1 **Development of Localized Surface Plasmon Resonance biosensors for the detection of**

2 ***Brettanomyces bruxellensis in wine***

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Abstract

Incident light interacting with noble-metal nanoparticles with smaller sizes than the wavelength of the incident light induces Localised Surface Plasmon Resonance (LSPR). In this work a gold nanostructured surface was used for the immobilization of a 5' end Thiol modified DNA probe to develop a LSPR nanobiosensor for the detection of the spoiler wine yeast *Brettanomyces bruxellensis*. Gold was evaporated to obtain a gold thickness of 4 nm. 2 μL of the DNA from the target microorganism and the negative control at various concentrations were used to test the specificity and sensitivity of the LSPR technique. Changes in the optical properties of the nanoparticles due to DNA-probe binding are reflected in the shift of LSPR extinction maximum (λ_{max}). The results obtained using as target microorganism *B. bruxellensis,* and as negative control *S. cerevisiae* demonstrated the specificity of both the DNA-probe and the protocol. The LSPR spectrophotometry technique detect 0.01 ng/μL DNA target confirming the possibility to utilise this system for the detection of pathogen microorganisms present in low amount in food and beverage samples.

1. Introduction

In the last decade the optical properties of noble metal nanoparticles that exhibit unique extinction spectra induced various authors to explore alternative strategies for the development of optical biosensors. The interaction between metals and biomolecules opened a wide field of applications for biosensors, from ecology, to medicine and food analysis. Moreover, optical properties of gold nanostructures, which show high chemical stability, allowed the utilization of Localized Surface Plasmon Resonance (LSPR) for the construction of sensitive biosensors [1].

LSPR biosensors, analogously to SPR sensors, based on LSPR spectroscopy, transducing small changes in the refractive index near a nanoscale noble metal surface into a measurable wavelength shift [2, 3]. LSPR is a collective oscillation of the conduction band electrons at the nanoparticles' surface that develops when incident electromagnetic radiation is of appropriate frequency. The properties of the particles (i.e. size, shape and dielectric function) influence the plasmonic oscillation that occurs at a specific resonance wavelengths [4]. The possibility to change these parameters allows the application on various fields such as DNA detection. Biological molecules can be detected as their presence induces a modification of refractive index near the metal surface. All conditions and parameters have to be optimized to obtain repeatability and sensitivity of the LSPR biosensors. Metal nanoparticles size, shape, interparticle separation, and metal nanoparticle fabrication parameters (time, temperature, thickness of metal deposition layer, etc.) are important points to optimize for the obtained specificity and sensitivity. Thus the homogeneity of the nanoparticles has to be evaluated, mostly by using a Scanning Electron Microscope (SEM).

One important characteristic of the LSPR biosensors is the high sensitivity that they can reach due to the interaction of the biomolecules with highly localized fields, one application being the detection of DNA. As demonstrated by Spadavecchia et al. [5] using labelling strategies such as the utilization of gold nanorods (AuNRs) and gold nanostars (AuNSs) the LSPR biosensor reach a high sensitivity.

One other characteristic of these biosensors is that they can be useful for high throughput monitoring both in proteomics and DNA research [6] promising screening platforms in a highly minaturized format that requires small volumes of analyte solutions [7, 10]. The common way to detect microorganisms present in samples using DNA as target is based on the utilization of one labeled DNA molecules, mostly by fluorescent tags, to increase sensitivity.

Common methods based on ELISA assays, and PCR using the specific hybridization of the target DNA to one or two specific probes, while when biosensors use the amplicon as a target, the amplification step cause a delay in the achievement of the results [8].

The aim of this work was the development of LSPR nanobiosensors for the rapid and sensitive detection of *Brettanomyces bruxellensis*, a spoiler yeast worldwide well-known to produce unpleasant aromas in wine. As Au NPs have been the system-of-choice in preparation of LSPR biosensors, a gold nanostructured surface was prepared and used for the immobilization of either a specific or non-specific DNA probe used as receptor for the target DNA molecule extracted from wine yeasts.

2. Materials and methods

2.1 Materials and equipment

The reagents utilised for the preparation of the buffer solutions listed below were acquired from Sigma–Aldrich (Switzerland). Three buffers: 1X PBS (10X, NaCl 1.5 M, Na_2HPO_4 anhydrous 81 mM, NaH_2PO_4 anhydrous 19 mM); 1X SSPE (20X: NaCl 3M, NaH_2PO_4 anhydrous 230 mM, EDTA x $2H_2O$ 25 mM and 1X TRIS-HCl (10X, Tris-HCl 0.5 M) were compared for their contribution over the biofunctionalization steps of the gold nanostructured surfaces. A complex made using the Thiol capture probe at 100 ng/µL and DNA from the target yeasts at 10 ng/µL were used to select the buffer for the hybridisations.

All LSPR measurements were obtained by using a home-built optical extinction setup [9].

2.2 Preparation of gold nanoparticles on glass slides and Scanning Electron Microscope (SEM) measurements

Glass slides (Carol Roth & Co. KG, Germany) were cut at the size of about 25×8 mm^2 with a diamond tip, washed in a solution of Decon 90 and deionized water (18.2 Ω) (2:8, v/v ratio) into an ultrasonic water bath at 50°C for 15 min according to [10].

Commercial TEM copper grids (200 mesh, diameter of 3.05 mm grids)(Strata Tek^{TM} Double Folding Grids, Ted Pella, Inc.) with double folding were fixed with scotch-tape onto the processed glass slides for a maximum of 3 double grids located on the same glass slide prior to gold evaporation, to obtain a pattern of 100 or 200 well per grid. Gold was evaporated by using a MEB 400 (PLASSYS, France) evaporator at the following conditions: 1.0×10^{-6} Torr at 40°C, and an evaporation rate of 0.08 nm/s to obtain a gold thickness of 4 nm. After evaporation, the TEM masks were removed and the gold covered glass slides were transferred in an oven (Naberthem, Germany) at 500 °C for 8 h to allow the formation of the gold nanopaticles (NPs) by thermal annealing before a washing step with an acetone-ethanol (1:1) mixture in an ultrasonic water bath at 30 °C for 30 min. To verify the gold surface of bare gold nanoparticles and their interspatial distance Scanning Electron Microscope analysis were made using a field emission scanning electron microscopy (Raith, SEM-FEG-eLine, France) at the following conditions: accelerating voltage of 10 kV, working distance of 8.8 mm, samples covered with an ultrathin layer (3 nm) of palladium by sputter-coating to suppress the charging effect.

2.3 DNA probes

The specific DNA probe for *B. bruxellensis* (Thiol-Brett-probe)(MWG, GmbH Ebersberg, Germany) [11, 12] was modified at 5' end by the addition of a thiol group: 5'-ThiC$_6$-TGTTTGAGCGTCATTTCCTTCTCAThiC6TGTTTGAGCGTCATTTCCTTCTCACTATTTAGT GGTTATGAGATTACACGAGG -3'.

116   A DNA sequence complementary to the Thiol-labelled probe was used as a positive control. The

117   Thiol-Brett-probe was used at 100 ng/µL, while the sequence complementary to the capture probe

118   was standardized at 0.01, 0.1, 1 and 10 ng/µL prior to be used.

119   *2.4 Biofunctionalization of gold nanoparticles*

120   The immobilization of the DNA probes on the surface of the gold nanoparticles (NPs) was realized

121   through thiol chemistry. The Thiol-Brett probe (capture probe) was used at 100 ng/µL.Then, 3 µL

122   of the capture probe, previously denatured at 95°C for 5 min, were allowed to bind to the gold

123   nanoparticles using 1X SSPE buffer at 4°C for 1, 2, and 6 h each, in order to optimize the probe-

124   NPs hybridization conditions for the biofunctionalization of the glass slides.

125   *2.5 Microorganisms and DNA samples analysis*

126   Pure yeast strains cultures of *Brettanomyces bruxellensis* DSM70726 (Deutsche Sammlung von

127   Microorganismen und Zellkulturen GmbM, Braunschweig, Germany) as positive sample, and

128   *Saccharomyces cerevisiae* DSM70424 as negative sample, were used for testing the specificity and

129   sensitivity of the biosensor. The yeasts were cultured in Malt Extract broth (Oxoid, Milan, Italy)

130   and Malt Extract Agar (Oxoid) prior to be used for the experiments. The DNA of the reference

131   strains was extracted and purified from one millilitre of overnight broth culture using the Wizards

132   Genomic DNA Purification Kit (Promega, Milan, Italy) [11]. The purity and concentration of the

133   DNA samples were evaluated by using a Varian Cary 100 UV-Vis spectrophotometer (Agilent

134   Technologies). DNA samples were standardized using sterile ddwater.

135   LSPR experiments were carried out using 2 µL of the DNA sequence complementary to the capture

136   probe (positive control) at   0.01, 0.1, 1 and 10 ng/µL to optimize the sample-capture probe

137   hybridization step. Finally, 2 µL of the DNA samples from two selected yeasts (*B. bruxellensis* and

138   *S. cerevisiae*) at 0.01, 0.1, 1 and 10 ng/µL were used to test the specificity and sensitivity of the

139   LSPR technique. Both the complementary sequence and DNA samples from *B. bruxellensis* and *S.*

140   *cerevisiae* were added to the biofunctionalized glass slide after denaturation at 95°C for 5 min, and

141   allowed to hybridize to the capture probe for 2 h at room temperature.

142   *2.4 LSPR measurements and processing data*

143   The LSPR measured the extinction spectrum (absorption + scattering) of the nanoparticles obtained

144   by recording the intensities of transmitted radiation for the different wavelengths of a white light

145   source. The wavelength shift and optical density for bio-functionalization steps were compared.

146   Changes in the optical properties of the nanoparticles due to DNA-probe binding are reflected in the

147   shift of LSPR extinction maximum (λ_{max}). Each different concentration of the DNA was deposited

148   onto one TEM grid. Specifically, four patterns were chosen for each grid located on the glass slide,

149   and LSPR measurements on the same patterns after bio-functionalization steps were made. The

150   resulted LSPR spectra were measured and compared with those ones of clean NPs (NPs with no

151   linked DNA-probe). SpectraSuite-Spectrometer Operating Software was used to acquire LSPR data

152   and register the maximum spectra extinction. Furthermore, all the aquired data were processed with

153   software Origin Pro 8.5.

154

155   **3. Results and Discussions**

156   *3.1 Optimization of the buffer for LSPR measurements*

157   The standard extinction spectrum of bare NPs measured from four patterns /TEM grid, among 3

158   independent grids, had an average of 552.76 nm with an Optical Density (OD) of 0.2094.

159   Generally, after each biofunctionalization using the DNA-probe complex of Au NPs, the three

160   resulted resonant wavelengths had a red shift compared to bare NP's. Thus, the average wavelength

161   of the DNA samples in the presence of TRIS-HCl was 557.43 nm, of PBS was 566.33 nm and of

162   SSPE was 574.45 nm, respectively (Table 1). Such results imply a difference of the peak shift when

163   using gold nanoparticle of 4.67 nm, 13.57 nm and 21.69 nm, for TRIS-HCl, PBS, and SSPE buffer,

164   respectively. Interestingly, the 1X SSPE buffer showed the highest amplitude peak with 0.3545 OD

165   and the greatest increase of LSPR spectra, thus it was selected for the subsequent experiments. The

166   LSPR extinction spectra measured for pure NPs (Au NPs) and biofunctionalized Au NPs using the

167   TRIS-HCl, PBS and SSPE buffers at the same conditions are reported in Figure 1.

168

3.2 SEM characterization

By using Scanning Electron Microscopy (SEM) imaging characterization, it was found that gold nanoparticles are homogeneous prepared at 500°C. Thus, such gold structured substrates were chosen for LSPR experiments (Figure 2).

3.3 Effect of the time and temperature on biofuncionalization

The average wavelength extinction for the bare AuNPs before functionalization was 555.75 nm. The average wavelength red shift recorded increased accordingly after the capture probe immobilization onto the AuNPs, (Figure 3). After 1 h the average wavelength was 560.73 nm, while after 2 h it was 566.50 nm and finally, after 6 h it was 569.23 nm. In other words the shift calculated from bare NPs was 4.98 nm, 10.75 nm and 13.48 nm respectively (Table 2). On other hand, the OD values increased from 0.2928 OD for AuNPs to 0.3873 OD after hybridization of the probe after at 6 h. Based on the data reported previously the time selected for the immobilization of the capture probe was 1 h because it was considered sufficient to obtain a shift that could indicate the binding of the DNA probe to the NPs.

3.4 Short complementary sequence

The average wavelength extinction was 554.72 nm and the OD was 0.2597 OD for Au NPs. The average wavelength extinction for the biofunctionalized glass surface was 557.5 nm, and after short sequences hybridization at 0.01 ng/µL, 0.1 ng/µL, 1 ng/µL and 10 ng/µL was, 557.93 nm, 558.01 nm, 559.37 nm and 560.89 nm respectively, as reported in Table 3. Slight shifts were obtained for the short sequences as shown in Figure 4.

3.5 Specific detection of B. bruxellensis

The average wavelength for bare NPs was 546.00 nm, and 549.87 nm after biofunctionalization.

194   The average wavelength extinction corresponding to the hybridization of *B. bruxellensis* DNA

195   concentrations of 0.01, 0.1, 1 and 10 ng/µL was 551.23 nm, 553.36 nm, 562.78 nm and 568.27 nm

196   respectively, as reported in Table 4. The red shift obtained from the described concentrations was

197   3.87 nm. 5.23 nm, 7.36 nm, 16.78 nm and 22.27 nm, as shown in Figure 5.

198

199   *3.6 Control experiments using DNA probe from S. cerevisiae*

200   The average wavelength obtained for *S. cerevisiae* used as negative control was 564.59 nm for the

201   Au NPs (Optical Density of 0.236), followed by a value of 568.62 nm after biofunctionalization,

202   and it was 569.57 nm, 568.77 nm, 569.58 nm and 569.23 nm using 0.01, 0.1, 1 and 10 ng/µL DNA

203   respectively, as reported in Table 5. The red shift obtained for the concentrations was 4.03 nm, 4.98

204   nm, 4.18 nm, 4.99 nm and 4.64 nm. The value of the extinction was not affected by the DNA

205   concentrations used, and the red shift was not relevant as shown in Figure 6**.** The results obtained

206   using as target microorganism *B. bruxellensis,* and as negative control *S. cerevisiae* demonstrated

207   the specificity of both the probe and the protocol. Moreover, the washing steps with ddH$_2$O, avoid

208   false signals due to the presence of target DNA not bound to the Au NPs annealed probe, A cut off

209   value for the extinction wavelength values obtained was defined due to the lck of a proportional

210   shift wavelength and optical density related to DNA concentrations used during the experiments.

211   This value allows to distinguish the positive samples from the negative one. The utilization of the

212   LSPR technique for the detection of the DNA extracted from the wine spoiler yeast allowed to

213   avoid the utilization of fluorescence molecules in the protocol, and to reduce from two to one the

214   number of DNA probes commonly utilised. In fact, it was sufficient to modify the 5'-end of the

215   probe to bind it to the gold surface to obtain a high sensitvity.

216

217

218   **4. Conclusions**

219   An optimized protocol for the LSPR-detection of *B. bruxellensis* onto gold nanostructured

220   substrates by using a specific Thiol-labelled DNA probe is reported. Moreover, the LSPR

221   spectrophotometry technique confirmed it is possible to detect 0.01 ng/μL DNA target. A clear

222   discrimination between the specific hybridization with DNA *B. bruxellensis* and the nonspecific

223   binding with *S. cerevisiae* DNA was successfully demonstrated using the genomic DNA extracted

224   from the pure cultures and without the requirement of probe labeling procedures. Due to its high

225   sensitivity, the LSPR nanobiosensor could be an elegant alternative for the detection of pathogen

226   microorganisms present in low amount in food and beverage samples.

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277

278 **Table 1: Plasmonic properties (extinction and wavelength) were measured in 4 patterns /grid**

279 **for each buffer using gold nanoparticle and the gold NPs annealed to DNA (10 ng/µL) - probe**

280 **(100 ng/ /µL).**

281

	Resonant wavelength (nm), maximum extinction of the patterns:				Average wavelength extinction	Relative Standard Deviation %	
Buffer	**1**	**2**	**3**	**4**		**Wavelength**	**Extinction**
TRIS-HCl	556.27	557.05	557.05	559.37	557.43	0.24	5.31
	0.25	0.22	0.24	0.24	0.24		
PBS	565.56	567.11	564.79	567.88	566.33	0.25	0.46
	0.29	0.29	0.29	0.28	0.29		
SSPE	574.07	575.61	574.84	573.30	574.45	0.17	1.70
	0.34	0.36	0.35	0.36	0.35		

282

283 **Table 2. Plasmonic properties (extinction and wavelength) of the biofunctionalization steps**

284 **(hybridization of the probe at 100 ng/ /μL) to the glass golden surface) were measured in 4**

285 **pattern after 1, 2 and 6 h.**

Time	measure	Resonant wavelength(nm), maximum extinction of the patterns				Average wavelength extinction	Relative Standard Deviation %	
		1	**2**	**3**	**4**		Wavelength	Extinction
1 h	λ	560.15	562.47	560.15	560.15	560.73	0.20	2.32
	OD	0.32	0.34	0.32	0.32	0.32		
2 h	λ	567.88	567.11	566.24	564.79	566.50	0.23	2.55
	OD	0.34	0.33	0.33	0.32	0.33		
6 h	λ	568.66	570.2	570.2	567.88	569.23	0.20	2.38
	OD	0.38	0.39	0.38	0.37	0.38		

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287

288 **Table 3. Plasmonic properties (extinction and wavelength) of the short sequences (SS)**

289 **(sequences complementary to the capture probe) were measured in four patterns after 1, 2**

290 **and 3 h hybridization. Capture probe (CP) was used at 100 ng/µL. Values of the short**

291 **sequences are expressed in ng/µL.**

292

sample	measure	Resonant wavelength(nm), maximum extinction of the patterns:				Average wavelength extinction	Relative Standard Deviation (RSD%)	
		1	2	3	4		Wavelength	Extinction
CP	λ	555.5	560.15	560.4	553.95	557.5	0.58	6.62
	OD	0.24	0.28	0.25	0.25	0.25		
SS 0.01	λ	557.82	557.05	558.6	558.27	557.93	0.12	4.98
	OD	0.29	0.31	0.29	0.28	0.29		
SS 0.1	λ	556.27	559.37	559.37	557.05	558.01	0.28	7.51
	OD	0.27	0.31	0.32	0.29	0.30		
SS 1	λ	558.6	559.37	560.15	559.37	559.37	0.11	7.24
	OD	0.27	0.32	0.32	0.31	0.3		
SS 10	λ	560.15	560.82	560.92	561.69	560.895	0.11	5.82
	OD	0.33	0.29	0.30	0.33	0.31		

293

294

Table 4. Plasmonic properties (extinction and wavelength) are measured in four patterns for each DNA *B. bruxellensis* concentration used. Values of DNA samples are expressed in ng/µL. Capture probe (CP) was used at 100 ng/µL.

sample	measure	Resonant wavelength(nm), maximum extinction of the patterns:				Average wavelength extinction	Relative Standard Deviation %	
		1	2	3	4		Wavelength	Extinction
CP	λ	548.52	550.07	550.07	550.85	549.87	0.17	1.85
	OD	0.18	0.18	0.18	0.18	0.18		
DNA 0,01	λ	552.4	550.07	552.4	550.07	551.23	0.24	1.22
	OD	0.18	0.18	0.18	0.18	0.18		
DNA 0,1	λ	553.17	553.95	553.17	553.17	553.36	0.07	6.25
	OD	0.19	0.19	0.21	0.19	0.19		
DNA 1	λ	563.24	560.15	565.27	562.47	562.78	0.37	8.61
	OD	0.25	0,24	0.22	0.27	0.24		
DNA 10	λ	567.11	569.43	567.11	569.43	568.27	0.23	2.32
	OD	0.26	0.27	0.26	0.27	0.26		

299 **Table 5. Plasmonic properties (extinction and wavelength) are measured in four pattern for**

300 **each *S. cerevisiae* DNA concentration. Values expressed in ng/µL. Capture probe was used at**

301 **100 ng/µL.**

302

303

sample	measure	Resonant wavelength(nm), maximum extinction of the patterns:				Average wavelength extinction	Relative Standard Deviation %	
		1	2	3	4		Wavelength	Extinction
CP	λ	570.75	568.72	567.88	567.13	568.62	0.27	1.67
	OD	0.25	0.25	0.25	0.25	0.25		
DNA 0,01	λ	570	567.88	568.66	571.75	569.57	0.29	1.80
	OD	0.25	0.25	0.24	0.25	0.25		
DNA 0,1	λ	568.34	567.88	567.88	570.98	568.77	0.26	4.51
	OD	0.24	0.25	0.25	0.27	0.25		
DNA 1	λ	568.66	569.43	570.11	570.12	569.58	0.12	0.85
	OD	0.25	0.25	0.25	0.25	0.25		
DNA 10	λ	571.75	567.54	568.66	569.00	569.23	0.31	3.79
	OD	0.26	0.24	0.25	0.25	0.25		

304

305 **Figure 1. LSPR spectra for bare gold nanoparticle and the gold NPs annealed to DNA (10**

306 **ng/μL) - probe (100 ng/ /μL) using SSPE, PBS and Tris-HCl buffer at the same experimental**

307 **conditions.**

308

309 **Figure 2. Scanning Electron Microscope (SEM images) of bare are gold nanoparticles.**

310

311 **Figure 3. LSPR spectra for immobilized probe at 100 ng/μL after 1, 2 and 6 h. Proportional**

312 **increase of the redshift wavelength with the time.**

313

314 **Figure 4. LSPR spectra of short sequence complementary to the Thiol - Brett probe (100**

315 **ng/μL)used at 0.01, 0.1, 1 and 10 ng/μL. A slight wavelength shift caused from the short length**

316 **of the sequences is present.**

317

318 **Figure 5. *Brettanomyces bruxellensis* LSPR spectra of bare Au NPs, *B. bruxellensis* DNA at**

319 **0.01, 0.1, 1 and 10 ng/μL. The hybridisation between the gold surface linked DNA Thiol probe**

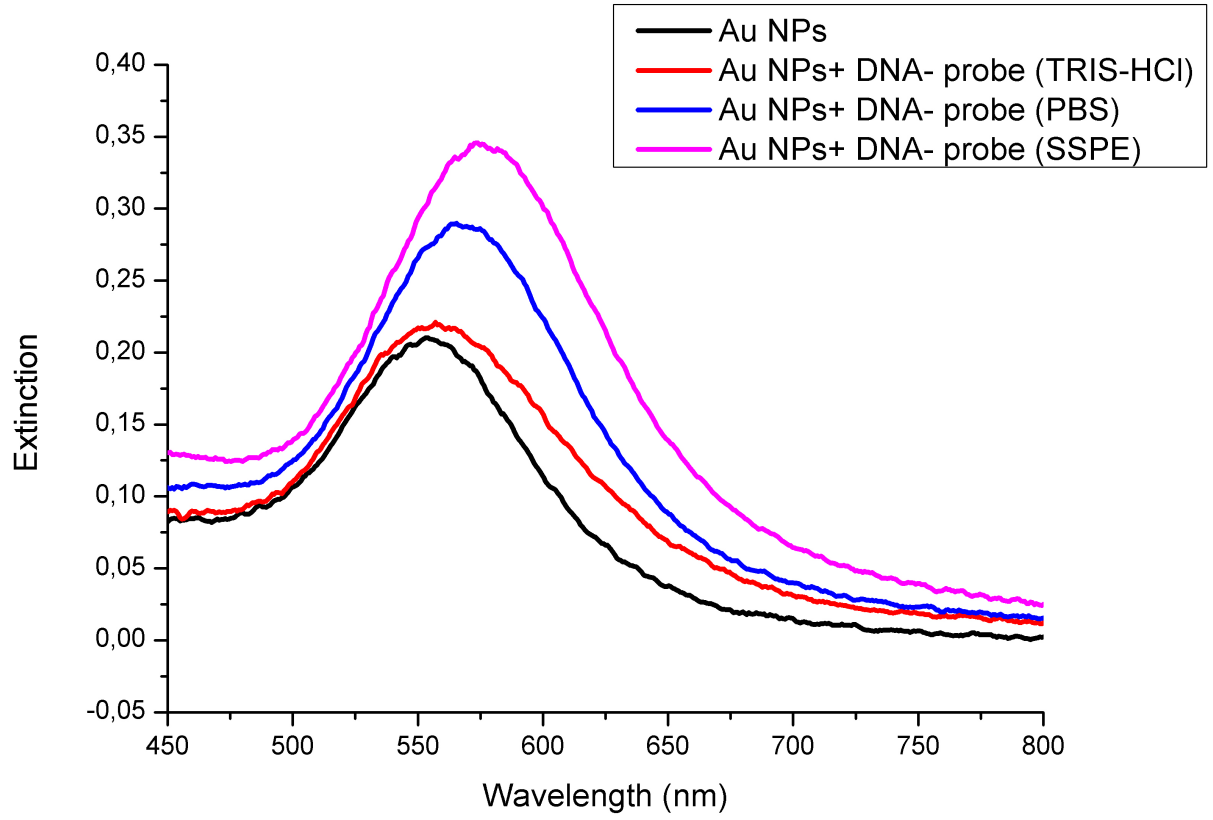320 **and the yeast DNAs was conducted at room temperature for 2 h.**

321

322 **Figure 6. *S. cerevisiae* LSPR spectra of bare Au NPs, *S. cerevisiae* DNA at 0.01, 0.1, 1 and 10**

323 **ng/μL. The hybridisation between the gold surface linked DNA Thiol probe and the yeast**

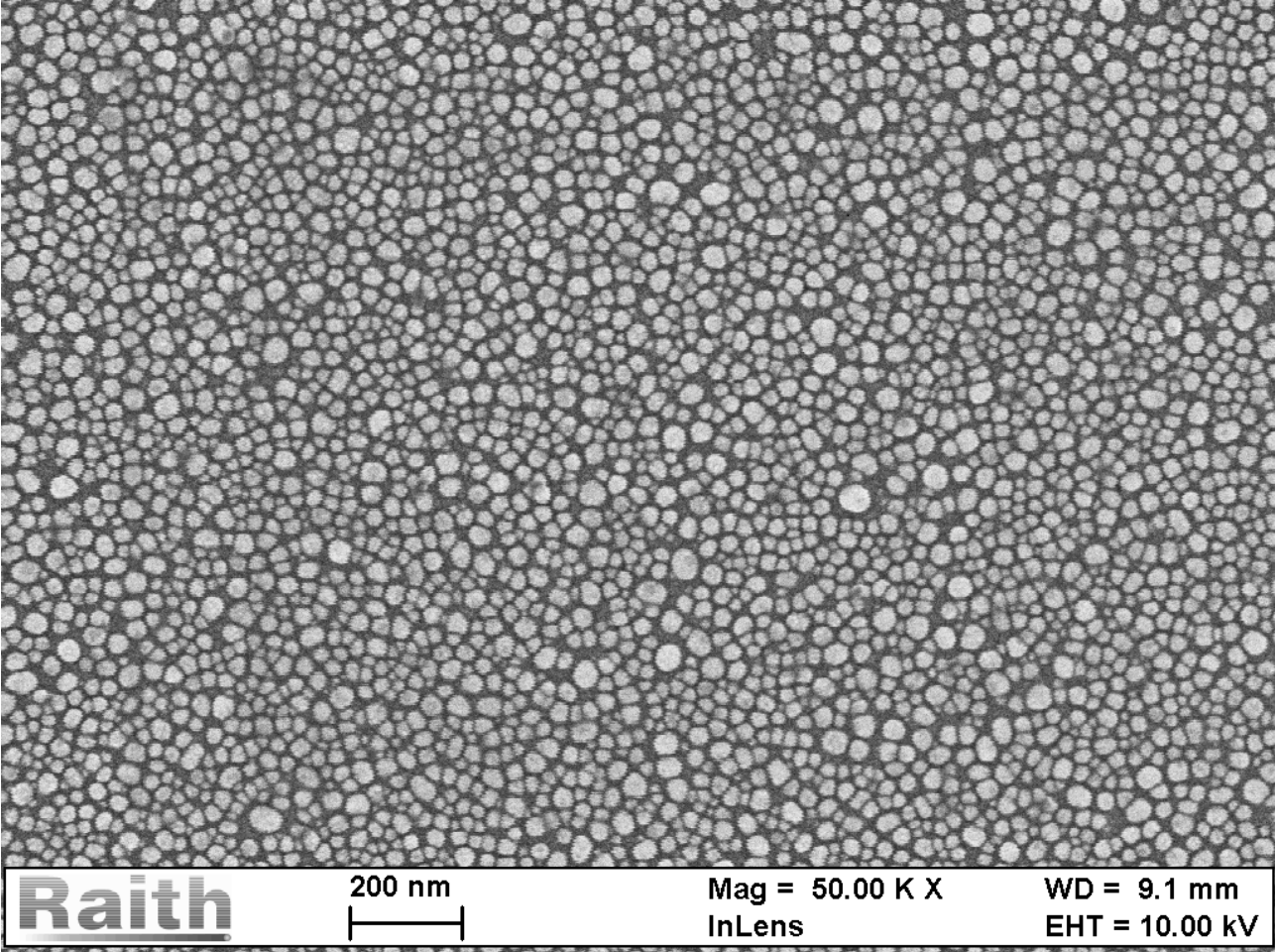324 **DNAs was conducted at room temperature for 2 h.**

325

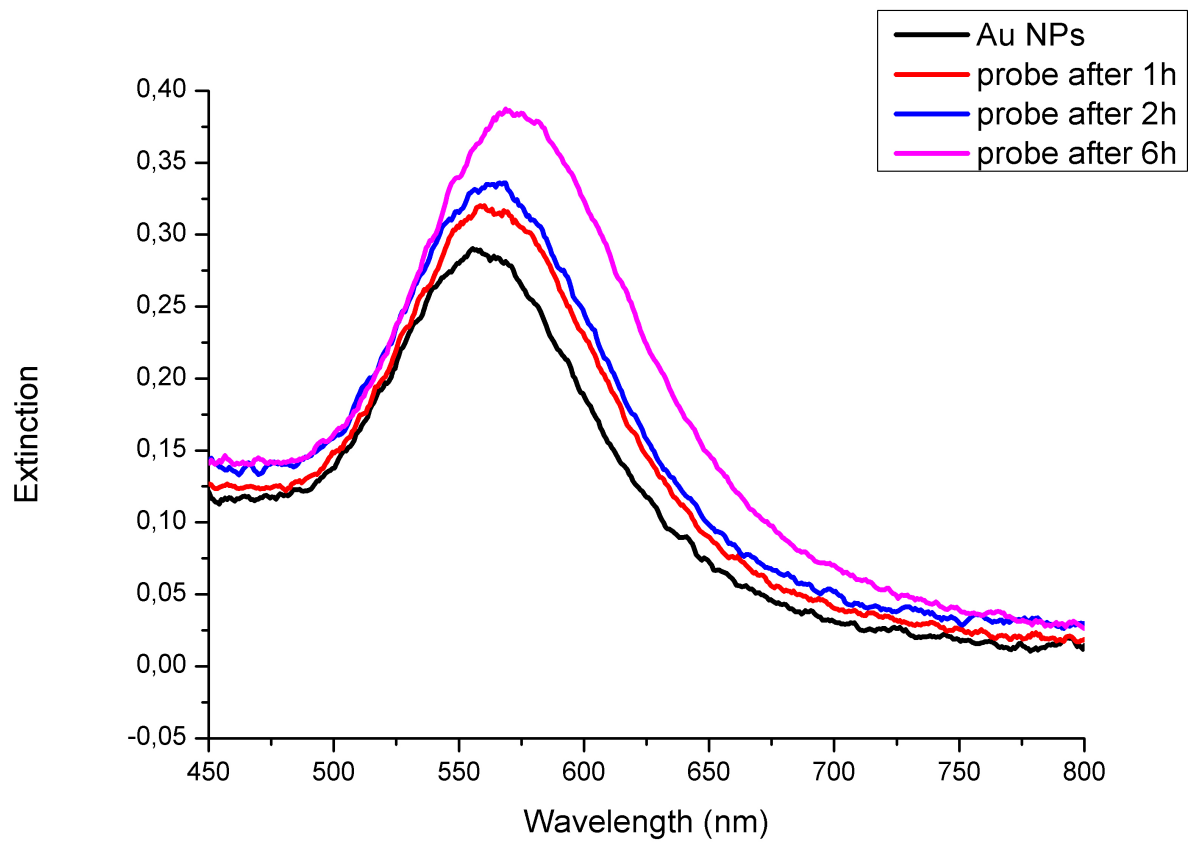326

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328 **Figure 1**

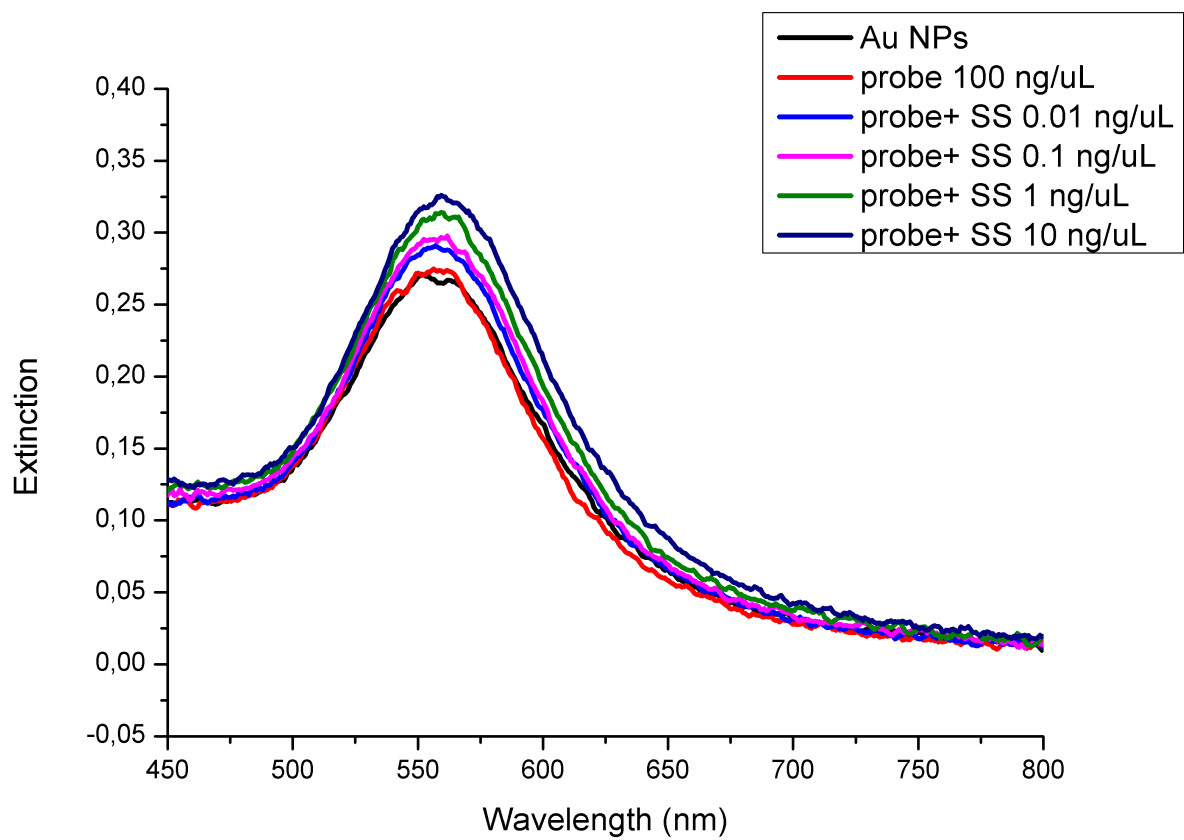329

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333 **Figure 2**

334

Figure 3

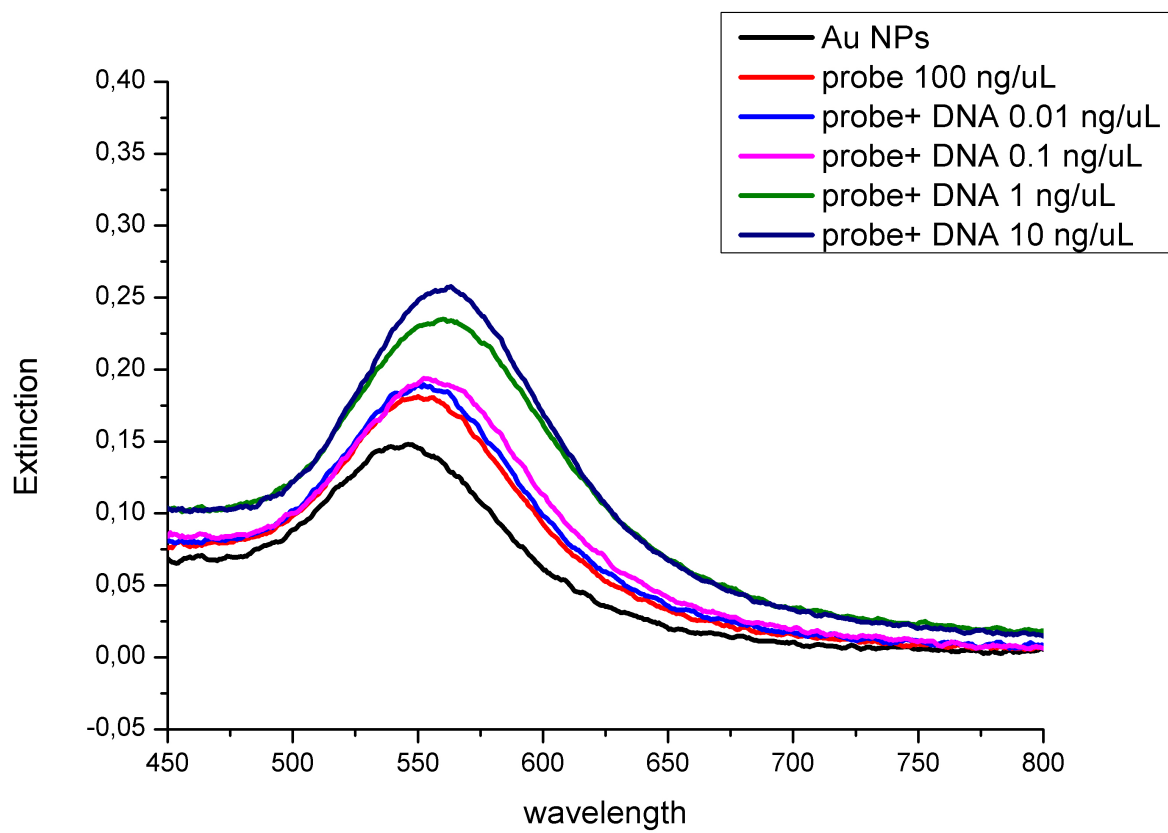336

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Figure 4
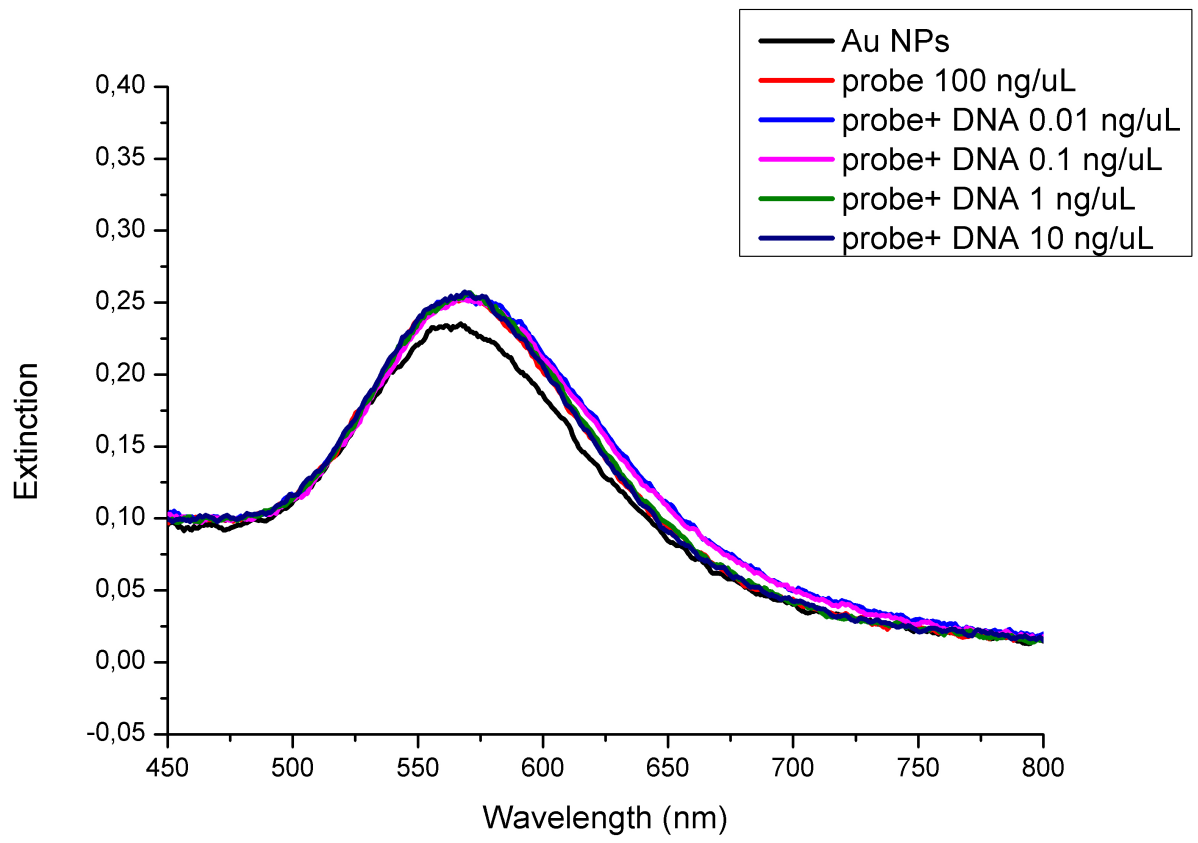
341   **Figure 5**

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Figure 6

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