Development of biocompatible plasmonic thin films composed of noble metal

nanoparticles embedded in a dielectric matrix to enhance Raman signals

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Abstract

This work focused on the production of nanocomposite thin films, composed of noble nanoparticles

embedded in a dielectric matrix, to be tested as biocompatible plasmonic platforms for detection of

molecules using Surface Enhance Raman Spectroscopy (SERS). Three different thin films systems

were deposited by reactive DC magnetron sputtering, namely Au-Al<sub>2</sub>O<sub>3</sub>, Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub>.

The depositions were followed by a thermal treatment at different temperatures to promote the

growth of the nanoparticles. Localized Surface Plasmon Resonance (LSPR) bands appeared already

at 300 °C, related to the presence of Au nanoparticles, and at 500 °C in the case of Ag nanoparticles.

Furthermore, at 700° C, the Ag-TiO<sub>2</sub> films showed a broadband optical response due to the

formation of Ag clusters at the film's surface. The biological experiments showed that the presence

of the thin films didn't affect the growth of C. albicans, which is very convenient if one needs to

detect low concentrations of this microorganism using SERS platforms. As for the SERS

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measurements, an enhancement of R6G Raman spectra intensity was clearly perceivable, but only for the  $TiO_2$  matrix. Furthermore, the application of a plasma treatment allowed to better expose the nanoparticles, providing a further enhancement of Raman signals.

# **Keywords:**

Thin films; Nanoparticles; Oxide matrix; Localized Surface Plasmon Resonance; *C. albicans*; Surface Enhanced Raman Spectroscopy.

# **Highlights:**

- Plasmonic thin films of Au-Al<sub>2</sub>O<sub>3</sub>, Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> were prepared;
- Annealing treatments affect the microstructure and plasmonic response;
- Cellular growth of *C. albicans* in not affect by the presence of the films;
- Plasma treatments on Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> enhances Raman signals of R6G molecules;
- A relative SERS enhancement factor of 10<sup>2</sup> was obtained.

#### 1. Introduction

Nanoscale tailoring of materials allows to obtain physical and chemical properties that differ from bulk counterparts [1]. Among several examples, are the nanostructures composed of noble metals (e.g. gold and silver nanoparticles), which present nanoplasmonic properties that can be used in different applications [2,3]. In a metallic surface, collective electronic excitations in the metallic/dielectric interface, associated with an oscillating electromagnetic field, originate surface plasmons [4,5]. If these surface plasmons propagate along a continuous metallic surface, they are designated as surface plasmon polaritons (SPP) [6,7]. When confined to a metallic nanostructure or nanoparticle are mentioned as localized surface plasmons (LSP) [8]. In this regard, Localized Surface Plasmon Resonance (LSPR) became a subject of great interest in research due to the multitude of applications that arise from this phenomenon [9,10]. Aside from decorative purposes, many technological applications of LSPR can be found in the literature, such as plasmonic sensors for gas and biomolecular detection, plasmon based photo-detectors, phototherapies and surface enhanced Raman spectroscopy (SERS) [11,12].

LSPR is characterized by strong electromagnetic field enhancements near the nanostructure and strong extinction (absorption + scattering) bands, and these properties can be tuned by changing some nanostructural characteristics [13,14]. Some of them are the geometry, size, shape and distribution of the nanoparticles embedded in a dielectric medium, which are known to affect the LSPR absorption band [15–18]. Furthermore, the atomic number and concentration of metal composing the nanoparticles also affect the LSPR properties, as well as the dielectric function of the surrounding medium [9,15]. Tailoring these parameters allows to obtain plasmonic nanostructures with absorption bands in the visible range of the electromagnetic spectrum, revealing a great potential to the construction of optical biosensors.

It is well-known that Surface Plasmon Resonance (SPR) sensors have high sensitivities, due to the long decay length of its evanescent field, and are already widely applied in biosensing [19]. On the

other hand, LSPR presents highly confined and intense electromagnetic fields around the nanostructure. This leads to a lower sensing volume, comparing to SPR sensing, but, consequently, the analyte occupies a much larger portion of the sensing area [13,20]. Another benefit of LSPR is that it can be excited by passing-through light (transmittance mode T-LSPR) [21], and this allows the use of ordinary optical equipment to observe the T-LSPR band and monitor its evolution in the presence of different analytes [22].

Furthermore, using LSPR in biosensing isn't limited to optical sensors. Applying LSPs to Raman spectroscopy allows the enhancement of commonly low signal intensity, resulting in Surface-Enhanced Raman Spectroscopy (SERS) [23,24]. Raman spectroscopy relies on the phenomenon of inelastic scattering, defined by the energy difference between incident photons and vibrational modes [25–27]. As consequence, each (bio)molecule will have a unique Raman spectrum, acting as a fingerprint for that molecule [28]. In this respect, the high intensity and confinement of electromagnetic fields near the nanoparticles in LSPR, can benefit Raman spectroscopy, by enhancing the Raman signal intensity of a molecule placed in the sensing area of a nanoparticle [29]. This electromagnetic enhancement enables the detection of very low concentrations of molecules as it can reach enhancement factors up to  $10^{14}$  [30–32].

One of the possible use of SERS technology is the quick detection and identification of individual cell types, from microorganisms to cancer tissue and stem cells [33–35]. The produced molecular fingerprints by Raman spectroscopy can be used to identify and differentiate compounds, and can possibly be used to create a signature database for Raman spectroscopy identification [36]. As the spectra are reproducible, they are highly adequate for identification of fungi [37,38]. More specifically, in the case of microorganisms, the rapid identification of significant species is of utmost importance to choose the appropriate therapy, which can be related to the morbidity and mortality rates [39]. The identification of *Candida spp.* by Raman spectroscopy is already described in the literature [40,41].

In terms of nanoplasmonic transducers, most research is done in nanoparticles produced by chemical methods or nanolithography in the production of LSPR platforms [42]. However, these techniques reveal problems like high production cost or environmental problems [9,43]. In this field, some research has been done in magnetron sputtered thin films consisting of noble metal nanoparticles embedded in a dielectric matrix [44,45]. Changing the deposition parameters of these films, as well as the post-deposition thermal treatment conditions, allows to tune LSPR bands within the visible spectra by changing some nanostructural parameters (size, shape and distribution of the nanoparticles), the noble metal concentration, and also the dielectric matrix (usually an oxide) [46–49]. However, to achieve high Raman signal enhancements, it is necessary to ensure that a high number of nanoparticle "hotspots" are found in the surface. It was shown by Rodrigues *et al.* that using plasma etching it is possible to promote the erosion of the matrix surface, allowing for the exposure of more metallic nanoparticles [50].

In this work, different series of plasmonic thin films were prepared, in order to study the influence of plasmonic metal (Au or Ag) and dielectric matrix (TiO<sub>2</sub> and Al<sub>2</sub>O<sub>3</sub>) on the main characteristics and properties of the films. They consist of Au-TiO<sub>2</sub>, Ag-TiO<sub>2</sub> and Au-Al<sub>2</sub>O<sub>3</sub> deposited by reactive magnetron sputtering. To induce the growth of the nanoparticles and thus to obtain LSPR bands, observable in the transmittance spectrum, the films were submitted to post-deposition thermal annealing. In terms of applications, the thin films were evaluated as SERS platforms using R6G as well as in cellular cultures of *Candida albicans* to explore the possibility of using the plasmonic thin films as SERS chips to detect microorganisms.

## 2. Experimental details

2.1 Preparation and microstructural characterization of the nanoplasmonic thin films

The thin films were prepared by reactive DC magnetron sputtering and deposited in glass lamellae, fused silica and Si (p-type, Boron doped) substrates. For TiO<sub>2</sub> matrix thin films, the cathode was a titanium target (99.99 % purity) with rectangular dimensions,  $200\times100\times6$  mm<sup>3</sup>, on which gold or silver pellets were placed in the preferential erosion zone of the target, as the objective was to produce Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> thin films, with a total area of 32 mm<sup>2</sup>. A current of 2 A was applied (current density of  $100 \text{ A.m}^{-2}$ ), and the generated plasma was composed by argon (partial pressure of  $3.3\times10^{-1}$  Pa) and O<sub>2</sub> (partial pressure of  $7.8\times10^{-2}$  Pa). For Al<sub>2</sub>O<sub>3</sub> matrix, the cathode was an aluminum target (99.99% purity), also with a total area of Au pellets of 32 mm<sup>2</sup> incrusted in its preferential erosion track. A current of 1.5 A was applied (current density of  $75 \text{ A.m}^{-2}$ ) in a plasma composed by argon (partial pressure of  $4.1\times10^{-1}$  Pa) and O<sub>2</sub> (partial pressure of  $9.2\times10^{-2}$  Pa). The substrates were placed in a grounded sample holder, rotating at 5 rpm.

To promote the growth of the nanoparticles in the matrix, the thin films were subjected to post-deposition annealing treatments, with heating ramps of 5 °C/min and different isothermal temperatures (300, 500 and 700 °C), followed by a free cool down to room temperature.

The in-depth chemical composition profile was evaluated by Rutherford Backscattering Spectrometry (RBS). The measurements were performed with a 2 MeV <sup>4</sup>He<sup>+</sup> ion beam at an incidence angle of 0°. The detection system was composed by three detectors: a standard at 140° and two pin-diode detectors located symmetrical to each other, both at 165° (with detector 3 on the same side as the standard detector 2). The collected data were analyzed with IBA DataFurnace NDF v9.6a [51–53]. Morphological features were analyzed by Scanning Electron Microscopy (SEM), in a Dual Beam SEM/FIB FEI Helios 600i microscope. The thickness was also measured through the observation of the cross-sections of the as-deposited thin films.

The crystalline structure of the films was obtained by *in situ* high temperature X-ray diffraction (HT-XRD), in a Bragg–Brentano configuration using the Brucker D8 diffractometer equipped with Cu-K $\alpha$  radiation ( $\lambda$  = 0.154 nm). The measurements were conducted using the same conditions as the annealing step. Furthermore, the size of the Au or Ag crystalline domains was determined by fitting the preferential growth diffraction peaks with *Pearson VII* functions, using the *Winfit* software [54]. The films' surface roughness was obtained by Atomic Force Microscopy (AFM), using a MultiMode SPM microscope, controlled by a NanoScope IIIa from Digital Instruments, in tapping configuration. Transmittance spectra, in the range of wavelengths from 300 to 900 nm, with a step of 0.5 nm, were measured using a Shimadzu UV-2450(PC) spectrophotometer.

## 2.2 Growth behavior of C. albicans on Au-Al<sub>2</sub>O<sub>3</sub>, Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> thin films

The growth behavior was evaluated by the interaction of the yeast cells with the surface of the plasmonic thin films, annealed at different temperatures. A pre-culture of yeast cells was prepared from a cell colony and inoculated in a culture tube with 20 mL of Yeast Peptone Dextrose (YPD) medium (1% yeast extract (w/v), 1% bactopeptone (w/v), 2% Glucose (w/v)). This pre-culture was incubated overnight at 30 °C. The thin films (in Si wafer of 2.25 cm²) were sterilized with 70% ethanol for 2 hours and then washed using sterile water. On the next day, the cells were transferred to culture tubes with 20 mL of YPD medium with the thin films, at an initial optical density (OD) of 0.1 at 600 nm of wavelength. The cells were incubated at 30 °C and shaken at 120 rpm. The OD at 600 nm in a Spectronic 20 spectrophotometer was monitored during the 25 hours of the experiment. For further evaluation of the interaction between cells and the thin films' surface, two time points were selected, one in the microbial exponential growth phase (8 hours) and another in the later stationary growth phase (25 hours). At these selected time points the number of viable cells after the interaction of the thin film was estimated by counting the colony forming units (CFUs).

CFUs data from three independent experiments were analyzed using one-way ANOVA, with Dunnett's multiple comparisons test, with 95% confidence level, using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The objective was to test if the results from the incubation of *C. albicans* with the thin films were significantly different from the control group growth over the thin films substrate (silicon wafer).

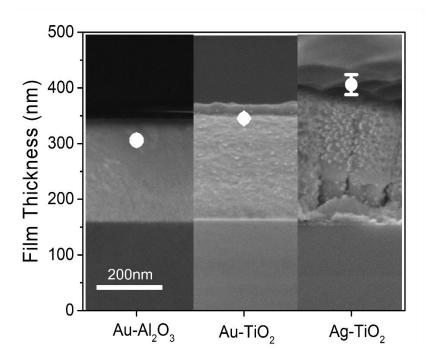
## 2.3 RAMAN measurements in plasmonic thin films

Plasma treatment was performed in all samples at 700 °C annealing to increase the number of nanoparticles partially exposed to the surface, as showed by Rodrigues *et al.* [50]. For surface-enhanced Raman spectroscopy measurements, a 20  $\mu$ L droplet of a 2×10<sup>-4</sup> M Rhodamine 6G solution in ethanol was placed on top of the samples and left to dry overnight. The SERS measurements were performed at room temperature with a Horiba Jobin Yvon T64000 triple Raman spectrometer, equipped with liquid nitrogen cooled charge coupled device (CCD) Symphony II 1024 × 246 FIVS detector, with a resolution better than 1 cm<sup>-1</sup>. The excitation line, 514.5nm, of an argon ion laser, was focused onto the sample using a ×50 MS Plan objective of an Olympus Microscope BHSM, in a back-scattering geometry. The spectra were obtained with a measured power of about 0.4 mW onto the sample, during an integration time of 30 s, with 5 measurements per sample.

#### 3. Results and discussion

# 3.1 Composition and microstructure of the as-deposited films

RBS analysis allowed to determine the atomic concentration of noble metal in each deposited thin film series. In the films with aluminum oxide matrix, Au-Al<sub>2</sub>O<sub>3</sub>, the atomic concentration of Au was 17 at.%. Regarding the TiO<sub>2</sub> matrix, 13 at.% of Au was found in the Au-TiO<sub>2</sub> films and 19 at.% of Ag for the Ag-TiO<sub>2</sub> films. RBS analysis showed that there was a higher gold content in the Al<sub>2</sub>O<sub>3</sub> matrix, comparing to the TiO<sub>2</sub> matrix, for the same amount of gold in the target (two pellets). This could be due to the difference in the applied current to the sputtering target, as previously shown, a higher current causes a lower effective Au sputtered area [50], and also the lower sputtering yield of Al<sub>2</sub>O<sub>3</sub> compared to TiO<sub>2</sub> [55]. Changing the noble metal into the TiO<sub>2</sub> matrix from Au to Ag displayed different noble metal concentrations in the thin films. In fact, Ag-TiO<sub>2</sub> thin films have a higher noble metal content, as the silver has a higher sputtering yield compared to gold [56]. As for the SEM analysis, it allowed to observe the morphology, as well as the thickness of the asdeposited thin films. The micrographs in Figure 1 show a featureless-dense morphology for the Au-Al<sub>2</sub>O<sub>3</sub> and a thinner film. The microstructure of Au-TiO<sub>2</sub> thin film is also relatively dense, though more granular and thicker, while the Ag-TiO<sub>2</sub> thin film reveals a more porous microstructure. This behavior is not only related to the higher content of plasmonic metal dispersed in the TiO<sub>2</sub> matrix, but also due to the growth of more disordered microstructures associated to the presence of Ag instead of Au [57].



**Figure 1** - Thickness measurement and SEM micrographs of as-deposited thin films: Au-Al<sub>2</sub>O<sub>3</sub>; Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub>.

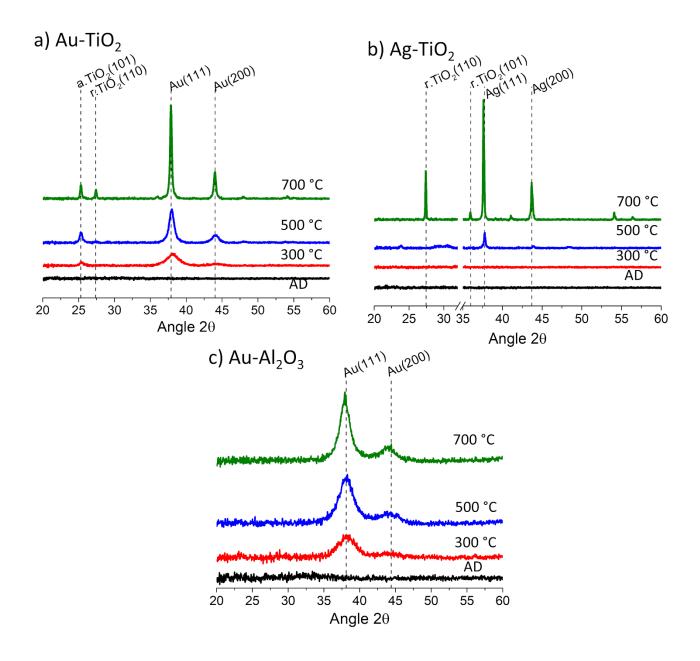
### 3.2 The growth of plasmonic nanoparticles via thermal annealing

### 3.2.1 Structural evolution of the films using in situ XRD annealing

X-Ray diffraction showed an amorphous structure for all as-deposited thin films (Figure 2). In the Au-TiO<sub>2</sub> thin films (Figure 2a) Au starts to crystallize in fcc structure after annealing at 300 °C, as evidenced by the diffraction peak (111) appearing at 38.1°. At this temperature the TiO<sub>2</sub> matrix also starts to crystalize into the anatase phase, as revealed by the faint peak (101) at 25.3° [ICDD: 44882]. The anatase phase is characteristic of the Au-TiO<sub>2</sub> thin films annealed to temperatures above 300 °C, as shown in previous works [58,59]. At 500 °C, the (111) diffraction peak becomes more intense, while the (200) diffraction peak related to the crystallization of Au appears at 44.4°. Finally, at 700 °C, the diffractions peaks (111) and (200) related to Au crystallization become even more intense and sharper since the crystalline domains of Au are larger. As for the matrix, the diffractograms show the crystallization of TiO<sub>2</sub> in its rutile phase for temperatures of 700 °C,

showed by the diffraction peak positioned at 27.4°, indexed to the (110) orientation of the rutile structure [ICDD: 62677]. Furthermore, at 700 °C both phases coexist in the film, anatase and rutile. Concerning the Ag-TiO<sub>2</sub> films (Figure 2b), it is possible to differentiate from the Au-TiO<sub>2</sub> system as the diffractograms don't show any diffraction peaks related to the crystallization of Ag or TiO<sub>2</sub> at 300 °C. Increasing the temperature to 500 °C led to the crystallization of Ag in the fcc structure, as a sharp diffraction peak appears for a 2Θ angle of 38.1° [COD-1100136] related to the (111) orientation of the fcc structure of crystallized Ag. However, contrarily to thin films of Au-TiO<sub>2</sub>, where it is possible to observe the anatase phase at 500 °C, the presence of Ag in the TiO<sub>2</sub> matrix inhibits the formation of anatase phase [47,60]. Further increase of the annealing temperature to 700 °C led to the appearance of sharp and intense peaks, corresponding to the (111) and (200) orientations of Ag structure, the latter appearing at a 2Θ angle of 44.4° [COD-1100136]. Concerning the TiO<sub>2</sub> matrix, after annealing at 700 °C, the matrix crystallizes into the rutile phase, with an intense and well-defined diffraction peak at 27.4° [ICDD: 62677], correspondent to the (110) orientation of this phase. Therefore, when compared to Au, the Ag atoms promote the formation of rutile instead of anatase [47].

Finally, in Au-Al<sub>2</sub>O<sub>3</sub> thin films (Figure 2c), and similarly to Au-TiO<sub>2</sub> system, it is possible to observe the crystallization of Au in the face centered cubic structure at 300 °C, with the appearance of the (111) diffraction peak at a 2Θ angle of 38.2° [ICDD: 04-0784]. At 500 °C, the (111) diffraction peak increases in intensity, and the diffraction peak related to the (200) orientation of the fcc structure of Au appears at 44.4° [ICDD: 04-0784]. After annealing at 700 °C, both diffraction peaks increase in intensity and become slightly narrower. However, the annealing process didn't induce any crystallization of the Al<sub>2</sub>O<sub>3</sub> matrix.



**Figure 2** - XRD diffractograms of the as-deposited and annealed thin films: (a) Au-TiO<sub>2</sub>, (b) Ag-TiO<sub>2</sub> and (c) Au-Al<sub>2</sub>O<sub>3</sub>.

Using the preferential growth orientation of the noble metals (Au or Ag), the grain sizes were estimated for the thin films annealed from 300 to 700 °C. The simulation consisted of fitting Pearson VII functions to the diffractograms of the thin films in the (111) diffraction peak for Au and Ag. The grain size of Au-Al<sub>2</sub>O<sub>3</sub> thin films suffers a slight increment from 2 to 4 nm with the

increase of annealing temperature from 300 to 700 °C. For the same temperature interval, in the Au-TiO<sub>2</sub> system, the grain size variation is more significant, changing from 3 to 9 nm as the temperature is increased from 300 °C to 500 °C, and increases even further to 33 nm, with an annealing temperature of 700 °C. The differences in growth of the Au nanoparticles between the Al<sub>2</sub>O<sub>3</sub> and TiO<sub>2</sub> matrixes is most likely related to the structural evolution of the matrixes during the annealing. It has been shown that the crystallization of TiO<sub>2</sub> increases the number of grain boundaries [61], leading to a higher diffusion of gold trough these boundaries, where the nanoparticles can more easily aggregate and suffer coalescence and/or Ostwald ripening phenomena, resulting in the formation of larger nanoparticles. On the other hand, the lack of crystallization of the Al<sub>2</sub>O<sub>3</sub> matrix prevents the formation of grain boundaries, hindering the diffusion of Au throughout the matrix thus inhibiting the growth of large crystalline domains of Au [62].

Table 1 - Mean grain size of Au and Ag crystalline domains for the different thin films annealed at different temperatures.

Annealing	Noble metal grain size (nm)				
temperature	Au-Al <sub>2</sub> O <sub>3</sub>	Au-TiO <sub>2</sub>	Ag-TiO <sub>2</sub>		
300°C	2	3	-		
500°C	3	9	61		
700°C	4	33	141		

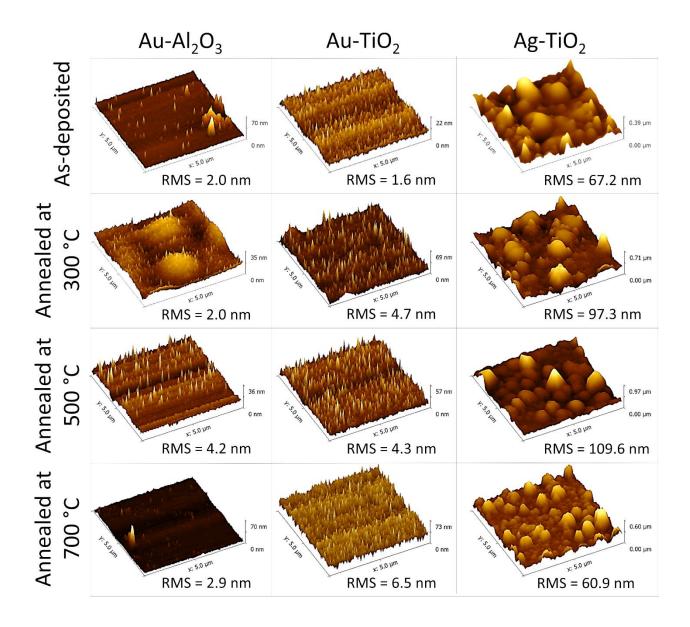
Finally, since the Ag-TiO<sub>2</sub> thin films don't present crystallization of the silver atoms at 300 °C, no value of grain size is obtainable for this annealing temperature. However, at 500 °C, the grain size of the crystalline domains of Ag is considerably higher than the Au systems: 61 nm. Moreover, it further increased to 141 nm when the annealing temperature was 700 °C. These values strongly

suggest that Ag crystals have diffused to the surface of the thin films, a typical behavior of this kind of films [47,60].

### 3.2.2 Surface roughness of the films

An important thin film's feature when they interact with different analytes such as cells is the surface roughness [63]. Using AFM, it was possible to measure the surface roughness of the thin films at the different annealing temperatures. As-deposited Au-Al<sub>2</sub>O<sub>3</sub> films have a very smooth surface, with a root mean square (RMS) roughness of approximately 2.0 nm. The annealing treatment at 300 °C didn't affected the roughness of the films but increasing the temperature to 500 °C promoted an increase of roughness to 4.2 nm. Nevertheless, unexpectedly the increase of the annealing temperature from 500 °C to 700 °C led to a small decrease in roughness of the films from 4.2 to 2.9 nm. This decrease in surface roughness could be due to the densification of the Al<sub>2</sub>O<sub>3</sub> matrix, as already reported [55].

Considering the Au-TiO<sub>2</sub> thin films, the surface roughness of the thin films increases with the annealing temperature (Figure 3). From as-deposited to 300 °C, the roughness increases from 1.6 to 4.7 nm, respectively. This increase is most likely due to the beginning of the crystallization of Au and TiO<sub>2</sub> into anatase. From 300 °C to 500 °C, the roughness remains approximately the same, despite the crystallinity improvement. Only when the rutile phase appears, at 700 °C, it is possible to observe another increment of the film's roughness from 4.3 to 6.5 nm.



**Figure 3** - Topographic images obtain by AFM for the as-deposited and annealed thin films with RMS roughness measurements.

Finally, Ag-TiO<sub>2</sub> thin films reveal a very different surface topography, in comparison with Au-Al<sub>2</sub>O<sub>3</sub> and Au-TiO<sub>2</sub> thin films. The film as-deposited has a relatively much higher roughness, about 67.2 nm, as the presence of Ag seems to greatly affect the thin film microstructure. Further increasing the annealing temperature to 500 °C led to an increase of roughness to 109.6 nm. This may be due to the crystallization of Ag embedded in the TiO<sub>2</sub> matrix. From 500 °C to 700 °C of annealing temperature, the surface roughness decreases, from 109.6 to 60.9 nm, as the matrix crystallizes in

the rutile phase and crystallization of Ag increases in intensity. This lower surface roughness could also be due to the formation of large Ag crystals in the surface of the thin film, in contrast with the film's surface, as seen by Borges *et al.*, due to the coalescence of silver [57]. In the AFM 3D images, the presence of large structures is clear.

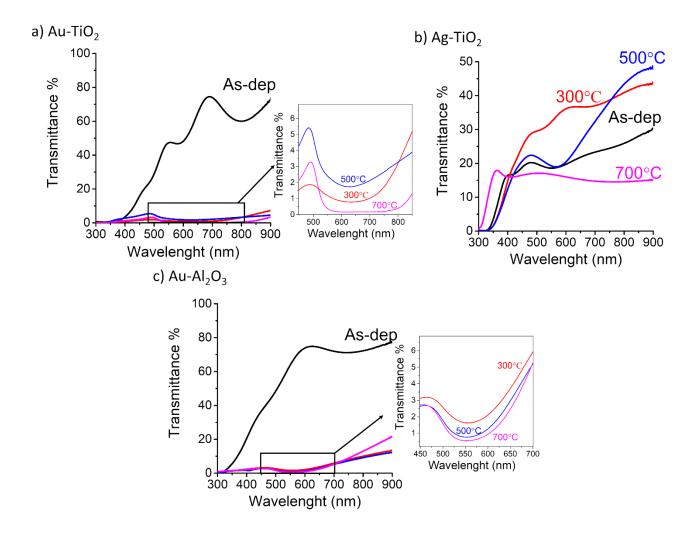
### 3.3 Optical response of the films (LSPR bands)

Regarding the Au-TiO<sub>2</sub> thin films (Figure 4a), interference behavior appears for the as-deposited film, with no LSPR band observable in the transmittance spectrum. Through the annealing process, it was induced the growth of Au nanoparticles that stayed embedded into the TiO<sub>2</sub> matrix. At 300 °C, the transmittance drastically decreases to below 6%. A broad LSPR absorption band is observable in the wavelength range from 500 nm to 750 nm. Further increasing the thermal treatment temperature to 500 °C induced a higher crystallization of the Au embedded in the anatase the matrix. In terms of the LSPR absorption band, the film annealed at 500 °C showed an increase of overall transmittance and narrowing of the band itself, presenting a more defined LSPR band due to the increase of gold nanoparticles. Finally, when the annealing temperature reaches 700 °C, the LSPR band became broadened, and the band minimum reached zero transmittance, extending from 550 nm of wavelength to 800 nm. This is in accordance with the coalescence of the gold nanoparticles into large spheroidal shaped clusters, as predicted in theoretical studies of this plasmonic system [64].

As for the Ag-TiO<sub>2</sub> thin films (Figure 4b), the as-deposited thin film shows a lower transparency, when compared to the gold counterpart, yet presenting an interference-like behavior. The annealing temperature of 300 °C results in an increase of transmittance but maintaining the interference behavior observed in the as-deposited film. This is due to the lack of crystallization and aggregation of Ag in nanoparticles, as the XRD results had previously shown. The transmittance spectra of the film annealed at 500 °C shows a faint LSPR band at about 565 nm. The appearance of the LSPR

effect is related to the crystallization of Ag and its aggregation into nanoparticles. Nevertheless, at 700 °C, where the crystallization of Ag presents a diffraction peak of high intensity, and the matrix also is crystallized in the rutile phase, the film has an almost constant absorption throughout almost the whole spectra. This optical response is a consequence of the diffusion of Ag towards the surface of the films, aggregating in large crystals or even fractal structures [57].

Finally, the changes induced in the transmittance spectra of Au-Al<sub>2</sub>O<sub>3</sub> thin films due to thermal annealing are presented in Figure 4c. The as-deposited thin film shows an interference pattern, with no evidence of a T-LSPR band. This behavior is in agreement with the amorphous structure of the film showed by XRD, suggesting that gold is dissolved in the matrix or might be present in the form of very small clusters. Annealing the film at 300 °C induces the crystallization of Au and its aggregation in nanoparticles. At this temperature, it's possible to observe an accentuated decrease in transmittance, near 560 nm compared to the as-deposited sample, due to the appearance of a LSPR band. However, this band starts at 475 nm until 650 nm, making a wide and low intensity LSPR band. Further increasing the annealing temperature to 500 °C and then to 700 °C led to an increase in Au crystallinity and grain size, and these morphological changes decreased the overall transmittance spectrum and thus the LSPR band, which also became broader with the increase of the temperature.



**Figure 4** - Evolution of the transmittance spectra with the annealing treatment at different temperatures for the deposited thin films: (a) Au-TiO<sub>2</sub>; (b) Ag-TiO<sub>2</sub> and (c) Au-Al<sub>2</sub>O<sub>3</sub>.

## 3.4 Interaction of C. albicans with plasmonic thin films

Characterizing the plasmonic thin films in terms of interfering with cell viability may provide useful information when considering the construction of a biosensor to detect live cells or monitoring the growth of a certain species.

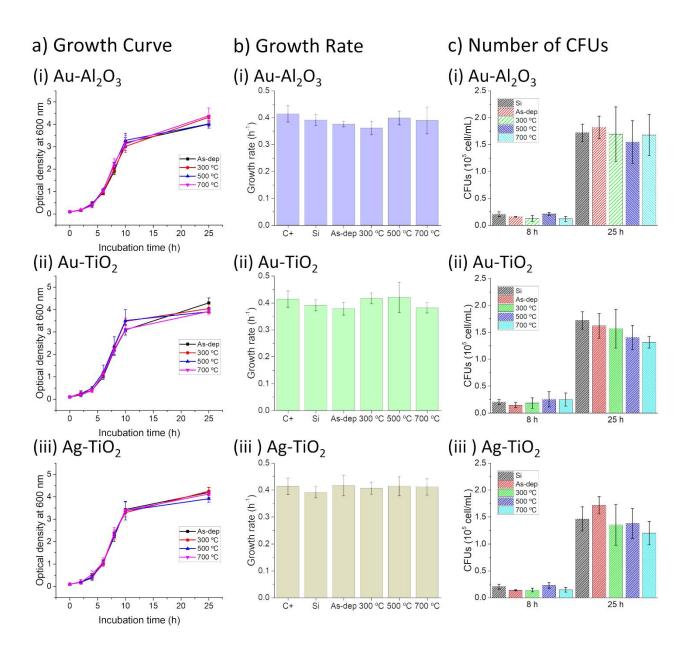
*C. albicans*, a pathogenic yeast species, was incubated for 25 hours in YPD medium in the presence of Au-Al<sub>2</sub>O<sub>3</sub>, Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> thin films at different annealing temperatures (as-deposited, 300, 500 and 700 °C) to evaluate cell viability, and results are presented in Figure 5.

Firstly, growth curves were plotted as optical density measurements, at 600 nm wavelength, at different timepoints for 25 hours, Figure 5a. From the growth curves, it is possible to perceive that the presence of the plasmonic thin films in the growth medium didn't affect significantly the growth of the yeast cells, when compared to the control group.

Using the exponential growth phase of the growth curve, it is possible to determine the specific growth rate of the cells in the presence of the thin films and compare it to the control group, Figure 5b. Since the analysis of the growth curve of *C. albicans* didn't show any significant differences, the specific growth rate was similar among the thin films, as expected.

Nonetheless, colony forming units were counted at two distinct time points: one in the exponential growth phase (8 hours) and another at the final growth phase (25 hours) to evaluate cells viability (Figure 5c), for each series of thin films with different annealing temperatures. Comparing the results with the control group, the thin films had no significant effect on the viability of the yeast cells, both after 8 hours and 25 hours of incubation, with no signs of cellular death or growth inhibition.

Therefore, the results show that the proposed thin films have no effect on the growth and viability of the tested microorganisms and may be used for creating biosensors for cellular entities.



**Figure 5** - Cellular growth of *Candida albicans* interacting with the thin films at different annealing temperatures: a) growth curve given at optical density at 600 nm; b) growth rate for yeast cultures in contact with the film's surface; c) number of CFUs counted for the selected time points, 8 and 25 hours of incubation (one, two, three and four \* are related to the statistical significance of the results and correspond to P < 0.05, P < 0.01, P < 0.001 and P < 0.0001, respectively).

### 3.5 Surface enhancement of Raman spectra using the plasmonic thin films

Plasmonic thin films present interesting applications for Raman spectroscopy, as they can enhance the signal by several orders of magnitude [65]. The enhancement factor is an important metric to compare the performance between different plasmonic systems, but quantifying the enhancement isn't a trivial endeavor. The enhancement factor depends on several factors, some of them related to the material itself, but also with the studied probe and the experimental apparatus [65]. However, if the measurements are carried out in the same experimental conditions, the difference of peaks' amplitude between samples is the same as the difference of enhancement for those specific conditions. Thus, it is possible to compare the performance of the thin films, at least for this restrictive set of experimental conditions.

The SERS spectra bring a second difficulty, as it's common to observe significant non-uniform fluorescence, which makes their analysis challenging but needs to be considered to get an accurate fit. For this, Moores *et al.* developed a fitting algorithm for SERS spectra that assumes the spectrum as a series of peaks plus a smoothly varying baseline typical of fluorescence [66].

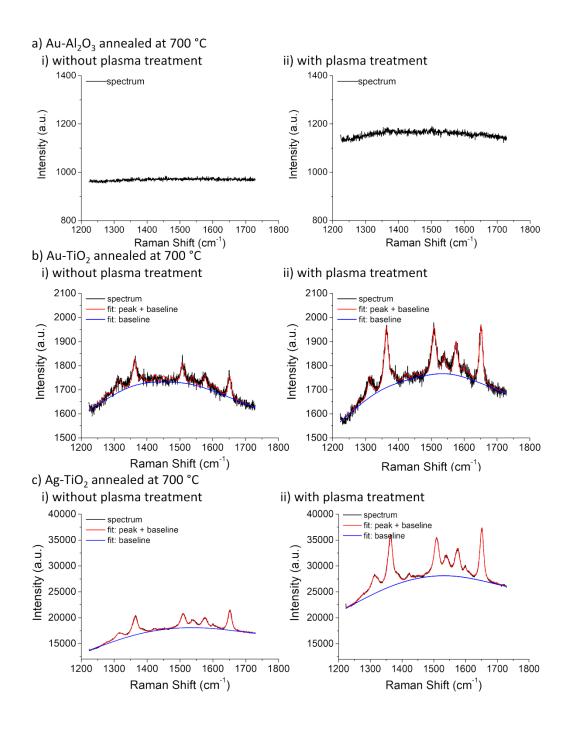
Density-functional theory (DFT) calculations can be used to predict the vibrational frequencies and assign the vibrational modes. The DFT and experimental results obtained by Watanabe *et al.* for Rhodamine 6G adsorbed on silver were used to identify peaks [67], as well as the provided R package implementation of the algorithm provided by Moores *et al.* [66] to fit the spectra. The peaks were modeled using pseudo-Voigt functions.

The experimental results and the resulting fits are displayed in Figure 6. The Au-Al<sub>2</sub>O<sub>3</sub> system did not show a clearly measurable signal related to the presence of R6G molecules, even after the films being subjected to plasma treatment. The behavior of this system is surely related to the poor crystallization of Au nanoparticles throughout the Al<sub>2</sub>O<sub>3</sub> matrix, in agreement with the structural analysis.

For Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub>, with and without plasma treatment, it is possible to observe peaks related to the presence of R6G. Figure 6b-i shows the Raman spectrum for Au-TiO<sub>2</sub>, without plasma treatment. When compared to the corresponding spectra of Ag-TiO<sub>2</sub> (Figure 6c-i), the latter have much higher intensities for the same Raman peaks (the plots are not in the same intensity scale). Therefore, the replacement of Au by Ag into a TiO<sub>2</sub> matrix enhances Raman signals, and the reason for this behavior is related to the higher nanoparticles' size that were obtained after the thermal treatment.

Furthermore, both plasmonic systems have shown enhancement of the R6G signals after plasma treatment. The effect of the plasma treatment is to further expose some of the embedded nanoparticles to the environment, increasing adsorption areas. This turns the thin films more sensitive to the presence of analytes, as can be confirmed by the respective Raman spectra.

From the obtained fits, the intensity of the Raman signal at 1363 cm<sup>-1</sup> on the Ag-TiO<sub>2</sub> thin films is around one order of magnitude below the fluorescence and around the same order on Au-TiO<sub>2</sub>. The R6G molecules have conventional Raman cross section of  $10^{-30}$  cm<sup>2</sup> per molecule, however, at the excitation wavelength used of 514.5 nm, the fluorescence cross section of the R6G is on the order of  $10^{-16}$  cm<sup>2</sup> per molecule [68]. So, it is possible to infer that the measured Raman signal of R6G has a cross section of around  $10^{-15}$  cm<sup>2</sup> and an enhancement of  $10^{15}$  on the Ag-TiO<sub>2</sub> system, while a cross section of  $10^{-16}$  cm<sup>2</sup> and an enhancement of  $10^{14}$  was estimated for the Au-TiO<sub>2</sub> system. However, the used wavelength is within the absorption band of the R6G, and therefore the signal enhancement comes from two sources: the substrate with metallic nanoparticles and the R6G Resonant Raman Scattering. At the used wavelength of 514.5 nm, the enhancement from Resonant Raman Scattering for R6G molecules is in the order of  $10^4$  [69]. Subtracting the resonant enhancement leaves the contribution of  $10^{11}$  for Ag-TiO<sub>2</sub> and  $10^{10}$  for Au-TiO<sub>2</sub>.



**Figure 6** - Observed SERS spectra and the obtained fits for the baseline and total spectrum on a region dominated by R6G modes: (a) Au-Al<sub>2</sub>O<sub>3</sub>; (b) Au-TiO<sub>2</sub> and (c) Ag-TiO<sub>2</sub>, (i) without and (ii) with plasma treatment.

The results for the Raman peak position and relative intensities of the peaks are consistent among the different samples and also to the results reported by Watanabe *et al.* for R6G on Ag [67]. With the obtained intensities, the relative enhancement between the thin films was determined. Table 2

shows the results for the four most intense peaks and the corresponding enhancement factors. Only the results of the four most intense peaks were used, since they provided a more accurate fit than the remaining peaks that don't exhibit a so discernible profile.

Table 2 - Relative Raman intensities of the four most intense R6G peaks, and relative enhancement of each plasmonic thin film.

	Relative Intensity (%)				Relative
Sample	Peak 1	Peak 2	Peak 3	Peak 4	Enhancement
	(1363 cm <sup>-1</sup> )	(1509 cm <sup>-1</sup> )	(1575 cm <sup>-1</sup> )	(1651 cm <sup>-1</sup> )	Zimaneement
Ag-TiO <sub>2</sub>	91	71	51	100	36.6
Ag-TiO <sub>2</sub> w/plasma treatment	95	71	51	100	96.2
Au-TiO <sub>2</sub>	100	74	47	80	1.0
Au-TiO <sub>2</sub> w/plasma treatment	100	78	55	96	2.5

These results show that the plasma treatment itself provided a further enhancement of 2.6 times for the Ag-TiO<sub>2</sub> and 2.5 for the Au-TiO<sub>2</sub>, in relation to the same thin film without treatment. Yet, by comparing all the tested films, it is clear that the differences between the two systems are much higher, with relative enhancements that can be almost 2 orders of magnitude.

#### 4. Conclusions

In this work, three different systems of nanocomposite plasmonic thin films were deposited by reactive DC magnetron sputtering, changing the dielectric matrix and/or the nanoparticles' noble

metal. These changes allowed to produce the three plasmonic systems: Au-Al<sub>2</sub>O<sub>3</sub>, Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> and to study their differences in terms of chemical, morphological and optical responses.

The content in Au was different for the Al<sub>2</sub>O<sub>3</sub> and TiO<sub>2</sub> matrix, about 15 and 13 at.% respectively. The atomic content of Ag in the TiO<sub>2</sub> matrix was higher than Au for the same deposition conditions, about 17 at.%, as silver has a higher sputtering yield.

The structural analysis showed an amorphous Al<sub>2</sub>O<sub>3</sub> matrix independently of the post-deposition thermal annealing applied to the Au-Al<sub>2</sub>O<sub>3</sub> thin films, while the average size of Au crystalline domains was incremented very smoothly with the increase of annealing temperature. The TiO<sub>2</sub> matrix behaved differently from Al<sub>2</sub>O<sub>3</sub>, since crystalline domains of anatase, and rutile for the highest temperature (700 °C), were formed. The Au crystallization in the TiO<sub>2</sub> matrix started also at 300 °C, however the grain size suffered higher increments when compared to the Al<sub>2</sub>O<sub>3</sub> matrix, reaching average values 1 order of magnitude higher. Moreover, an even significant increase was observed in the average grain size of Ag in TiO<sub>2</sub>, where the Ag aggregates with sizes in the order of few hundred nm were estimated. Consequently, only the roughness of Ag-TiO<sub>2</sub> films suffered a significant increment due to the migration of Ag cluster to the surface.

Despite the relative low size of Au crystalline domains in Al<sub>2</sub>O<sub>3</sub> matrix, these films showed a plasmonic behavior already at 300 °C, showing narrow LSPR bands and a slight decrease of transmittance minimum with annealing temperature. The Au-TiO<sub>2</sub> films also exhibited a plasmonic behavior at 300 °C, with a LSPR peak shifted to higher wavelengths since the refractive index of TiO<sub>2</sub> is higher. A broadening of the LSPR band due to the contribution of larger (scattering) nanoparticles over smaller ones was also perceivable. In the thin films with Ag nanoparticles, a well-defined LSPR band was only visible at 500 °C, as this band gives place to a spectrum of almost constant transmittance (broadband absorption) after annealing at 700 °C, associated to the existence of fractal structures on the surface.

Due to their plasmonic behavior, all films were tested as SERS platforms. However, in order to select the best candidates for detection of *e.g.* microorganisms such as *C. albicans* it was pertinent to evaluate if contact with the surface of the films affect cell growth or viability during time. The results suggested no influence in growth behavior of the yeast cells as well as in viability, showing that is possible to use these thin films in biosensing approaches with living microorganisms.

As such, the performed SERS experiments showed that the presence of the Au-TiO<sub>2</sub> and Ag-TiO<sub>2</sub> thin films allowed the observation of R6G Raman scattering, with enhancements of 10<sup>10</sup> and 10<sup>11</sup>, respectively, while in Au-Al<sub>2</sub>O<sub>3</sub> films, this was not possible. The application of the plasma treatment provided a further enhancement of Raman signals, and the use of Ag nanoparticles instead of Au into a TiO<sub>2</sub> matrix provides a relative enhancement factor close to 10<sup>2</sup>.

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