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Trends and Challenges of Refractometric

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Nanoplasmonic Biosensors

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10 **Abstract**

11

12 Motivated by potential benefits such as sensor miniaturization, multiplexing opportunities and higher
13 sensitivities, refractometric nanoplasmonic biosensing has profiled itself in a short time span as an
14 interesting alternative to conventional SPR biosensors. This latter conventional sensing concept has
15 been subjected during the last decades to strong commercialization, thereby strongly leaning on well-
16 developed thin-film surface chemistry protocols. Not surprisingly, the examples found in literature
17 based on this sensing concept are generally characterized by extensive analytical studies of relevant
18 clinical and diagnostic problems. In contrast, the more novel LSPR alternative finds itself in a much
19 earlier, and especially, more fundamental stage of development. Driven by new fabrication
20 methodologies to create nanostructured substrates, published work typically focuses on the novelty of
21 the presented material, its optical properties and its use – generally limited to a proof-of-concept – as a
22 label-free biosensing scheme. Given the different stages of development both SPR and LSPR sensors find
23 themselves in, it becomes apparent that providing a comparative analysis of both concepts is not a
24 trivial task. Nevertheless, in this review we make an effort to provide an overview that illustrates the
25 progress booked in both fields during the last five years. First, we discuss the most relevant advances in
26 SPR biosensing, including interesting analytical applications, together with different strategies that
27 assure improvements in performance, throughput and/or integration. Subsequently, the remaining part
28 of this work focuses on the use of nanoplasmonic sensors for real label-free biosensing applications.
29 First, we discuss the motivation that serves as a driving force behind this research topic, together with a
30 brief summary that comprises the main fabrication methodologies used in this field. Next, the sensing
31 performance of LSPR sensors is examined by analyzing different parameters that that can be invoked in
32 order to quantitatively assess their overall sensing performance. Two aspects are highlighted that turn

33 out to be especially important when trying to maximize their sensing performance, being 1) the targeted
34 functionalization of the electromagnetic hotspots of the nanostructures, and 2) overcoming inherent
35 negative influence that stem from the presence of a high refractive index substrate that supports the
36 nanostructures. Next, although few in numbers, an overview is given of the most exhaustive and
37 diagnostically relevant LSPR sensing assays that have been recently reported in literature, followed by
38 examples that exploit inherent LSPR characteristics in order to create highly integrated and high-
39 throughput optical biosensors. Finally, we discuss a series of considerations that, in our opinion, should
40 be addressed in order to bring the realization of a stand-alone LSPR biosensor with competitive levels of
41 sensitivity, robustness and integration (when compared to a conventional SPR sensor) much closer to
42 reality.

43

44 **Keywords**

45 Optical biosensors, plasmonic sensors, nanoplasmonics sensors, bioanalytical applications, surface
46 biofunctionalization

47

48 **1. Introduction**

49 Driven by the increasing need for sensitive, fast, cost-effective, low-reagent-consumption and ease-of-
50 use biosensors for applications in the clinical and biomedical field, a myriad of biosensing configurations
51 and devices have appeared in the literature during the last decades. In connection to this, a major
52 unmet diagnostic demand is the necessity of reliable compact Point-of-care (POC) devices, which can
53 provide instant results in any place at any time, offering the possibility of personalized care that may
54 result in an improved health outcome. From the currently well-defined technologies, optical biosensors
55 show unquestionable advantages as compared to other biosensing technologies, including high
56 immunity to electromagnetic (EM) interferences, better stability in aggressive environments, and above
57 all, the ability of providing label-free measurements combined with their potential for multiplexing and
58 miniaturization, offering a great prospective for highly integrated devices. Among the different optical
59 sensing platforms, those based on the use of plasmonic structures meet many of these benefits, and
60 hence, are considered to be key components for the creation of advanced biosensing platforms.

61
62 Plasmonics is the field that studies the interaction of EM radiation with metals. Resonant coupling of
63 optical waves to the free electrons of a metal can give rise to surface bound EM modes that are
64 commonly referred to as Surface Plasmons (SPs). These plasmonic modes are typically excited at the
65 interface of a noble metal and a dielectric, thereby complying to the SP excitation condition that
66 demands the presence of two adjacent materials with oppositely signed optical constants. SPs exhibit
67 their maximum field intensity at the metal-dielectric interface, while decaying evanescent waves
68 penetrate into both adjacent media. The evanescent field that penetrates into the surrounding dielectric
69 provides the SP with a sensing probe that is extremely sensitive to changes of the refractive index (RI)
70 close to the metal surface. It is this property that is exploited when plasmonic structures are used as

71 refractometric sensing platforms: changes that occur in the vicinity of the metal-dielectric interface,
72 such as the attachment or recognition of biomolecules, induce RI changes that alter the excitation
73 conditions of the SP. These changes can be tracked over time, providing a measurable quantity for the
74 label-free detection of biomolecular interactions. SPs generally come in two varieties: propagating SPs
75 excited on thin metal films, commonly referred to as Surface Plasmon Polaritons (SPPs) or Surface
76 Plasmon Resonances (SPRs), and Localized Surface Plasmon Resonances (LSPRs), the latter being SPs
77 excited on sub-wavelength-sized metal nanoparticles (see Figure 1).

78 LSPRs provide metal nanoparticles with exemplified absorption- and scattering cross-sections at specific
79 wavelengths, opening up a world full of bright and vividly colored nanostructures in the VIS and NIR
80 region of the light spectrum. Theoretical interest in the optical properties of metal nanoparticles dates
81 back to the 20th century [1]. However, it has not been until recently, that, accompanied by the eruption
82 of nanotechnology, providing new methods to fabricate, structure and measure nanoscale materials,
83 that nanoplasmonics has experienced an enormous experimental boost leading to a deeper
84 comprehension of these light-metal interactions. As a consequence, the optical properties of metal
85 nanoparticles have led to many new applications in either new or already existing fields of interest, such
86 as photovoltaic devices [2, 3], nanophotonics applications [4], biomedical applications such as imaging,
87 drug delivery, photothermal therapy and therapeutics [5, 6] and, of course, biomolecular sensing [7].

88 In contrast, propagating SPRs have been around for approximately half a century now, providing SPR-
89 based biosensors with sufficient time to position themselves as a landmark label-free biosensing
90 platform. Nowadays, the initial potential of SPR sensors has surpassed all expectations, establishing this
91 sensing concept as a routine analytical instrument. Motivated by both its simplicity and versatility, its
92 scope of applications has spread into a wide range. In this regard, affinity and kinetic studies or simple
93 detection of compounds have met a systematic, easy, fast, real-time and usually sensitive manner to be

94 done. Its validity as a reference optical biosensor is reflected from the number of yearly publications
95 covering application areas ranging from environmental monitoring and food quality to safety, and
96 clinical diagnostics. The implementation of SPR imaging (SPRi) as an alternative SPR-based approach that
97 promotes in-parallel analyses, has expanded its use into the pharmaceutical research and the overall
98 medical field, with applications including high throughput screening, protein-protein interaction studies
99 and drug discovery, amongst others. Besides, the continuous progress in physics, engineering, material
100 science and nanotechnology has allowed the introduction of performance-enhancing modifications to
101 conventional SPR sensor configurations. Of particular importance is the pursuit of improvements in the
102 three most reported weaknesses of SPR biosensors: sensitivity, throughput capabilities and potential for
103 miniaturization.

104 To this end, LSPR-based biosensing platforms are considered to be the next-generation plasmonic
105 sensing platforms. Their inherent advantages over conventional SPR sensors are expected to fill in the
106 gaps left open by SPR sensors. Judging by the exponentially increasing number of publications in this
107 topic during the last decade (see Figure 2), it is not a surprise that the field of nanoplasmonic sensing
108 has been subjected to a great scientific interest. So far, most effort has been directed towards the
109 fabrication and development of the employed nanostructures, the evaluation of its physical and optical
110 properties and its potential to perform biosensing, although this latter concept is typically limited to a
111 proof-of-concept point of view.

112 In literature, several works have already extensively covered the field of refractometric LSPR sensing in a
113 very extensive and general manner [8-10]. In this review, we mainly focus on the progress made in this
114 field during the last 4-5 years, paying special attention to the use of refractometric nanoplasmonic
115 sensors for real biosensing applications, while identifying the implications, requirements and pending
116 challenges in order to achieve fully operative devices with appropriate levels of sensitivity, robustness

117 and integration potential, that can make them ultimately competitive with conventional SPR-based
118 devices.

119 **2. SPR Biosensing: Improving Performance and Design**

120 Despite being the most widely used label-free optical biosensor, SPR sensors suffer from several
121 limitations when compared with other optical label-free sensing techniques, which can be traced back to
122 fundamental properties of SPRs. First of all, the penetration depth of a SPR's evanescent field into its
123 neighboring dielectric is typically hundreds of nm, thereby being much larger than typical sizes of
124 biomolecules [11]. Hence, in some analytical and clinical areas where concentrations to be detected are
125 particularly low or when the target structure is much smaller than this penetration depth, the molecules
126 occupy only a fraction of this evanescent field that acts as a sensing probe, thereby offering a resolution
127 for the detection of analytes that is typically not sufficient. Furthermore, the characteristic SPR wave-
128 vector exceeds that of light traveling through the same dielectric. Excitation of SPRs is therefore only
129 possible when this momentum mismatch is overcome, something that can be achieved by enlarging the
130 wave-vector of the excitation light. Generally, this is accomplished by using a prism-coupled
131 Kretschmann excitation scheme, making the sensor configuration significantly bulky. Upon addition of
132 the microfluidics, optical components and other hardware, most commercialized SPR biosensors are still
133 portable, but their size and weight is not optimal, and far from ideal for LOC or POC devices. Finally, the
134 large propagation distances of SPRs (10-100 μm) limit the minimum sensing area, thus strongly reducing
135 high-throughput capabilities for multiplexed measurements. Not surprisingly, much effort is being put in
136 overcoming these that are considered the weakest points of these biosensors. In the following
137 paragraphs an overview is given of recent works that point toward this direction.

138 Although highly useful for real-time detection and with a proven effectiveness in the monitorization and
139 characterization of biomolecular interactions, the sensitivity of refractometric SPR sensors usually

140 ranges between 10^{-6} - 10^{-7} refractive index units (RIUs) [11, 12] and a limit of detection (LOD) of
141 approximately 0.5-1 pg/mm². In terms of concentration sensitivities, these values would correspond to
142 LODs in the pM-nM range, when optimal surface biofunctionalization has been previously done and
143 high-quality biological reagents are employed. This limited sensitivity, which can be beaten by other
144 label-free optical configurations [13], becomes a more critical factor for the direct detection of small
145 analytes at very low concentrations, for clinical application where the concentrations can range from pM
146 to fM, or for single-molecule detection. Most of the proposed strategies to overcome this problem tend
147 to be based on the expansion of the detection assay with additional steps, such as the addition of
148 successive reagents or compounds in a specific layer-based system. These approaches induce an overall
149 increase of molecular weight and hence, a significant enhancement of the measured signal. An
150 illustrative example comprises sandwich formats where secondary recognition elements are added,
151 either free or labeled with for instance a nanoparticle [14-19]. Despite the improved sensing
152 performance, this methodology complicates overall procedures, since more reagents are required, and
153 analysis times are inevitably lengthened.

154 In the case that simplicity and rapidity are considered to be essential features, sensitivity enhancing
155 strategies that affect the optics, the metallic surface, or the SPR excitation methodology, might be much
156 more appropriate. For an extensive overview on these different approaches, we refer to the work
157 carried out by Homola *et al.* [11]. The use of alternative SPR excitation methods, different from the
158 conventional prism-based coupling scheme, such as the use of waveguide-[20, 21], fiber-optic-[20, 21],
159 or grating-based light-coupling [22], have been considered as sensitivity enhancing strategies [23].
160 However, a recent theoretical study demonstrated that the sensitivity of SPR sensing is reaching its limit
161 regardless of the employed coupling configuration and/or modulation technique (wavelength, angle,
162 intensity) [24]. This work proclaims that most improvements may come from the optimization of the SPR
163 itself, that is, by changing properties of either the employed thin metal films, or their dielectric

164 surroundings. To this end, it has been demonstrated that the use of multilayers built out of noble and
165 ferromagnetic metals (Au/Co/Au), which exploit magneto-optical activity in combination with SPRs,
166 result in a significant four-fold enhancement of the sensing performance of a conventional SPR
167 biosensor [25, 26]. Also, Long-Range Surface Plasmon Resonance (LR-SPR) sensors have proven
168 pronounced sensitivity improvements, reaching values of approximately $2.5 \cdot 10^{-8}$ RIU [27]. Compared to
169 conventional SPR sensors, where the metal substrate is comprised between different dielectrics, LR-
170 SPRs can only be excited when the thin metal film is comprised between two dielectric media with
171 similar RI (i.e. the sensing medium - typically being the buffer used for biosensing applications - and a
172 thin layer of Teflon). LR-SPRs propagate along the metal film exhibiting much larger penetration depths
173 (200-1400 nm), which becomes especially relevant for the detection of large targets such as cells and
174 bacteria yielding 2.5- to 5.5-fold better sensitivities [28], or to deeply study cellular response such as cell
175 volume changes [29].

176 Also the microfluidics and the sample transport to the gold sensing surface have a strong impact on the
177 sensing performance in terms of sample dispersion and overall response time. Homola's group has
178 designed a dispersionless microfluidic system which minimizes the mixing of samples and enhances the
179 sample transport directly to the surface by incorporating two pairs of in- and output ports for sample
180 injection. Controlled valve-mediated port-switching allows for the regulation of the sample injection,
181 assuring that the change of sample volumes takes place near the sensing surface [30]. These
182 modifications improve the sensing performance, exhibiting a RI resolution of $1.3 \cdot 10^{-7}$ RIU [31].
183 Application of this strategy to oligonucleotide hybridization assays has led to a significant detectability
184 improvement when compared to traditional microfluidics, achieving LODs as low as 70 pM [30], while its
185 successful implementation has also been expanded to protein detection in diluted plasma [31, 32].
186 Besides, as recently demonstrated by Lynn *et al.*, geometrical aspects of the flow cell, such as the
187 channel height, can also have a pronounced impact on the sensitivity of SPR sensing [33]. Finally, by

188 decreasing the size of the read-out area to a minimum, Kvasnička *et al.* have shown that the LOD can be
189 pushed down to detection levels of a few hundreds of molecules [34].

190 Next to sensitivity-enhancing strategies, much interest is also still focused on the improvement of the
191 multiplexing and miniaturization capabilities of these sensors. SPR sensors can be noticeably
192 miniaturized and/or integrated if the prism-coupled excitation scheme is replaced by one of the
193 previously mentioned alternatives such as dielectric waveguides, end-fire coupling, or diffraction
194 gratings grafted into the plasmonic substrate. Nevertheless, each of these methodologies adds more
195 complexity to both the fabrication process and the user-friendliness of the device. Other strategies that
196 proclaim smaller and more integrated devices include those that leave out the pump that delivers
197 solutions to the flow cell (an external pump is still necessary), or the use of integrated microfluidics. The
198 latter option not only leads to more compact devices, but also opens up possibilities to improve the
199 sensor's capabilities to carry out multiplexed measurements. In this line, some of the reported SPR
200 sensors incorporate several measurement channels [11], although most of them are limited to at most
201 10 channels. A second approach that provides the ability of carrying out multiplexed measurements is
202 embodied by the SPR imaging (SPRi) concept [35, 36]. This technique, which is considered a large
203 qualitative step forward in the field to microarray-based sensing, has become a valuable tool in
204 proteomics, facilitating the simultaneous analysis of hundreds of biomolecular interactions. Essentially,
205 a SPRi consists of an expanded light source projected onto a patterned gold surface, where the reflected
206 light is imaged onto a CCD camera. Image processing algorithms allow for real-time contrast
207 measurements of all the active spots, providing a quantitative measure for the amount of adsorbed
208 molecules (refractive index change) on each sensing area. Different SPRi instruments are currently
209 commercialized but, unfortunately, the sensitivity of these devices appear to be typically lower than the
210 conventional SPR biosensors [24], and eventually, amplification steps are included in order to enhance
211 the detectability. From a practical point of view, SPRi instruments commonly have restrictions in terms

212 of sample delivery, since they are limited to a single analyte/solution flow at a time on a multiple-
213 ligand spotted surface. The use of appropriate microfluidics facilitate the evaluation of parallel and
214 simultaneous analyte solutions and in case of being highly required, for internal referencing [37]. A few
215 attempts have been done in order to develop microfluidic flow cell arrays with the aim of performing
216 parallel and individualized throughput delivery. Eddings *et al.*[38, 39] have developed a 3D microfluidic
217 flow cell array for the independent delivery of up to 48 different analyte solutions, either for the in-situ
218 patterning of the spot or for the secondary delivery of the target solutions. More complex is for instance
219 the flow cell developed by Ouellet *et al.* [40] consisting of a PDMS microfluidic flow array of 264
220 independent chambers with individual volumes of up to 700 pL. This system is also designed to allow for
221 the recovery of bound sample for further downstream processing. Recently, both nanostructures and a
222 digital droplet-based 2D microfluidic interfaces have been combined in a SPRI, to enhance both
223 sensitivity and improve the automation of simultaneous analyses requiring ultra-low sample volumes
224 [41-43].

225 **3. Nanoplasmonic Biosensing**

226 **3.1 Motivation, Instrumentation and Fabrication**

227 One could argue that the current increase in interest in nanoplasmonic sensing platforms is nothing
228 more than the logical consequence of nanotechnology pushing conventional SPR sensing towards new
229 frontiers. Either way, the most important question that needs to be answered is whether this evolution
230 is worth the effort. Inherent benefits of metal nanostructures offer possibilities that can difficultly be
231 met by conventional SPR sensors. Compared to SPRs, where the propagating nature of the plasmonic
232 mode assures large effective sensing areas, the strong EM field confinement and the localized nature of
233 LSPRs limits the minimum sensing area of metal nanostructures to their size. Combined with the

234 possibility of exciting the LSPRs with direct EM illumination, and thus becoming unnecessary the use of
235 bulky coupling methodologies, the use of metal nanoparticles offers very promising opportunities for
236 sensor miniaturization and multiplexing. Besides the strong EM field confinement of LSPRs ensures
237 smaller penetration depths of the evanescent field into the surrounding dielectric. As a direct
238 consequence, biomolecules attached to the nanoparticle surface occupy a much larger fraction of the
239 evanescent field, raising the expectations of exceptional sensitivities for the detection of tiny
240 biomolecules in low amounts.

241 In general, a nanoplasmonic biosensor consists of a nanostructured substrate with compatible
242 microfluidics. The LSPR of the nanostructures can be excited by a UV-VIS light source, while a
243 spectrometer collects the necessary light. For high nanostructure surface densities, extinction
244 measurements are the easiest way to characterize the optical properties (See Figure 3.A). In this case,
245 light is shed on the plasmonic nanostructures and the transmitted light is analyzed with a spectrometer.
246 However, in the limit of single particle sensing, a much higher contrast is needed between the excitation
247 light and the light absorbed by the nanoparticles. In those cases, scattering measurements are
248 preferred. These high signal-to-background levels can be achieved by dark-field (DF) microscopy or total
249 internal reflection (TIR) spectroscopy. As the size of the particles is reduced, the scattering cross section
250 becomes smaller and absorption becomes dominant, making extinction measurements more desirable.
251 In DF microscopy (transmission configuration) a DF condenser is used to focus a hollow – high numerical
252 aperture – cone on the nanostructured substrate. Then, the scattered light dispersed by the
253 nanostructures can be collected by a microscope objective with a lower numerical aperture (Figure 3.B).
254 In contrast, in TIR microscopy, the LSPR is excited in a prism-coupled TIR configuration (Figure 3.C),
255 thereby also using a microscope objective to collect the scattered light, but, in this case, without any
256 restriction on its numerical aperture.

257 Nanostructured substrates employed in the nanoplasmonic biosensors can be divided in those based on
258 *top-down* or *bottom-up* fabrication methodologies. While the former group relies on lithographical
259 patterning techniques, the latter one is based on chemically synthesized colloidal nanoparticles that are
260 further deposited on substrates. Herein, we only point out the basics of both fabrication concepts. For a
261 more detailed and extensive information on the fabrication of nanoplasmonic structures, we refer to the
262 review by Jones *et al.* [44]. The current variety and extraordinary optical properties of synthesized
263 nanoparticles can be attributed to great advances in nanotechnology, providing researchers with the
264 necessary wet chemistry methods that enable precise geometrical nanoparticle engineering. In this
265 regard, next to spheres [45], rods [46-48], plates [49], triangles [50, 51], (bi)pyramids [52-54], cubes [55,
266 56], tubes [57], stars [58] or prisms [59], also hybrid- and alloy nanoparticles have been fabricated [60],
267 such as for example core-shell particles [61-64], nanoflowers [65] or nanorice [66]. In all these cases, the
268 nature of colloidal nanoparticles imposes serious drawbacks on their use as biosensors in solution (i.e.
269 colorimetric aggregation-based assays). Changes in the ionic strength, pH or buffer temperature, can
270 lead to the particle precipitation. Besides, surface biofunctionalization protocols can screen or modify
271 the charge distribution yielding a rupture of the colloidal equilibrium. To avoid these difficulties,
272 attachment of the colloidal nanostructures to a solid support can be the best alternative or even a
273 prerequisite. An additional benefit is its compatibility with microfluidics for in-flow sensing assays. To
274 this end, different methodologies have been developed that aim at attaching the colloidal nanoparticles
275 to previously functionalized surfaces via either covalent or electrostatic linkage strategies. Thiol- or
276 amino- modified glass surfaces can strongly attach gold nanoparticles to the surface, although
277 sometimes this functionalization step can be especially tricky with particles that require a stabilizer layer
278 on the surface to avoid aggregation, like in the case of nanorods, nanoplates or other kind of structures
279 [49, 54]. These surface modifications usually hinder an efficient and reproducible coverage of the solid
280 support. In an attempt to overcome this problem, additional steps are typically required involving the

281 exchange of this protective layer with other functionalized compounds (i.e. PEGylated compounds,
282 thiolated compounds with carboxylic acid, biotin, etc). This step subsequently allows for binding to
283 appropriately modified surfaces (amino-, thiol-, avidin-modified or opposite-charged surfaces so that
284 electrostatic interactions occur [58, 67, 68]). An attractive alternative consists of directly growing the
285 nanostructure on the substrate [69]. The surface density of immobilized nanoparticles can be controlled
286 by optimizing parameters such as the concentration of the colloidal nanoparticle solution, the
287 incubation time, or the temperature, making it possible to obtain highly-dense or very sparse surface
288 concentrations. For sparse density, inter-particle discrimination becomes possible, enabling the spectral
289 monitoring of a single nanostructure. It should be noted that almost all these immobilization strategies
290 lead to a random nanoparticle surface distribution, yielding low control of both position and orientation.
291 This issue can be overcome by carrying out a previous ordered functionalization of the substrate, for
292 instance, by carefully modifying the surface at specific positions that finally results in an arrayed-based
293 distribution.

294 The most typical top-down fabrication approaches include conventional lithography such as
295 photolithography, electron beam lithography (EBL) or focused ion beam lithography (FIB), allowing for
296 the formation of ordered arrays of nanometric structures with well-defined shapes and sizes. Although
297 widely used, these techniques are slow and high-cost, and despite high levels of resolution, typically
298 limit the patterning area to only a few μm^2 . On the other side, conventional photolithography permits
299 faster, parallel and large-scale fabrication at the expense of lower resolution. A different approach for
300 large-scale and low-cost creation of plasmonic nanostructures is offered by colloidal lithography
301 techniques, such as nanospheres lithography (NSL) [70-72], or hole-mask colloidal lithography (HCL)
302 [73]. In both methods, the self-assembled layer of nanospheres onto the substrate is used as a sacrificial
303 mask for the generation of nanostructured substrates. With NSL, hexagonal self-assembly of
304 nanospheres in close-packed layers renders highly ordered patterning, whereas HCL, characterized by

305 short-range ordered arrays of nanostructures, offers more versatility in terms of particle geometry.
306 Another fabrication methodology that currently receives much attention is nanoimprint lithography.
307 This technique relies on the use of reusable master stamps, which can be either hard (rigid) [74], or soft
308 (elastomeric) [75], that are used to imprint or transfer predefined patterns onto almost any desired
309 substrate. These patterns are typically used as a mask for successive fabrication steps. Due to the
310 reusability of the master stamps, and even their tunability in the case of soft stamps [76], nanoimprint
311 lithography is considered a low-cost technique with potential for high-throughput fabrication of sub-
312 micron structured substrates. Using this fabrication methodology, large nano-patterned areas of domes
313 [77], cavities [78], holes [79], and dots/disks [80-82] have been reported. A different approach is offered
314 by nanostencil lithography. Based on shadow mask deposition, and having the additional benefit of not
315 requiring any resist-processing, baking, or solvent-use, this technique fabricates dense nanostructured
316 substrates with high resolution [83], such as nanodots [84], or nanorods[85], and can even be extended
317 for the creation of nanoplasmonic structures on flexible substrates[86, 87]. Next to these
318 abovementioned approaches, other top-down methods that are worth mentioning include the use of
319 porous alumina templates for the creation of vertical nanorods [88] and nanotubes [89], the direct seed-
320 mediated growth of nanoplates directly on top of surfaces [69], the use of interference lithography [90],
321 or the creation of nanoparticle cluster arrays using a hybrid top-down/bottom-up approach [91].

322 **3.2 Sensing Performance of Refractometric LSPR Biosensors**

323 **3.2.1. Sensitivity Considerations**

324 The ability with which metal nanostructures can detect RI changes is generally expressed in terms of
325 their bulk sensitivity η_B , that is, the linear dependence of resonance wavelength λ_{LSPR} on the
326 homogeneous bulk RI changes of the dielectric environment:

327 Next to η_B , a second property that strongly influences the sensing performance is the ability with which
328 these spectral shifts can be discriminated, something that is normally taken into account by considering
329 the full-width-half-maximum (Γ) of the resonance peak. Both quantities are often combined in a
330 generalized performance-assessing figure-of-merit (FOM) parameter, defined as:

331
$$FOM_B = \frac{\eta_B}{\Gamma},$$

332 This parameter, both valid in wavelength and energy scale [92], provides an easy way for the
333 performance assessment of nanoplasmonic structures whose spectral response is described by a
334 Lorentzian peak. However, to expand its use to other nanometric structures that exhibit more complex
335 spectral responses, Becker *et al.* proposed an alternative *FOM*-quantity by considering relative peak
336 intensity changes at the wavelength where the slope $dl/d\lambda$ is maximized [93]:

337 Both η_B , FOM_B and FOM_B^* parameters are extensively used and considered to be good indications for
338 the bulk sensing performance of nanoplasmonic sensors [78, 92-99]. Depending on the specificity of the
339 employed sensing platform, different, but equally useable FOM definitions can be found. In this regard,
340 Offermans *et al.* devised a generalized scaling law for lattice-based nanoplasmonic sensing schemes, in
341 which the employed FOM parameter is uniquely determined by geometrical lattice properties [100].

342 However, from a biosensing point-of view, it is much more interesting to probe the EM field distribution
343 of a nanoplasmonic sensor close to the metal surface, which is the region where the biomolecular
344 interactions take place. For this, a surface sensitivity parameter η_S can be defined that accounts for the
345 near-field sensing performance:

346 In this expression, d , is the thickness of a very thin adsorbed layer that homogeneously coats the metal
347 nanostructure, with a refractive index that is representative for organic molecules. Again, the
348 accompanying figure-of-merit can be defined by: $FOM_s = \eta_s / \Gamma$, which in the limit of $d \rightarrow 0$ can be used to
349 mutually compare the surface sensing performance of different (nano-)plasmonic sensing schemes.
350 When considering the simplest type of metal colloids, the most archetypical example of a
351 nanoplasmonic sensor is based on spherical gold nanoparticles. However, the strongly blue-shifted LSPR
352 inherent to this particle-shape ($\lambda_{LSPR} < 600$ nm) results in a spectral overlap with the interband transition
353 of gold. As a consequence, sensors based on spherical nanoparticles not only exhibit low bulk
354 sensitivities ($\eta_B < 120$ nm-RIU⁻¹), but also broad peak widths ($\Gamma > 100$ nm) [101]. The resulting FOM
355 parameters, which are an order of magnitude smaller than those corresponding to SPR sensors, limit
356 their potential use as label-free refractometric LSPR nanobiosensors.

357 In contrast, a much better sensing performance is obtained when rod-shaped nanostructures are
358 considered. For these particles, variation of their longitudinal axes allows for the spectral tuning of their
359 LSPR, while maintaining relatively low particle volumes [102]. Red-shifting of their plasmon resonance
360 not only assures larger sensitivities, but also distances their LSPR from the interband transitions of the
361 employed plasmonic material. The combination of both factors results in enhanced bulk and surface
362 sensing performances when compared to spherical nanoparticles. However, most interestingly, it has
363 been shown that although conventional SPR sensors outclass these gold rod-shaped LSPR sensing
364 platforms in bulk sensing performance by approximately one order of magnitude, their surface sensing
365 performance dictates a very different behavior: gold nanorods exhibit a surface sensing performance
366 that is 15% better than that of their SPR-counterpart, while theoretical calculations predict an even
367 larger improvement [92]. Besides, nanorods exhibit an optimized spectral sensing region when their

368 aspect ratios lies between 3 and 4 ($\lambda_{LSPR} = 700-800 \text{ nm}$), which can be accessed through precise
369 nanoparticle engineering [92, 93, 103]. Moreover, the overall sensitivity enhancement of single rod-
370 based LSPR sensors can be duplicated when silver is used as the plasmonic material[47], although silver
371 has the strong disadvantage of its chemical instability. These results not only demonstrate the potential
372 of LSPR sensors, profiling them as alternative for conventional SPR sensors, but also emphasize the fact
373 that when comparing both plasmonic sensing schemes, distinction between bulk and surface FOMs is
374 mandatory. This discrepancy finds its roots in the different EM field distributions of both plasmon types.
375 Due to the much larger penetration depths of SPRs into the surrounding dielectric, SPRs possess much
376 larger sensing volumes for the detection of bulk RI changes. However, when it comes to biosensing, the
377 spatially more confined EM field distribution of LSPRs assures a larger fractional occupancy of molecules,
378 assuring better surface sensing performances. For a real comparative study involving both sensing
379 platforms, only an assessment based on the surface sensing performance paints a realistic picture of the
380 actual biosensing performance.

381 Unfortunately, different nanoparticles are usually compared through their bulk sensing performance,
382 often leaving their surface sensing characteristics out of the equation. For conventional spheroidal
383 nanostructures, such as spherical and rod-shaped particles, bulk sensitivities tend to range between 100
384 and 500 nm/RIU . These values can significantly increase for more complex structures [104], such as
385 bipyramids (540 nm/RIU), nanoprisms (583 nm/RIU) [59], ring-disk nanocavities (648 nm/RIU) [105] or
386 nanocrosses (1000 nm/RIU) [106]. Either way, the lack of information of their surface sensing
387 performance is not that surprising, especially when taking into account the work carried out by Piliarik *et*
388 *al.*, in which a gold nanorod is used as an example to illustrate the strong correlation that exists between
389 the surface sensing performance and the EM field profile of the LSPR [107]. From this work it can be
390 concluded that for a good assessment of the surface sensing capability of a nanostructure, it becomes
391 necessary to assure a homogeneous coverage of the nanoparticle's surface with biomolecules, or, even

392 more important, to channel molecule binding uniquely to those regions of the particle that exhibit the
393 highest sensitivity. This latter approach becomes especially desirable to enhance the surface sensitivity
394 but simultaneously is really difficult from an experimental point of view, especially when considering
395 that nanoparticle surface chemistry still lags behind thin film surface modification protocols. As a
396 conclusion, we can point out that in order to fully exploit the biosensing potential of more complex
397 nanostructures, the functionalization on the so-called EM hotspots of the structure, typically being sharp
398 edges, or small cavities, should become the main challenge to be surpassed.

399 **3.2.2. Sensitivity Improvements**

400 As briefly introduced in the previous section, two aspects that turn out to be especially relevant when
401 trying to maximize the biosensing performance of nanoplasmonic sensors are: (i) the directed
402 biofunctionalization of the metallic nanostructures leaving the supporting substrate unaltered and (ii)
403 overcoming intrinsic negative influences that the substrate itself (typically glass/silicon-based materials)
404 can have on the sensing performance.

405 ***Directed Functionalization***

406 The field that offers most margin for the improvement of the LSPR sensing performance is related to the
407 proper surface functionalization of the plasmonic nanostructures, that is, *directed functionalization*.
408 However, this aspect is often under-highlighted in published studies of refractometric LSPR biosensing.
409 Before going into further detail, it must be mentioned that the functionalization process for SPR sensors
410 is much more simplified: whereas in thin film sensing biomolecules *see* a homogeneous gold surface at
411 which binding can take place, in LSPR sensors part of the underlying substrate is typically exposed,
412 making undesired non-specific binding events to this substrate easy to occur. In this latter case, ideally
413 all molecules reaching the nanostructured surface should be anchored solely to the nanostructures, and
414 preferably, only to those places that find themselves in the vicinity of EM hotspots, where RI sensitivity

415 is maximized. However, only few examples exist in literature that proactively attack this problem. Most
416 probably, inherent complexities related to accurate orthogonal modification of both materials lie at the
417 heart of the problem: in an ideal system one would functionalize the metallic nanostructures with the
418 biomolecules at a given desired surface density concentration, while the underlying substrate is blocked
419 with materials or compounds that assure its inertness to both the immobilized biomolecules and its
420 corresponding target. A study that exemplifies this very well was carried out by Feuz *et al.* [108]. In this
421 work, thin gold nanohole films fabricated on top of a TiO_2 substrate are used in combination with a
422 proof-of-concept protein sensing assay (detection of Neutravidin by biotinylated surfaces), to provide a
423 comparable study that demonstrates that by assuring both previously mentioned demands, the LOD of
424 this particular system can be extremely improved, offering an approach that can be extrapolated to
425 other nanoplasmonic sensing platforms. By only exposing the highly sensitive inner walls of the
426 nanoholes to the surrounding dielectric, the authors assure the binding of molecules to the most
427 sensitive regions of the transducer surface (see Figure 4.A.). Furthermore, a material-selective
428 poly(ethylene glycol)-based surface chemistry limits the binding of NeutrAvidin only to surface
429 immobilized biotin that finds itself on the exposed gold regions. By doing this, a 20-fold enhancement of
430 the sensor response time is reported. It should be noted that the controlled immobilization onto the
431 gold areas with highest sensitivities is not achieved by surface chemistry but by the nanostructure
432 fabrication process itself. The latter is done by sandwiching the gold nanohole substrate between two
433 TiO_2 -layers. This strategy clearly simplifies the chemistry and reduces the complexity to discriminate
434 between TiO_2 and gold. In an alternative, but conceptually similar methodology, the same authors have
435 developed a more complex material-selective surface chemistry protocol [109]. In this case, gold
436 nanodisk dimers were fabricated on SiO_2 substrates using bioactive TiO_2 layers located in the gaps
437 between the gold disks (see Figure 4.B.). By appropriately choosing pegylated compounds that
438 selectively react with Au, TiO_2 or SiO_2 , biomolecules could be immobilized exclusively in these high EM-

439 field gaps, assuring enhanced sensitivities. When comparing this sensitivity to the case where the
440 molecules are uniquely bound to the gold nanodisks, a four-fold larger signal per bound molecule was
441 obtained (Figure 4.B.).

442 Another interesting strategy takes advantage of thiol place-exchange processes. Using gold nanoplates
443 grown on glass or silicon surfaces, Beeram *et al.*[110, 111], followed this procedure by first covering the
444 surface with a non-reactive thiol. Then, subsequent addition of another thiol-modified compound (in
445 this case incorporating a reactive carboxylic group) induces an exchange process of reagents, occurring
446 preferentially at edge- or vertex sites of the nanostructures (which turn out to be approximately 2-3
447 times more sensitive than terrace sites) (see Figure 4.C). Following this path, the binding of antibodies
448 (anti-IgG) exhibited a higher sensitivity compared to the case in which the nanoplates were entirely
449 covered with the reactive thiol group. Moreover, the detection of target IgG resulted in a LOD 500 times
450 lower than the one obtained with conventional antibody coverage of the nanoplates, even using much
451 higher concentrations of anti-IgG receptor molecules (10x). Also, the length of the thiol linkers, used to
452 control the distance from the surface, have a significant effect since larger spectral shifts are obtained
453 for short thiols [111].

454 *Substrate Effects*

455 The required attachment of lithographically fabricated nanostructures to underlying substrates imposes
456 significant intrinsic drawbacks on the sensing performance of LSPR sensors. To assure a robust binding
457 of the nanostructures, often, extremely thin metal adhesion layers are employed to form a bridge
458 between the plasmonic material and the substrate. However, the use of these metal adhesion layers
459 increases the LSPR dephasing time, reducing the scattering amplitude and inducing peak broadening.
460 Judging from a biosensing point of view, this aggravates the signal to noise ratio of LSPR sensors,
461 resulting in inferior sensing performances. Therefore, a proper choice of material and geometry has

462 proven to be of critical importance to provide competitive sensor performances [112, 113]. In thisline,
463 plasmonic resonances can be improved by replacing the metal adhesion layer by a thin molecular layer
464 (i.e. (3-mercaptopropyl)trimethoxysilane) which binds both the glass substrate (through the silane
465 group) and the metal (through the thiol group)[114].

466 Furthermore, the supporting substrates (typically glass) possess a RI that is much higher than those
467 corresponding to the buffer solutions normally used in bioassays. As a consequence, the symmetry of
468 the EM around the nanostructures is broken, shifting a much larger part of this EM field towards the
469 metal/glass interface. Here, this EM field is almost entirely insensitive to RI changes of the external
470 dielectric medium, significantly lowering the overall sensitivity of the nanoplasmonic structures [115]. To
471 overcome this problem and improve the sensing features of the nanostructures, the effective RI
472 surrounding the nanostructures has to be decreased. The use of low RI materials as supporting
473 substrates presents itself as a very straightforward method to overcome this problem. Following this
474 route, *Brian et al.* showed that a Teflon ($n=1.32$) substrate supporting a thin gold film perforated with
475 nanoholes yielded a 40 % improvement of the bulk sensitivity [116]. Another strategy relies on placing
476 the nanostructures on nanopillars, distancing them with respect to the underlying glass substrate. These
477 dielectric pillars can be created *during* the lithographical fabrication process [117, 118], or *afterwards*,
478 using an isotropic etch of the glass substrates (see Figure 5). Following this path, all the hotspots of the
479 nanostructures are exposed to the surrounding dielectric, increasing the particle surface available for
480 biosensing, leading to sensitivities that are comparable to that of *free* nanostructures in a homogeneous
481 dielectric medium [112]. The latter was achieved via DNA hybridization measurements.

482 **3.3 LSPR Biosensing: Applications and Issues**

483 The previous sections have highlighted fundamental aspects that researchers are implementing to
484 improve the performance and the capabilities of nanoplasmonic biosensors. However, its transfer to

485 real applications is not that straightforward. When aiming at the detection of low amounts of molecules
486 and pushing high throughput capabilities to the limit, single particle sensing is the preferred option.
487 Currently, array-based LSPR biosensors are most commonly used. Some results reported in the literature
488 using array-based nanostructures in which their sensing performance is directly compared to
489 conventional SPR sensing, seem to be encouraging, while simultaneously, other publications reveal
490 aspects of refractometric LSPR sensing that inevitably generate serious doubts about its real potential.
491 For instance, Homola's group developed a high-resolution LSPR setup and did some experiments on
492 ordered nanorod arrays determining their surface coverage with DNA sequences. They concluded that
493 although LSPR-based biosensors can detect a number of molecules (i.e. number of interactions) two
494 orders of magnitude lower than SPR-based sensors, which is clearly advantageous, the resulting
495 analytical performance is very similar compared to other high-resolution SPR setups [119]. This result is
496 most probably due to the kinetics of the interaction as the probability of the biomolecular interaction is
497 also proportional to the number of interacting molecules. Linked to this result, and putting more focus
498 on the influence of the fluidics and the kinetics of the reaction, a recent study by Sipova *et al.* [120]
499 compared the performance of SPR and LSPR-like sensors to detect interactions events in flow-through
500 formats. The study was done by comparing a flat surface covered with receptors, resembling an SPR
501 system, with a situation in which a single receptor is immobilized on the surface, for the detection of
502 single binding events, being approximately analogous to an LSPR-like system. The estimation concluded
503 that for common biomolecular interactions (antigen-antibody, DNA-DNA, etc.) and for typical detection
504 times (10 min) the probability of positive response, that is, of detecting a single molecular interaction in
505 the case of a single receptor (i.e. in a single nanostructure) is much lower (between 10^{-1} and 10^{-3})
506 compared with flat surfaces homogeneously covered with receptors. The reason behind this is precisely
507 due to the low number of recognition elements available in LSPR sensing and only seems to approach
508 SPR performance when dissociation rates are extremely high and/or when the analytes are small.

509 Although for static end-point analyses these conclusions may not be the same, these results exemplify
510 the existing debate about the real necessity of pushing forward single event detection with
511 nanoplasmonic platforms as next-generation biosensors from a strictly practical analytical point of view.
512 In the following we discuss some recently reported relevant works that include both the use of single
513 nanoparticles and array-based nanostructures as sensing platforms for real biosensing experiments.

514 3.3.1 Single Particle Sensing

515 The detection of discrete binding events with single nanostructures has positioned itself as the ultimate
516 goal for future biosensors which share high throughput capabilities, low sample consumption and
517 extremely low limits of detection among their main characteristics. However, from a strict biosensing
518 perspective, where detecting low concentrations of target is the main goal, the potential and feasibility
519 of label-free single particle sensing is probably more limited. Whereas the spectral readout setup can be
520 easily adapted to single particle detection using microscopy, there are still some general issues related
521 to poor signal-to-noise ratios. Although this can be significantly improved by implementing near-normal
522 incidence DF microscopy instead of more conventional large incidence angle schemes [121], DF
523 microscopy generally provides low signal levels and insufficient time resolution for the detection of
524 few/single analytes. Also, it usually results in fair sensitivities and long-time analysis [122], caused by
525 mass transport limitations [123, 124]. Recently, by expanding a conventional DF microscopy scheme
526 with a broadband laser source and an intensified CCD camera, Ament *et al.* demonstrated the label-free
527 single protein detection with an individual metallic nanostructure [125]. Contrary to prior work, their
528 technique offers impressive levels of time-resolution (ms time scale, with between 4 and 6 orders of
529 magnitude better levels than previous reported works) and measurement noise (tens of pm).
530 Furthermore, Zijlstra *et al.* demonstrated that the detection of individual target analytes can also be
531 achieved by making use of photothermal microscopy [126]. Gold nanorods - biotinylated mainly at the
532 tips [127] - were immobilized on the surface and three biotin-binding proteins with different molecular

533 weight were discretely detected, observing step-like signal-enhancements proportional to their sizes
534 [126]. Also, in order to allow for multiplexed spectral interrogation of single nanoparticles, much effort
535 is being put in devising techniques for parallel read-outs. We can distinguish techniques based on the
536 use of conventional DF microscopy, in which the spectral readout is either carried out with the use of a
537 liquid crystal tunable crystal [128], gratings [129], or advanced image processing algorithms [130] for the
538 parallel spectral read-out of multiple nanostructures. The latter approach is especially interesting, since
539 next to parallel read-out, this methodology normalizes the LSPR of geometrically different nanoparticles.
540 This minimizes the influence of inherent differences in the same batch of nanostructures, avoiding the
541 necessity of averaging several nanoparticles while improving fitting parameters in biosensing assays. But
542 despite the benefits of these methods, a great challenge lies ahead in order to provide these techniques
543 with improved signal levels, and hence, better time resolution, to make them really useful to study the
544 real-time kinetics of surface binding events.

545 In terms of applications, a variety of recent examples have appeared in literature. Until recently, single
546 molecule/event detection has been restricted to the use of labels, due to the limited resolution of peak
547 shift resolution in most common LSPR-based technologies. Amplification schemes based on the use of
548 gold nanoparticles, which monitor single DNA hybridization events [131] have been reported. Another
549 approach is based on exploiting the catalytic activity of the enzyme Horseradish Peroxidase (HRP), which
550 in this proof-of-concept study was used as target itself [132]. Exploiting the catalytic activity, few or even
551 one HRP molecule can be detected on conical nanoparticles, by adding a substrate which produces a
552 precipitated product. The binding of the enzyme on the surface of the nanostructures induced the
553 localization of the precipitate on the very same structure, significantly enhancing the overall signal, and
554 improving the detectability. The strategy could be adapted to detect other type of molecules which
555 incorporated HRP as labels in an ELISA-like assay, which inevitably prevents a label-free approach. On
556 the other hand, strict label-free single-particle sensing has already been attempted to evaluate self-

557 assembled monolayer formation, in this case, on silver nanoparticles [133] and also for proof-of-concept
558 biotin-streptavidin detection either with nanoparticles [58, 134, 135] or nanoholes [136].

559 Some examples of protein detection by immobilizing aptamers or antibodies on nanoparticle surface
560 (either nanospheres [130, 137] or nanorods [138, 139]) have also been reported, showing its potential.

561 In some cases, sensitivities in the attomolar range are achieved [138], although it should be mentioned
562 that this involved end-point measurements. Huang *et al.* [140] have detected TNF α protein using
563 immobilized Ag nanoparticles (2.6 nm) functionalized with its specific antibody using DF single
564 nanoparticle optical microscopy and spectroscopy (SNOMS). Although acquisition times of several hours
565 were necessary, the high sensitivity of these small-size NPs assured single molecule detection and the
566 study of the binding kinetics. Song *et al.* [141] employed single particle sensing to study DNA-protein
567 interaction, in particular, to estimate the relative promoter activity, by immobilizing the DNA sequence
568 (SP6 promoter and single point mutation variants) and detect promoter Polymerases (SP6 RNA
569 polymerase). What adds special interest to this work is that, contrary to conventional methodologies,
570 real-time kinetics of the reaction was studied. Mayer *et al.* demonstrated the detection of single
571 capturing and unbinding events using single gold bipyramids in real time, paying special attention to the
572 dissociation rather than to the binding events, since it is slow enough to detect it in the time resolution
573 frame, and should not be affected by initial concentration or diffusion effects [53]. The relatively long
574 timescale of the process (10^5 s) turns out to be an advantage to detect single events. This approach can
575 provide valuable fundamental information regarding nanostructure behavior and protein interaction
576 dynamics in comparison with labeled methodologies such as FRET (fluorescence energy transfer
577 processes) [142], which require modification of molecules and limits time scale to the stability of the dye
578 used, being sometimes short to some purposes (i.e. 10^2 s).

579 3.3.2 Particle Array Sensing

580 As previously discussed, whereas single particle sensing offers outstanding potential from a multiplexing
581 point of view, and avoids inherent “signal-averaging effects” when dealing with nanostructured
582 surfaces, this concept exhibits some important drawbacks which prevents its extensive use for routine
583 biosensing analysis. In this regard, biosensing schemes based on arrays of nanostructures not only offer
584 improved possibilities for cheap and mass-scale chip production, but also hold the advantage of keeping
585 the required instrumentation very simple. It comes therefore as no surprise that most nanoplasmonic
586 biosensing studies currently rely on the use of nanostructured substrates. As is also the case for single
587 particle sensing, besides some relevant examples published some years ago[143, 144], the majority of
588 recent publications keeps being limited to a proof-of-concept based on a routine biomolecule
589 immobilization and subsequent target detection. More in-depth analytical study, including a complete
590 optimization of the bioassay to set its reproducibility, sensitivity, specificity and viability to detect
591 targets in complex samples is yet rare to find. Besides, often end-point analyses are carried out instead
592 of real-time measurements that monitor the reaction kinetics. Even more, end-point measurements are
593 generally carried out in air after performing successive incubation/washing steps, thereby inevitably
594 affecting their reproducibility and increasing measurement times. On the contrary, in the case of real-
595 time monitoring, the use of a fluidic cell prevents the samples from drying, ensuring that biomolecules
596 remain under favorable aqueous environment which minimizes their denaturation and also facilitates
597 the kinetics analysis [145]. The most recent works, which are characterized by a proactive strategy that
598 addresses one or more of these previously mentioned aspects are discussed in the remainder of this
599 section and summarized in Table 1.

600 When considering nanostructured arrays, one can initially distinguish between particle- and hole-based
601 nanostructures, being the particle based one the more extensively used for nanoplasmonic biosensing.
602 Most of publications employ antibody-based strategies, either for the detection of proteins or viruses,

603 DNA interactions or with more unusual approaches such as the study of supported lipid bilayer
604 formations. Among them, some stand out for handling interesting issues related to biosensing. For
605 instance, although no in-depth optimization has been performed, Zhou *et al.* used an integrated LSPR
606 sensor based on Ag nanotriangles fabricated by NSL to detect p53 protein levels in serum from cancer
607 patients (patients with head and neck squamous cell carcinoma, HNSCC), which is commonly
608 overexpressed when compared to healthy patients. Specific antibody against p53 was covalently bound
609 to the nanoparticle surface, and detection was done with incubation steps and static measurements.
610 Unfortunately, no protocol optimization, reproducibility estimations or studies related to the influence
611 of serum were presented. Similarly, but including real-time dynamics analysis, Chen *et al.* [146],
612 demonstrated the detection of two different proteins (PSA and Extracellular adherence protein EAP)
613 using specific antibodies immobilized on the surface of gold nanodisks. In this case, estimated LODs
614 ranging between 1-8 pM were reported (compliant with the requirements for clinical applications).
615 However, also in this case, neither specificity studies nor detailed assay optimization were addressed.

616 More in-depth biosensing analyses have been reported using Au-capped nanoparticles [147], fabricated
617 using a dense monolayer of silica nanoparticles on top of a gold substrate as a core template for the
618 subsequent deposition of a thin Au film (tens of nm). Their ease of fabrication, strong LSPR and
619 integration in simple optical setups yields very compact nanoplasmonic biosensing schemes which are
620 typically based on static absorbance measurements done after intermediate incubation steps. Despite
621 the fact that no cell- or microfluidics are employed, the assay optimization and the reproducibility
622 studies confirm the robustness of the approach. This type of substrate has been exploited for a variety
623 of biosensing applications, most of them with a clinically relevant goal, either using antibodies or nucleic
624 acids as active recognition elements. A selection of successful results are gathered in Table 1, where one
625 can highlight for instance the detection of proteins in complex media (detection of casein protein in milk
626 with antibodies immobilized on the surface using Protein A as orienting molecule [147]). Another

627 remarkable example is based on the detection of cell activity and cell function (i.e. determination of cell
628 metabolites such as Interleukin-2, IL-2). This is achieved by immobilizing specific antibodies against these
629 metabolites. Afterwards the cells are simply deposited and appropriately stimulated in order to trigger
630 the metabolism to produce and secrete certain molecules which can be captured by the antibody [148]).
631 Other biosensing examples are focused on the detection of relevant biomarkers for Alzheimer's disease
632 using specific antibodies [149], the interaction of toxins with membranes which are immobilized on the
633 surface [150] or fibrinogen detection, mediated by antibodies which are immobilized in an oriented
634 manner using a specific aptamer that recognizes the Fc (constant fraction) of the immunoglobulin [151].

635 Multiplexed analyses can be implemented through the use of biodeposition systems [152] or by
636 adapting the fabrication to obtain multiple areas of gold-capped nanoparticles, (multispot gold-capped
637 nanoparticle arrays, MG-NPA) which can be individually immobilized and interrogated (i.e. between 15-
638 60 spots and sizes in the mm range), assuring higher levels of throughput. This approach allowed for the
639 detection of antigens related to the hepatitis B virus [153], the detection of antibodies recognizing
640 influenza virus [154] or the detection of DNA point mutations related with corneal dystrophies [155],
641 which was validated with real patients' samples. Furthermore, Cu-capped nanostructures have been
642 used to carry out a complete multiplexed biosensing experiment in which pathogenic bacteria DNA was
643 directly detected in real isolates coming from samples as blood, pus, urine or sputum exhibiting
644 sensitivities in the fM range [156].

645 Recently, alternative biosensing approaches based on specific recognition events have been reported
646 that offer interesting results beyond clinical diagnosis. For instance, using self-assembled gold nanorods,
647 the detection of chiral compounds has been showed using enantioselective sensitive receptors [67],
648 being a good example of LSPR sensing in the field of drug-protein interactions. In this work, the
649 nanostructures, immobilized on the inner walls of a microfluidic channel, were functionalized with

650 human α -thrombin that was used as the selective receptor. Then, a complete optimization of the
651 protocol for the detection of the drug *RS*-melagatran was performed, achieving discrimination of the
652 enantiomeric counterpart (*SR*-melagatran). Besides, although at the cost of a diminished sensitivity, the
653 target could also be detected in human serum. This concept has been further expanded for the
654 discrimination of a racemic mixture (in this case for (*R*) and (*S*)- 1,2,3,4,-Tetrahydro-1-naphtylamine,
655 TNA), using weak or non-enantioselective receptors instead of strong ones. In this case, the authors
656 used a dual channel microfluidic chip, with a weak chiral receptor in one channel and a nonselective
657 receptor in the other one [157]. By combining the information extracted from both channels it was
658 possible to determine the individual concentration of each enantiomer of TNA in a racemic mixture.

659 Another biosensing concept that has been recently exploited involves the detection of conformational
660 changes in proteins, which is more plausible to be detected using the strong EM field confinement of
661 LSPRs than with the more deeply penetrating evanescent fields of conventional SPRs. In this case the
662 focus does not lie on the direct detection and quantification of a specific target, but on the study of the
663 protein structure or interactions against external stimuli or interactions with small molecules, which,
664 due to their low molecular weight, would otherwise be difficult to detect by simply binding them to the
665 surface. This is the case recently reported by Hall *et al.* [158], where Ca^{2+} was detected in a label-free
666 manner, by monitoring the conformational changes of the protein calmodulin immobilized on the
667 surface of Ag nanoprisms. The changes in the conformation affect the density and overall height of the
668 protein immobilized layer, resulting in spectral LSPR shifts. A low-noise level (0.002 nm and S/N levels of
669 500), together with a well-controlled immobilization of calmodulin, which ensures the proper
670 orientation of the globular domains responsible for the interaction with the ion, resulted in Ca^{2+}
671 detection at concentrations as low as 23 μM . Furthermore, the strong EM field confinement of LSPRs,
672 which can be exploited to increase the sensitivity to detect small molecules, can be further enhanced by
673 taking advantage of another phenomena, derived from the strong coupling between molecular

674 resonances of chromophores and the LSPR, resulting in larger spectral shifts [159]. This has been used to
675 study the interaction of small drugs with human membrane-bound cytochrome P450 3A4 (CYP3A4),
676 which contains a heme chromophore group [160]. In this work, CYP3A4 was first stabilized in the form of
677 soluble nanodisks with membrane scaffold proteins, and then immobilized onto Ag nanoparticles. The
678 binding of a variety of small drugs (MW~ 100-700 Da) has been tested and the observed spectral shift
679 (blue shift, red shift or no shift) identifies different interaction types.

680 Whereas particle arrays are solely dominated by the LSPR of the nanostructures, nanohole substrates
681 present additional peculiarities due the co-existence of surface plasmons that propagate along the metal
682 film and localized plasmons excited inside individual nanoholes, whose interaction can give rise to
683 exciting optical phenomena such as Extraordinary Optical transmission (EOT) [161] or interacting anti-
684 symmetric plasmons [162]. Over the years, nanohole substrates have been extensively studied, both
685 from a fundamental and practical point of view. Since the spectral interrogation of nanohole films relies
686 on simple transmission measurements, thereby lacking the need of prism-coupling, and hence,
687 noticeably simplifying the optical measurement schemes [163, 164], their use for biosensing has been
688 widely suggested [165-169]. Similarly to particle-array this particular measuring scheme is also
689 compatible with imaging configurations [3], opening up facile routes towards multiplexed sensing
690 assays, as demonstrated in some biosensing applications, including quantification of ovarian cancer
691 biomarkers with an integrated microfluidic platform[170], studies of antibody-ligand binding kinetics
692 [166] or simultaneous detection of antibody-target binding events in temperature regulated flow cells in
693 microarrays based substrates [171]. However, these measurements generally tend to exhibit moderate
694 sensitivities, even lower than SPR-based biosensors, as demonstrated in these previous examples (in the
695 nM range [166, 170]. Better sensing performances for high throughput measurements can be achieved
696 introducing dual-color filter imaging [90], resulting not only in better sensitivities, but also in increased
697 accuracy and signal-to-noise ratios. On the other hand, when studied in a conventional Kretschmann

698 configuration it turns out that these nanohole array films exhibit enhanced bulk sensitivities compared
699 to planar gold surfaces. These sensitivity enhancements can be precisely tuned using the periodicity of
700 the holes and their geometry as variables [172], although resulting in lower resolutions [173]. Besides
701 this enhancement of sensitivity on thin gold film, strong localized plasmons are generated in the hole
702 [174-176], providing an enhanced sensitivity in these areas. Due to the coexistence of propagating and
703 localized excitations at the same time, the behavior of these LSPRs is quite complex being the shape and
704 size of the hole, the lattice periodicity and the substrate material important parameters governing both
705 phenomena [116, 167, 177]. Biosensing schemes that exploit the LSPRs excited in the nanohole itself
706 possess the attractive advantage of minimizing the required sample volumes, as long as accurate
707 immobilization in these areas occurs. The resulting reduction of the sensing area will eventually proclaim
708 better sensitivities, opening up pathways towards very low LODs [163]. Besides the biosensing examples
709 described above, other relevant applications have been based on the formation of supported lipid
710 bilayer (SLB) formation. The addition of a silicon-based layer over nanohole structures permits the
711 formation of SLB and has allowed studies that involve cell membrane related biorecognition reactions
712 [178, 179]. Besides, SLBs have been used as myelin-mimicking bilayers to kinetically characterize
713 autoantibodies involved in neurological disorders [180]. Nevertheless, as of today, the advancements
714 made in the fabrication of these nanoholes structures and the reported studies that discern their optical
715 features have not been fully transferred to a routinely used biosensing scheme and the majority of the
716 examples in the literature do not surpass the proof-of-concept level in which a simple
717 biofunctionalization of the surface followed by a preliminary detection assay is carried out, thereby fully
718 neglecting any further optimization of the addressed biosensing assay.

719 Currently, one of the most promising uses of nanohole arrays for the creation of refractometric
720 nanoplasmonic sensors relies on suspending these structures, such that the holes act as nanochannel-
721 based structures. In this configuration, the analytes are forced to flow through the nanometer-sized

722 sensing channels, where the nanohole concentrates the most sensitive areas. This approach does not
723 only enhance the analyte delivery, but also improves the binding efficiency and reduces the sample
724 consumption. Gordon's group [181-183] demonstrated the applicability of these structures as
725 optofluidic sensors achieving a significant enhancement in adsorption kinetics compared with flow-over
726 strategies and a 2-fold LOD improvement by concentrating the target in the nanohole proximity [184].
727 Biosensing was demonstrated as proof-of-concept by binding an antibody to a previously functionalized
728 surface. Real time measurements can be performed due to the introduction of a fluidic cell integrating
729 the sensing structure. Altug's group designed a very similar structure with a bulk sensitivity of 535
730 nm/RIU [185]. Furthermore, a 14-fold improvement in the mass transport rate constants was achieved
731 [186]. Virus detection based on the immobilization of specific antibodies onto the surface (not restricted
732 to the in-hole section but over all the metal surface) has been demonstrated with this design, although
733 the measurements were based on end-point analyses, requiring long-time incubations (60-90 min) and
734 no microfluidics were used [187]. Hook *et al.* have also fabricated so-called nanopores based on
735 suspended arrays of nanoholes [188]. However, besides the optimization of the structure and its
736 characterization, these authors have devoted a great effort in the controlled functionalization of gold
737 areas while protecting the rest of the substrate (SiN) from non-specific adsorptions: the use of
738 appropriate antifouling compounds such as pegylated compounds with thiols or with poly-lysines, allow
739 the particular binding to each region (gold or silicon, respectively), thereby minimizing nonspecific
740 adsorptions. If the thiol-pegylated compounds also contain additional functionality (i.e. biotin groups),
741 subsequent biofunctionalization can take place and can be controlled at the areas of interest (gold and
742 not silicon). These experiments showed a 10-fold faster response as compared with non-suspended
743 holes with diffusion-controlled binding.

744 3.4 Integration, Microfluidics and Multiplexing

745 Despite the reported advantages of LSPR-based sensors in terms of multiplexing and integration, only
746 few works have dealt with these aspects. So far, to our knowledge, only one product has reached
747 commercial implementation [189]. Lamdagen Corporation has launched a device based on LSPR for
748 laboratory use. The device allows real time measurements using syringe pumps and nanostructured
749 metallic films as sensing chips. Four or eight-spot arrayed substrates can be used which can be
750 simultaneously monitored, yielding a medium degree of throughput. The device consists of light sources
751 connected to optical fibers to direct the light onto the surface and output optical fibers which collect the
752 reflected light directly to individual spectrometers, permitting individual channel monitoring. The
753 equipment also incorporates software to analyze the data. The reproducibility and robustness of the
754 system seems to be high and complex media such as serum and saliva can be analyzed. Despite this
755 potential, the sensitivity levels in a label-free configuration without the aid of amplification are still fair,
756 lying in the nM range. Nevertheless, this is an interesting example of integration LSPR system, and can
757 be considered an initial step towards point-of-care devices.

758 Nanoplasmonic point-of-care devices inevitably require three crucial aspects: compactness, ease-of-use
759 and potential for high-throughput. Removing the need of a spectrophotometer and using light emitting
760 diodes (LEDs) as LSPR excitation source can specifically contribute to simplification of the system, as
761 recently demonstrated by Huang *et al.* [190]. The authors have designed a setup where a LED ($\lambda=530$
762 nm) is used for plasmon excitation, while a quadrant photodetector is used to continuously measure the
763 change of the transmitted light at a fixed wavelength (see Figure 6.A.). Dual-channel microfluidics (for
764 sample and reference) has been fabricated and integrated with the nanoplasmonic chip. This flow cell
765 incorporates an automated sample delivery system consisting of an off-chip micropump and microvalves
766 [191]. Calibration curves of the interaction of Anti-biotin antibody to biotin-coated nanoparticle surface

767 were demonstrated as proof-of-concept, although with limited sensitivity, mainly due to the properties
768 of the substrate (gold nanoparticles that rendered a sensitivity of 10^{-4} RIU and a LOD of 270 ng/mL of
769 antibody).

770 A similar approach was presented by Neuzil *et al.* [192], in which a palm-size reflectance-based LSPR
771 sensing scheme was fabricated, that also uses LED illumination and a photodiode-based detection
772 scheme, yielding a simplified setup (see Figure 6.C.). In this case, instead of a single LED, four different
773 LEDs were combined, to improve either data normalization or to simply allow for a more efficient
774 selection of the most suitable one for a specific application. The reflected light coming from each LED is
775 lead to a single photocurrent output, after which the signals are de-multiplexed and digitized, before
776 being displayed on an incorporated LCD display. Although the device undoubtedly shows good potential
777 for future point-of-care devices, the lack of incorporated microfluidics forces the measurements to be
778 based on static incubation. However, up to this moment, no biosensing demonstration of this device has
779 been presented.

780 More recently, another portable transmission-based nanoplasmonic sensor has been reported [193],
781 based on the use of three individual LEDs combined with on-board signal amplification (Figure 6.B). This
782 scheme uses the monitorization of spectral LSPR shifts, using an algorithm that extracts the spectral
783 position of the resonance both before and after molecular interaction events. Immobilization of single-
784 stranded DNA was carried out as a proof-of-concept. However, the straightforward subsequent
785 detection of complementary target has not been yet tested, and moreover, the measurements were
786 performed in air under static conditions. Also aiming at increasing the compactness of the sensing
787 devices, Mazzota *et al.* [194] have presented an integrated detection based on the use an array of small
788 photoactive diode regions (i.e. silicon p/n junctions) that act as independent photodetectors. In this
789 case, two of them have a nanostructure patterned on their surface (nanodisks on Si_3N_4 coated glass),

790 while the other two are used as references. Changes in the extinction spectra caused by binding
791 interactions then result in real-time measurable changes of the photocurrent output. By incorporating
792 the nanoplasmonic chip in a flow cell, its biosensing capability was shown by preparing a biotinylated
793 surface and subsequently monitoring the real-time binding of Neutravidin. Furthermore, also
794 implementation of a Vertical-cavity surface-emitting laser (VCSEL)- optical excitation, combined with a
795 CCD camera for detection, significantly reduces the dimensions of the setup [195]. In this work, the
796 biosensing measurements based on the study of biotin-neutravidin interactions, showed similar
797 outcome when compared to the use of a spectrophotometer.

798 The advantage of using a CCD camera for multiplexed measurements with an LSPR-based device has
799 recently been demonstrated by Ruummele *et al.* [196]. They have reported the first example of full
800 spectral imaging of a macroscale LSPR sensor array by modifying and adapting a commercial SPRi with a
801 liquid crystal tunable filter (LCTF), a flow cell and by aligning the camera with the illumination path (See
802 Figure 6.C.). The LCTF, which is used to filter the white light illuminating the nanoplasmonic chip,
803 enables the monitorization of either visible or near-IR wavelengths. While scanning, a camera
804 simultaneously captures images whose intensity maps are then correlated with wavelength, enabling
805 the extraction of region-specific LSPR spectra, whose spatial resolution is theoretically diffraction-
806 limited. Substrates with sizes of 6.45 cm^2 containing different nanodisks areas, which can be individually
807 biofunctionalized and monitored were fabricated. Simultaneous binding measurements could be
808 performed over an area of around 1.1 cm^2 and as proof-of-concept two approaches were demonstrated:
809 (i) homogeneous coverage of the nanodisk surface with biotin at a unique concentration and anti-biotin
810 detection at different concentrations, using static measurements whose results yielded a calibration
811 binding curve; (ii) the controlled immobilization at different areas using different DNA probe
812 concentrations and a subsequent in-flow delivery of the complementary sequence at a single
813 concentration; in this case, multiplexing throughput is incorporated under dynamic conditions. Although

814 not compact enough to be denominated as “lab-on-chip”, this is an attractive approach that attempts to
815 expand LSPR sensing with multiplexed capabilities, similar to the transition of SPR to SPRi.

816 The previous examples aim at the integration of the optical components and those needed for the
817 different detection read-outs; in this regard, some of them use static measurements while others allow
818 for in-flow sample injection via the use of custom-made single-channel flow cells.

819 However, the successful implementation of more complex microfluidics, that assure both
820 miniaturization and improved throughput capabilities, still remains a challenging task. In literature some
821 examples can be found of nanoplasmonic biosensing with more elaborated PDMS microfluidics, ranging
822 from a few fluidic channels, (1-2 channels [197, 198]) to high throughput chips (nanohole substrate with
823 50 channels monitored in parallel by a CCD camera [199]). Next to PDMS, other polymeric materials
824 offer more compatibility with mass production and low-cost fabrication such as cyclic olefin copolymer
825 (COC). COC has profiled itself as an interesting alternative material for the fabrication of microfluidic
826 systems, illustrated by the previously described dual-channel system designed by Huang *et al.* [190]. A
827 more complex configuration that incorporates up to 64 differentiated incubation chambers is described
828 by Malic *et al.* [200]. They have designed and fabricated monolithic thermoplastic microfluidics which is
829 based on three layers: the nanostructured bottom layer (flow layer), which, next to the chambers (8x8)
830 and the fluidic channels, also incorporates the plasmonic nanostructures (nanogratings); the top layer
831 (control layer) which is built out of a thermoplastic elastomer (TPE) and allows the integration of
832 reservoirs and active fluidic elements such as an array of pneumatic valves to ensure the delivery of
833 fluids; finally, an intermediate membrane whose main function is the assembly of the entire device. This
834 apparatus operates under pressure driven flow supplied by a multichannel syringe pump equipped with
835 switching valves for sample loading. The design allows both differentiated functionalization and
836 detection in a row/column configuration. This is done by opening/closing the corresponding valves of

837 each functionalization/sample loading channel in such a way that up to 64 different conditions can be
838 tested. The experimental setup is designed to perform sequential transmission measurements, by using
839 a XYZ rotational stage to individually align the illumination source with each chamber. The authors have
840 characterized the device in terms of bulk sensitivity and more interestingly, they have done kinetic real
841 time measurements for the detection of CD44, a clinically relevant biomarker. They have monitored all
842 the steps, from the surface derivatization with a thiol-based reagent, to the attachment of specific
843 antibody anti-CD44 and the subsequent detection of CD44 at different concentrations, enabling the
844 determination of binding and affinity constants. Direct detection (with a LOD of 5.26 nM) and also
845 amplification with a secondary antibody to improve sensitivity (up to a LOD of 10.53 pM) were
846 performed, with reproducibility studies included. This impressive high-throughput approach would gain
847 additional merit, especially when viewed from a POC perspective, if parallel read-out of all chambers
848 could be performed.

849 **4. Considerations, Future Trends and Conclusions**

850 As reflected in this review, the work published in the field of refractometric nanoplasmonic biosensing is
851 often of a very fundamental nature. Without a doubt, the main reason behind this is the relative youth
852 of this ever-expanding field of research. Examples in literature of nanoplasmonic sensing schemes that
853 report interesting biosensing capabilities are typically accompanied by novel nanoplasmonic structures
854 that often rely on new material properties. In most cases, the novelty of these *new* materials relegates
855 their use as biosensing platforms to a secondary plane. In this regard, these studies are often
856 accompanied by – far from optimized – preliminary proof-of-concept biosensing assays.

857 Several of these works clearly illustrate the great potential offered by nanoplasmonic structures as
858 biosensing platforms. On this subject, one should emphasize several - previously discussed - studies at a
859 single particle level that attribute better sensing performances to specific nanoparticle geometries, such

860 as nanorods, when compared to conventional SPR sensors. The most eye-catching example is
861 undoubtedly the successful accomplishment of single-molecule detection, often considered the most
862 important milestone of nanoplasmonic sensing. However, the voluminous and expensive experimental
863 setups needed for the spectral interrogation of single nanoparticles, infringes the demands required for
864 the creation of compact and low-cost platforms that can be used out of the laboratory.

865 To achieve the latter, more opportunities are offered by sensing schemes based on nanostructured
866 substrates, which, accompanied by facile experimental setups, possess much more opportunities for the
867 fabrication of compact and integrated devices, with real possibilities of technological transfer into
868 commercial products. However, on the downside, the reported biosensing performances of
869 nanoplasmonic sensors based on this concept are typically of the same order as standard SPR sensors
870 [97], thereby not adding any significant benefits when it comes to sensing performance. Although this
871 can be compensated by other interesting properties, like miniaturization, integration and multiplexing
872 capabilities, the sensitivity typically is considered the key factor that makes or breaks the potential of a
873 sensing scheme.

874 During the last lustrum, an ever increasing scientific interest can be observed involving the use of
875 plasmonic metamaterials for sensing purposes. These metamaterials, consisting of precisely tailored
876 nanostructured substrates that exhibit optical properties not seen in nature, can give rise to exciting
877 optical responses with high FOM-values, suggesting their potential use as nanoplasmonic sensing
878 platforms. In this category, a special interest goes out to Fano resonances, that is, optical modes caused
879 by interference of continuum broad optical modes and strong localized plasmons. The optical modes are
880 excited in precisely engineered or even self-assembled nanostructures [201], and exhibit either sub- or
881 super-radiant optical modes with asymmetric and sharp spectral fingerprints [202], making a strong
882 argument for their employment as refractometric biosensing schemes [105, 106, 203-209]. Also,

883 metamaterials based on ordered arrays of nanoparticles, which support a guided mode which can be
884 caused either by near-field interactions or diffractive far-field effects, have appeared to be interesting
885 for refractometric sensing applications [88, 89, 210-213]. Furthermore, also arrays of randomly ordered
886 gold nanodisks have been shown to act as thin layers of meta-atoms with very high effective RI, allowing
887 the guidance of in-plane EM modes [214]. Through the use of these guided modes, the overall
888 biosensing performance of isolated gold nanodisks can be improved by more than one order of
889 magnitude, which was shown with direct label-free antibody detection.

890 However, also in works involving metamaterials, the focus is typically pin-pointed on the novelty of the
891 material itself, whereas the presented biosensing evaluation (if presented) is often under-highlighted
892 and relegated to a secondary role. We have no doubt that this situation will change in the next ten to
893 fifteen years, but it will not be until then, that nanoplasmonic sensors will reach a point where their
894 massive use as commercial biosensing platforms becomes attractive. In order to accelerate this
895 technology-transfer process, we argue that more research effort should be focused on one of the most
896 underexposed aspects of nanoplasmonic biosensing: *the surface chemistry*. As discussed in this review,
897 proper biofunctionalization of the surface (with or without complementing microfluidic systems) has led
898 to significant sensitivity enhancements of nanoplasmonic sensing platforms, mainly caused by
899 suppressing non-specific interactions of molecules and forcing biomolecular interactions to take place
900 solely at EM hot-spots of the nanostructures. Examples that point towards this direction are still few in
901 number.

902 Therefore, in order to dethrone conventional SPR sensing (and its well-studied planar surface
903 chemistry), the current knowledge of nanoparticle biofunctionalization should expand drastically. It will
904 not be until then that, when accompanied by low cost and large-scale fabrication techniques,

905 commercial nanoplasmonic sensing platforms with integrated microfluidics hit the market, and should
906 be considered as viable technological alternatives for conventional SPR sensors.

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Tables

Table 1. Examples of LSPR biosensing assays

Substrate	Features	Bioassay	Sensitivity/LOD	Ref.
Au Nanoholes	<ul style="list-style-type: none"> Flow-through nanoholes; static measurements in air 	<ul style="list-style-type: none"> Viruses (VSV, Ebola and Vaccinia) Direct detection with Ab oriented via Protein G Cell-growth media measurements 	10^6 - 10^9 pfu/mL-	[187]
Au Nanoholes	<ul style="list-style-type: none"> Flow-through nanoholes ; real-time measurements Controlled gold surface modification preserving SiN from nospecific adsorption 	<ul style="list-style-type: none"> Neutravidin binding Biotin-PEG immobilized on gold 	n.r.	[188]
Au Nanoholes	<ul style="list-style-type: none"> Non-suspended nanoholes. Flow real-time measurements SiO_x layer on top of gold 	<ul style="list-style-type: none"> Study of supported lipid bilayer (SLB) formation Protein binding Conformational changes 	n.r.	[179]
Au Nanoholes	<ul style="list-style-type: none"> Non-suspended nanoholes. 	<ul style="list-style-type: none"> Autoantibodies detection on SLB 	n.r.	[180]

	<ul style="list-style-type: none"> • Flow real-time measurements • SiO₂ layer on top of gold 	<ul style="list-style-type: none"> • Complete kinetics characterization 		
Au nanodisks	<ul style="list-style-type: none"> • Flow real-time measurements • No specificity studies • Not detailed assay optimization 	<ul style="list-style-type: none"> • Extracellular adherence protein (EAP) • Direct detection with Ab 	8pM (estimated)	[146]
		<ul style="list-style-type: none"> • Prostate specific antigen (PSA) • Direct detection with Ab 	1pM (estimated)	
Au nanodisks	<ul style="list-style-type: none"> • Flow cell to deliver samples • Static measurements after incubation • No specificity studies • Not detailed assay optimization 	<ul style="list-style-type: none"> • Prostate specific antigen (PSA) • Direct detection with Ab 	>280 pM	[81]
		<ul style="list-style-type: none"> • Sandwich assay + substrate precipitation 	83 fM (3pg/mL)	
Au capped NPs	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • Casein detection in milk • Direct detection with Ab oriented via Protein G 	10 ng/mL	[147]
Au capped NPs	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • IL-2 secreted from cells after stimulation • Direct detection with Ab Oriented via Protein A 	10 pg/mL	[148]
Au capped NPs	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • Tau protein detection in • Direct detection with Ab oriented via Protein G 	10 pg/mL	[149]
Au capped NPs	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • Melittin (peptide toxin) 	10 ng/mL	[150]

		<ul style="list-style-type: none"> • Interaction study with Hybrid bilayer membrane (HBM) 		
Au-capped NPs	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • Fibrinogen • Direct detection with Ab oriented via RNA aptamer which recognizes Fc fraction 	0.1 ng/mL	[151]
Au-capped NPs (Multispot)	<ul style="list-style-type: none"> • Static measurements after incubation • Sequential spot measurement 	<ul style="list-style-type: none"> • Antibody against Avian influenza antigen (Ala) • Gold binding preptide-Ala fusion protein (GBP-Ala) immobilized on the surface 	1 pg/mL	[154]
Au-capped NPs (Multispot)	<ul style="list-style-type: none"> • Static measurements after incubation • Sequential spot measurement 	<ul style="list-style-type: none"> • Hepatitis B (HB) antigen (HBsAg) detection • GBP-scFv fusion protein immobilized on the surface 	100 pg/mL	[153]
		<ul style="list-style-type: none"> • Anti-HBsAg deetection • GBP-HBS-Ag fusion protein immobilized on the surface 	1 pg/mL	
Au-capped NPs (Multispot)	<ul style="list-style-type: none"> • Static measurements after incubation • Sequential spot measurement • Real samples analysis 	<ul style="list-style-type: none"> • Single point mutation detection of BIGH3 gene (related to Corneal Dystrophy) • Complementary sequence immobilized 	1pM target DNA	[155]

Cu-capped NPs (Multispot)	<ul style="list-style-type: none"> • Static measurements after incubation • Sequential spot measurement • Real samples (Clinical Isolates) 	<ul style="list-style-type: none"> • Bacterial DNA detection • Complementary sequence immobilized 	10 fM target DNA	[156]
Au NPs	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • Stazonolol (steroidal hormone) detection • Indirect competitive immunoassay with specific Ab 	2.4 nM	[215]
Au deposited on nanoporous structure (Anodicaluminum oxide substrates AAO)	<ul style="list-style-type: none"> • Static measurements after incubation 	<ul style="list-style-type: none"> • CRP detection • Direct detection with Antibody immobilized 	1 fg/mL	[216]
Ag NPs	<ul style="list-style-type: none"> • Static measurements 	<ul style="list-style-type: none"> • P450 Cytochrome (CYP3A4) interaction with drugs CYP3A4 stablized and immobilized on NP 	Qualitative	[160]
Ag nanotriangles	<ul style="list-style-type: none"> • Static measurements after incubation • Serum samples • No optimization 	<ul style="list-style-type: none"> • Detection of p53 • Direct detection with Antibody immobilized 	n.r	[217]
Au nanorods	<ul style="list-style-type: none"> • Flow cell to deliver samples 	<ul style="list-style-type: none"> • Enantioselective detection of RS-melagatran 	0.9 nM of RS-	[67]

	<ul style="list-style-type: none"> • Static measurements after incubation • Reproducibility studies 	<ul style="list-style-type: none"> • Chiral recognition using human α.thrombin • Serum matrix studies 	melagatran	
Au nanorods	<ul style="list-style-type: none"> • Flow cell to deliver samples • Static measurements after incubation • Reproducibility and accuracy studies 	<ul style="list-style-type: none"> • Chiral discrimination of racemic mixture of TNA • Protein immobilized on the surface or • Specific antibody immobilized on the surface 	20-100 nM of chiral TNA(depending on the receptor)	[157]
Ag nanoprisms	<ul style="list-style-type: none"> • Flow real time measurements • Accurate surface immobilization for correct orientation 	<ul style="list-style-type: none"> • Conformational changes of calmodulin upon ion interaction • Cqalmodulin-ligand interaction study • Kinetics studies of the conformational change 	600 fmol $\text{Ca}^{2+}/\text{cm}^2$ 23 μM of Ca^2	[158]

Figure Legends

Figure 1. Schematics showing the detection principle of plasmonic biosensors based on (A) Surface Plasmon Polaritons (SPPs or SPRs) propagating along the interface of a metal and a dielectric. (B) Localized Surface Plasmon Resonances (LSPRs) strongly confined to the surface of sub-wavelength metal nanostructures.

Figure 2. Scientific publications centered on the topic of nanoplasmonics (1990-2012). Source: Web of Knowledge.

Figure 3. Diagrams illustrating nanostructure-based biosensing setups: (A) Extinction measurements, (B) Dark-field microscopy and (C) Total Internal Reflection (TIR) microscopy.

Figure 4. Examples of controlled directed functionalization of metal nanostructures (A) Gold nanoholes of TiO₂ functionalized with different surface-selective compounds. The control on the modification of the most sensitive material (gold) leads to signal enhancement [108]. (B) Controlled binding on the hotspots (between two gold nanodisks) and comparison with whole gold nanodisk surface. A 4x signal per molecule enhancement is achieved [109]. (C) Wavelength shift achieved on gold nanoplates after IgG binding on the terrace or in the edges (more sensitive areas) [111]. Reproduced from [108, 109, 111]. Copyright (2010, 2012 and 2011) American Chemical Society.

Figure 5. Schematic representation of short-range ordered arrays of gold nanodisks located on isotropic dielectric pillars, providing a strategy that can be used to increase the refractometric sensing performance of these nanostructures [112]. Single stranded DNA molecules attached to the nanodisks are used as receptor probes for the specific detection of complementary DNA strands.

Figure 6. Examples of integrated LSPR systems. (A) Microfluidics with two cells, the setup scheme and the automated sample delivery system [191]. (B) Compact small size transmission based LSPR using three LEDs as light source. The system incorporates the sample and photo detector [193]. (C) Palm-size reflectance-based LSPR with four LEDs as light source and a photodiode as detector. It incorporates a LCD touch screen display [192]. Reproduced from [191-193], Copyright (2008) American Chemical Society.

Figures

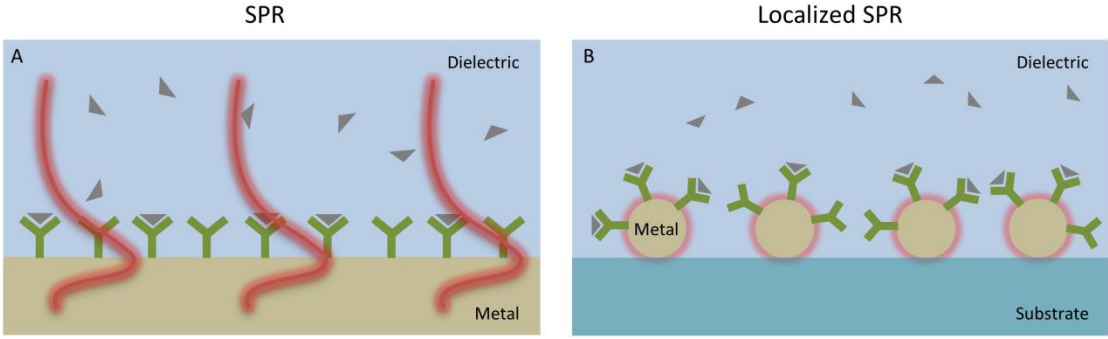


Figure 1

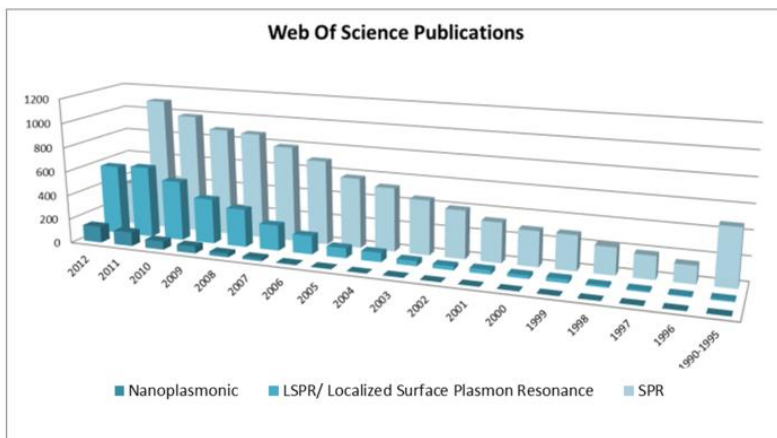


Figure 2

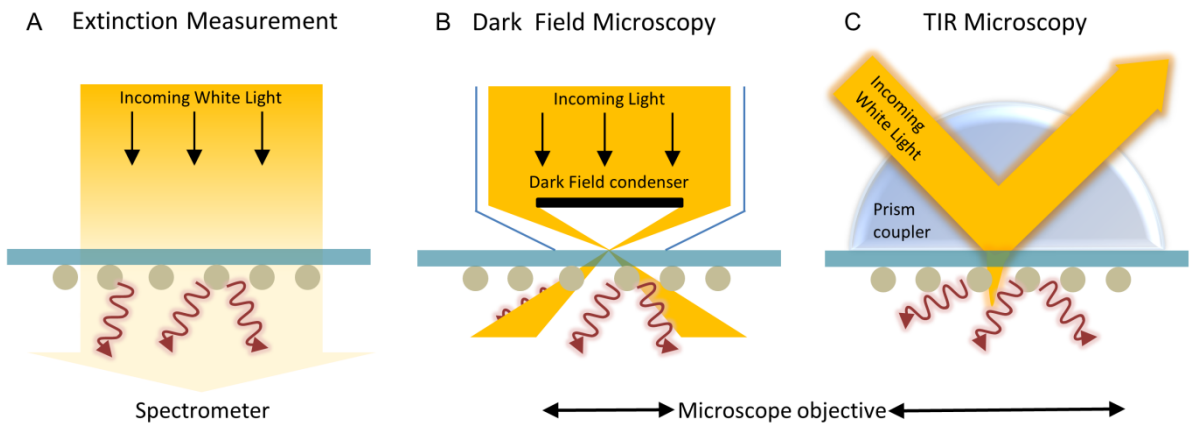


Figure 3

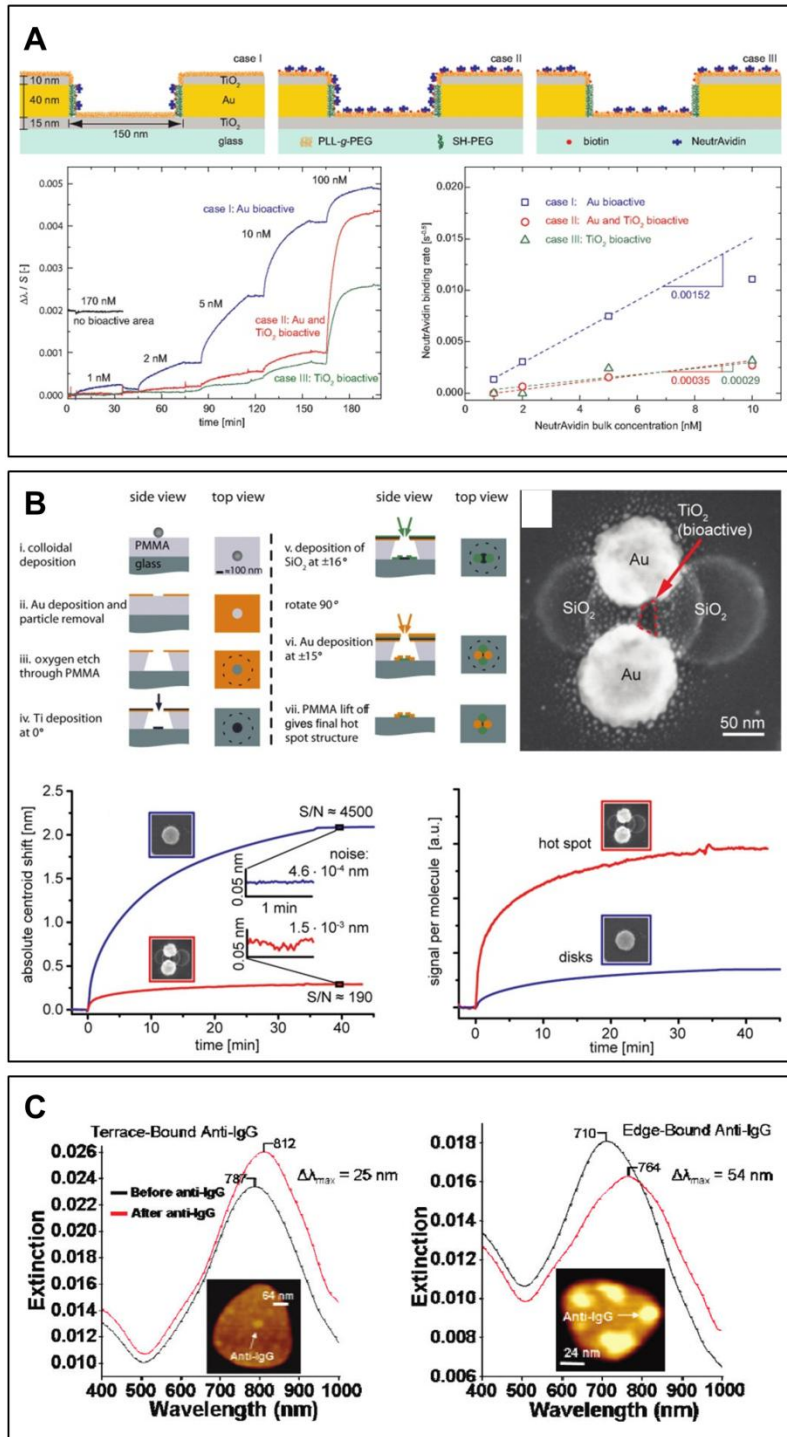


Figure 4

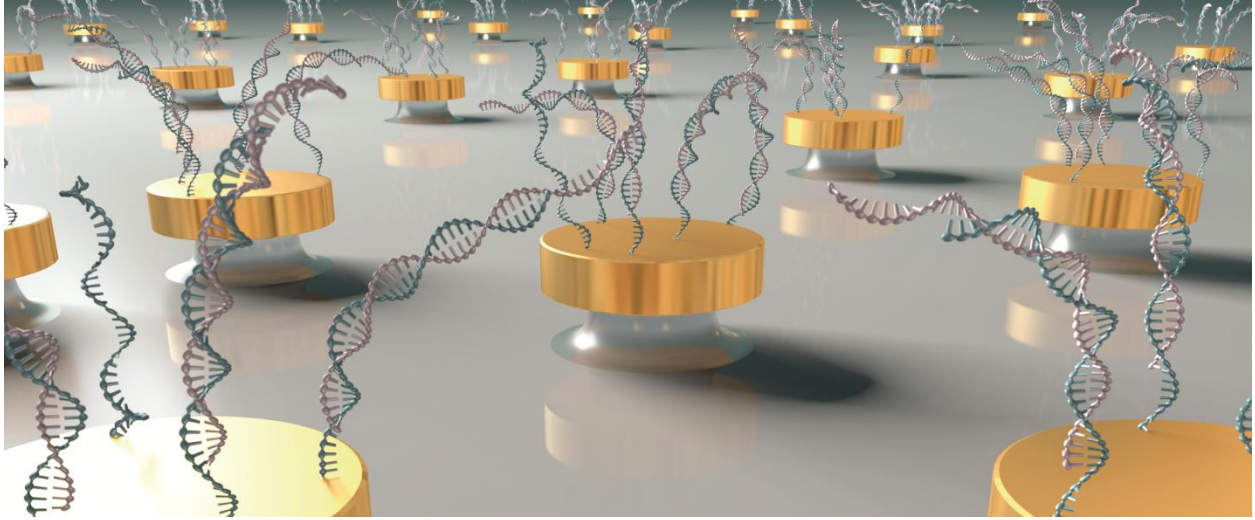


Figure 5

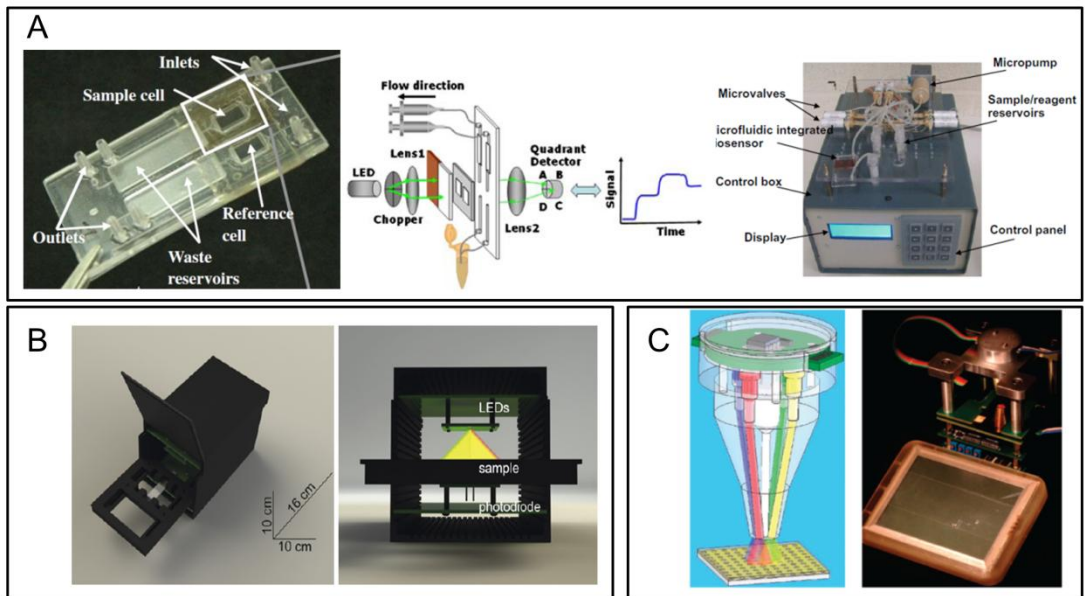


Figure 6