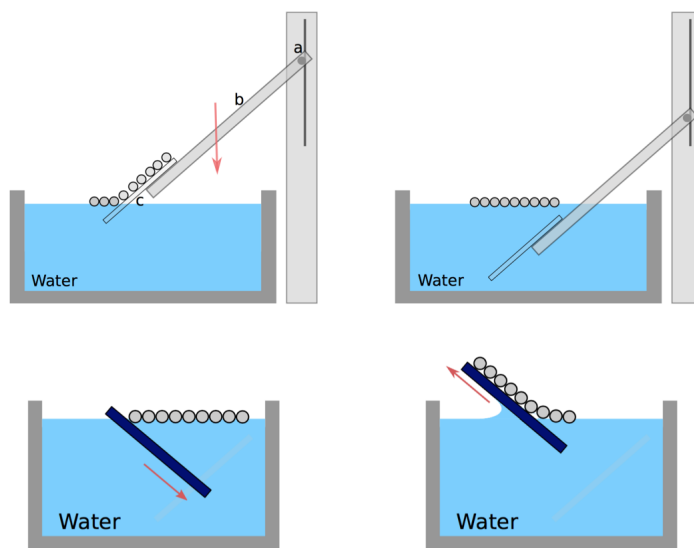


Figure 3.2: Representation of the motorized dipper (a) and the T-shaped arm (b) on which the substrate (c) with the colloidal solution is fixed. The entire process is represented in four steps: the dipping of the first substrate, the SAM on the water surface, the dipping of the second substrate, the adhesion of the mask.



Figure 3.3: Scheme of the drying process: evaporation of the water and capillarity forces which compact the NSs into a mask (*left*). Scheme of the hexagonal closed-packed lattice of the NSs SAM after the drying process (*right*).

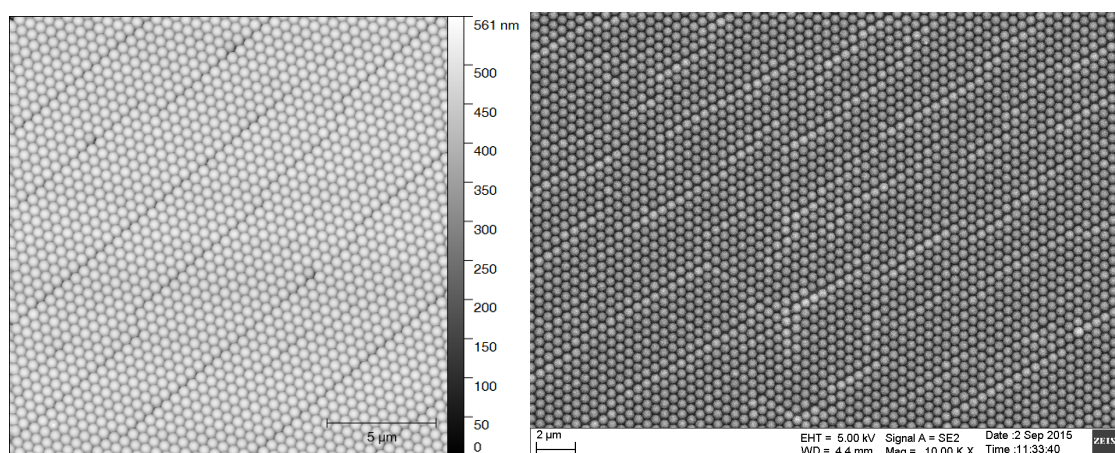


Figure 3.4: Images of the self-assembled monolayer of nanospheres taken with AFM (*left*) and SEM (*right*).

rate in a dry high-energy ion-assisted chemical etching process through use of a chemically reactive plasma, generated at low pressure, which removes material from the substrate by collision and chemical reaction.

RIE System The RIE system consists of a cylindrical vacuum chamber with a wafer plate for storing the substrate situated at the bottom. This plate is electrically isolated from the rest of the chamber, which is kept at ground potential. The gases employed for the reactive plasma flow into the chamber through a valve at the top, while a second valve situated at the bottom, connected to a vacuum pump, serves as a way out for the byproduct of the etching. This process depends primarily on the etched medium, which determines the type and proportions of the etching gases.

Procedure Firstly, vacuum is created in the chamber, maintaining the inner pressure in the typical range of $10^{-3} \div 10^{-1}$ mbar. Then, the etching gas flows in the chamber, producing a plasma through application of a radio-frequency (RF) electromagnetic field to the substrate holder. This ionizing field usually works at 13.56 MHz frequency and at few tens Watts of power. Thus, the plate gains a negative charge and the electrostatic field thus generated attracts the positive ions of the plasma towards the substrate. Now, two processes may take place: high chemically reactive ions reacts directly with the sample, eroding it, while low chemically reactive ions physically etch the material in a kinetic process. Byproducts from the first process are desorbed, while that of the second diffuse away from the substrate, exiting from the bottom valve introduced previously. RIE is an anisotropic and thus highly directional process, since the plasma ions are driven by the electric field. Hence, the sample is etched mainly in the vertical direction. Figure 3.5 illustrates the RIE system and a simple process scheme.

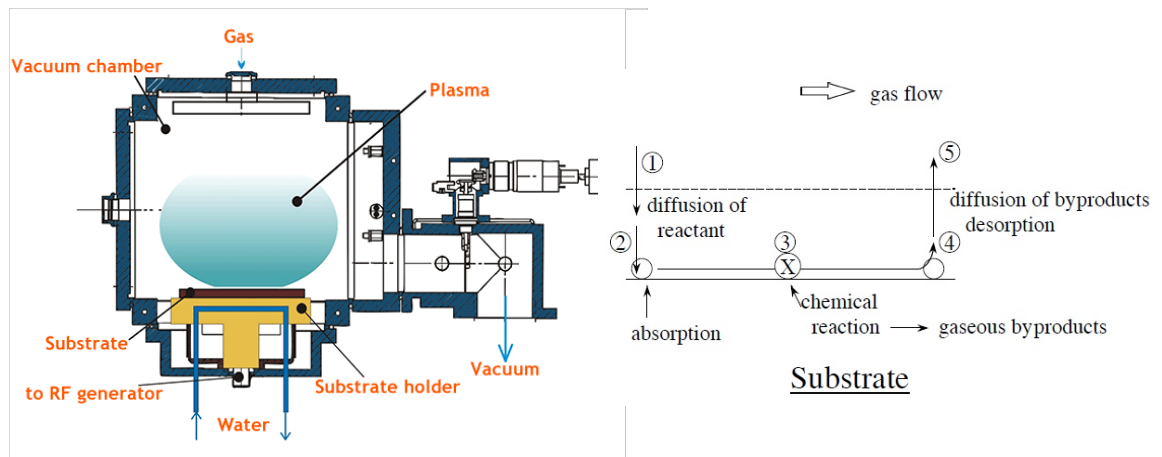


Figure 3.5: Representation of the RIE system (*left*) and the RIE process (*right*).

RIE in NanoSphere Lithography

To shrink the PS spheres of the mask, a mixture of Oxygen (O_2) and Argon (Ar) is employed. O_2 is chemically reactive, thus it just erodes the nanospheres, while the Ar gas, being chemically inert, enrolls the physical process, sputtering material away from the sample. Hence, the latter causes the nanospheres to become wrinkled, while the former tends to smooth the surface. However, two pressure regimes in the RIE process are possible: *high pressure* and *low pressure*. In the first regime, higher gas flow leads to an isotropic etching process, while in the low pressure regime we have less gas flow, which implies that ions can easily diffuse towards the sample, in a vertical motion due to the driving field. The latter process is the one employed for the production of nanohole arrays and the highly directional nature of this regime leads the PS spheres to gain a lenticular shape (fig. 3.6).

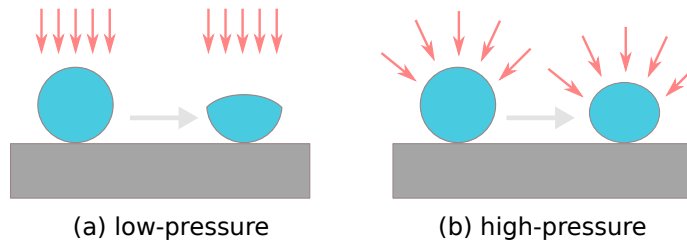


Figure 3.6: Representation of the two RIE pressure regimes. Notice the lenticular shape of the PS nanosphere in the low pressure regime.

Once the pressure, flux, voltage and power parameters are fixed, the only parameter left to set is the time, which determines the final diameter of the spheres and hence the nanoholes diameter. All of the former parameters have been optimized in order to obtain the best shape for our purposes (see table 3.1), while the timing to obtain the desired diameter has been calibrated by means of SEM observation and analysis. An example of the different final dimensions of the spheres after different etching times are shown in figure 3.7.

O_2 flux	9.2 sccm
Ar flux	3.6 sccm
Pressure	$1.6 \cdot 10^{-3}$ mbar
Bias	66 V
Power	15 W

Table 3.1: Values of the multiple parameters involved in the RIE, optimized for this work.

RIE is characterized also by a time limit, due to the fact that an excessive etching time might cause the collapse of the spheres and for this reason the final diameter is always greater than the half of the initial one.

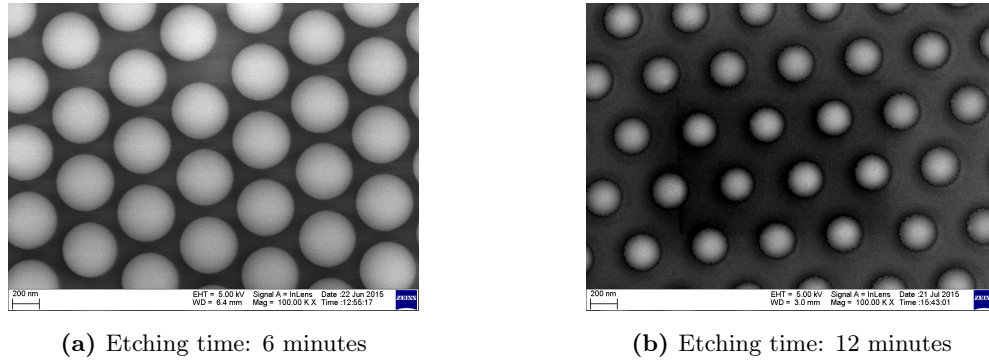


Figure 3.7: SEM images of the non-closed-packed nanosphere mask after different RIE times, with the conditions of table 3.1.

3.1.4 Magnetron sputtering

Now the mold is in its final configuration, hence it is possible to obtain the nanohole array through metal deposition. There are different ways to deposit a thin metal layer, like evaporation or sputtering, among the others. The technique used in this work is the latter, for reasons that will be explained later. In a sputtering process, which somehow looks to be the opposite of the RIE process, an ionized gas erodes material from a metal target by collision between the gas atoms with the metal ones. Thus, the transferred kinetic energy in the impact allows the ejection of the target material in all directions, reaching the sample conveniently placed in front of the metal target. The emission of material from the target, in fact, due to its finite dimension, has neither spherical nor uniform distribution. Thus, the diffusion is not perpendicular to the sample, causing shadow effects. A way to obtain a flat deposition is to place the sample properly close to the target, so it may see the source to be uniform. Before describing in detail the process, let us say that in the non-perpendicular metal deposition which characterizes the sputtering process resides the advantage of using this technique, with respect to the evaporation: the metal reaches the substrates zone lying under the etched spheres, narrowing the hole diameters, thus, enhancing the EOT signal.

Magnetron Sputtering System The Magnetron sputtering system is similar to that of the RIE: it is composed by a vacuum chamber, a sample holder electrically connected to a metal target and a valve to let gas flow inside the chamber. At first, vacuum is created inside the chamber to a prepressure of $\sim 3 \cdot 10^{-6}$ mbar. Then, Argon gas flows inside the chamber through an inlet, reaching a higher working pressure of the order of $\sim 10^{-3}$, to create the plasma which will erode the target. The choice of the gas is due to the fact that Argon is inert and hence it does not react with the target neither with the growing metal layer. By applying a high negative DC voltage to the target and grounding the sample holder, the former acts like a cathode while the latter as an anode. This intense electric field ignites the Argon plasma by ionizing its atoms which are thus driven towards the cathode: the metal target. Being our system a Magnetron sputtering, also a magnetic field

is employed by means of magnetrons placed inside the target holder. This field increases and confines the plasma intensity in the region close to the target, speeding up the erosion process and hence improving the sputtering efficiency.

The sputtered material from the metallic target consists of neutral atoms which diffuse, colliding with other atoms in the atmosphere. This collision rate depends on the chamber pressure, linked to the gas flow. A lower pressure decreases the collision rate, but also leads to a decrease in the efficiency of the sputtering process, because of the lower number of ionized atoms which may hit the target. On the other hand, a higher pressure would increase the sputtering efficiency, while decreasing the deposition efficiency, due to the growing collision rate and deflection of the sputtered particles. Figure 3.8 shows the magnetron sputtering system with a schematic view of the process. In our case, we have three target holders, with their own confinement system, which allows the deposition up to three different materials. It is thus possible to deposit metal alloys by co-sputtering or make multi-layer deposition by consecutively sputtering different materials.

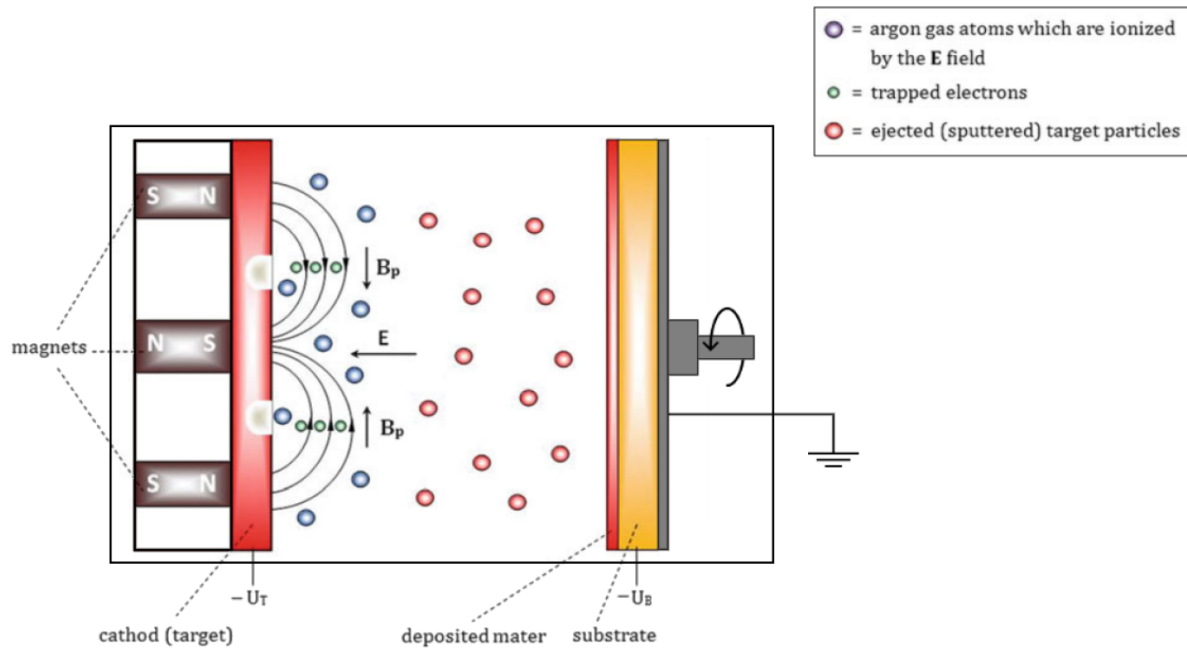


Figure 3.8: Scheme of the magnetron sputtering system.

As for the RIE case, the chamber pressure and the power and voltage applied to the targets parameters were first optimized, thus leaving the time as a free parameter which calibration gives the deposition rate of each material.

In this work, NHA of gold are used, thus a gold (Au) deposition is performed. Unfortunately, gold adhesion to silica glass is very weak, thus an adhesion layer is needed. This task is carried out by a thin layer of chromium (Cr) of few nm. Chromium, by oxidizing, is able to bind with the SLG, while on the other side, being a metal, exposes a suitable surface to receive gold. To preserve the optical properties of gold, only ~ 5 nm are deposited. Thus, a gold layer, between $50 \div 100$ nm, is sputtered. The reason why Au is preferred

to another excellent plasmonic metal like silver (Ag) is because Ag can be oxidized, thus modifying its interface and its optical spectrum.

As we said previously, the thickness of the metal layer is a crucial parameter, hence, it must be large enough to prevent transmission through the film, i. e., to become opaque for the incident radiation, allowing only funneling through the hole, giving rise to the EOT. On the other hand, if the thickness is too large, the PS spheres cannot be removed anymore, being submerged by the metal film.

The next step consists in removing the NS from the metal film, to finally obtain a NHA. The PS spheres dissolve in toluene, hence, immersing the sample in this solvent and sonicating it for two minutes leads to the final desired structure. Figure 3.9 shows SEM and AFM images of the NHA after mask removal.

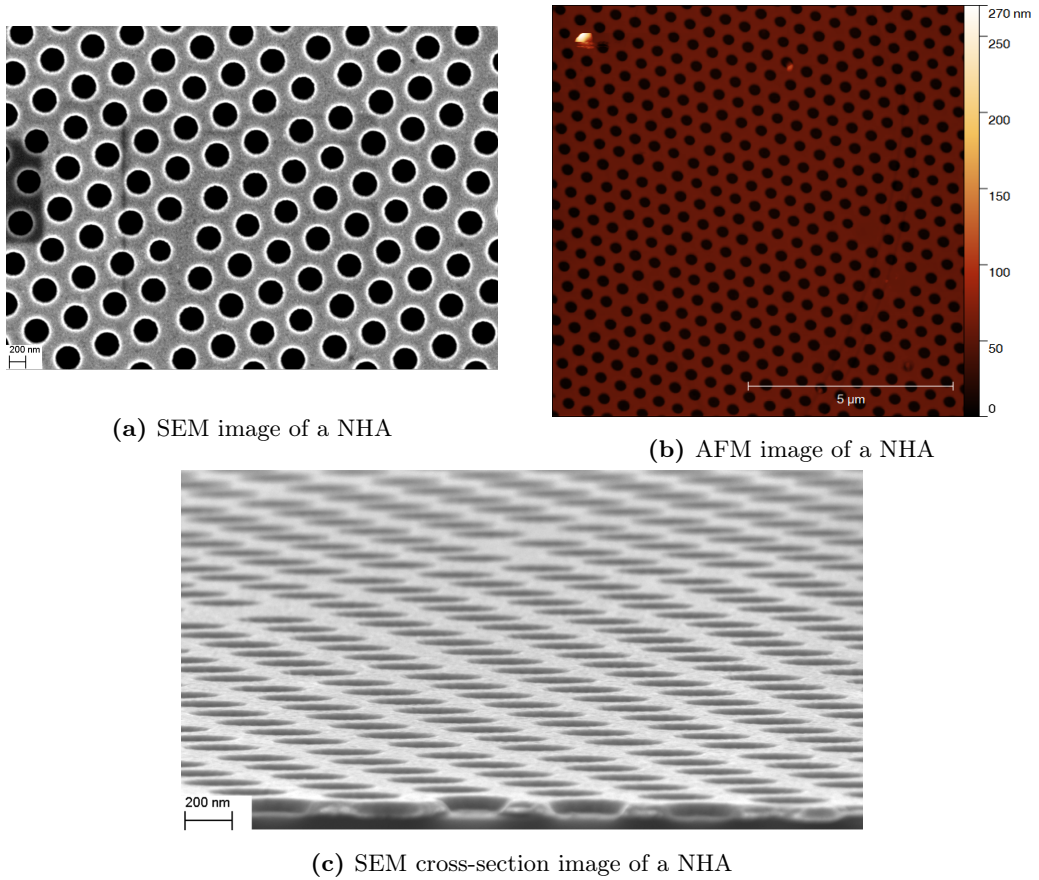


Figure 3.9: SEM and AFM images of a NHA after mask removal. The PS spheres had the initial diameter of 522 nm, before being etched to a final diameter of $\sim 300 \div 330$ nm. The adhesion layer of Cr is 5 nm thick, while the gold film thickness measures 55 nm.

Therefore, a nanohole array is finally obtained on a chosen substrate and it is ready to use. It is important to stress the simplicity in the geometrical parameters tuning thanks to this technique, i. e., the NSL. The most important parameters are:

- the periodicity of the hexagonal closed-packed lattice a , which corresponds to the initial diameter of the PS nanospheres;

- the diameter of the holes d , which correspond to the final diameter of the NS, which in turn depends on the RIE time;
- the metal thickness t , which depends on the metal deposition time.

Chapter 4

Label-free biosensing with NHA

A biosensor is a device able to detect and recognize a specific biological substance, called *analyte*. The principal components of such device are the sensitive part, called *receptor*, able to selectively recognize the analyte through binding, and the physical component which translates the occurred interaction in a simple, measurable and quantifiable signal, for example an electrical or an optical signal: the *transducer*. In this work, the transducer role is carried out by the NHA, which translates the interaction in an optical signal, i. e., EOT, thanks to its strong dependence on the dielectric environment, introduced in chapter 2. The biological receptor can be an antibody, antigen, enzyme or nucleic acid, depending on the specific analyte we intend to reveal, which moreover determines the specificity of the device.

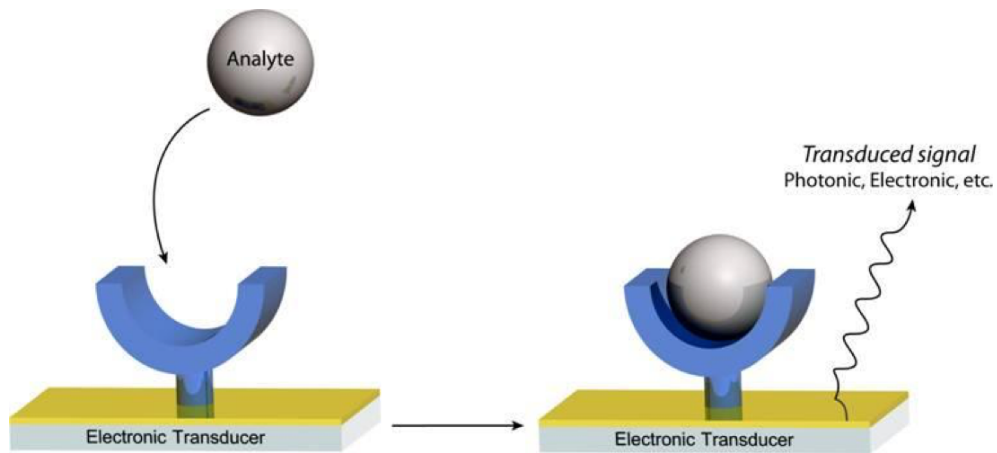


Figure 4.1: Representation of a biosensor and its main components.

The advantage in employing a plasmonic device is that no optically-active marker is required to label the analyte in order to reveal the occurred binding, thus, making the NHA a *label-free* biosensor. So no chromophoric groups or other type of labels, which may contaminate the signal, are needed. Moreover, the evanescent field, which expires in few tens nanometers, permits the detection of low concentrated small biological molecules. Finally, plasmonic devices provide real time detection, making these type of biosensors

suitable for fast biological detection.

Therefore, plasmonic devices represent a great choice of high-sensitivity, high-specificity and label-free biosensors for real time detection of biological substances and monitoring of biological interactions.

In 1983, Liedberg et al. [31] demonstrated the applicability of SPR for biosensing, feeding an always growing interest in the possible application of SPR-based biosensors, from medical to environmental issues. Since then, SPR biosensors have been involved in diagnostic and monitoring of diseases, being able to detect proteins, DNA, bacteria, viruses, toxins, cancer markers, heart attack protein markers, antibodies, hormones, drugs and so on.

4.1 SPR biosensors features

As briefly revealed in advance, the strenght of SPR devices resides in its very high sensitivity, due to the strong dependence of SPP excited at the metal-dielectric interface on a change of the refractive index of the adjacent environment. In fact, if we take eq. 1.25, together with the relation between the refractive index of the dielectric medium n_d and the dielectric constant ε_d , given by $n_d = \sqrt{\varepsilon_d}$, the dependence of the plasmonic properties on a local variation of n_d is straightforward. Actually, a variation in the refractive index, Δn , leads to a variation in ε_d in eq. 1.25, which yields a variation in the resonance condition between the impinging radiation and the SPPs. As previously seen in chapter 1, to reach the resonance condition, the coupling condition between SPPs and light must occurs. To accomplish this condition, two coupling techniques have been introduced: (i) ATR with prism or waveguides and (ii) diffraction through grating and periodic structures. In this work, only the latter will be considered.

The main features that describe the performances of SPR sensors are:

Sensitivity There are two types of sensitivity of a sensor: the *bulk* sensitivity and the *local* sensitivity. The first, S , is defined as the ratio of the output signal variation, dY , to that of the refractive index, dn , of the sensed medium:

$$S = \frac{dY}{dn} \quad (4.1)$$

where dY represents the measured physical quantity, like the angle or the wavelength shift, while dn is defined in RIU (Refractive Index Units). Another way to express bulk sensitivity is to express it in terms of the figure of merit (FOM), linked to the width of the resonance (Full Width Half Maximum) by the following equation:

$$FOM = \frac{S}{FWHM} \quad (4.2)$$

The local sensitivity, S_0 , is defined considering that the change in the refractive index, dealing with small molecules occurs close to the device surface, in a layer thinner than

the penetration depth of the SP:

$$S_0 \equiv \frac{1}{\Delta n} \left. \frac{\partial \lambda_{peak}}{\partial d_a} \right|_{d_a=0} \quad (4.3)$$

where d_a is the thickness of the analyte layer.

Resolution The resolution of a biosensor represents the smallest detectable variation in the bulk refractive index appreciable in the output signal. In our case, the resolution is determined by the sensor instrumentation noise.

Limit of detection (LOD) The limit of detection, contrarily to the resolution, refers to the local sensitivity and is defined as the smallest detectable analyte concentration by the biosensor. By plotting the output signal variation to different analyte concentrations, it is possible to estimate the LOD.

The main advantage of these structures is that, by tailoring the geometrical features, plasmonic characteristics can be controlled conveniently. Although the NHA can be interrogated in reflection mode measurements, in this work they will be used in transmission mode, taking advantage of the extraordinary optical transmission, which has been discussed in details in section 2.2. Thus, as the analyte adsorption occurs at the NHA surface, the local dielectric environment becomes altered and with it the refractive index and the dielectric function. Hence, the resonant condition changes, leading to a redshift of the EOT peak. On this principle the sensing mechanism is based. It is possible also to reveal the binding event by means of intensity measurements, keeping light at a fixed wavelength above the resonance condition and measuring the variation of the transmitted light. Figure 4.2 represents both measuring methods.

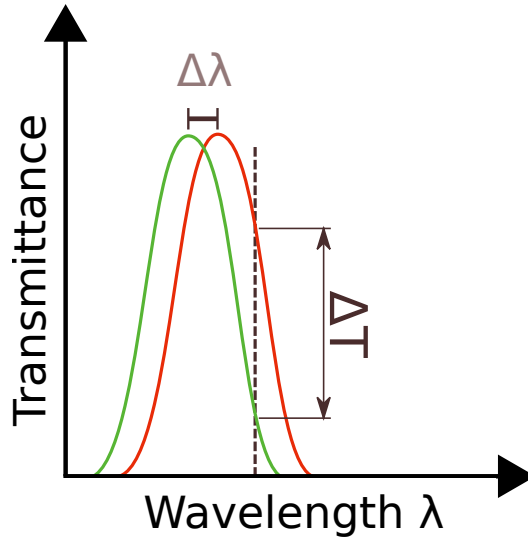


Figure 4.2: Representation of wavelength redshift and increasing intensity measurements.

Differently from prism-based SPR and reflection mode measurements, which require an accurate setup and control of the incident angle, NHA-based optical setup can be way more

simple if zero-order transmission measurements are performed, i. e., at normal incidence with respect to the NHA surface. This configuration is suitable for miniaturization, in fact, thanks to the simplicity of the setup, which only necessitate of a source of white light normally incident to the biosensor and collinear to a detector, like a spectrophotometer or a fiber optics, nanohole arrays show the best choice for a lab-on-a-chip device, as shown by De Leebeck et al. [32] in microfluidic devices. Gordon et al. [33] used a particular setup which is displayed in figure 4.3 as an example.

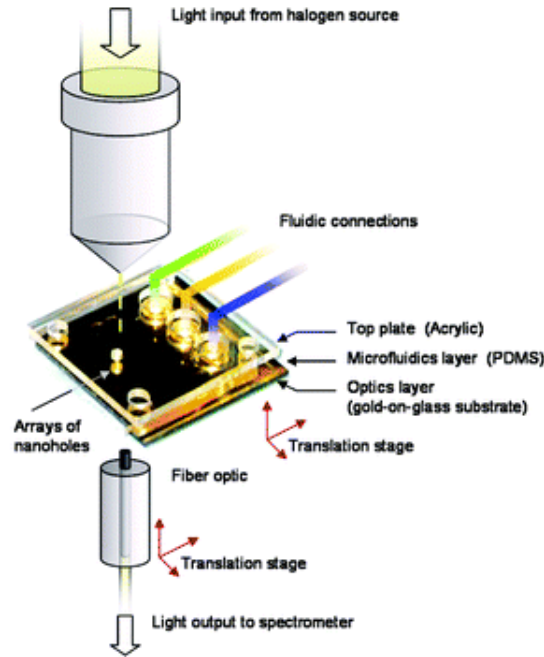


Figure 4.3: Gordon et al. sensing setup [33]. Microfluidic channels bring solutions to the NHA surface while a white light source normally illuminates the sensor. Transmitted light is then collected by a collinear fiber optics and EOT peak redshift can be monitored.

This lab-on-a-chip devices has been widely used, demonstrating the efficiency of their performances, in medicine [34, 35], public health [36, 37] and so on. Other configurations and applications are possible, to which we hint at for the sake of completeness: NHA can be used as free-standing flow-through biosensors [11, 38, 39], which efficiency is limited by the high fluidic resistance occurring in nanoscaled channels, and for enhanced spectroscopy like SERS [3, 40–42].

4.2 Functionalization protocol

The model system which has been employed in this work is the Biotin-Streptavidin system. This system has been largely studied and used to test biosensors, thanks to the high specificity and strength of the binding. In this work, Biotin, a vitamin, will play the role of the receptor, while Streptavidin (SA), a protein, that of the analyte. However, Biotin cannot simply bind to the NHA gold surface, thus, an additional ligand-layer is needed to allow the receptor to bind with the surface: thiols. Thanks to a SAM layer of

thiols, we can finally functionalize our nanohole array, making it a proper biosensor (fig. 4.4).

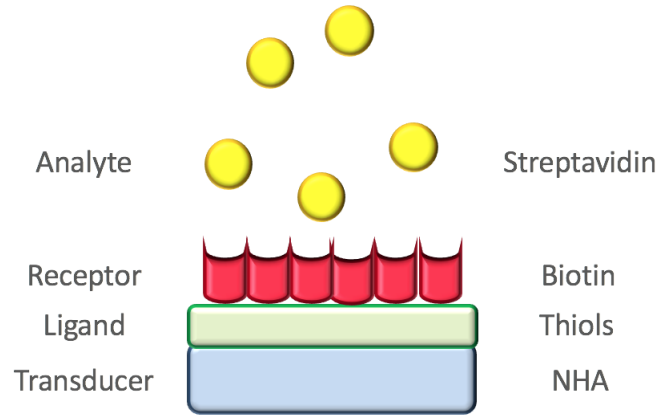


Figure 4.4: Schematic representation of the constituents of the NHA biosensor.

The protocol we followed is the Van Duyne's functionalization protocol [43]. The procedure is divided in three main steps, as shown in figure 4.5: (a) self-assembly of a monolayer of two different types of thiols on the NHA surface, one acting as a spacer, the other as a link for the Biotin, (b) binding of the receptor to the SAM of thiols by means of a crosslinking reaction, (c) sensitivity and specificity test by exposure to different SA concentrations and aspecific molecules.

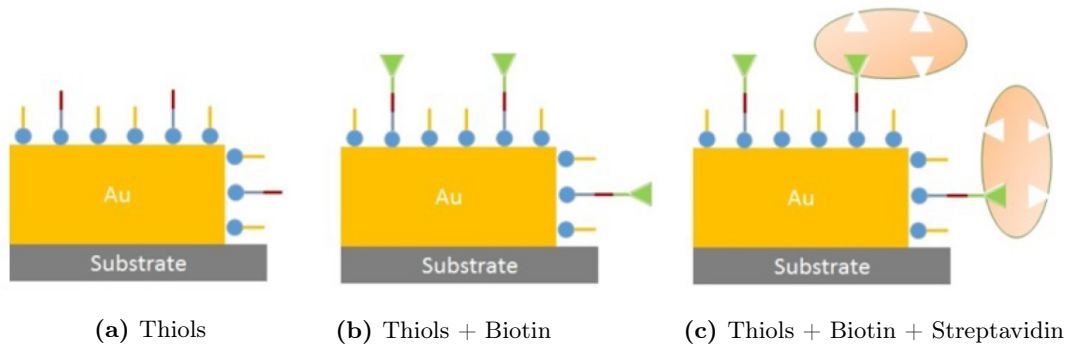


Figure 4.5: Schematic representation of the Van Duyne functionalization protocol.

4.2.1 Thiols SAM

The thiols employed as ligands in this work are alkanethiols, whose chemical formula is $SH-(CH_2)_n-R$, thus, it is an organosulfur compound that, thanks to the sulfur atom (S) at one end, display high binding affinity with gold, while on the other side there is an " R " group. By dipping the sample in a bath solution of thiols, the anchoring group (SH) adsorb on the gold surface and the CH_2 groups contribute to form the SAM due to the repulsing Van der Waals forces, finally exposing the head group (R), ready to bind to Biotin molecules. In the Van Duyne protocol, the bath consists of a 1 mM thiols solution

3:2 of 1-Octanethiol (1-OCT):11-Mercaptoundecanoic acid (11-MUA) in ethanol and lasts at least 24 h to let the SAM form. 11-MUA thiols ($HS(CH_2)_{10}CO_2H$) are the linking thiols: the R group is the $-COOH$ carboxylic group (represented in figure 4.5 by the red stems), able to bind with an amino-terminated biomolecule, in our case Biotin, while 1-OCT thiols ($CH_3(CH_2)_7SH$) are the spacers (in figure 4.5, blue dots with the yellow stem), having an inert R group (CH_3 , methyl group) at the end, thus preventing aspecific bindings, while separating 11-MUA thiol molecules, in order to maximize the receptor binding process: if the 11-MUA molecules are too close, two Biotin molecules, being larger than that of thiols, would not have enough space to bind to both of them, hence, not filling all the linking points. Once the bath is performed, the samples are rinsed with ethanol and Milli-Q water and then dried with N_2 flow.



Figure 4.6: 11-MUA and 1-OCT thiols representation.

A thiol molecule occupies an area of 0.214 nm^2 [44], so a coarse estimate of the number of thiols expected to bind to the NHA surface can be made. Since we are working with 2 mm NHA spots, whose geometrical parameters have been introduced in the previous chapter, we find an exposed metallic surface of $3.44 \cdot 10^{12} \text{ nm}^2$, leading to an expected number of $16 \cdot 10^{13}$ bound thiols.

4.2.2 Biotin

The second steps concerns the Biotin ($C_{10}H_{16}N_2O_3S$, a small vitamin) exposure of the samples. For our purposes, instead of using simple Biotin, we used a Biotin-PEG₂-Amine, which binds to the 11-MUA by means of a crosslinking agent, EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), which activates the carboxylic group to bind with the Amine (NH_2). Thus, an amide bond takes place and the sample is finally functionalized and becomes a proper biosensor. The PEG₂ serves as a further spacer and protection from aspecific binding. To perform this second functionalization step, a 1 mM solution of Biotin-PEG₂-Amine with 10 mM of EDC in PBS solvent (Phosphate Buffered Saline) is prepared. The exposure consists in depositing droplets of $2.7 \mu\text{L}$ volume on each spot, letting it slowly evaporate ($\sim 1 \div 3$ hours). Then the spots are rinsed with PBS and Milli-Q water and dried with N_2 flow.

The average number of Biotin molecules contained in $2.7 \mu\text{L}$ of solutions are of the order of $\sim 10^{15}$, while that of 11-MUA thiols were about $\sim 10^{12}$, thus, all the Biotin molecules should be able to bind to the linking points.

The molecular weight of Biotin is 244.3 Da while the Biotin-PEG₂-Amine has a molecular weight of 374.5 Da. A comparison between the two formulas is given in figure 4.7.

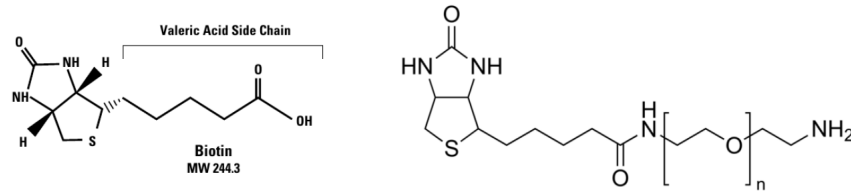
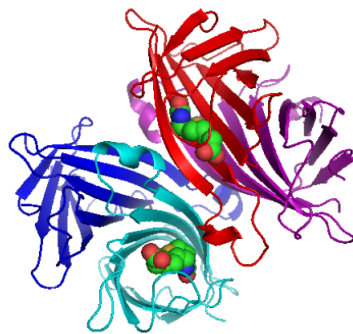


Figure 4.7: Comparison between the Biotin molecule (*left*) and the Biotin-PEG₂-Amine molecule (*right*).

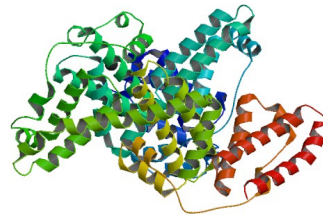
4.2.3 Streptavidin

Streptavidin (SA) is a protein of molecular weight 52.8 kDa, thus, a very heavy and big molecule. The Biotin-Streptavidin system displays one of the strongest non covalent interaction found in nature, with a binding affinity $K_a = 10^{15} \text{ M}^{-1}$). The sample are exposed to different SA concentrations ($10^{-5} \div 10^{-10} \text{ M}$) prepared in PBS 10 mM, in a manner similar to that of Biotin: a droplet of 2.7 μL of volume for each spot, ~ 2 hours of evaporation wait and finally PBS and Milli-Q rinse and N_2 flow, to remove the unbound molecules. The Streptavidin molecule is represented in figure 4.8a.

Bovine Serum Albumin: aspecific protein The protein used for the selectivity test, thus, the aspecific protein, is Bovine Serum Albumin (BSA), whose molecular structure and weight are similar to that of SA, as can be seen in figure 4.8. The aspecific solution consists of $1.67 \cdot 10^{-7} \text{ M}$ of BSA in 10 mM PBS. The BSA molecule is represented in figure 4.8b.



(a) Streptavidin molecule.



(b) Bovine Serum Albumin molecule

Figure 4.8: Comparison between the Streptavidin molecule (*left*) and the BSA molecule (*right*).

4.3 Simulations

In order to have some reference values for the bulk and the local sensitivities, simulations of the plasmonic properties of the NHA have been performed with the EMUstack

software, using the Finite Elements Method (FEM) method [45]. First, near-field simulations result, performed with the same method, are presented.

4.3.1 Near-field response

When EM radiation impinges on the NHA, a perpendicular electric field $E_{\perp} \equiv E_z$ is generated, due to the plasmonic resonance, at the top and bottom interfaces. Figure 4.9a shows E_z behaviour at the top interface (metal/air), while that on the bottom interface is similar, with inverted signs. It is possible to clearly notice that a strong localization of the field occurs at the hole edges, leading to an enhancement of the transmission of the impinging radiation in far-field. While the incident radiation field has been set equal to 1 V/m, the near-field enhancement causes E_z to be equal to 5 V/m in the hotspots. Figure 4.9b shows E_z trend against the distance (z) from the interface at both interfaces.

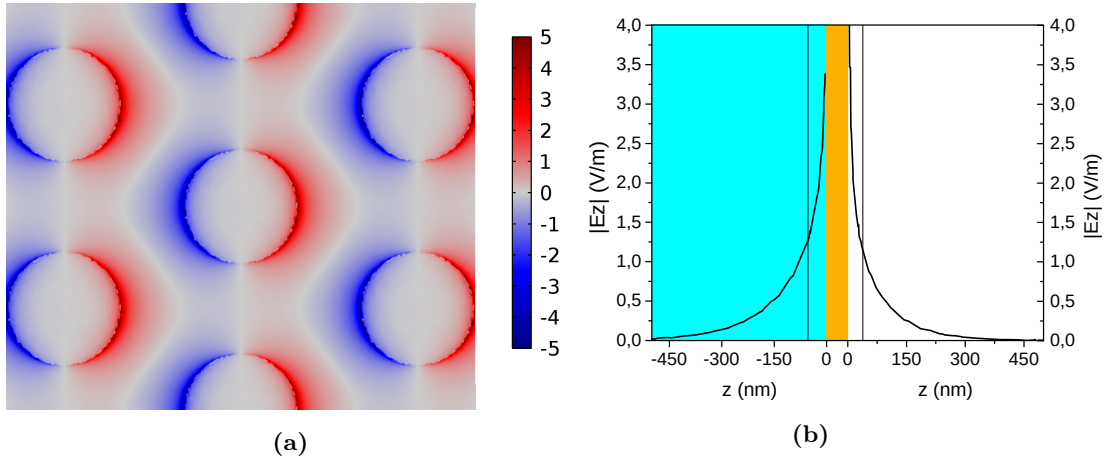


Figure 4.9: (a) Representation of E_z behaviour at the NHA-Air interface from a top view. (b) Decay of the electric field E_z as a function of the distance from the interfaces: silica-metal and metal-air. The two vertical lines represent the decay lengths δ of E_z in the two media.

The two curves obtained are then fitted with an exponential decay function, providing two decay lengths values (δ_{SiO_2} and δ_{air}), which correspond to the distance at which E_z is a $1/e$ of the field at zero distance:

$$\delta_{SiO_2} = (53 \pm 3)nm \quad \delta_{air} = (38 \pm 2)nm \quad (4.4)$$

4.3.2 Bulk sensitivity

The bulk sensitivity is the parameter which links the EOT peak shift to the change of the dielectric environment refractive index, when the thickness of the dielectric medium is greater than the decay length of the electric field (see eq. 4.4). Evaluations of a NHA bulk sensitivity have been performed by replacing the environment above the NHA, air ($n_{air} = 1$), with another dielectric medium with different refractive index (for example

water, $n_{H_2O} = 1.33$) and assessing the EOT peak shift as a function of the refractive index variation (RIU), as shown in figure 4.10 (and related data in table 4.1):

n	PEAK POSITION [nm]
1	818.1 ± 1.0
1.33	901.8 ± 1.0
1.5	962.1 ± 1.0

Table 4.1: Refractive index and corresponding EOT peak positions.

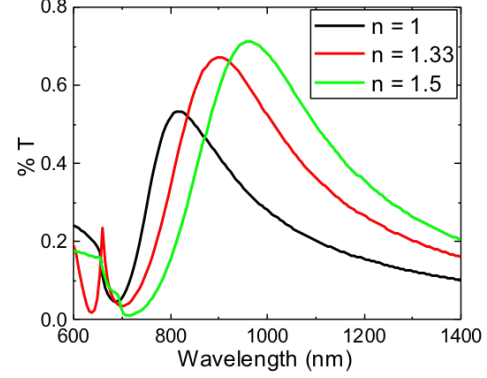


Figure 4.10: EOT peak with $n = 1$, $n = 1.33$ and $n = 1.5$ refractive index media.

Thus, the bulk sensitivity obtained from simulations is:

$$S_{bulk}^{simul} = \frac{\Delta\lambda_{centroid}}{\Delta n} = 290nm/RIU \quad (4.5)$$

4.3.3 Local sensitivity

For the local sensitivity, simulations with increasing coating layer of refractive index $n \sim 1.5$, typical of biological material (in the interest of the present work), have been performed. Thus, a silica layer (SiO_2 , $n_{SiO_2} = 1.45$) have been considered as probe layer, with increasing thickness $t = 1, 2, 4, 6, 8$ nm (smaller than the electric field decay length, see eq. 4.4) and the corresponding EOT peak shifts have been analyzed (see figure 4.11 and related data in table 4.2).

LOCAL SENSITIVITY	
THICKNESS t [nm]	SHIFT [nm]
1	1.5 ± 1.0
2	2.9 ± 1.0
4	6.0 ± 1.0
6	9.1 ± 1.0
8	11.3 ± 1.0

Table 4.2: EOT peak positions as the SiO_2 layer thickness increases.

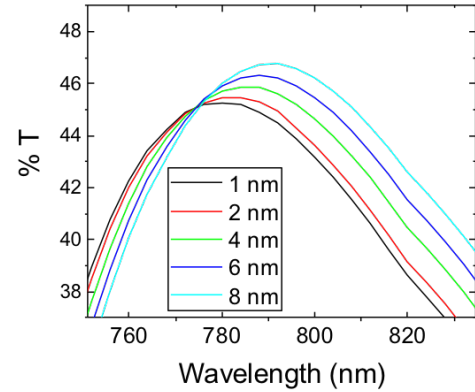


Figure 4.11: EOT peak shifts as the SiO_2 layer thickness increases.

Hence, from simulations results, by means of a linear fit of function $\Delta\lambda = m \cdot t + q$ (shown in figure 4.12 with related fit parameters in table 4.3), dividing the slope m , which represents the local sensitivity with silica, for the variation of the refractive index $\Delta n = n_{SiO_2} - n_{air}$, NHA local sensitivity is obtained.

FIT PARAMETERS	
$\Delta\lambda = m \cdot t + q$	
m	q
1.44 ± 0.05	0.1 ± 0.2

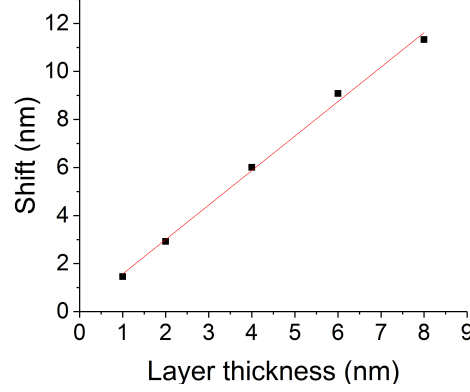


Table 4.3: Fit parameters for local sensitivity estimate.

Figure 4.12: Fit of the EOT peak shifts as a function of the SiO_2 layer thickness.

$$S_{loc}^{simul} = \frac{m}{n_{SiO_2} - n_{air}} = 3.2 RIU^{-1} \quad (4.6)$$

Actually, the curve should be an exponentially saturating function, whose characteristic length is the near-field decay length. Since the thickness of the coating silica layer is shorter than the decay length, it is sufficient to use the linear approximation.

These results will be compared with the experimental NHA bulk and local sensitivities in chapter 6.

Chapter 5

Results: synthesis and characterization of NHA

In this chapter we will describe the experimental results obtained in the present work and we will provide further details to those given in the previous chapters, from the synthesis of a NHA to data analysis and results of the fabrication process.

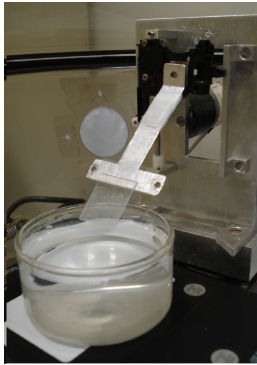
5.1 Self-assembled masks

To produce the mask which will be involved in the NSL process, we used monodisperse polystyrene nanospheres of diameter 522 ± 2 nm in aqueous solution, purchased from Microparticles GmbH. Firstly, a 1:1 solution of NS:isopropyl alcohol (2-propanol, purchased from Sigma Aldrich) is prepared. The proportion has been empirically investigated in order to find the best working conditions. The SAM method for PS nanosphere has been presented in section 3.1.2.

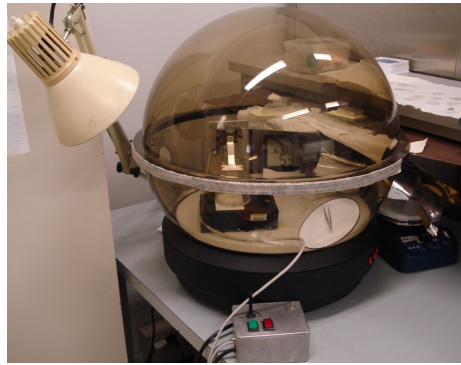
The substrate employed are Soda Lime Glass (SLG) and monocrystalline silicon wafer with $\langle 100 \rangle$ lattice orientation. The immersion substrate is always an SLG, while the picking-up substrate can be both an SLG or a Si wafer, the latter used only as a check sample for better SEM analysis. The substrates, after they have been conveniently cut, are placed in a teflon support carousel that holds the substrates in a vertical position, in order to maximize the exposed area to the cleaning process. The cleaning process consists of a bath in acid piranha first, which cleans all organic residuals and powder from the surface, while making it hydrophilic, and then a basic piranha bath, that improves further the hydrophilicity of the substrates. The former solution is a 3:1 ratio of sulfuric acid (H_2SO_4), 90 mL, and hydrogen peroxide (H_2O_2), 30 mL. The carousel is immersed in the solution, kept at $90^\circ C$ by means of an heating plate, for about 1 hour. Then, the substrates are rinsed in Milli-Q water, placed in another carousel, and immersed in the basic piranha. This one is as well a 3:1 solution of 90 mL of ammonium hydroxide (NH_4OH) and 30 mL of hydrogen peroxide, and the cleaning, which is also kept at a temperature of $90^\circ C$ with the heating plate, lasts about 20. Once again, the substrates are then rinsed with Milli-Q

water and dried with air flow.

Therefore, the substrates, highly hydrophilic, are now ready for the self-assembling process. This is a crucial step, because the quality of the mask determines that of the NHA, therefore, many tests and adjustment of the multiple parameters involved in the process have been made, in order to optimize the mask quality and dimension of the mask. The immersion substrate is fixed to the T-shaped arm and exposed to $20 \div 26 \mu\text{L}$ of the NS solution by means of a micropipette, so as to fill the entire surface of the substrate homogeneously. The amount of solution is experimentally determined, depending on the air humidity and on the substrate response. The substrate is then slowly dipped into Milli-Q water with a 45° angle, letting the nanospheres float and thanks to the meniscus between water and the alcoholic solution they rearrange in a close-packed self-assembled monolayer at the water surface. It has been found that the best conditions for the mask to form is at $\sim 70 \div 80\%$ air humidity, thus, the motorized system is placed inside a chamber which allows to control the air humidity. A schematic view of the process was previously depicted in figure 3.2 and a picture of the motorized dipper and the chamber is now provided in figure 5.1.



(a)



(b)

Figure 5.1: (a) Picture of the motorized T-arm and the dipping substrate. (b) Picture of the chamber.

Moreover, producing controlled vibrations enable the nanospheres to rearrange better in a mono-domain hexagonally close-packed lattice, hence, further improving the quality of the mask. Then, it is now possible to collect the mask, by manually immersing another substrate with a tweezer. The mask is attracted by the highly hydrophilic substrate and by slowly moving backwards and upwards, a SAM of NS on a substrate is obtained. Finally, the masks undergo the drying process, placing the substrates in a vertical position, still in the wet environment, to slow the process and let the water capillarity forces further compact the nanospheres. A good mask is characterized by a strong iridescence, typical of the scattering of white light encountering an ordered structure, as shown in figure 5.2, with a typical extent of few cm^2 .

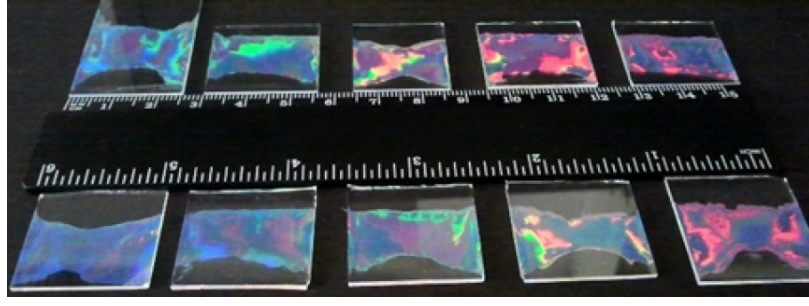


Figure 5.2: Pictures of some substrates with the correct assembled mask, characterized by the iridescence.

Mask characterization To investigate the quality of the masks, both a morphological and optical characterization of the mask are performed. The optical measurements have been performed with a *Jasco V-760 UV-VIS-NIR* spectrophotometer, by means of a normal incidence non polarized light beam, in absorbance. The absorbance A is linked to the transmittance T through the following relation:

$$A = 100(2 - \log_{10}(T)) \quad (5.1)$$

where both A and T are expressed in percent. A typical mask spectrum is shown in figure 5.3, where we see that in the NIR range the mask is almost transparent to radiation ($A \lesssim 1\%$), while for $\lambda < 700$ nm it starts to absorb radiation, displaying a narrow peak at $\lambda \sim 650$ nm, whose width is associated to the overall degree of order of the mask: a narrow peak implies a well-ordered mask.

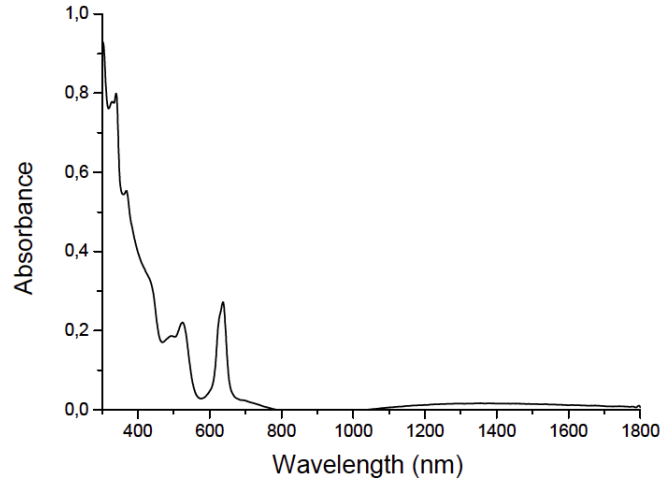


Figure 5.3: Absorbance spectrum of a well-ordered SAM of 522 nm nanospheres on SLG.

The morphological measurements have been acquired with SEM (*Zeiss SIGMA HD SEM-FEG Microscope*) and AFM (*NT-MDT Solver PRO-M Microscope*) microscopes, and analyzed with their respective softwares, *ImageJ* and *Gwyddion*, as shown in figure 5.4, where is clearly displayed the hexagonal closed-packed 2D monolayer.

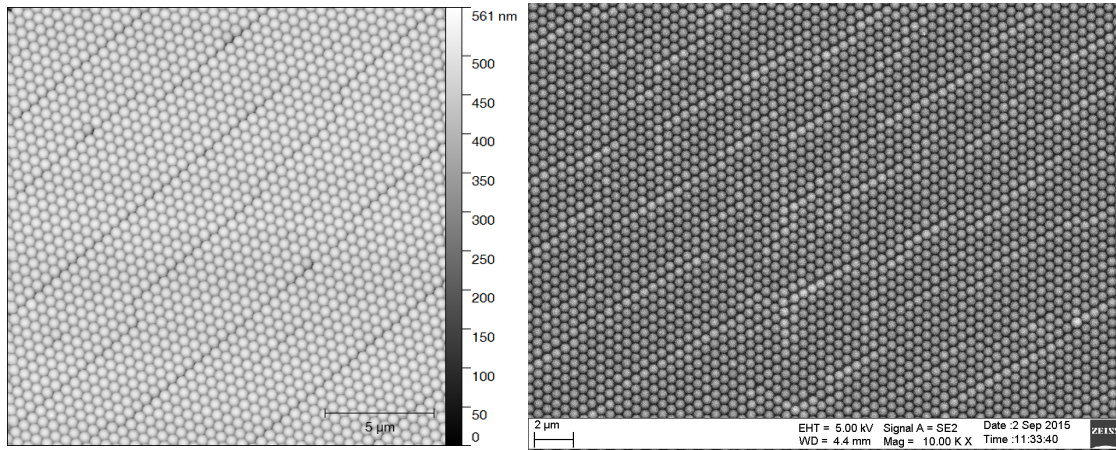


Figure 5.4: Images of a well-ordered SAM of 522 nm nanospheres taken with AFM (*left*) and SEM (*right*).

5.2 Reactive Ion Etching

As described in details in section 3.1.3, the NHA periodicity is determined by the NS diameter, while the holes diameter is chosen by setting the proper parameters of the etching process. The PS nanoparticles are hence shrunk by means of the RIE process, in which a reactive atmosphere composed by a mixture of O_2 and Ar gases erodes the nanospheres. Optimal parameters were empirically determined, like the working pressure of $1.6 \cdot 10^{-3}$ mbar and the 3:1 gas ratio between O_2 and Ar . RIE parameters, shown in table 3.1 are below reported:

PRESSURE	O_2 FLUX	Ar FLUX	BIAS	POWER
$1.6 \cdot 10^{-3}$ mbar	9.2 sccm	3.6 sccm	66 V	15 W

Table 5.1: RIE parameters for 522 nm PS NS etching.

The only free parameter is hence time. Time calibration is thus performed in order to control the etching rate, by testing different RIE times and checking the spheres shape and dimension through SEM observations, with the constraint that etching the NSs below half of their initial diameter dimension causes them to collapse.

RIE characterization Even in this case both an optical and morphological characterization is performed. Optical measurements provide a spectrum similar to that of the initial mask, but now it will be obviously less absorbing the presence of the peak will suggest that the mask has not been compromised by the RIE process and no collapse of the NSs occurred.

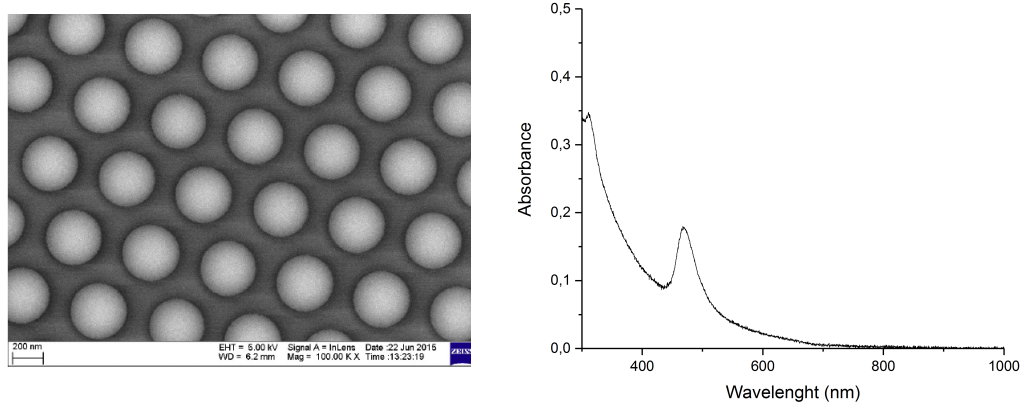


Figure 5.5: SEM image of a 522 nm nanospheres mask after the RIE process (*left*) and its absorbance spectrum (*right*).

5.3 Magnetron Sputtering

The height or thickness (t) of the NHA is another crucial parameter on which the quality and characteristics of the biosensor depends. In this work we are dealing with two different metallic layers: Cr and Au. The Cr layer, which is an adhesion-layer, is characterized by a thickness of ~ 5 nm, enough to let the gold layer adhere solidly to the substrate, without affecting the plasmonic properties and hence the EOT spectrum. The Au layer thickness instead measures about ~ 65 nm, in order to avoid the plasmonic coupling between the two sides and making the structure opaque in the NIR-VIS, without excessively damping the SPPs propagations and preventing to the NS to remain stuck in the metal which would prevent the mask removal. The two layers have been deposited in sequence, by means of a magnetron sputtering, widely displayed in section 3.1.4. Our sputtering system is characterized by three sources, 1 DC and 2 RF, thus, it is possible to sputter 3 different elements. Since the metal targets are fixed, only the sample holder is free to move, being fixed to a rotating plate. Hence, it is possible to place each sample in front of each source and deposit different metals, one by one. Our sample holder is a three arm plate, allowing to place multiple samples for each sputtering process. Also a positional calibration on the sample holder has been investigated, finding the best condition in the center of each arm, where a homogeneous sputtered layer is obtained.

First vacuum is created in the sputtering chamber, reaching a pressure of $3 \cdot 10^{-6}$ mbar. Then, Ar gas flows inside the chamber, increasing the chamber pressure to $5 \cdot 10^{-3}$ mbar, the proper working pressure to ignite the plasma which will erode the metal targets. Thus, the sources are powered on to the working values, depositing metal for a proper time interval, obtained in previous calibrations. The working values of the sputtering system are reported in table 5.2. Therefore, the time values corresponding to the desired thickness have been set (see table 5.3).

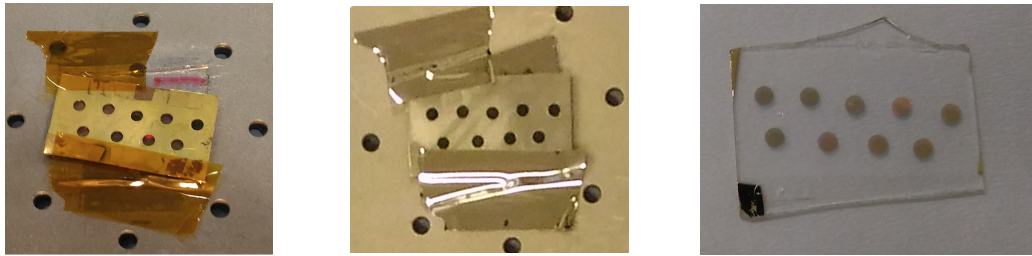
METAL TARGET	Cr	Au
POWER SOURCE	DC	RF
POWER VALUE [W]	20	50
VOLTAGE BIAS [V]	362	167
DEPOSITION RATE [nm/s]	0.15	0.70

Table 5.2: Magnetron sputtering parameters.

	Cr	Au
TIME [s]	33	93
THICKNESS [nm]	5	65

Table 5.3: Metal deposition parameters.

In order to make samples practical for biosensing tests and in order to maximize the reproducibility of measurements, a perforated foil, with 9 holes of 2 mm diameter, is applied to each sample ¹, so to obtain 9 spots and hence 9 biosensors per each sample after the deposition, as shown in figure 5.9.

**Figure 5.6:** Picture of a mask with applied perforated foil (*left*); picture of the sample after the sputtering process (*center*); Picture of a typical 9-spots sample (*right*).

Sputtering characterization To inspect the thickness of the deposited metal, the substrate is signed with a marker (see figure 5.9 (*left*), in the right top corner of the sample) and after each deposition, each sample is cleaned in ethanol and toluene, which remove the marker sign and the metal above, leaving a groove. Thus, it is now possible to measure t with the AFM and analyzed with Gwyddion software.

5.4 Mask removal

Then mask removal is performed by sonicating the samples in toluene bath for 2 minutes. Finally, the samples are further cleaned in Au etchant and Cr etchant, for $3 \div 5$ s each, in order to remove Cr and Au grains at the hole bottom, rinsing in Milli-Q water at each step. Below are presented some figures and spectra of the NHA at each step and

¹The adhesive tape employed is a Kapton tape, which does not release gas in vacuum.

eventually treating the sample with the UVO cleaner or $3 \div 5$ s of acid piranha, in order to remove PS or other organic residuals and restore the hydrophilicity of the sample, favorable to the functionalization procedure. Removing PS and metal residual from the hole bottom is fundamental, if we aim to reach the most cleaned signal possible and a sharp peak. Thus, a NHA is obtained.

NHA characterization Optical characterization is made with the Jasco spectrophotometer. A micrometrical sample holder 5.7 has been elaborated to take advantage of the spot configuration previously introduced and to maximize the reproducibility of measurements. The holder can be moved in vertically and horizontally, in the plane perpendicular to the light beam by means of two micrometric screws. In this way each spot can be precisely centered on the beam, which is further reduced by a cleavage ($2.5 \text{ mm} \times 5 \text{ mm}$), resulting in a $1 \text{ mm} \times 2.5 \text{ mm}$ beam. The spectrum acquisition starts when the spot is centered on the beam, namely, when a minimum in the transmittance is detected. Figure 5.7c shows the centering operation.

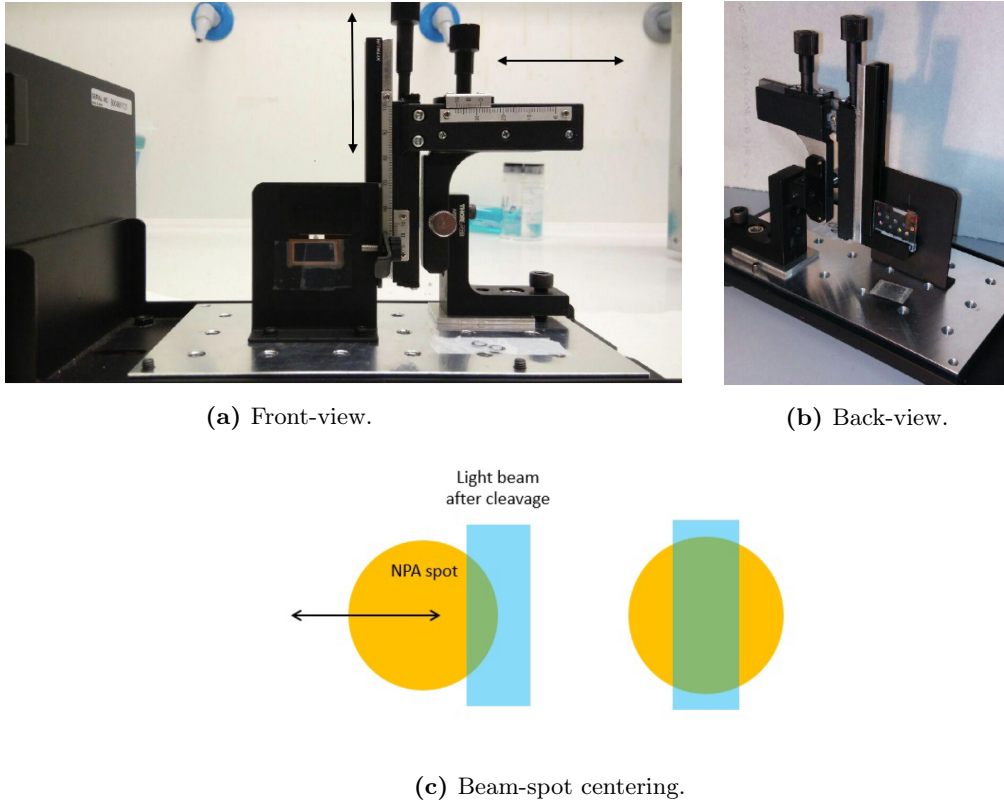


Figure 5.7: Pictures of the micrometrical sample holder and of the beam-spot centering operation.

The measurements are this time performed in transmission T and the wavelength range is $500 \div 1500 \text{ nm}$, with the EOT peak, corresponding to the $< 1, 0 >$ SPP Bloch-wave excitation, occurring at $\lambda \sim 850 \div 870 \text{ nm}$. Figure 5.8 shows the height growth, sharpening and blue-shift of the EOT peak through the cleaning process.

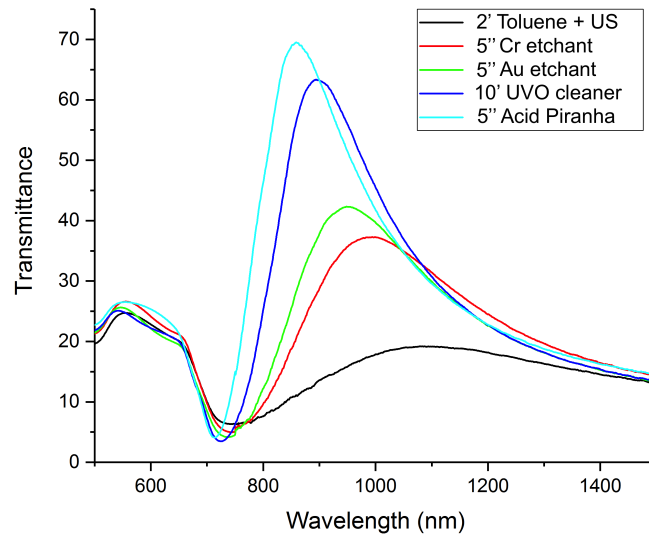
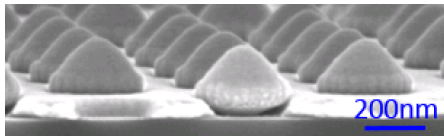
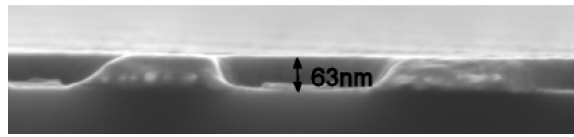


Figure 5.8: NHA spectrum after the cleaning steps. Notice the height growth and the blue-shift of the peak at each step.

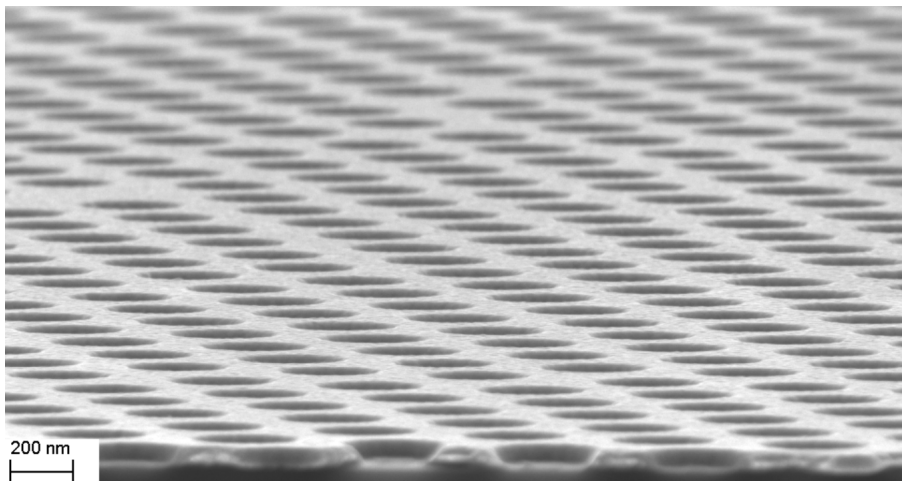
Also in this case, after this procedure, the morphological features of the samples are investigated by means of the AFM (metal layer thickness, not reported, see figure 3.9b as an example) and SEM microscopes and images are shown here below.



(a) NHA cross section after sputtering. Notice the deformed coated PS nanospheres.

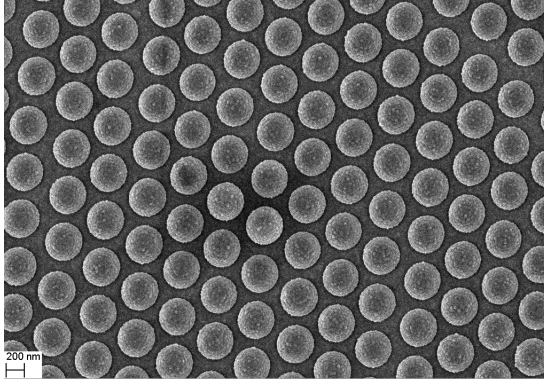


(b) NHA cross section after cleaning (hole depth of 63 nm is reported).

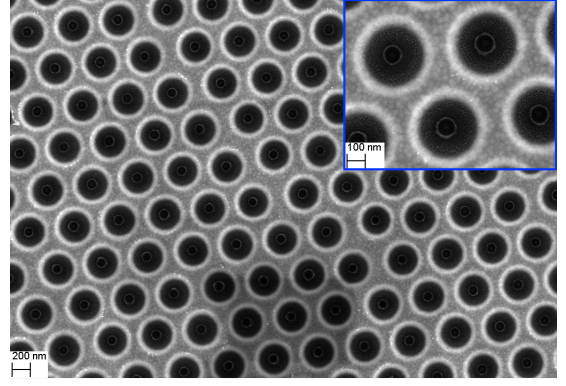


(c) NHA cross and top view of a NHA sample after cleaning.

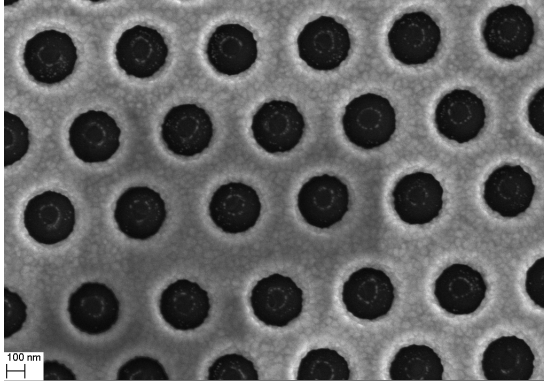
Figure 5.9: SEM images in cross section of a NHA sample.



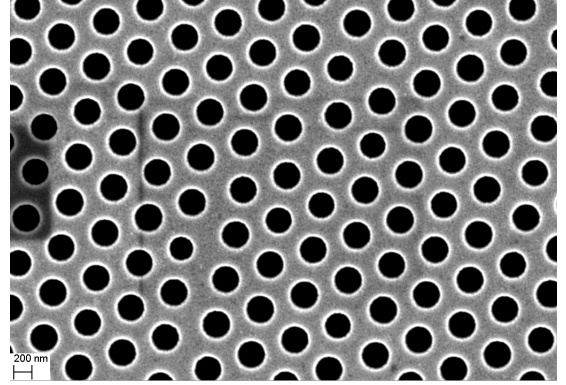
(a) NHA sample after sputtering. PS nanospheres are still in sight, coated with Cr and Au.



(b) NHA sample after toluene bath in US. It is possible to see PS residuals and Cr and Au grains in the hole bottom (*see insight*).



(c) NHA sample after Cr and Au etching. The hole bottom looks almost cleaned.



(d) NHA sample after acid piranha. The holes are now perfectly cleaned.

Figure 5.10: SEM images in top-view of a NHA sample through the mask removal and cleaning processes.

5.5 EOT analysis

The NHA spectra have been analyzed by means of the Fano function, whose shape and characteristics have been presented in section 2.2.2. The function form have been conveniently rewritten as a function of the wavelength, more precisely, as a function of λ_{max} , namely, the wavelength at which the maximum in transmittance $T(\lambda)$ occurs, leading to:

$$T(\lambda) = T_0 + A \frac{q + \sigma \cdot \left(\frac{1}{\lambda} - \frac{q \cdot \sigma - \lambda_{max}}{q \cdot \sigma \cdot x_{max}} \right)^2}{1 + \sigma^2 \cdot \left(\frac{1}{\lambda} - \frac{q \cdot \sigma - \lambda_{max}}{q \cdot \sigma \cdot \lambda_{max}} \right)^2} \quad (5.2)$$

This method has been preferred to the centroid method, which is a weighted average of the wavelengths with respect to the transmittance, because it provides a global fit, characteristic of the Fano function, which is independent on the wavelength range considered,

thus making it independent of the transmittance noise oscillations. The wavelengths λ_{max} have been estimated in a range from 844 nm to 889 nm, with calculated mean value of 860 nm and semidispersion of 23 nm.

It is possible to calculate the filling factor f of the NHA defined as follows:

$$f = \frac{\text{Holes area}}{\text{NHA Area}} = \left(\frac{d}{a_0} \right)^2 \frac{2\pi}{\sqrt{3}} \approx 0.28 \quad (5.3)$$

where d is the hole diameter and a_0 the array periodicity. The filling factor is used to calculate the normalized transmission, i. e., the amount of light which passes through the NHA with respect to the light that would pass through a single hole of area equal to the sum of all the nanoholes. From equation 5.3 we obtain that, neglecting the plasmonic properties, only 28% of light would be transmitted by the NHA. Thus, dividing the transmittance spectrum by f , the normalized transmittance is obtained. We still obtain transmittance exceeding 100%, stressing the fact that actually extraordinary optical transmittance phenomenon through NHAs occurs and is due to SPPs. In figure 5.11 a normalized transmittance spectrum of a NHA is reported, together with the fitting Fano-type function and its fitting parameters in table 5.4.

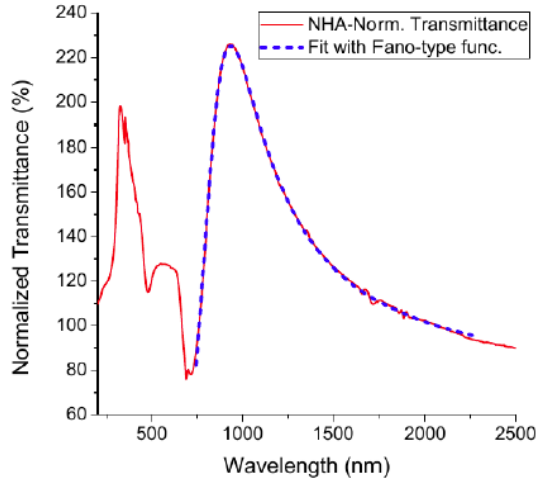


Figure 5.11: Normalized transmittance spectrum of a NHA with the fitting Fano-type function superimposed.

Avg. λ_{max}	860 ± 23 nm
T_0	2.2 ± 0.5
A	26.2 ± 0.2
q	-2.74 ± 0.01
λ_{max}	855.7 ± 0.5
σ	3724 ± 6

Table 5.4: Fitting parameters from fig. 5.11.

EOT peak error The error in the EOT peak position determination is $\sigma_\lambda = \pm 1$ nm, resulting from the squared sum of the systematic error due to beam-spot repositioning (~ 1 nm) and the fit error ($\ll 1$ nm).

Chapter 6

Results: biosensing tests

This chapter describes the results on the sensitivity tests for both bulk and local sensitivity. Also a specificity test will be shown at the end.

6.1 Bulk sensitivity

Bulk sensitivity measurements have been performed measuring a NHA sample EOT peak in air and after depositing a PMMA ($n_{PMMA} = 1.48$) layer. In figure 6.1 and table 6.5 are reported a graphic and the related data of the bulk sensitivity test.

n	PEAK POSITION [nm]
1	861.0 ± 1.0
1.48	996.2 ± 1.0

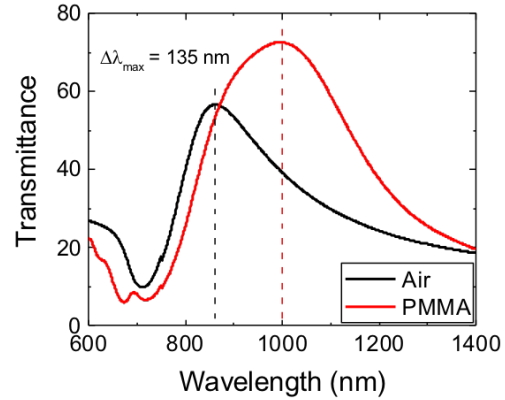


Table 6.1: Refractive index and corresponding EOT peak position.

Figure 6.1: Measured EOT peak with $n = 1$ and $n = 1.48$ refractive index media.

Thus, the bulk sensitivity has been estimated by means of eq. 4.5:

$$S_{bulk}^{exp} = 281nm/RIU \quad (6.1)$$

This result is in good agreement with the simulated one, from eq. 4.5, that is:
 $S_{bulk}^{simul} = 290nm/RIU$.

6.2 Local sensitivity

The main aim of this work is to prove the NHA suitability for biosensing applications. Thus, we are speaking of small analyte molecules and therefore, of change in the refractive index within few nanometers from the structure surface. Hence, we recall the local sensitivity, introduced in section 4.1, which represents how much the EOT peak shifts, as the amount of bounded analyte at the NHA surface increases. The master equation defining the sensitivities, as a function of a d_a thick layer of analyte, is the following [4, 46]:

$$\Delta\lambda_{peak}(d_a) = \lambda_{peak}(d_a) - \lambda_{peak}(0) = S_{bulk}\Delta n \left[1 - \exp\left(-\frac{2d_a}{l_d}\right) \right] \quad (6.2)$$

where $\Delta\lambda_{peak}(d_a)$ represents the EOT peak shift, S_{bulk} is the bulk sensitivity, $\Delta n = n_a - n_e$ is the change in the refractive index, defined as the difference between the refractive index of the analyte, n_a , and that of the former environment, n_e , and l_d is the effective decay length of the local field (from eq. 4.4, $\delta \sim 55$ nm). If we take $d_a \gg l_d$, the bulk sensitivity relation is recovered:

$$\Delta\lambda_{peak}(\infty) = S_{bulk}\Delta n \quad (6.3)$$

In the opposite limit $d_a < l_d$, approximating the exponential to the first-order series expansion, a linear expression is obtained:

$$\lambda_{peak}(d_a) \approx \lambda_{peak}(0) + S_{bulk}\Delta n \left[1 - \left(1 - \frac{2d_a}{l_d} \right) \right] = \lambda_{peak}(0) + \left(\frac{2S_{bulk}\Delta n}{l_d} \right) \cdot d_a \quad (6.4)$$

To test the local sensitivity of the NHA, a thin layer of silica, through use of the magnetron sputtering, can be deposited on its surface and the resulting optical transmittance spectra will be then analyzed.

t_{SiO_2} [nm]	PEAK POSITION [nm]
0	837.1 $\pm$ 1.0
13	853.0 $\pm$ 1.0
26	864.9 $\pm$ 1.0
39	878.0 $\pm$ 1.0
FIT PARAMETERS	
m	1.29 $\pm$ 0.04
q	(1.0 $\pm$ 1.0) nm

Table 6.2: Different SiO_2 layers with the corresponding peak positions (*top*). Fit parameters (*bottom*).

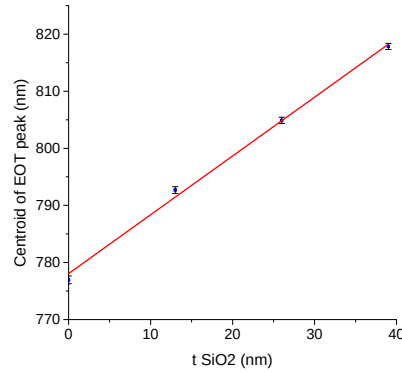


Figure 6.2: EOT peak shifts as the silica layer increases.

Thus, the experimental local sensitivity can be calculated:

$$S_{loc}^{exp} = \frac{m}{n_{SiO_2} - n_{air}} = 2.87 \pm 0.07 RIU^{-1} \quad (6.5)$$

which results to be slightly different from the simulated result: $S_{loc}^{simul} = 3.2 RIU^{-1}$, likely due to the local defects in the nanofabrication which affect more strongly the local sensibility with respect to the bulk one.

6.3 Biosensing Test

Biosensing tests were performed exposing the NHA spots to different concentrations of the analyte, after having accomplished the functionalization protocol and measuring the transmittance spectrum of each spot after every step, in order to monitor the occurred binding of the different substances. In the case of thiols and biotin, since in the functionalization protocol one stock solutions concentration for thiols and for Biotin are employed, the procedure is the same for every spot, thus similar EOT peak shifts should be observed after thiols and Biotin functionalization steps.

Then, the spots can be exposed to different Streptavidin concentrations, from $1.67 \cdot 10^{-6}$ M to $1.67 \cdot 10^{-11}$ M, with $2.7 \mu L$ droplets.

Figure 6.3 and table 6.3 show the EOT peak spectra and data at each step of the functionalization and sensing test. It is thus possible to observe a peak shift of about ~ 3 nm between thiols (black curve) and Biotin (red curve) expositions, less than 1 nm shift after SA $3.60 \cdot 10^{-10}$ M exposition (green curve) and the last spectrum corresponds to the maximum SA concentration (or saturation) of $1.67 \cdot 10^{-6}$ M exposition (blue curve).

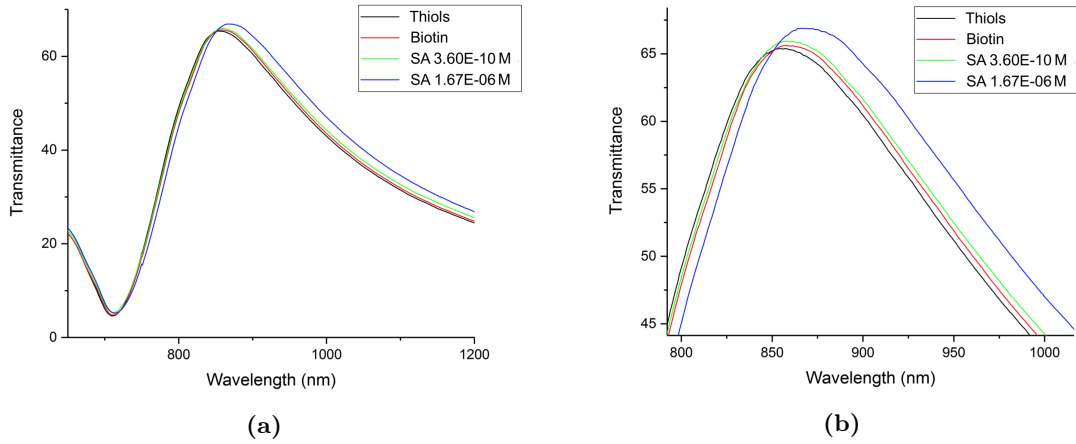


Figure 6.3: (a) Measured spectra after each functionalization step. (b) Insight of the peak shifts.

At each step, stability tests have been performed, in order to verify the robustness of bindings and the stability of the NHA structure. Thus, after the thiols exposition, once the EOT peak shift has been observed, certifying the occurred binding of thiols to the NHA, the

STEP	λ_{max} [nm]	CONSECUTIVE SHIFT [nm]	Biotin-SA shift [nm]
<i>Thiols</i>	856.6 ± 1.0	/	/
<i>Biotin</i>	859.9 ± 1.0	3.3 ± 1.0	/
<i>SA</i> $1.67 \cdot 10^{-10}$	860.0 ± 1.0	0.1 ± 1.0	0.1 ± 1.0
<i>SA</i> $1.67 \cdot 10^{-16}$	870.2 ± 1.0	10.2 ± 1.0	10.3 ± 1.0

Table 6.3: Measured EOT peak shifts after each functionalization step.

sample has gone through multiple rinsing without displaying any additional shift (neither forwards, nor backwards), proving the stability of our system after the thiols step. The same procedure has been applied to Biotin, leading to identical results: no further shifts have been observed. Even after SA exposition, no modification after multiple rinsing have been detected and the EOT peak preserved its features even after several days, assessing the stability and reliability of the produced NHA, as shown in figure 6.4.

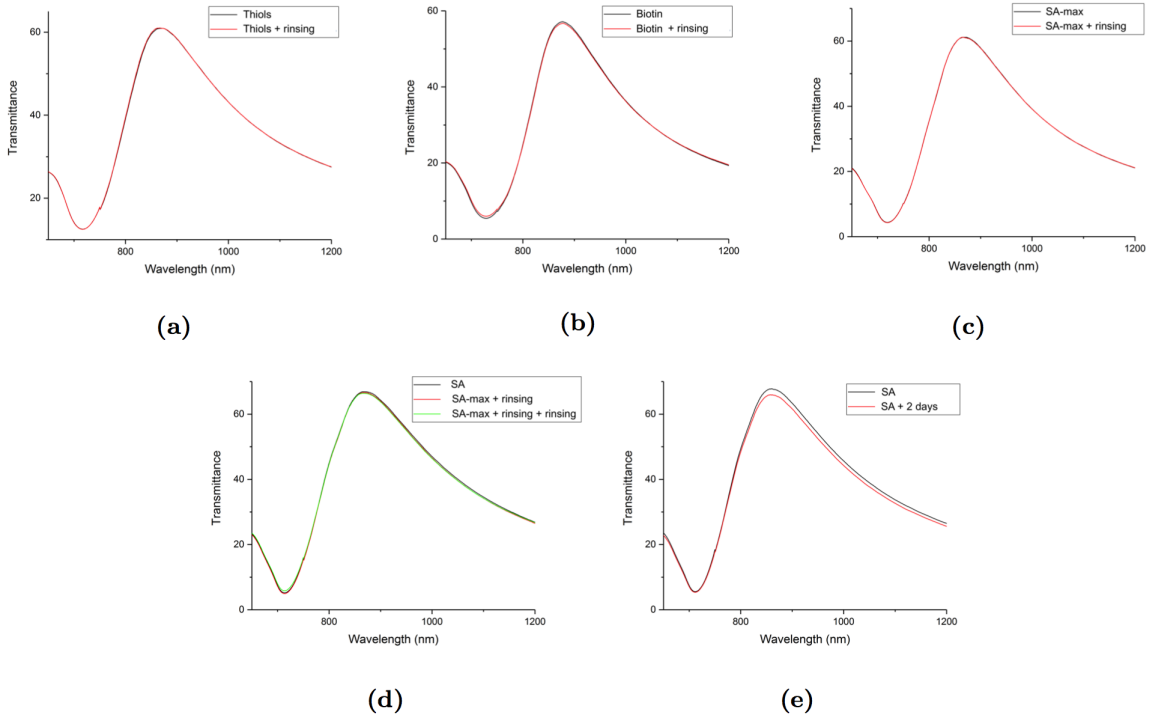


Figure 6.4: (a) Measured spectra after thiols bath (black) and after an additional rinsing (red). (b) Measured spectra after Biotin exposition (black) and after an additional rinsing (red). (c) Measured spectra after SA exposition (black) and after an additional rinsing (red). (d) Measured spectra after SA exposition (black) and after two consecutive rinsings (red, green). (e) Measured spectra after SA exposition (black) and after two days (red).

Notice the superpositions of the different spectra.

Finally, the sensing properties of a biosensor are resumed in the sensing curve (or calibration curve), which relates the EOT peak shifts as a function of the streptavidin concentrations [SA] in log-scale, fitted with the Langmuir isotherm function [5], from the

Langmuir adsorption model, given by:

$$\Delta\lambda_{peak} = \frac{\Delta\lambda_{sat} \cdot K_{aff} \cdot [SA]}{1 + K_{aff} \cdot [SA]} \quad (6.6)$$

where $\Delta\lambda_{sat}$ is the saturation shift value of $\Delta\lambda_{peak}$, which is the maximum output signal that the NHA biosensor can produce, while K_{aff} is the effective affinity constant. The higher is its value, the higher is the affinity between the receptor and the analyte, leading to an irreversible binding reaction. This latter parameter displays a high value, stressing the high affinity between streptavidin and biotin and the irreversibility of the binding. Figure 6.5 shows the sensing curve obtained, while in table 6.4 are reported $\Delta\lambda_{sat}$ and K_{aff} resulting from the fit.

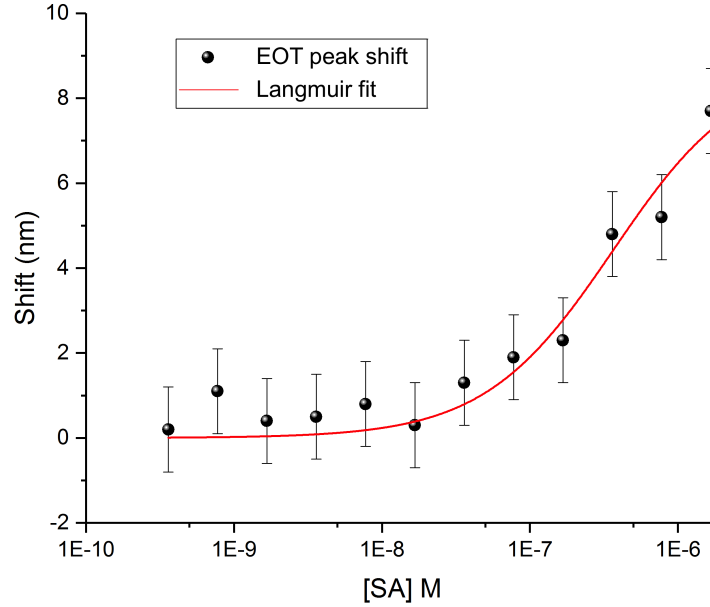


Figure 6.5: Langmuir sensing curve.

LANGMUIR FIT	
$\Delta\lambda_{sat}$ [nm]	8.8 ± 1.9
K_{aff} [M ⁻¹]	$(2.7 \pm 0.9) \cdot 10^6$

Table 6.4: Measured EOT peak shifts after each functionalization step.

From this graph we can obtain another important parameter, the *Limit of Detection*, LoD, which corresponds to the lowest analyte concentration, that the NHA biosensor is able to detect. Since the error bar in our measurements is 1 nm and being the EOT peak shift corresponding to the blank solution exposition ~ 0 nm, we estimate the Limit of

Detection at the intersection between the Langmuir isotherm fitting curve and $y = 1$ nm, as shown in figure 6.6.

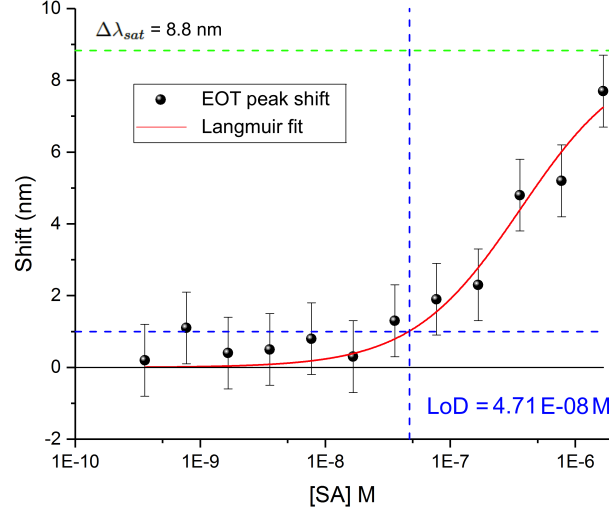
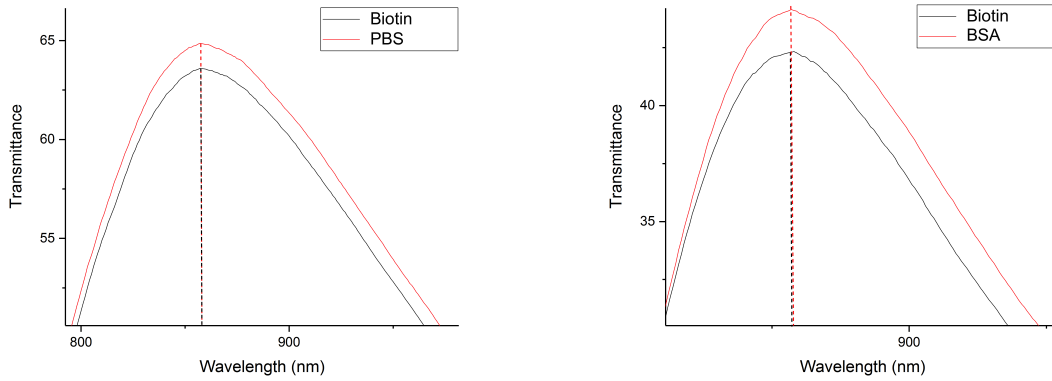


Figure 6.6: Langmuir sensing curve and respective Limit of Detection (LoD).

leading to a Limit of Detecton of:

$$LoD \sim 4.7 \cdot 10^{-8} M. \quad (6.7)$$

Specifity test A crucial characteristic of a biosensor is its specificity, i. e., the capability of detecting only the desired analyte. Therefore, expositions to a blank solution, i. e., solution with no analyte (PBS) and to an aspecific solution containing another common protein like BSA (Bovine Serum Albumine) have been performed (see figure 6.7).



(a) An under 1 nm shift is detected after blank solution exposition.

(b) A 2.5 nm shift is observed after aspecific solution exposition.

Figure 6.7: Specificity test spectra.

The blank solution tests provided no shifts within the experimental errors (< 1 nm), meaning that no solvent remains fixed to the structure and also it does not remove already bounded molecules (thiols or biotin), as a consequence of the previously introduced stability test.

The specificity test, on the other hand, provided EOT peak shifts around $\sim 2 \div 3$ nm. In principle no shift should be expected after BSA exposition, considering the fact that no binding should occur between BSA and Biotin. Therefore, this aspecific signal might be due to BSA binding with the SLG at the hole bottom (being BSA a protein, as such it tends to bind to glass surfaces). To overcome this problem there are different possibilities, three of which are likely the most feasible: (i) protecting the substrate surface with appropriate substances, e. g., mPEG silane (mPEG-Si) [47], (ii) RIE etching of the substrate in order to lower the glass surface with respect to the NHA plane, thus decoupling the nanostructure from the aspecific molecules at the glass surface and (iii) bring the structure to BSA saturation as a last functionalization step before performing the biosensing tests. However, the two latter solutions have not already been tested in the present work, while the former will be discussed later.

6.3.1 Sensing issues

Specificity problem of our biosensing system have already been discussed above. Furthermore, from figure 6.5 it is possible to notice that low Streptavidin concentrations are characterized by shift fluctuations. Moreover, exposing once more to $[SA]_{max}$ the spot already exposed to the maximum concentration, a further shift is observed, shown in figure 6.8 (blue dot).

STEP	$[SA]$ [nm]	SHIFT TOT
I exposition	$1.67 \cdot 10^{-10}$	7.7 ± 1.0
II exposition	$1.67 \cdot 10^{-10}$	9.4 ± 1.0

Table 6.5: Biotin-[SA] measured shifts after two consecutive expositions to $[SA]_{max}$.

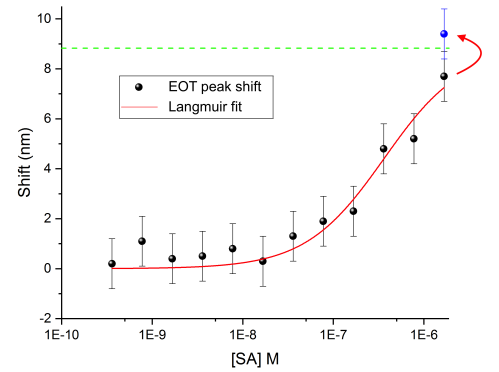


Figure 6.8: Additional shift of ~ 2 nm (blue dot) displayed by a further exposition of the spot already exposed to $[SA]_{max}$.

Even if the last shift value is closer than the former to that expected from local sensitivity simulations (~ 15 nm), this result indicates that the functionalization has to be improved, likely due to a non optimal control of the exposure of NHA to the molecules

triggered by hydrophobicity issues. In fact, this means that once we expose a spot to a certain concentration, only a percentage of the analyte falls on the spot. It has indeed been observed that the SA solution drops tend to evaporate spreading on the SLG rather than on the spot. This is probably due to the higher hydrophilicity of the substrate, compared to that of the nanostructure [48]. Thus, drop expositions to SA may not lead to a complete deposition of the desired substance entirely in sight, also due to the natural inclination of proteins to stick to glass surfaces.

In the case of low Streptavidin concentrations instead, since the electric field is more intense at the hole top and bottom rims of the holes, while on the top surface the signal results weaker (as discussed in section 4.3.1), the hydrophobicity displayed by the NHA structure may cause no analyte deposition at the hole walls and bottom rim, providing a more feeble response than expected, hence explaining the fluctuations observed in figure 6.5.

Moreover, aware of these facts, a probing test with Biotin at saturation has also been performed: each NHA spot have been exposed multiple times to Biotin solution drops. As a result, in many cases a further red-shift have been observed, meaning that a single exposition did not bring to saturation each spot, leading to insufficient number of receptor sights available. Therefore, the fluctuations observed during the biosensing tests may be ascribable also to this problem. However, two consecutive Biotin drop expositions have revealed to be sufficient to saturate the spots, not providing any further shift to successive expositions. Other possibilities then double Biotin exposition, not already tested in the present work, are to operate a thiols-like bath, immersing the sample for few hours in a proper Biotin solution, or to employ silicone wells, in order to keep the solutions drops inside, avoiding its spreading on the substrate surface. On the other hand, since a reliable biosensing signal must be obtained from a unique exposition to the analyte, consecutive drop expositions to the analyte solution does not represent a suitable option for sensing tests. This also applies for the analyte solution bath option, since analyte molecules of medical interest are often expensive and of limited availability. Hence, the silicone wells might represent the most feasible solution.

Another attempt has been performed, in order to reduce sensing instabilities: treating the samples with a chemical protection, mPEG Si. These molecules bind to the substrate surface, both reducing its hydrophilicity and preventing aspecific bonding. Actually, after treating the substrate with mPEG-Si, Biotin and Streptavidin drops tend to remain on the spots more favorably during the evaporation. Therefore, an improvement in the mPEG-Si protocol may lead to better results.

6.4 Comparison with another biosensing system

In this section, a comparison between the performances of the ordered NHA biosensor and a disordered NHA device is presented.

6.4.1 Disordered NHA

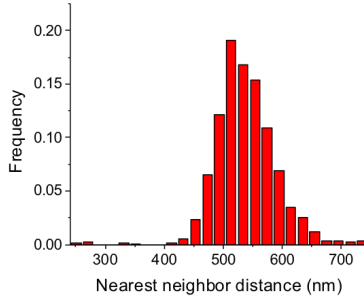
In order to evidence the importance of an ordered array of nanoholes with hexagonal symmetry for the extraordinary plasmonic properties of noble metal NHAs, we compared its sensing performances with a sample with a disordered distribution of nanoholes drilled in a thin Au film. In particular, we fabricated a sample with filling factor (ratio of the hole surface to the sample area), hole diameter and metal film thickness similar to the one of the ordered NHA. For this purpose, a protocol described by Hanarp et al. [49] was used to deposit a disordered distribution of nanometric PS nanoparticles on the sample surface. Before discussing the performances results, a brief description of the fabrication process of a disordered NHA is presented.

Synthesis and characterization PS nanoparticles (diameter $d = 300$ nm) with a charged surface functionalization were purchased from Life Technologies. The diameter was chosen in order to match the diameter of the PS nanospheres that we reach by the reactive ion etching step in the NanoSphere Lithography protocol described in section 3.1.3. A SLG substrate was rinsed with a series of solution with polymer electrolyte and ultra-pure water (Milli-Q) to allow a disordered deposition and to avoid aggregation of the PS charged nanospheres. First SLG substrates were cleaned with acid and basic piranha solutions for respectively 60 min and 20 min (same procedure discussed in section 3.1.1). Then they were exposed for 30 s in sequence to three different electrolyte solutions - PDDA, PSS and PAX - and after each functionalization step they were thoroughly rinsed with Milli-Q water. After water evaporation, a solution of surface charged polystyrene nanospheres was dispersed on the substrate surface and left for 15 min in a closed environment to avoid fast evaporation of the solution. Finally a thermal treatment for 1 hour at 120 °C was done in order to improve the adhesion of the nanospheres on the surface. The disordered pattern of PS nanospheres was used as masks for the deposition of a 3/77 nm *Cr/Au* bilayer performed by means of the magnetron sputtering (section 3.1.4). Thus, the main geometrical parameters such as hole diameter, nearest neighbor average distance and holes filling factor were transferred on the metal thin film. The PS nanospheres were then removed by sonication in toluene.

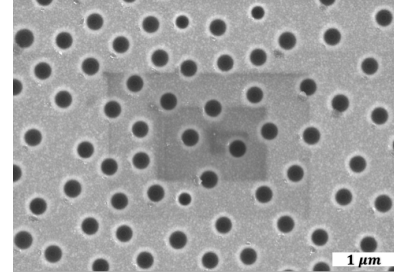
Figure 6.9a presents the frequency distribution of the distance of the nearest neighbor and a gaussian fit of this distribution gives a 530 nm average distance with 40 nm of standard deviation, while figure 6.9b shows a SEM view of the sample.

Optical properties The optical properties of the disordered nanohole array were investigated. The lack of order avoids the grating coupling between the incident light and the surface plasmons that provides the Extraordinary optical transmission and therefore no Fano-like transmission resonance arises in the transmittance spectrum. However, in the transmittance spectrum of the disordered NHA in figure 6.10 a transmittance peak can still be observed (red line).

This transmission peak in the NIR wavelengths range is due the localized plasmon



(a) Frequency distribution of the nearest neighbor.



(b) SEM image of a disordered NHA.

Figure 6.9: Morphological characterization of a disordered NHA.

NHA PEAKS	ORDERED	DISORDERED
T_{max}	58	20
HEIGHT	49	11
FWHM	296	358

Table 6.6: Comparison between the T_{max} of an ordered NHA and a disordered NHA.

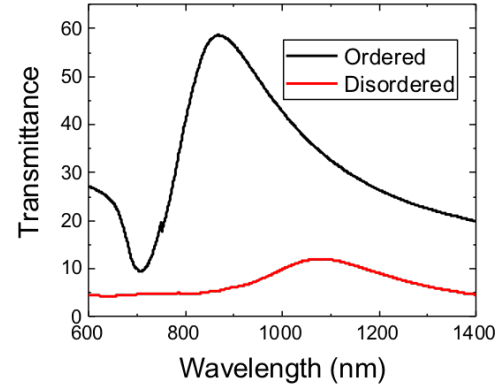


Figure 6.10: Comparison between the transmittance spectra of an ordered NHA (black) and a disordered NHA (red).

resonance that arises at the edges of the disordered and non-interacting nanoholes [50,51]. This transmittance peak results much broader and less intense than the EOT transmission peak of the equivalent hexagonally ordered NHA (see table 6.6). Nevertheless, the plasmonic origin of the EOT peak makes it sensitive to the variation of refractive index at the interface of the disordered NHA. For this purpose we measured the bulk and local sensitivity to compare the sensing performances of ordered and disordered NHAs.

Sensing Performances For the bulk sensitivity, first the transmission of the NHA in air was measured. Then, a 0.5 mm thick film of 1:10 PMMA solution was spin coated and cured at 90 °C on a heating plate for 5 minutes and the transmittance spectrum was measured again. In figure 6.11 the two transmission spectra are plotted and the related shift of PMMA spectrum to the air one can be estimated.

Thus, the bulk sensitivity can be obtained:

$$S_{bulk} = \frac{\Delta x_{max}}{\Delta n} = 156nm/RIU \quad (6.8)$$

BULK SENSITIVITY	
MEDIUM	λ_{max}
Air	1085.0 ± 1.0
PMMA	1160.0 ± 1.0

Table 6.7: Transmittance peak positions before and after PMMA deposition.

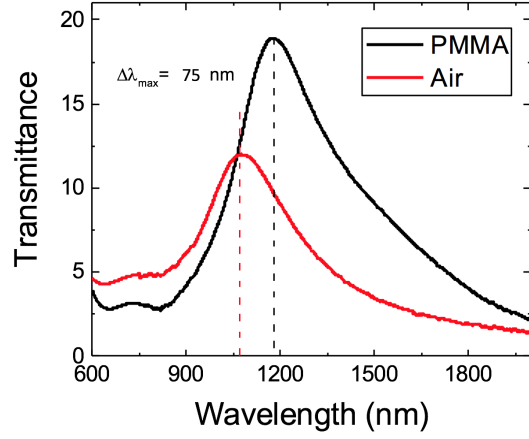


Figure 6.11: NHA Transmittance spectra before (red) and after (black) PMMA deposition.

Furthermore, the local sensitivity of the disordered NHA was estimated by depositing incremental thin layers of silica on the sample surface. Layers of thickness $t = 6, 18, 30$ nm of silica were deposited by magnetron sputtering and the redshifts of the transmittance peaks were monitored (see figure 6.12).

THICKNESS t [nm]	SHIFT [nm]
0	0
6.1	3.8 ± 1.0
18.4	14.9 ± 1.0
30.7	23.8 ± 1.0

Table 6.8: SiO_2 layers thickness and related Transmittance peak shifts.

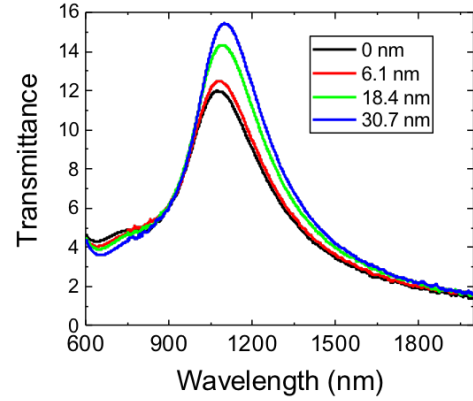


Figure 6.12: Transmittance spectra of the disordered NHA as the SiO_2 layer thickness increases.

A linear fit of the transmittance peak wavelength shift versus dielectric layer thickness was calculated, as for the case of ordered NHAs, and a local sensitivity was thus estimated:

$$S_{loc} = \frac{m}{n_{Air} - n_{SiO_2}} = 1.7 RIU^{-1} \quad (6.9)$$

returning a smaller value than the one obtained with the ordered structures and approximately a half value with respect to the simulated one and pointing out that the hexagonal

FIT PARAMETERS	
$\Delta\lambda = m \cdot t + q$	
m	q
0.79 ± 0.03	-0.3 ± 0.6

Table 6.9: Fit parameters for local sensitivity estimate.

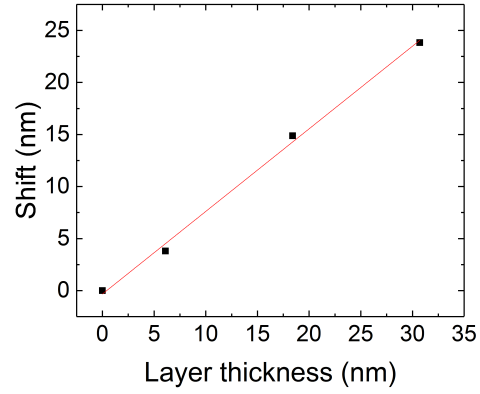


Figure 6.13: Fit of the Transmittance peak shifts of the disordered NHA as a function of the SiO_2 layer thickness.

symmetry of the holes pattern strongly enhances the sensing performances of such plasmonic nanostructures, making them reliable sensing devices.

Chapter 7

Conclusions

The aim of this thesis work was to investigate the physical properties of the ordered NanoHole Array plasmonic nanostructure as optical transducer for biosensing experiments.

First of all, an efficient protocol for NHAs fabrication has been presented and optimized, proving that Nano Sphere Lithography represents a reliable technique to produce high throughput, cost-effective well-ordered NHA. Moreover, employing this technique, morphological NHA parameters can be easily finely tuned, underlining the versatility of this technique. A stable nanostructure has been obtained, demonstrated by the resistance that NHAs display when treated with the acid piranha.

Physical optical properties of the synthesized NHA, like the Extraordinary Optical Transmission, EOT, due to plasmonic resonance, was observed measuring transmittance spectra at normal incidence and the Fano-like nature of this phenomenon has been studied. In fact, EOT is the result of an interference phenomenon of two different channels: a continuum one, represented by the surface plasmon resonance, and a discrete one, due to the Bragg-diffraction channel of the NHA structure. The physical property which allows the NHA to be employed as a transducer in a biosensing system, namely, the strong dependence of the optical properties and in particular of the plasmonic resonance condition, on the variation of the refractive index of the surrounding environment, have been studied and observed. Thus, the occurred binding of an analyte with a specific receptor at the NHA surface, which causes a change in the refractive index, is translated into a red-shift of the EOT peak. This signal has been analyzed by means of a Fano-like function, corroborating the theoretical interpretation of the phenomenon.

Bulk and local sensitivities have been tested and compared to simulated results. As expected, a good agreement is found in the case of the bulk sensitivity, since we are working with E-SPR, whereas further improvements are need, in order to obtain comparable results for the local sensitivity.

$$S_{bulk}^{simul} = 290nm/RIU \quad vs \quad S_{bulk}^{exp} = 281nm/RIU$$

$$S_{loc}^{simul} = 3.2RIU^{-1} \quad vs \quad S_{loc}^{exp} = 2.87 \pm 0.07RIU^{-1}$$

Also a functionalization protocol has been investigated, outlining issues in the procedure. Above all, SLG hydrophilicity versus NHA hydrophobicity issue seems to be the main obstacle to high sensitivity sensing experiments, preventing efficient deposition of the desired substances. Possible solution to overcome the different problems encountered have been tested and suggested.

A biosensing test, using the Biotin-Streptavidin as receptor-analyte system, demonstrated the suitability of NHA as transducer. NHA samples have been exposed to different analyte solution concentrations, measuring the resulting spectra. The measurements reproducibility and the stability of the biosensors have been demonstrated. Thus, a sensing curve has been obtained, reaching a Limit of detection of:

$$LoD \sim 4.7 \cdot 10^{-8} M. \quad (7.1)$$

close to the nM value of commercial biosensors. The sensing curve shows that at low concentrations the shift signals exhibit still some fluctuations. These results, together with bulk and local sensitivity results (both simulated and measured) suggest that although the NHA investigated exhibit performances close to the state-of-the-art, probably they can be used even more effectively for sensing larger objects like bacteria, thanks to their high bulk sensitivity, linked to the long near-field decay length. Nevertheless, regarding the fluctuations of the shifts, which are probably due to the previously mentioned issues in the solutions exposition procedure, further optimization is needed to improve the NHA reliability. In this sense, a chemical protection test of the substrate, in order to reduce its hydrophilicity, through use of mPEG-Si, has been attempted, but still with unstable results. Further improvement in the mPEG-Si protocol are thus needed. However, the most feasible and reliable solution suggested might be the employment of silicone wells around the NHA spots, to maximize the exposition efficiency to the desired substances.

Despite these issues, the ordered NHA suitability as label-free, high-sensitivity, cost-effective and high throughput transducer for biosensing applications has been demonstrated. This fact has been corroborated by a comparison with a disordered NHA device, which further stood out the unique and fascinating properties of the ordered NHAs.

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