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**Development of Localized Surface Plasmon Resonance biosensors for the detection of
Brettanomyces bruxellensis in wine**

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23 **Abstract**

24 Incident light interacting with noble-metal nanoparticles with smaller sizes than the wavelength of
25 the incident light induces Localised Surface Plasmon Resonance (LSPR). In this work a gold
26 nanostructured surface was used for the immobilization of a 5' end Thiol modified DNA probe to
27 develop a LSPR nanobiosensor for the detection of the spoiler wine yeast *Brettanomyces*
28 *bruxellensis*. Gold was evaporated to obtain a gold thickness of 4 nm. 2 μ L of the DNA from the
29 target microorganism and the negative control at various concentrations were used to test the
30 specificity and sensitivity of the LSPR technique. Changes in the optical properties of the
31 nanoparticles due to DNA-probe binding are reflected in the shift of LSPR extinction maximum (λ
32 $_{max}$). The results obtained using as target microorganism *B. bruxellensis*, and as negative control *S.*
33 *cerevisiae* demonstrated the specificity of both the DNA-probe and the protocol. The LSPR
34 spectrophotometry technique detect 0.01 ng/ μ L DNA target confirming the possibility to utilise this
35 system for the detection of pathogen microorganisms present in low amount in food and beverage
36 samples.

37
38 **Keywords:** Localized Surface Plasmon Resonance (LSPR), *Brettanomyces bruxellensis*,
39 genosensor, Scanning Electron Microscopy

40

41

42 **1. Introduction**

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

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

62 One important characteristic of the LSPR biosensors is the high sensitivity that they can reach due
63 to the interaction of the biomolecules with highly localized fields, one application being the
64 detection of DNA. As demonstrated by Spadavecchia et al. [5] using labelling strategies such as the
65 utilization of gold nanorods (AuNRs) and gold nanostars (AuNSs) the LSPR biosensor reach a high
66 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 miniaturized 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, Na₂HPO₄ anhydrous 81 mM, NaH₂PO₄ anhydrous 19 mM); 1X SSPE (20X: NaCl 3M, NaH₂PO₄ anhydrous 230 mM, EDTA x 2H₂O 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].

92 2.2 Preparation of gold nanoparticles on glass slides and Scanning Electron Microscope (SEM) 93 measurements

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

97 Commercial TEM copper grids (200 mesh, diameter of 3.05 mm grids)(Strata Tek™ Double
98 Folding Grids, Ted Pella, Inc.) with double folding were fixed with scotch-tape onto the processed
99 glass slides for a maximum of 3 double grids located on the same glass slide prior to gold
100 evaporation, to obtain a pattern of 100 or 200 well per grid. Gold was evaporated by using a MEB
101 400 (PLASSYS, France) evaporator at the following conditions: 1.0×10^{-6} Torr at 40°C , and an
102 evaporation rate of 0.08 nm/s to obtain a gold thickness of 4 nm. After evaporation, the TEM masks
103 were removed and the gold covered glass slides were transferred in an oven (Naberthem, Germany)
104 at 500°C for 8 h to allow the formation of the gold nanoparticles (NPs) by thermal annealing before
105 a washing step with an acetone-ethanol (1:1) mixture in an ultrasonic water bath at 30°C for 30
106 min. To verify the gold surface of bare gold nanoparticles and their interspatial distance Scanning
107 Electron Microscope analysis were made using a field emission scanning electron microscopy
108 (Raith, SEM-FEG-eLine, France) at the following conditions: accelerating voltage of 10 kV,
109 working distance of 8.8 mm, samples covered with an ultrathin layer (3 nm) of palladium by
110 sputter-coating to suppress the charging effect.

111 2.3 DNA probes

112 The specific DNA probe for *B. bruxellensis* (Thiol-Brett-probe)(MWG, GmbH Ebersberg,
113 Germany) [11, 12] was modified at 5' end by the addition of a thiol group: 5'-ThiC₆-
114 TGTTTGAGCGTCATTTCTTCTCATHiC₆TGTTTGAGCGTCATTTCTTCTCACTATTTAGT
115 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/ μ L, while the sequence complementary to the capture probe
118 was standardized at 0.01, 0.1, 1 and 10 ng/ μ L 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/ μ L. Then, 3 μ L
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 GmbH, 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 μ L of the DNA sequence complementary to the capture
136 probe (positive control) at 0.01, 0.1, 1 and 10 ng/ μ L to optimize the sample-capture probe
137 hybridization step. Finally, 2 μ L of the DNA samples from two selected yeasts (*B. bruxellensis* and
138 *S. cerevisiae*) at 0.01, 0.1, 1 and 10 ng/ μ L 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

169 3.2 SEM characterization

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

173

174 3.3 Effect of the time and temperature on biofunctionalization

175 The average wavelength extinction for the bare AuNPs before functionalization was 555.75 nm.
176 The average wavelength red shift recorded increased accordingly after the capture probe
177 immobilization onto the AuNPs, (Figure 3). After 1 h the average wavelength was 560.73 nm, while
178 after 2 h it was 566.50 nm and finally, after 6 h it was 569.23 nm. In other words the shift calculated
179 from bare NPs was 4.98 nm, 10.75 nm and 13.48 nm respectively (Table 2). On other hand, the OD
180 values increased from 0.2928 OD for AuNPs to 0.3873 OD after hybridization of the probe after at
181 6 h. Based on the data reported previously the time selected for the immobilization of the capture
182 probe was 1 h because it was considered sufficient to obtain a shift that could indicate the binding
183 of the DNA probe to the NPs.

184

185 3.4 Short complementary sequence

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

191

192 3.5 Specific detection of *B. bruxellensis*

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

The average wavelength extinction corresponding to the hybridization of *B. bruxellensis* DNA 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 respectively, as reported in Table 4. The red shift obtained from the described concentrations was 3.87 nm, 5.23 nm, 7.36 nm, 16.78 nm and 22.27 nm, as shown in Figure 5.

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

The average wavelength obtained for *S. cerevisiae* used as negative control was 564.59 nm for the Au NPs (Optical Density of 0.236), followed by a value of 568.62 nm after biofunctionalization, 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 respectively, as reported in Table 5. The red shift obtained for the concentrations was 4.03 nm, 4.98 nm, 4.18 nm, 4.99 nm and 4.64 nm. The value of the extinction was not affected by the DNA concentrations used, and the red shift was not relevant as shown in Figure 6. The results obtained using as target microorganism *B. bruxellensis*, and as negative control *S. cerevisiae* demonstrated the specificity of both the probe and the protocol. Moreover, the washing steps with ddH₂O, avoid false signals due to the presence of target DNA not bound to the Au NPs annealed probe, A cut off value for the extinction wavelength values obtained was defined due to the lack of a proportional shift wavelength and optical density related to DNA concentrations used during the experiments. This value allows to distinguish the positive samples from the negative one. The utilization of the LSPR technique for the detection of the DNA extracted from the wine spoiler yeast allowed to avoid the utilization of fluorescence molecules in the protocol, and to reduce from two to one the number of DNA probes commonly utilised. In fact, it was sufficient to modify the 5'-end of the probe to bind it to the gold surface to obtain a high sensitivity.

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.

227

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234

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 276
 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/ μ L) - probe**
 280 **(100 ng/ μ L).**

281

Buffer	Resonant wavelength (nm), maximum extinction of the patterns:				Average wavelength extinction	Relative Standard Deviation %	
	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/ μ L) to the glass golden surface) were measured in 4**
 285 **pattern after 1, 2 and 6 h.**

Time	measur e	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		

286

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.**

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		

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

298

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/ μ L) - probe (100 ng/ μ L) 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/ μ L 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/ μ L)used at 0.01, 0.1, 1 and 10 ng/ μ L. 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/ μ L. The hybridisation between the gold surface linked DNA Thiol probe**
320 **and the yeast DNAs was conducted at room temperature for 2 h.**

321

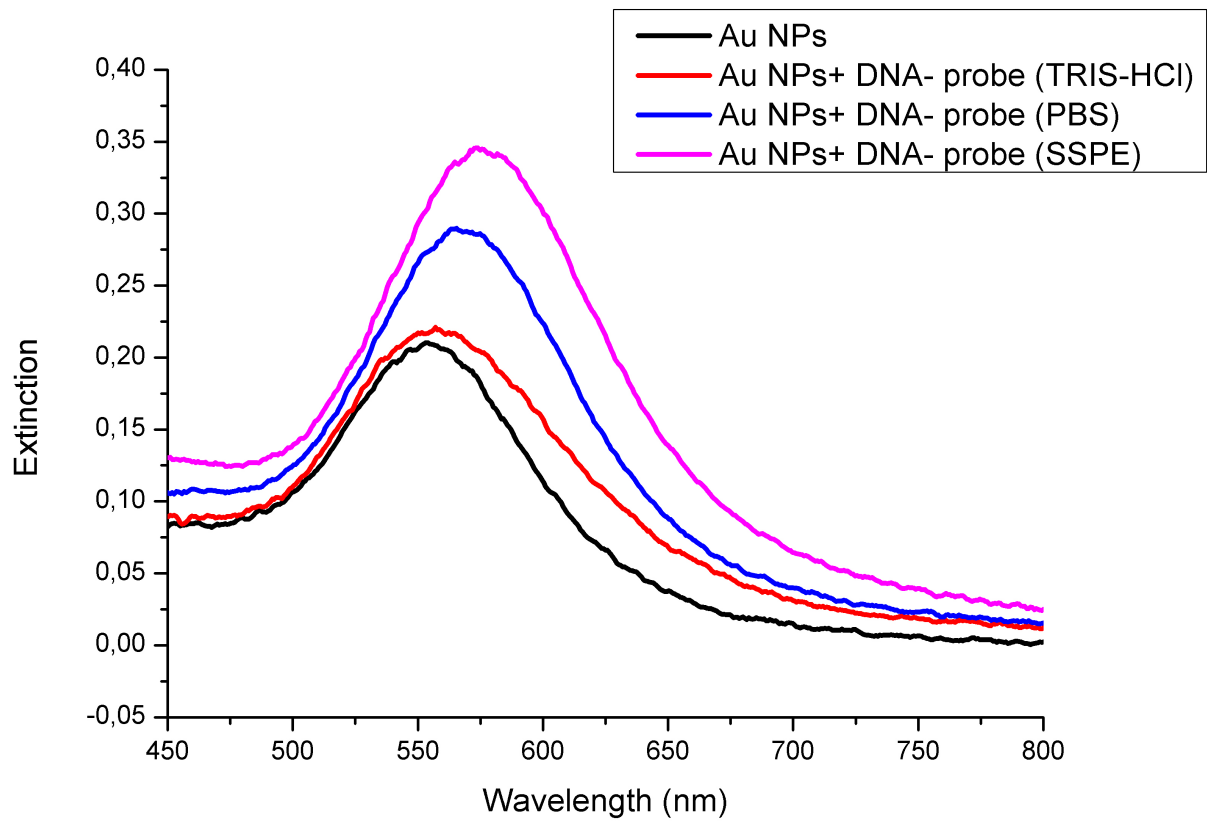
322 **Figure 6. *S. cerevisiae* LSPR spectra of bare Au NPs, *S. cerevisiae* DNA at 0.01, 0.1, 1 and 10**
323 **ng/ μ L. The hybridisation between the gold surface linked DNA Thiol probe and the yeast**
324 **DNAs was conducted at room temperature for 2 h.**

325

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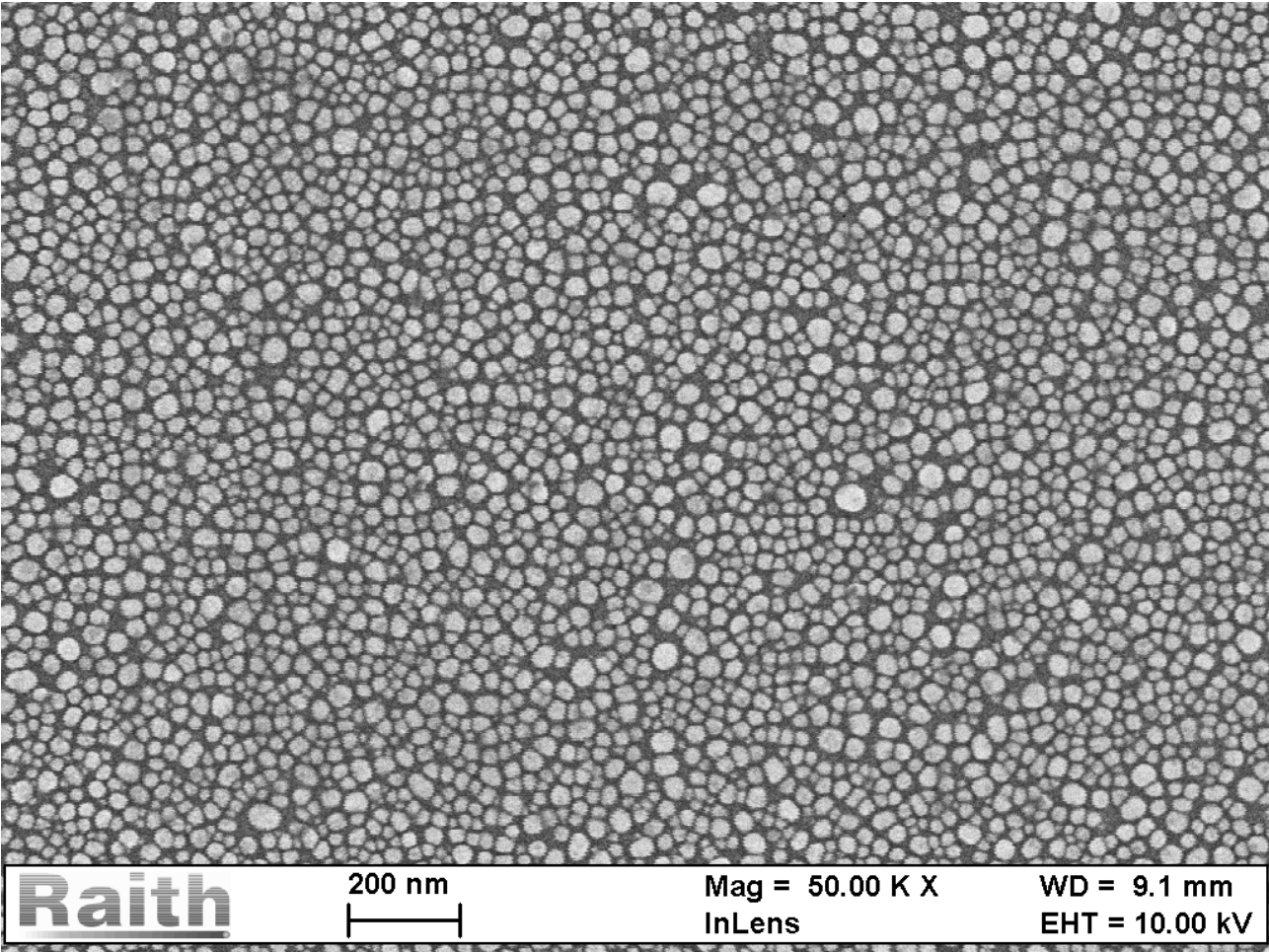
327

328 **Figure 1**



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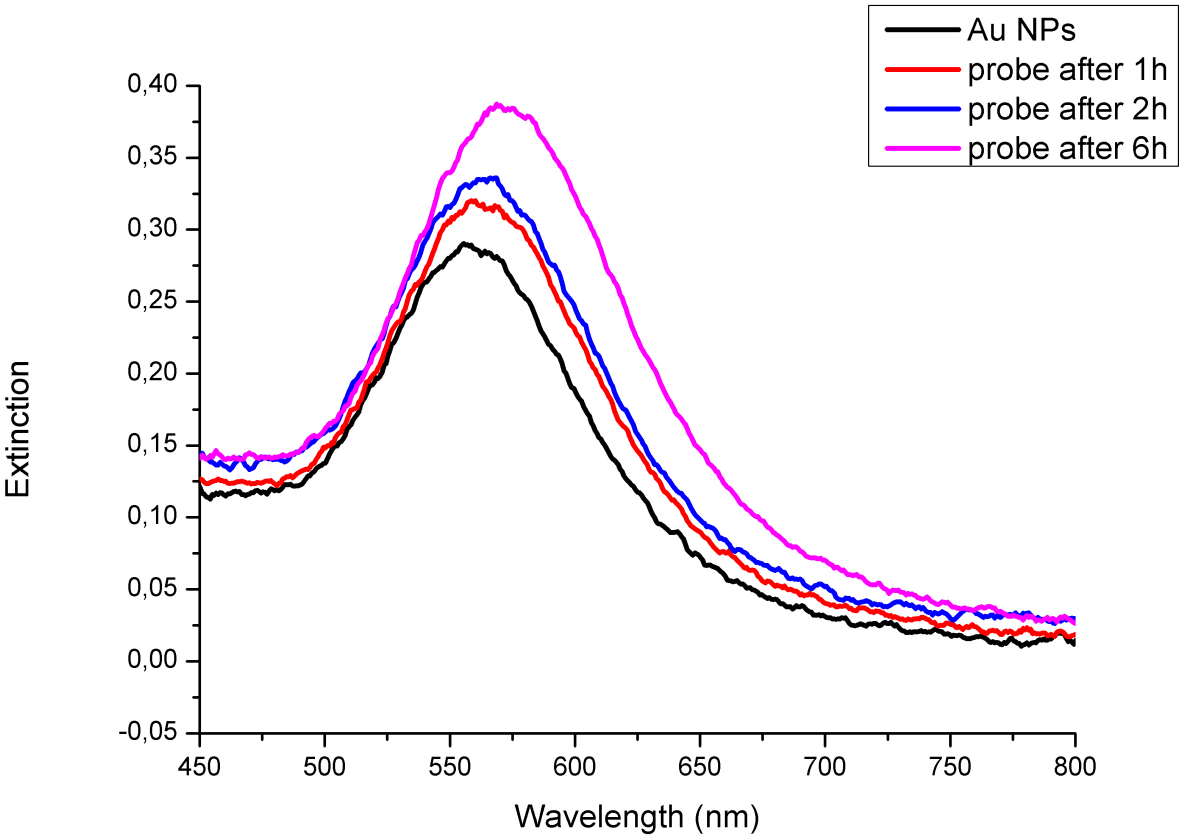
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333 **Figure 2**

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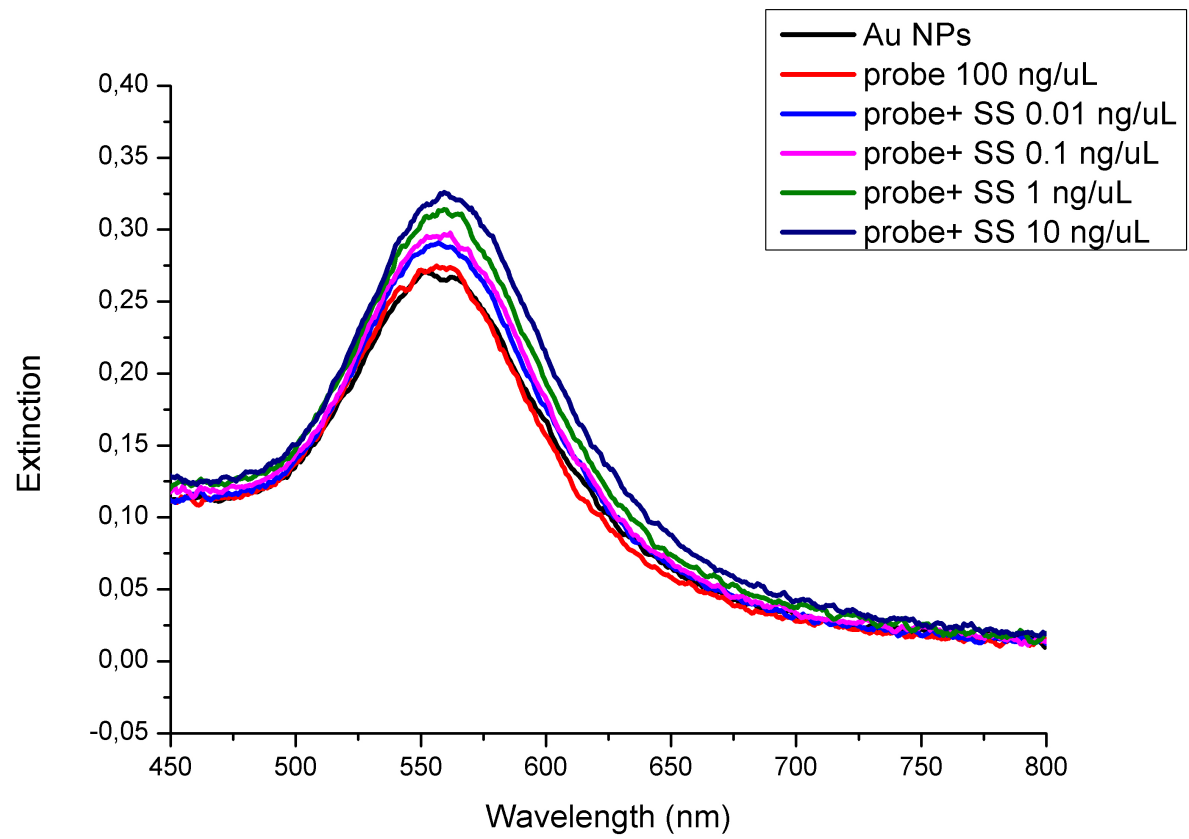
335 **Figure 3**



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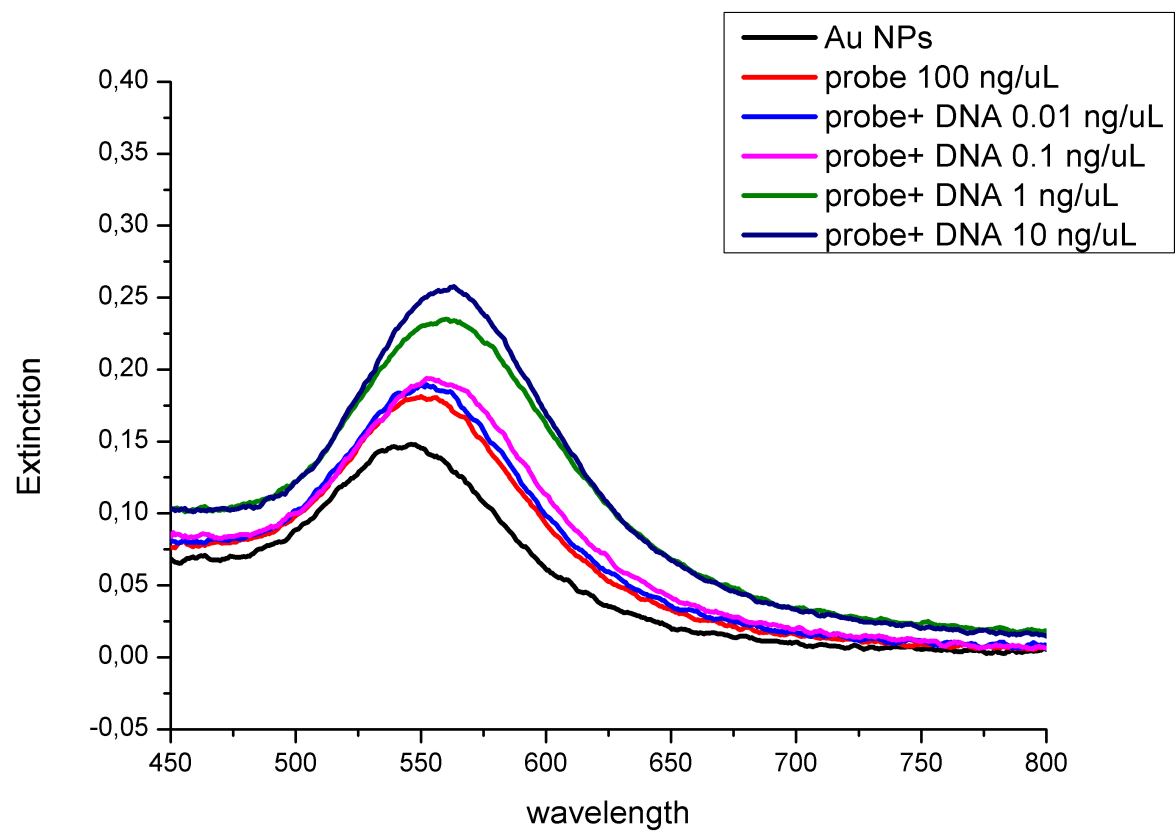
338 **Figure 4**



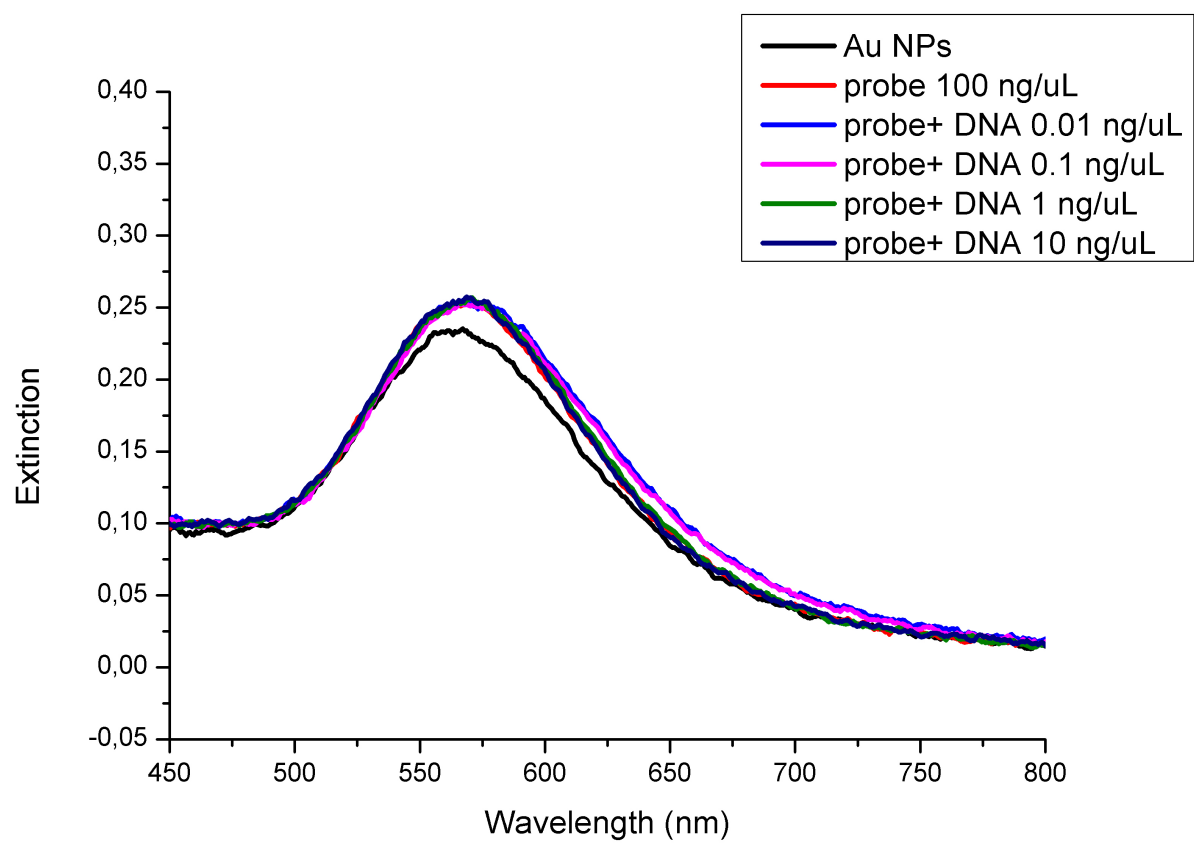
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341 **Figure 5**



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344 **Figure 6**

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