Principles, Technologies, and Applications of Plasmonic Biosensors

Maria Soler* and Laura M. Lechuga

Nanobiosensors and Bioanalytical Applications Group (NanoB2A), Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC, BIST and CIBER-BBN; 08193 Bellaterra, Barcelona (Spain)

ABSTRACT

Plasmonic materials and phenomena have been widely studied and applied in multiple fields since long. One of the most promising applications is in the engineering of biosensor devices, offering label-free and real-time analysis of biomolecular interactions with excellent performances. In this tutorial, we provide a pedagogical review of the working principles of plasmonic biosensors, main fabrication methods, instrumentation, and general guidelines for their development. Especial focus is placed on the biosensor performance characterization and assessment, as well as on the sensor surface biofunctionalization. At the end, we discuss the common procedure to develop and validate biosensors for relevant biomedical and environmental purposes, and future perspectives in terms of boosting capabilities and sensor integration in point-of-care platforms.

1. INTRODUCTION

Biosensors have been profiled as the most promising alternative for modernizing the biological and chemical analysis, which will have a decisive impact in the boost of healthcare and medical assistance - especially in point-of-care diagnosis - as well as in environmental control and monitoring. A biosensor is defined as a self-contained integrated device capable of providing specific quantitative or semi-quantitative analytical information using a biological or biomimetic recognition element, which is in direct spatial contact with a transducer (Figure 1). The biorecognition layer, typically composed of enzymes, antibodies, or nucleic acids, is designed to specifically interact with the target compound in a sample. When the biochemical interaction occurs, a series of physicochemical changes in the medium or the sensor surface are detected by the transducer and converted into discrete or continuous signals that can be read immediately. The engineering and integration of biosensors into the so-called point-of-care (POC) devices offer unique features to improve current analysis techniques in the biomedical or environmental fields.[1–4] The combination of the bioreceptor layer with the transducer in one single device confers the ability to detect the target analyte with high sensitivity and selectivity in a fast one-step. Moreover, biosensors could ideally overcome important limitations of conventional techniques, such as the need of analyte extraction or purification, or the use of additional equipment for signal readout, which is usually operated by specialized personnel. Label-free biosensors, in particular, can also monitor biological interactions in real time, allowing for the evaluation of the affinity and kinetics of the interaction and, thereby helping in elucidating the biochemical mechanisms involved in a disease or in the evaluation and characterization of drugs, for example. Finally, biosensors also benefit from a great versatility, being possible to evaluate a wide range of analytes just by selecting the appropriate biological receptor. Recent advances in nanotechnology further

provide interesting opportunities for biosensor miniaturization, high-throughput, and low-cost production, creating competitive alternatives for point-of-care analysis.[5,6]

The pursuit to obtain powerful biosensors has experienced an exponential growth in the last decades, encompassing the work of numerous disciplines, such as material sciences, physics, engineering, molecular biology, chemistry, or biotechnology. The multidisciplinary nature of biosensor research has led to a vast range of biosensor platforms based on different type of biorecognition elements (i.e., catalytic or affinity-based) or transducers (i.e., electrochemical, mechanic, optical, etc.). Optical biosensors based on plasmonics are among the most widely studied and employed, with an increasing interest in the area, which is continuously introducing novel materials and architectures or demonstrating new high-value applications.[4,7]

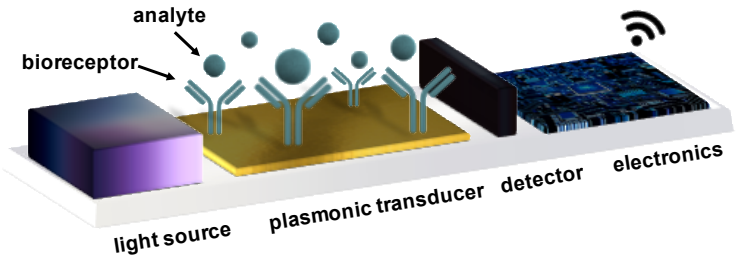

Figure 1. Illustration of a point-of-care biosensor based on plasmonic transducer. The integrated device contains a light source and detector, electronic system for signal readout, and the plasmonic sensor. The sensor surface is functionalized with specific bioreceptors (i.e., antibodies) for the selective interaction with the analyte of interest.

In this tutorial, we will provide a review of the most common plasmonic biosensors focusing on the description of the physics and working principles, the instrumentation of the sensing platforms, and the main performance characteristics. We will provide as well standard guidelines for a proper sensor surface biofunctionalization and a brief comment on the application of plasmonic biosensors to the biomedical and environmental fields.

2. SURFACE PLASMON RESONANCE (SPR) BIOSENSORS

The Surface Plasmon Resonance (SPR) systems are founded on an optical phenomenon that was first noticed between 1900 and 1912 by R.W. Wood.[8,9] He observed an unusual dark and light pattern in the reflected light of a metal-backed diffraction grating, and although he hypothesized about light-metal interactions, no clear answer was provided and the phenomenon was termed as Wood's anomalies. Along the following years, theoretical studies and analysis were performed by Rayleigh,[10] Wood,[11] and Palmer,[12,13] but was Fano in 1941 who led to the conclusion that these anomalies were related to surface waves (*surface plasmons*) supported by the grating structure.[14] In the 50s, further experimental research described the excitation of a surface plasma oscillation of the conducting electrons of the metal, which generates evanescent electromagnetic waves.[15–17] Soon after, in late sixties, the controlled excitation of surface plasmons was achieved by Kretschmann and Raether,[18] and Otto[19] by means of attenuated total reflection (ATR) using prism-coupler based systems; and its first application for sensing was demonstrated by Nylander and Liedberg.[20] On the other hand, Cullen *et al.* were first in developing an SPR based on grating-coupler systems,

demonstrating their sensing performance with immunoassays.[21] Later, in the 80s, the evanescent field SPR principle also found applications in spectroscopy, as for the interrogation of thin chemical and biological films, which was first demonstrated by Pockrand *et al.*[22]
In 1980, the first commercial SPR biosensor was launched (BIAcore. Pharmacia Biosensor AB, Sweden), and ten years later it was first resold.[23,24] Since then, the commercialization and use of SPR biosensor systems has widely expanded and numerous manufacturers are selling different platforms with improved capabilities that are routinely employed in research laboratories or the pharmaceutical industry for the analysis of biochemical compounds and interactions. It is worth mentioning that SPR sensors are sold as bare instrumentation, thus the application for specific biological or chemical assays has to be developed and optimized by the end user.

2.1. Physics and working principle

Surface Plasmon Resonance refers to the coherent oscillations of charge density that exist at the interface between two media with dielectric constants of opposite signs, such as a metal and a dielectric (Figure 2a). The surface plasmons are excited by coupling of an incident polarized light, and they propagate along the metal-dielectric interface as surface plasmon polaritons (SPP), behaving like a quasi-free electron plasma and generating an electromagnetic field with an exponentially decaying intensity into both media (i.e., evanescent field).[25] The SPP is a transverse-magnetic (TM) polarized mode, that is, the magnetic vector is perpendicular to the direction of propagation of the wave and parallel to the plane of the interface. Solving Maxwell's equations with appropriate boundary conditions, the SPP propagation component can be expressed as a function of both the metal and the dielectric permittivity:[25–27]

$$k_x^{SPP} = \frac{\omega}{c}\sqrt{\frac{\varepsilon_m \varepsilon_d}{\varepsilon_d + \varepsilon_m}}$$

In this equation, ω is the angular frequency, and c the speed of the light in vacuum; ε_m represents the frequency-dependent and complex dielectric function of the metal ($\varepsilon_m = \varepsilon_m' + i\varepsilon_m''$) and ε_d is the dielectric constant of the medium, which is directly related to the refractive index ($\varepsilon_d \approx n_d^2$). This direct dependency between the propagation vector and the refractive index of the dielectric constitutes the main principle of refractometric sensing platforms.

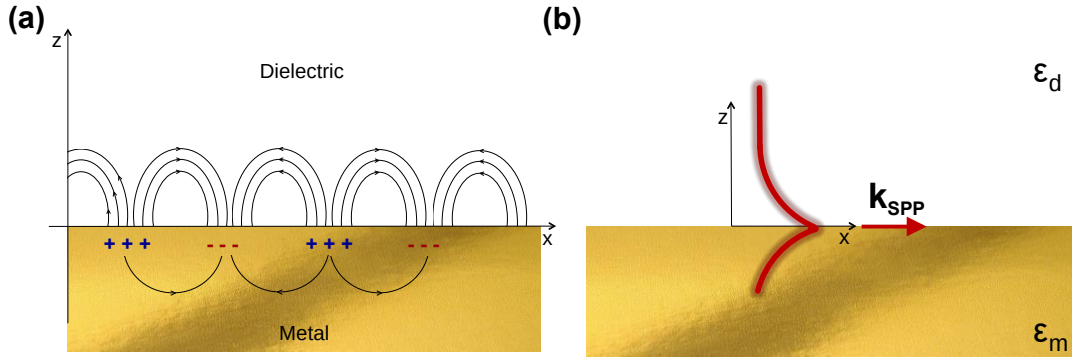

Figure 2. Schematics of Surface Plasmon Resonance (SPR) at the plane interface of a metal and a dielectric showing: **(a)** the collective charge oscillation at the surface, and **(b)** the transversal evanescent field distribution. Axis represent the Cartesian coordination system.

For the SPP to occur, the equation square-rooted denominator ($\varepsilon_d + \varepsilon_m$) needs to have an absolute positive value, therefore the real part of the ε_m must be negative and its absolute value smaller than ε_d. At visible wavelengths, this condition is fulfilled for several metals, from which gold, silver or aluminum are the most commonly used in plasmonics. Further, due to the relatively small imaginary part of the refractive index of these metals, they show strongly suppressed plasmonic attenuation when compared to other materials thus minimizing propagation losses. It is worth mentioning here that certain plasmonic behavior has also been observed in non-noble metals and dielectric materials (like graphene), introducing interesting advantages in terms of energy losses or reconfiguration, but their utilization for biosensing has not spread yet.[28–30] The evanescent field generated by an SPP is confined at the metal-dielectric interface and decreases exponentially into both media (Figure 2b). The field is distributed in a high asymmetric fashion and most of it is concentrated in the dielectric medium close to the metal surface, showing a typical penetration depth between 50-500 nm when working in visible or near-infrared (NIR) wavelengths.[27,31] This is particularly significant for SPR sensing, as it represents the depth probe, meaning that only refractive index changes occurring within the evanescent field penetration depth will alter the SPP propagation and can be detected. Therefore, when a biochemical interaction or biorecognition event takes place at the surface of the plasmonic metal, it can be directly monitored by interrogation of certain properties of the reflected light, like wavelength, angle, or intensity. The evanescent field-based working principle confers plasmonic sensors one of the most important assets: the label-free detection capability. This feature enables the direct monitoring of biochemical reactions and quantification of specific analytes without the need of tags (fluorescent or colorimetric), secondary or amplification steps, therefore simplifying the assay and greatly reducing the analysis turnaround time.

2.2. Instrumentation

The different SPR-based optical configurations can be classified upon the SPP excitation method or the detection scheme. The excitation of the SPR is achieved by coupling a light wave to the surface plasmons in such a way that the light's wavevector component parallel to the interface matches the propagation vector of the SPP:

$$k_x^{Light} = \frac{2\pi}{\lambda}\sqrt{\varepsilon_d}\sin\theta = k_x^{SPP}$$

Generally, the SPP propagation vector is considerably larger than the wavenumber of the light wave in the dielectric; therefore, surface plasmons cannot be excited by direct illumination on smooth surfaces. Common techniques employed for the incoming light coupling make use of prisms, gratings, or waveguides.[31] *Prism couplers* are the preferred method for the optical excitation of surface plasmons (Figure 3a). In the well-known Kretschmann configuration, based on the attenuated total reflection (ATR) phenomenon, light passes through a high refractive index glass prism and it is totally reflected at the prism base, generating an evanescent wave that penetrates the metal film. This evanescent wave propagates along the interface with a certain propagation vector, which can be adjusted to match that of the SPP by controlling the angle of incidence.[18] Excitation via *grating couplers* is based on the diffraction of the light waves (Figure 3b).[32] The component of the wavevector of the diffracted waves parallel to the interface is increased by an amount inversely proportional to the period of the grating and can be matched to that of the SPP. The excitation of SPP can also be

achieved by using optical *waveguide structures* (Figure 3c).[33] The light is guided by a high-refractive index planar optical waveguide - or cylindrical in the case of optical fibers - and, when entering the region with a thin metal layer on top of the waveguide, it evanescently penetrates through the metal exciting an SPP at its outer interface. In the last years, the use of photonic crystals has emerged as waveguides for SPP excitation and several devices have been realized employing periodic structures,[34] microstructured fibers,[35] or Bragg fibers.[36]

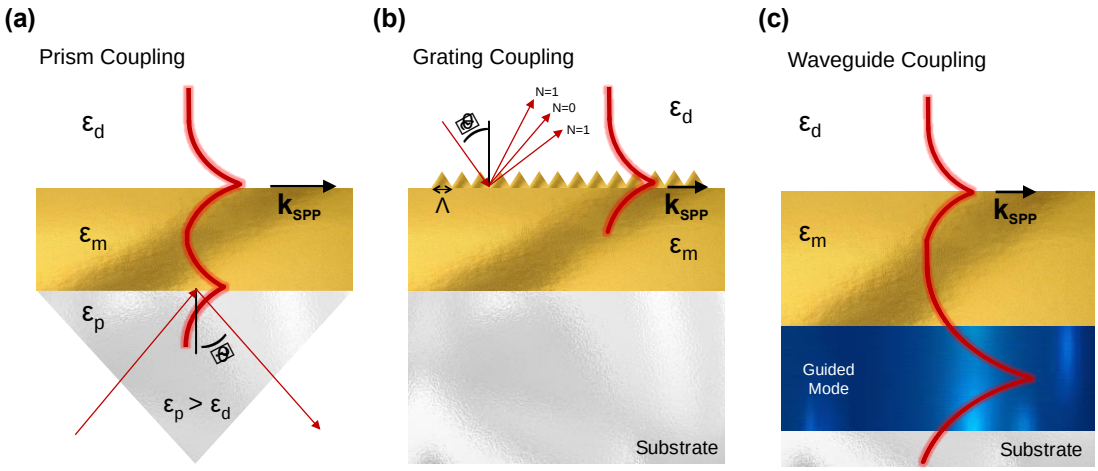

Figure 3. Schematics of most conventional SPR coupling methods, including **(a)** prism-coupled Kretschmann configuration **(b)** grating coupling, and **(c)** waveguide coupling. Thick red curves represent the distribution of EM evanescent fields. Red arrows represent light incoming and outputs.

Regarding the detection scheme, SPR sensors monitor changes or displacements of the spectral reflectivity dip, which strongly depends on the refractive index of the dielectric. Such interrogation can be based on angle, intensity, wavelength, or phase.

The *angle-based interrogation* is one of the most common methods employed in commercial SPR systems.[37] This configuration uses quasi-monochromatic light sources to excite the surface plasmons, and the SPR curve is obtained by representing the reflectivity as a function of the angle of incidence. Changes in the SPP can be observed by monitoring the entire curve displacements, the minimum value of the SPR dip, or by applying the centroid method, which calculates the center of mass of the spectral peak so that it minimizes possible interferences and background noise.

For *intensity-based interrogation*, both the light wavelength and the incidence angle are fixed. The angle is usually set at the point of maximum slope of the resonance dip. Therefore, changes in the SPP are detected as an increase or decrease of the reflectivity values. This approach has shown interesting advantages, especially for the development of SPR imaging (SPRi) systems.[38] By acquiring the reflectivity with two-dimensional detectors, such as charge-coupled devices (CCDs) or complementary metal-oxide sensors (CMOS), it is relatively easy to extend the label-free and real-time analysis to a multiplexed 2D array format. In regard of the detector type (CCD vs CMOS) much debate still exists in the research community. On one hand, CCDs have shown higher performances (e.g., sensitivity) and versatility (e.g., customization), especially when working in the near-infrared region. On the other hand, CMOS outperforms CCDs in the visible range, has shown a higher integration potential, and it is often more suitable for high-throughput applications. Nevertheless, the implementation of both technologies as SPR detectors can greatly enhance the multiplexing capabilities and

boost the miniaturization and integration of the instrumentation. Furthermore, since both wavelength and angle are fixed, this detection scheme avoids the possible noise background produced by mechanical rotors and it allows for using low-cost sources like laser or light emitting diodes (LEDs).

In the *wavelength interrogation* scheme, a broadband or polychromatic light source is employed for excitation and the SPR curve is obtained at the optimal fixed angle. The reflected light is usually coupled to a spectrometer, enabling the direct interrogation of the plasmonic dip wavelength position. This format provides a high sensitivity and it also allows the use of low-cost sources like halogen lights or LEDs, but the need of high-resolution spectrometers limits the miniaturization and integration capabilities.[39]

In recent years, *phase-based interrogation* methods have received a great attention. This technique evaluates the variation in the phase of the reflected light, which is known to change more abruptly than its intensity. However, measuring light high-frequency oscillations (around 10^{14} Hz) requires complex optical read-out schemes. The most common approach employs a polarizer in a configuration similar to ellipsometry. SPR excitation is associated with changes in the p-polarized light, while the s-polarized light remains invariant, therefore one can extract phase information from the interference of s- and p-polarized light. Other methodologies are based on heterodyne detection, shear interferometry, or spatiotemporal phase modulated interferometry.[40,41]

The latest research in SPR biosensors has been driven by the necessity of offering portable systems able to be deployed at the point of care or in resource-constrained settings. In this regard, the emergence of smartphones with high-performance cameras, LED flashlights, Internet connectivity, and high-resolution screens offers a unique opportunity to incorporate optical biosensors in our daily-use devices.[42] The CMOS camera of the smartphones can record not only the color (RGB or HVS model) but the light spectrum, so they can be used for interrogating the SPR transducer signal. Some smartphone-based SPR biosensors have been developed following this scheme; for example, an SPR imaging platform has been developed based on grating-coupled format using metal-coated Blu-ray disks as sensors. The LED flashlight is used to excite the surface plasmons and the reflected light is passed through a compact disk to spatially disperse the wavelength spectrum, which is imaged using the CMOS camera of the smartphone. Built-in lens on the front of the CMOS allow the image to be focused by touching the screen and the zoom-in of the camera enables magnification.[43] An angle-resolved prism-coupled SPR detection system has also been demonstrated using a disposable cartridge containing the gold sensor and assembled on the front camera for detection. The device uses the smartphone light source, and the spectral dip of the reflected light is traced on the screen as function of the angle and wavelength.[44] This demonstrates that although being a relatively mature technology, research on SPR biosensors is still ongoing actively and, in combination with other cutting-edge technologies, it might soon become a powerful diagnostic tool within reach of everyone.

3. NANOPLASMONIC BIOSENSORS

Amid the rise of nanotechnology, the research on plasmonic biosensing has discovered and incorporated new features and phenomena, only occurring at subwavelength dimensions, able to improve resolution, performance, and integration capabilities of the sensor systems.

Although nanoplasmonics is considered a relatively recent research field, the first uses of the exceptional effects of light-matter interaction at the nanoscale date back to the 4th century, being a clear example the Lycurgus Cup; an ancient Roman cage-cup currently exposed in the British Museum.[45] This vessel is made of dichroic glass containing silver nanoparticles. The cup appears green when viewed in the reflected light, whereas when light is transmitted from the inside, it appears to be red, illustrating the plasmonic phenomena of nanoparticles. However, controlled fabrication and characterization of nanoparticles was not achieved until few decades ago. With the implementation of nanofabrication techniques like e-beam litography (EBL) or focused ion beam (FIB) litography, high-precision nanostructured surfaces began to be evaluated as sensor transducers. These nanostructures show interesting advantages compared to propagating SPR, such as the excitation of plasmonic resonance by direct illumination, overcoming the need of complex light coupling systems, or the enhanced near-field evanescent field, which might increase the sensor surface sensitivity. Today, a few nanoplasmonic biosensors have arrived in the market (e.g., Nicoya in Canada, or LSPR AG in Switzerland) but their implementation as routine testing instrumentation in laboratories or clinics has not been yet accomplished. Research on nanoplasmonics is still on the way to incorporate all assets provided by the nanotechnology, making a truly operative and robust system that can bet the conventional SPR biosensor.

3.1. Physics and working principle

Light interaction with subwavelength-sized metallic nanoparticles arises the so-called Localized Surface Plasmon Resonance (LSPR), a non-propagating oscillation of the conducting electrons.[46,47] This effect is due to the accumulation of polarization charges on the surface of the nanoparticle, which is acting as a dipole (Figure 4). The dipolar field is responsible for the enhanced absorption and scattering of the light, as well as for the strongly enhanced EM field in the close vicinity of the nanoparticle surface.[48]

To simplify the theoretical description of the LSPR principle, we can consider a metallic spherical nanoparticle with $\emptyset \ll \lambda$, where $\emptyset$ is the diameter of the particle and λ is the wavelength of an incident light. Under this condition, the external EM field appears static around the nanoparticle and the charge oscillation behaves as a single dipole with amplitude that is strongly influenced by the distance between the surface charges.[46,47] Herein, the LSPR condition is related to the polarizability (α_0) of the particle, which is given by:

$$\alpha_0 = 4\pi\emptyset^3 \frac{\varepsilon_m(\lambda) - \varepsilon_d}{\varepsilon_m(\lambda) + 2\varepsilon_d}$$

The polarizability represents the distortion of the electron cloud in response to the external EM field and basically depends on the size of the particle ($\emptyset$), and the dielectric functions of the metal ($\varepsilon_m = \varepsilon'_m + i\varepsilon''_m$) and the surrounding medium ($\varepsilon_d \approx n_d^2$). The maximum polarizability is achieved when the absolute value of the denominator approaches zero, hence the LSPR is observed when $\varepsilon'_m = -2\ \varepsilon_d$. For noble metals, the dielectric function (ε'_m) shows a strong dependency on the electromagnetic wavelength, therefore the bright colors exhibited by nanoparticles strictly rely on the exact wavelength at which the LSPR resonance condition is satisfied. For the two most used plasmonic materials, gold and silver, this condition is satisfied in the visible region of the light spectrum, at 560 nm for Au and 450 nm for Ag, respectively.

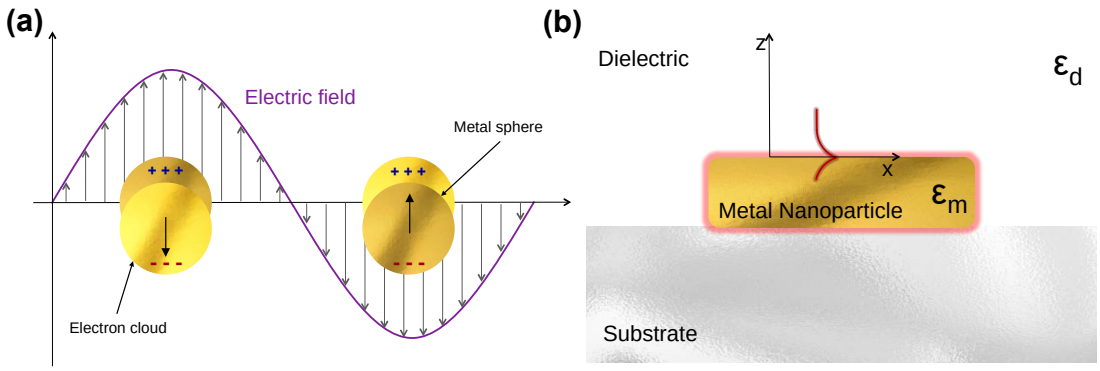

Figure 4. Schematic representation of **(a)** LSPR of spherical nanoparticles positioned in a static electric field, and **(b)** the evanescent field distribution of a metal nanostructured surface. Axis represent the Cartesian coordination system.

This theory can be extended to larger or nonspherical nanoparticles, revealing the appearance of LSPR modes with higher multipoles, where half of the electron cloud moves parallel and half anti-parallel to the external EM field. Especially for ellipsoidal nanoparticles, such as nanorods, surface plasmons split into two distinct modes due to surface curvature and symmetry exhibiting strong polarization-dependent spectra, where small changes in aspect ratio result in significant changes in the absorption band. This size and shape-dependency leads to another important property of plasmonic nanostructures: the spectral tunability. The morphology, size, and distance separation between the nanostructures contribute to the spectral signature of its resonance, dictating the bandwidth and peak position of the LSPR. By varying the size and shape of the plasmonic nanostructures, the LSPR can be tailored and tuned along the entire VIS and NIR regions of the spectrum, so it can fit the most suitable wavelength for specific applications.[49] On the other hand, extensive research has been placed into shaping the optical spectra of metallic nanostrucures by playing with constructive and destructive interferences of the plasmon modes in order to achieve the narrowest bands. In particular, Fano-type interferences have demonstrated a significant reduction of the spectral linewidth of plasmonic resonances.[50] Such narrow bands can be achieved by sculpting multi-particle assemblies (e.g., dimers, trimers, or oligomers) and metasurfaces that exploit the nanometer gaps between plasmonic resonators as dark modes. The unphased coupling of the two modes (bright and dark modes) leads to a narrow resonant band with typical asymmetric line-shape, which can enhance the biosensor sensitivity.

Besides of the material, size, and shape, the LSPR strongly depends on the dielectric constant of the medium surrounding the nanostructures. Changes in the RI of the medium within the evanescent field lead to changes in the polarizability, which results in displacements of the LSPR peak. In contrast to propagating SPR, the LSPR evanescent field is strongly confined to the particle surface exhibiting a rapid decay in the dielectric medium (typically of a few tens of nm). Due to the smaller penetration depth of the evanescent field, the bioreceptors attached to the nanoparticle surface occupy a much larger fraction of the field, which can confer to LSPR sensing high-resolution detection, even at the level of single particle analysis, for instance.

3.2. Fabrication of plasmonic nanostructures

The fabrication of nanoplasmonic sensors can be achieved by either top-down or bottom-up methodologies. The former group relies essentially on lithography patterning techniques while the latter consists on depositing chemically synthesized colloidal nanoparticles to a solid support, commonly glass substrates.

There is an endless variety of colloidal nanoparticles with different geometries, including spheres, rods, triangles, plates, cubes, pyramids, stars, prisms, tubes, etc., which can be fabricated with different synthesis techniques, like seed-mediated growth, anisotropic synthesis or template-assisted techniques. For a detailed description of colloidal plasmonic nanoparticle fabrication techniques, we refer to comprehensive reviews published elsewhere.[51,52] Our focus here is directed to the assembly of nanoparticles onto the solid support. Nanoparticles can be attached to previously functionalized glass surfaces via covalent binding or electrostatic interactions. Glass substrates can be modified with either thiol- or amine- functional groups via silanization or other polymeric procedures, which can strongly immobilize gold nanoparticles on the surface by chemisorption (i.e. thiol-gold interaction) or high-affinity interaction (i.e. amine-gold interaction). However, a major problem is nanoparticle aggregation that leads to low reproducible and low efficiency coverage. To solve this, nanoparticles can be previously covered with a functional protective layer (PEGylated compound), carrying carboxyl, amine, thiol, or biotin groups, for example. This step allows for chemically binding the particles to a functionalized substrate, avoiding aggregation and formation of multilayers. The surface density of nanoparticles can be ultimately controlled by optimizing the concentration of colloidal suspension, incubation time or temperature, etc. However, it is not possible to control the nanoparticle distribution over the surface for the formation of ordered periodic arrays.

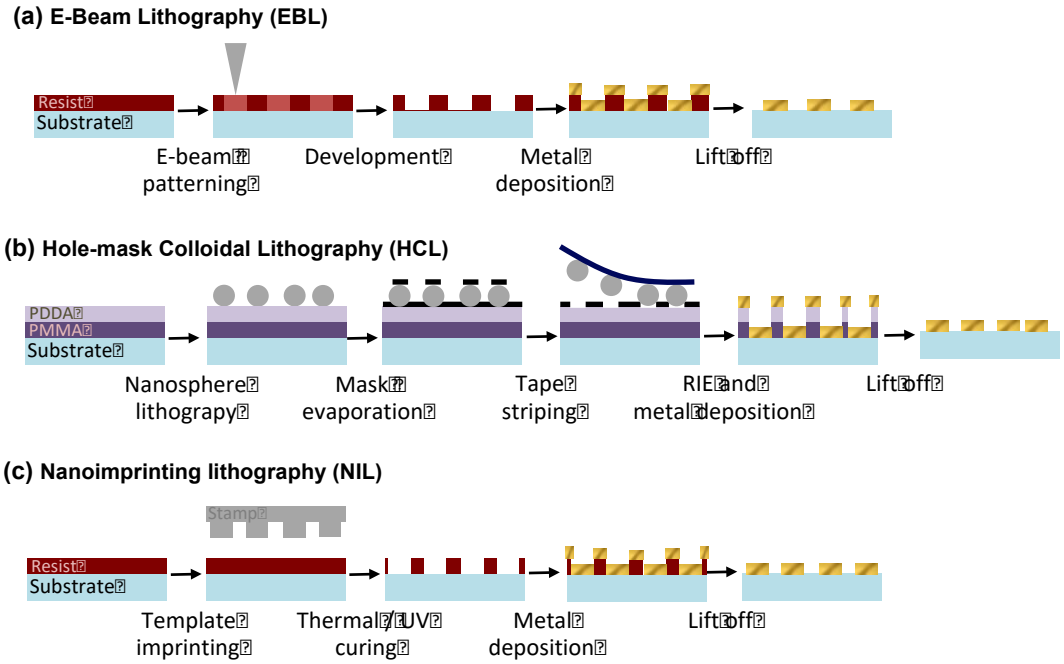

Figure 5. Schematics of common nanofabrication processes for nanoplasmonic sensors: **(a)** e-beam lithography, **(b)** hole-mask colloidal lithography, and **(c)** nanoimprinting lithography.

The fabrication of well-defined arrays of nanostructures can be achieved by top-down fabrication methods, such as photolithography, electron beam lithography (EBL) or focused

ion beam lithography (FIB) (Figure 5). FIB lithography consists in directly bombarding the metal sample with gallium ions to draw and sculpt the desired architecture. EBL uses electrons to write a pattern on a sensitive resist layer covering the sample, generally polymethyl methacrylate (PMMA). Then, the substrate is developed and the resist is lifted off, leading to the desired nanostructured metallic surface. Both FIB and EBL methods allow customized and highly precise pattern designs with resolutions of just a few nanometers and are widely used for systematic studies and evaluation of different geometries and architectures of nanoplasmonic sensors. However, both lithographic processes are extremely slow, require high-cost instrumentation, and are limited to patterning only a few μm^2 areas. Faster, larger scale and lower cost nanofabrication can be otherwise achieved by other photolithography approaches, such as nanosphere lithography (NSL) or hole-mask colloidal lithography (HCL). Both methods consist in forming a self-assembled layer of nanosphere particles onto the substrate employed as sacrificial mask for generating the nanostructured surface by subsequent etching and metal deposition steps. The difference between the two techniques is that NSL renders highly ordered patterning due to a close-packed layer of nanoparticles, while HCL results in short-ordered arrays of nanostructures. Another interesting technique is nanoimprint lithography (NIL). This technique employs a reusable master stamp to transfer predefined patterns to the desired substrate. The master stamp is usually fabricated through EBL, enabling sub-micrometer resolution patterning, and can be reused for more than one thousand times, making NIL a low-cost and scalable technique. Furthermore, it provides a high versatility for fabricating nanostructures of different sizes and shapes, such as disks or dots, holes, or domes. Finally, next to those approaches, other nanofabrication strategies worth to mention are the nanostencil lithography,[53] based on shadow-masked patterning of nanostructures, or the interference lithography,[54] where an interference pattern is written on the photoresist material, which are promising for large-scale and cost-effective production of nanoplasmonic sensors.

3.3. Instrumentation

There are two main categories of nanoplasmonic biosensor technologies: based on nanoparticles (i.e., LSPR biosensors, described in previous sections) and based on nanoapertures. The later generally consist in arrays of subwavelength apertures fabricated on plasmonic thin films, and its sensing principle relies on the so-called extraordinary optical transmission (EOT), which arise as combination of both propagating and localized SPR.[55,56] In both cases, the plasmonic resonance is characterized by the extinction wavelength peak (i.e., LSPR or EOT peak), corresponding to the maximum light absorption and scattering, which can be monitored to detect refractive index changes occurring onto the nanostructured surface. One of the major advantages of nanoplasmonic biosensors compared to conventional propagating SPR biosensors is the possibility to excite the plasmonic resonance by direct illumination, overcoming the need of complex prism- or grating-coupling schemes.

The configuration of nanoplasmonic biosensor platforms generally depends on the nanostructure surface density. For high-density nanostructured surfaces, extinction measurements are the easiest way to characterize the optical response (Figure 6a). Light is directly shed on the plasmonic nanostructures and the transmitted light is acquired with either a spectrometer, for wavelength interrogation, or a camera (CCD or CMOS), for intensity interrogation. A clear example of such configuration is the nanohole-array based sensor.[56,57]

Periodic arrays of gold nanoholes act as grating structures to couple normally incident light and excite the plasmon resonance. The EM field enhancement at the nanoholes enables the EOT, characterized by specific extinction peaks highly sensitive to dielectric refractive index changes. Portable optical readers can be assembled with off-the-shelf optical components, such as LED matching the EOT wavelength (usually in the VIS-NIR range), a microscope objective, and a CMOS sensor. This configuration is the preferred one for portable point-of-care LSPR biosensors, as it eliminates the optical components required for light coupling and allows the use of low-cost sources and detectors. Moreover, extinction measurements offer easy multiplexing and high-throughput capabilities, as large nanoplasmonic surface areas can be illuminated and interrogated simultaneously.

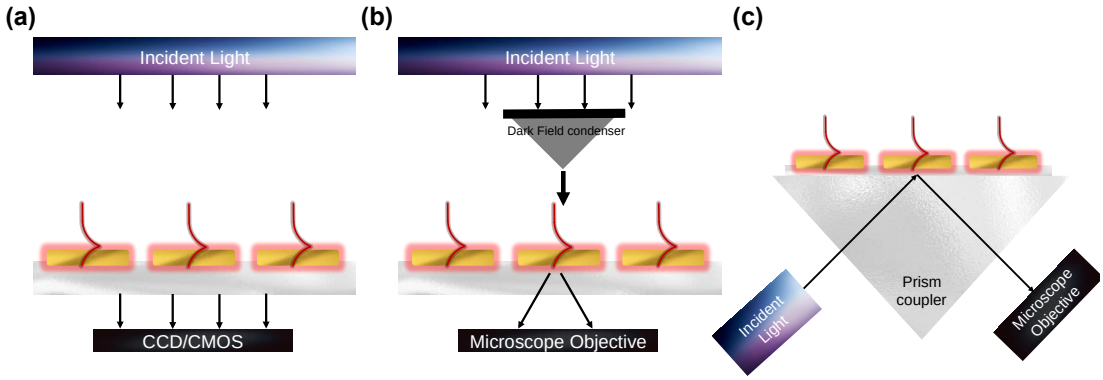

Figure 6. Diagrams illustrating nanoplasmonic-based biosensor setups: **(a)** extinction measurements **(b)** dark-field microscopy, and **(c)** total internal reflection (TIR) microscopy.

On the other side, for low-density nanostructured surfaces, a much higher contrast between the incident light and the light absorbed by the nanoparticles is needed. In those cases, scattering measurements offering higher signal-to-noise ratios are the most suitable. Dark-field (DF) microscopy or total internal reflection (TIR) microscopy are usually employed. DF microscopy works in transmission configuration, using a high numerical aperture condenser to focus the nanostructured surface (Figure 6b). The scattered light dispersed by the nanostructures is collected by a microscope objective with a lower numerical aperture. In TIR microscopy, the LSPR is excited through a prism-coupling scheme, also employing a microscope objective to collect the scattered light, without any restriction on the numerical aperture (Figure 6c). Generally, scattering measurements offer a higher control than extinction configuration and permit the evaluation at the single particle level. However, both of them require complex equipment and the integration in portable devices is not straightforward.

Finally, it is worth mentioning that nanoplasmonic sensors can also be employed in traditional Kretschmann-based SPR systems working in wavelength interrogation, just taking into account the different incident light polarization: transverse-magnetic (TM) mode is required for propagating SPR while transverse-electric (TE) mode should be used for LSPR nanostructures. This optical configuration can employ the already optimized instrumentation of advanced SPR biosensors, many of them already in the market, for enhancing certain analytical features (e.g., sensitivity, multiplexing, background noise reduction, etc.) as well as accurately compare the performance of novel plasmonic nanostructures with the standard SPR sensor.

4. PERFORMANCE OF PLASMONIC BIOSENSORS

Performance indicators and comparison between different sensor technologies are still subject to controversy in the field of plasmonic biosensors. The idea of developing novel platforms that outperform the conventional ones requires a prior establishment of the performance characteristics to be compared and reported on a benchmark basis, which unfortunately has not been fully established yet. The overall performance of a biosensor should be evaluated not only in terms of sensitivity but also in terms of robustness and reliability of the results (i.e., selectivity, reproducibility, etc.), which are influenced by several factors such as the biological interface, the quality of the sensor structures, or the setup instrumentation. This has raised the need of including numerous performance indicator parameters, and unfortunately, not all of them are determined and reported in published studies.

4.1 Performance indicators

The simplest sensitivity parameter in refractometric biosensors is the *bulk sensitivity* (S_B), which quantitates the capability to detect minute changes of the refractive index of the dielectric medium in contact with the sensor surface. The bulk sensitivity is calculated as the sensor response (i.e., $\Delta\lambda$, $\Delta\theta$, ΔI) per refractive index variation (Δn), expressed in refractive index units (RIU). To experimentally determine the bulk sensitivity, the sensor is exposed to different liquids (ethanol, acetone, water, etc.) or solutions at different concentrations (glycerol, HCl, etc.) and the signal is plotted versus the RI of the media. The slope of the calibration plot represents the S_B. The smallest detectable refractive index change determines the system limit of detection (LOD), also commonly referred as sensor resolution. The sensor resolution is calculated as three times the standard deviation of the baseline noise over the bulk sensitivity. Generally, the resolution of both plasmonic and nanoplasmonic biosensors is found between 10^{-5} to 10^{-6} RIUs, although some of the more advanced technologies have reached up to 10^{-7} RIUs. In terms of bulk sensitivity, however, significant differences can be observed depending on the system and structures.[58,59] For example, bulk sensitivity in the simplest plasmonic nanostructures (nanospheres, nanodisks, nanorods, etc.) tend to range between 300 and 500 nm/RIU, and these values increase for more sophisticated geometries, such as nanoprisms (600 nm/RIU),[60] ring-disk nanocavities (650 nm/RIU),[61] or nanocrosses (1000 nm/RIU).[62] Conventional SPR biosensors, in contrast, outperform nanoplasmonics in bulk sensitivity, reaching up to one order of magnitude higher values.[63] What might seem a discrepancy finds a logic reason when taking into account the confinement of the evanescent EM field. In propagating SPR, the larger evanescent field penetration depths allow for probing much larger volumes of the dielectric, therefore, increasing the S_B values.

From a biosensing point of view, however, the generic bulk sensitivity is not the most appropriate performance indicator. In plasmonic biosensors, the assay takes place on the sensor surface, where the evanescent EM field is more intense, and thereby the *surface sensitivity* (S_S) parameter is more useful. The S_S is defined as the sensor response to the surface coverage (i.e., number of molecules adsorbed on the sensor), and it is often expressed in terms of mass per area (e.g., pg/mm^2). To determine the S_S, different experimental approaches can be used, such as quartz crystal microbalance (QCM) analysis, but it needs to be carried out as additional independent measurements and the results tend to be

approximated. The surface sensitivity can also be determined in terms of adlayer thickness, which refers to the sensor response due to the addition of a homogeneous layer of certain material on the surface. It is usually calculated through layer-by-layer deposition of positively and negatively charged polymers, which density and refractive indices are well established and available in the literature. The layer thickness of the polymers can be accurately determined by ellipsometry and directly related to the sensor signal. For SPR biosensors, the best surface sensitivity has been reported for the newest Biacore systems (1 pg/mm^2). Theoretically, in LSPR biosensors, this value could be largely improved thanks to the spatial confinement of the EM field at the nanostrucure surface, which ensures a larger occupancy of the probe area by the biomolecular interaction. However, experimentally, only an improvement up to 15-20% has been demonstrated for some systems.[49,64] The challenge here is the necessity to assure a homogeneous coverage of the sensor for a good assessment of the surface sensitivity, which is especially complex for more sophisticated nanostructures and architectures.

Besides the different sensitivity indicators, the sensing performance of plasmonic biosensors is strongly influenced by the spectral shape and the background noise of the read-out system. Regarding the spectral shape, the most important parameter is the *Full Width Half Maximum* (FWHM), which is the width of the spectral peak (Lorentzian function) measured at half of its amplitude. Reasonably, a narrow spectral band will facilitate the quantitative detection of minimum displacements of the peak position. The FWHM is closely related to the propagation distance of SPR and the plasmon lifetime in LSPR, respectively, and it can be minimized through the rational design and optimization of the plasmonic nanostructure geometry and architecture. For example, structures supporting Fano resonances lead to sharp asymmetric peaks that have shown up to 2-fold enhanced sensitivity when compared to SPR sensors.[65–67]

The relation between the bulk sensitivity and the FWHM determines the Figure of Merit (FOM) of the sensor:

$$FOM = \frac{S_B}{FWHM}$$

The FOM is the most used parameter for comparing the performance of different biosensor systems. However, it is worth noting that the figure of merit is dependent on the metal film, the prism material, and the resonant wavelength. For instance, the SPR peak of silver structures tends to be sharper (i.e., it falls at shorter wavelength) than the gold ones. But also, at longer wavelengths, the evanescent field has a larger penetration depth, so it is more sensitive to RI changes and larger shifts of the resonance are expected. All in all, the optimum performance of a biosensor must take into account different factors that should be carefully studied, reaching a compromise between all parameters.

Finally, another interesting sensitivity indicator, especially for bioanalytical applications is the *concentration sensitivity* (S_C). This value relates the sensor response and the analyte concentration in solution. From this, the analytical limit of detection (LOD) can be determined, as the minimum amount of analyte that can be reliably detected. It is experimentally obtained through interpolation in standard calibration curves, i.e., triplicate assays of serial dilutions of the sample, and calculated as the analyte concentration corresponding to three or five times the standard deviation of the background (when considering a direct assay). It can be

expressed in molar (nM, pM), mass per volume (ng/mL, g/L), parts per million or billion (ppm, ppb), etc., depending on the analyte and sample types. Another interesting parameter is the limit of quantification (LOQ), which represents the minimum concentration that can be reliably quantified, usually the starting point of the linear range, and it is calculated as 10 times the standard deviation of the background. Both parameters depend on the bioreceptor quality and affinity, its orientation and density on the sensor surface, and the physicochemical conditions of the assay (pH, salinity, etc.), hence they cannot be broadly employed when comparing different biosensor platforms. Instead, the analytical sensitivity is often used when developing biosensor applications to evaluate the capability for addressing specific scenarios, for example, detection of certain levels of clinical biomarkers in blood or monitoring the levels of contaminants in water, and to compare the results with the ones from other analytical techniques like enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR) based assays.

4.2. Strategies for performance improvement

The most critical aspect to improve the biosensor performance is to increase the signal-to-noise ratio, which has a direct influence in the determination of both the sensor resolution and the analytical sensitivity. The intrinsic background noise of a sensor system can be due to multiple factors, including detrimental substrate effects and the system instrumentation. It has been reported that the underlying substrate of nanoplasmonic sensor impose significant drawbacks in the sensing performance. Often, the metal nanoparticles are attached to the substrate with aid of a thin metal adhesion layer (e.g., Ti or Cr). The presence of this adhesion metal increases the dephasing time of LSPR, which reduces the scattering amplitude, widening the resonance peak. To overcome this issue, some sensors opt for using a functional chemical matrix (e.g., glass silanization) for the immobilization of the nanoparticles on the substrate, although this approach may induce reproducibility and robustness problems.[68] But the background noise can also be directly related to the instrumentation, such as light fluctuations, detector resolution, or electronic processing of the signal. Reduction of this noise can be achieved by using high-quality equipment (spectrometers, lasers, etc.) but also with the incorporation of temperature controllers, vibration isolators, and advanced signal postprocessing. Data treatment with algorithms is commonly employed for improving the signal readout, such as using a polynomial fit or the centroid method instead of using the raw spectral data for tracking the peak position. It is also possible to apply electronic filters based on statistical data analysis to hinder and minimize intrinsic signal fluctuations due to the instrumentation.

Substrate effects are also important in the EM field distribution and intensity. Most of the supporting substrates are made of glass, which has a much higher RI than the bioassay sample (i.e., aqueous solutions). This contrast breaks the evanescent EM field symmetry around the nanostructures, which largely shifts towards the metal/glass interface (insensitive to external RI changes), therefore lowering the overall surface sensitivity. The straightforward approach to solve this is to use low refractive index materials as substrate, like Teflon (n = 1.32), which has been shown to improve the bulk sensitivity up to 40%.[69] Another strategy consists in distancing the plasmonic nanostructures from the surface by placing them on nanopillars or nanopedestals, and therefore increasing the particle surface available for binding. This approach has demonstrated to improve the surface sensitivity for biomolecular assays,

however it requires the introduction of delicate nanofabrication steps, such as the isotropic etch of the glass substrate.[70]

The ultimate strategy to achieve optimum biosensor performance relies on the sensor surface biofunctionalization. The plasmonic transducer surface needs to be modified for attaching the bioreceptor elements that will selectively capture and detect the analyte, but it is also important that the sensor surface can prevent and repeal non-specific adsorptions of non-relevant sample matrix components.[71] Carefully considering factors such as the bioreceptor orientation and density, a site-selective immobilization on the resonant hotspots, the final distance between the target biomolecule and the sensor surface, the antifouling properties or the use of specific blocking agents, and even the selection of the bioreceptor itself will be key for enhancing the analytical features of the plasmonic biosensor. This aspect is often under-featured in novel biosensors development, but it offers most margin to improve the sensing performance, therefore we consider that it deserves comprehensive and detailed discussion.

5. SENSOR SURFACE BIOFUNCTIONALIZATION

In label-free plasmonic biosensor, the analytical sensitivity and selectivity strongly depend on the biorecognition element tethered to the sensor surface. Typically, in affinity-based biosensors, the most employed biological receptors are antibodies, nucleic acids or cell membrane receptors, although specifically designed peptides, aptamers or molecularly imprinted polymers are can also be employed. These biomolecules show extraordinary affinity and specificity towards certain analytes, such as the corresponding antigen or the complementary oligonucleotide chain, allowing the selective capture of the target compound (analyte) with a high sensitivity. The immobilization procedure onto the sensor surface must take into account several factors for achieving an optimum biosensor: (i) the packaging density and orientation of the biorecognition element, (ii) the activity and stability during the analysis time, and (iii) the possible interferences or non-specific adsorption of other substances present in the sample matrix.

Sensor biofunctionalization procedures have been developed since many years ago, aiming to provide the optimal analytical performance. Physical adsorption (or physisorption) is the simplest strategy to attach the bioreceptor to the sensor surface, which takes advantage of intermolecular forces like electrostatic, hydrophobic and/or polar interactions (Figure 7a). Although it is a widely employed procedure in solid-based assays, such as ELISA, physical adsorption suffers from important drawbacks when dealing with biosensors. Flow-through assays or changes in the pH or buffer composition can lead to desorption of the biomolecules. Moreover, the uncontrolled interaction of the biomolecules with metallic surfaces can cause denaturation, unfolding or loss of affinity for the analyte. This is especially important for protein bioreceptors like antibodies, which can easily be adsorbed on gold surfaces due to the high content of amine groups, but their ability to capture and detect antigens essentially depends on their end-on orientation (with Fab regions available for antigen binding) and correct secondary structural conformation. A similar and better possibility is chemisorption. Thiol groups (-SH) are known to strongly interact with gold or silver, losing the hydrogen atom and forming a chemical bond. Thereby, bioreceptors with available thiol groups can be directly chemisorbed on the sensor surface, as it can be done for example with antibody fragments,

aptamers, or DNA probes. It is important to take into account though that lateral spacer molecules, such as short chain alkanethiols, might be used to control the bioreceptor density and reduce possible steric hindrance for analyte capture.

Another widely used and convenient strategy for bioreceptor immobilization is the covalent binding to a functional chemical matrix formed on the sensor surface (Figure 7b). For plasmonic sensors, the two most common scaffolds are dextran-based polymers and alkanethiol self-assembled monolayers (SAMs). In both cases, a carboxyl (COOH) functional group is usually incorporated in the chemical scaffold so that it can be activated for binding to terminal amine (NH_2) groups, largely available in protein bioreceptors. This chemical reaction consists in a well-established procedure employing a carbodiimide-based chemistry (i.e., EDC/NHS), and results in very stable amide bonds between the protein and the functionalized sensor surface. Other covalent coupling chemistries can also be used, for example amine-amine crosslinking or click chemistry, among others. This covalent binding methodology offers important advantages, such as the high control of bioreceptor density, by adjusting the number of functional groups or by including lateral spacers, and it also provides a good coverage of the metal surface for preventing non-specific adsorptions. The antifouling properties of the SAMs can be further enhanced by using PEGylated molecules, which due to their high hydrophilicity provide protein adsorption resistance. However, the direct covalent binding of bioreceptors generally occurs randomly, without any preferred orientation. To ensure a unidirectional immobilization of bioreceptors, it is recommended to make use of affinity ligands. For example, protein A/G are known to present a high affinity for the constant region of antibodies (Fc) therefore allowing its immobilization in the correct end-on orientation (Figure 7c). Another widely used strategy is the use of the biotin/streptavidin pair. Antibodies can be biotinylated specifically through the carbohydrate moieties of the Fc region and subsequently attach to a streptavidin layer formed on the sensor surface (Figure 7d). In addition to these methods, a vast number of different bioengineering strategies have been proposed for sensor functionalization, e.g., histidine or cysteine tags, calixarenes (Figure 7e), DNA-mediated coupling (Figure 7f), etc. for which we refer to specific articles and specialized reviews.[71–74]

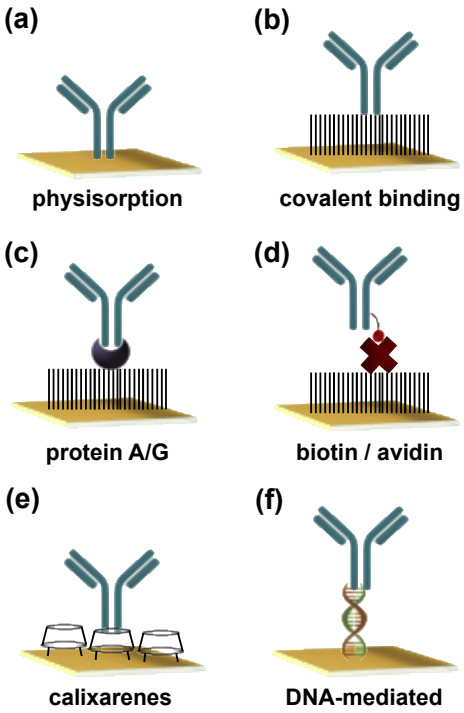

Figure 7. Schematic illustrations of common sensor biofunctionalization strategies: **(a)** direct physical adsorption, **(b)** covalent binding to a self-assembled monolayer, **(c)** via protein A or G, **(d)** via biotin/avidin pair, **(e)** calixarene-mediated, or **(f)** via DNA-hybridization.

Another important aspect in biofunctionalization is related to the surface regeneration, that is, the removal of the target analyte after the detection step without altering the immobilized bioreceptor layer. Efficient regeneration would provide reusability of the sensor, which is particularly important not only to save costs and time, but also to evaluate the stability and robustness of the bioactive surface. Typically, regeneration can be accomplished by introducing a low or high pH solution (e.g., HCl, NaOH, glycine, etc.) that disrupt the biochemical interaction between the analyte and the receptor. Other methods make use of ionic strength changes (e.g. high/low salt content buffers), temperature, or specific chemicals (e.g. formamide for DNA dehybridization) to break the interaction. Nevertheless, the regeneration procedures have to be evaluated empirically since the combination of binding forces is often unknown, and it is important to select the mildest regeneration conditions that assure the stability and integrity of the biorecognition layer.

Finally, one of the critical factors in biosensor functionalization is related to the selectivity and antifouling properties of the biolayer. Especially for label-free plasmonic sensors that detect changes in RI (and therefore changes of mass on the surface), the non-specific adsorption of sample matrix components is a major challenge for real applications. A number of strategies have been employed to reduce adsorption of non-relevant components: the use of PEGylated polymers as functional layers, the addition of blocking proteins like albumins or PEGylated polymers to saturate the surface, or a combination of both. However, the effect of these approaches is not fully controlled, and they are generally combined with sample dilution or buffer additives to minimize the background signal of real samples. In this regard, nanoplasmonic biosensors based on nanoparticle arrays might possess an interesting advantage compared to homogeneous thin film SPR biosensors: the site-selective biofunctionalization. By exploiting the different reactivity of two different materials (e.g., gold

and glass), it is possible to immobilize the bioreceptors specifically onto the plasmonic particles and blocking the exposed surface of the substrate to assure its inertness. In such way, all relevant biomolecular interactions will occur solely within the sensing probe areas and non-specific binding can be effectively minimized, which will increase the overall sensitivity and selectivity, improving the total bioassay performance.

6. APPLICATIONS OF PLASMONIC BIOSENSORS

Plasmonic biosensing technology is one of the most versatile analytical technique available nowadays. By choosing the appropriate biorecognition element, it can be applied to virtually any type of target molecule, including proteins and nucleic acids, but also small molecules like drugs or contaminants, pathogens like viruses or bacteria, and even human cells. It is so that a myriad of works has been reported demonstrating their application especially in the biomedical and environmental fields. Rather than mentioning and commenting several examples in the literature, for which we refer to excellent published reviews,[4,75,76] in this tutorial we will use a relevant case to illustrate and discuss the general procedure to be followed when designing and validating plasmonic biosensor applications for a specific purpose.

In medicine, the accurate diagnosis of specific diseases is crucial for the timely administration of correct treatment and appropriate clinical management of the patient. Further, the rapid and early identification of certain diseases, before the appearance of external symptomatology, can be extremely important. This is the case of cancer, for example, that has shown to have much better prognosis and patient survival rates when it is detected and treated at early stages.[77] Another clear case has been seen with the COVID-19 pandemics in 2020. This infectious disease emerged in late 2019 in Wuhan (China), caused by a coronavirus named SARS-CoV-2 (severe acute response syndrome coronavirus 2). Due to the fast and easy transmission of the virus (i.e., airborne transmission), the disease has rapidly spread worldwide, with pretty much no others options to control and stop it than population lockdown and social distancing. The availability of plasmonic biosensors for rapid and sensitive detection of SARS-CoV-2 viruses might be significantly useful in massive population screening, early detection of infected patients, and a more efficient management of the pandemic.[78]

To develop a biosensor for an emergent virus diagnosis, first steps to take into account are the bioassay approach and the specific bioreceptors, which will be based on the viral target molecule. Ideally, the preferred approach might be to target the external viral antigens, such as the spike (S) proteins of the coronavirus. This would allow directly detecting and quantifying the number of viruses in a patient. For this, it is required to produce *de novo* antibodies with sufficient affinity and selectivity towards the protein. This procedure can be relatively long and complex, since it needs to fully characterize the viral antigen, produce it (e.g. bacterial-based recombinant proteins), and then obtain the antibodies either by animal immunization and subsequent purification, or by recombinant methods, for example. Once an appropriate bioreceptor is achieved, the sensor surface biofunctionalization has to be designed and evaluated, controlling the orientation, density, and distribution of the specific antibodies to enhance the virus capture and detection. It is especially important to consider the viral particle sizes and mass transport issues, plus the physicochemical assay conditions, such as flow rates, pH, buffer composition, etc. In parallel, the target clinical sample has to be rationally selected

and established. It has been demonstrated that COVID-19 viruses are mostly present in respiratory fluids (nasal and nasopharyngeal) as well as in saliva. Pros and cons can be listed for the two types of clinical specimens. Respiratory fluids are collected with specific swabs, which might be a little disturbing for the patients (especially for children) but, when performed by trained sanitary personnel, ensure reliable virus sampling and storage. Saliva collection, in contrast, is non-invasive and could be easily done by the patient itself. However, saliva sampling may present a much higher variability, depending on the collection time and method, which can lead to assay reproducibility and reliability issues. Either way, both the sensor biofunctionalization and bioassay conditions would be assessed and finely optimized in terms of sample matrix effects (background signal and antibody-antigen recognition), to ensure the optimal analytical performance. Standard calibration curves will be obtained by spiking buffer and biological samples with known concentrations of the target (e.g., viruses obtained in microbiological culture), which will allow determining the analytical parameters, such as the concentration sensitivity, limit of detection and quantification, assay reproducibility and coefficients of variation, etc. Finally, the biosensor must be validated through the analysis of a number of blind clinical samples. It is recommended to challenge the novel biosensor versus standard techniques (e.g. ELISA, PCR), if available. With a large validation study ($n > 100$), the clinical sensitivity and specificity may be determined, besides of the accuracy, positive and negative predicted values, etc.

Owing to the versatility of plasmonic biosensors, it is also feasible to develop COVID-19 diagnostics based on other analytical approaches. For instance, it would be possible to target the genomic RNA sequence of the virus, instead of the viral antigens. Thanks to the advanced next-generation sequencing (NGS), in just a few weeks the exact genome of the SARS-CoV-2 virus was deciphered. This enabled the rapid development of highly specific genomic assays, based on RT-PCR, which have become the gold standard in COVID-19 diagnosis. Plasmonic biosensors can be applied for the direct and label-free detection of viral RNA by designing and immobilizing on the sensor surface single-stranded DNA probes with complementary sequence to specific SARS-CoV-2 gene fragments. Compared to the antigen-based recognition assay, genomic analysis is advantageous regarding the bioreceptor, which can be custom-designed and synthetically produced, but it requires the sample to be treated and processed for RNA extraction and fragmentation, and an outstanding sensor sensitivity would be needed if attempting direct RNA detection without previous PCR amplification. To further enhance the diagnostic capabilities, multiplexed plasmonic sensor systems could be implemented. Sensitivity and specificity of SARS-CoV-2 virus could be increased by combining different DNA probes targeting distinct genes of the same virus, but also it might be possible to realize one platform able to differentiate among several viruses sharing clinical symptomatology with COVID-19, e.g. human coronaviruses (hCoVs) responsible of common colds, or influenza viruses responsible of flu.

In this line, the same plasmonic biosensor technology could also be employed for environmental control and monitoring. The zoonotic origin of the COVID-19 pandemic has evidenced the need for accurately studying the presence and evolution of viruses in animals, especially for those colonies that share spaces with humans or are susceptible of being in close contact, eaten, or treated in some way. Similar cases occurred with the Ebola virus, the bird or swine flu, or more commonly with bacterial infections, like salmonellosis. Simple,

automated, and portable plasmonic sensors could enable a rapid detection of risky pathogens in animals, foods, or waters, and prevent from human transmission and pandemics outbreaks. For the particular case of COVID-19, it is known that bats and certain rodents are the main reservoirs of coronaviruses. Among them, one should distinguish the different families, since alpha-coronaviruses are mostly harmless for humans while beta-coronaviruses are prone to cause severe diseases, such as the SARS-CoV in 2003, the MERS-CoV in 2012, or the current SARS-CoV-2. Plasmonic biosensors targeting specific regions could rapidly and reliably detect the presence of these dangerous viruses. Furthermore, if integrated in point-of-care systems, plasmonic biosensors could be employed directly on field to carry out a close and routine monitoring of animal reservoir colonies, which would aid in early warning of communities and authorities, so they can urgently take the corresponding actions to minimize the risk of new pandemics.

Finally, it must be mentioned that developing applications for plasmonic biosensors should not only focus on the biochemical and analytical aspect, but also in the integration of microfluidic systems for sample handling as well as data analysis and interpretation. The engineering of microfluidics is continuously evolving, incorporating innovative designs and materials that can greatly facilitate and improve the biosensor performance. The idea of accomplishing a handy lab-on-a-chip system that includes sample processing and treatment, accurate and reliable analysis, and waste disposal management is still the most ambitious goal in biosensor research. In parallel, the fast development of artificial intelligence and machine learning tools will also effectively impact biosensor applications. The implementation of advanced algorithms for signal processing and interpretation could signify a breakthrough in biosensors, which would become smart systems not limited to detect and quantify specific analytes but also aiding in the decision-making process, even directly connected to therapy delivery systems or data management clouds.

7. SUMMARY AND FUTURE PERSPECTIVES

Plasmonic materials and nanostructures have a unique and vastly demonstrated potential for the realization of new enabling biosensor technologies with exceptional analytical capabilities. Plasmonic biosensors, through the evanescent wave based working principle, can offer label-free detection of any type of analyte (i.e., proteins, nucleic acids, pathogens, drugs, etc.) with outstanding sensitivities and in a real-time one-step format. Further, they can be integrated in small footprint, portable, and user-friendly devices to be employed directly at the point of need, which is key for their application in massive clinical analysis or for on-field environmental monitoring. The design and development of plasmonic and nanoplasmonic biosensors may take into account several factors, including the selection of the plasmonic metal, the particle geometry, the optical configuration and instrumentation, and also the biorecognition interface, which must be carefully optimized for each specific application. When all aspects are considered and finely engineered, plasmonic biosensor performance can meet the requirements for addressing relevant bioanalytical goals, such as the early diagnosis of serious diseases like cancer, massive population screening for virus infection detection, or for routine testing of contaminants in foods or waters.

In terms of biosensor performance, on-going research is already looking into new materials (e.g., high refractive index semiconductors) and sophisticated architectures that can provide the narrowest resonance bandwidth and largest refractometric sensitivity, as well as innovative nanofabrication methods and substrate supports that enable development of flexible and/or wearable sensors. The incorporation of novel bioengineered receptors and 2D materials as chemical biofunctionalization scaffolds might also enhance the analytical sensitivity and specificity of label-free plasmonic biosensors. Regarding device instrumentation, in the near future, plasmonic biosensors could become fully automated and smart miniaturized devices, integrated as powerful lab-on-a-chip tools. For that purpose, research in photonics engineering should synergize with other areas like materials and computer sciences, to implement cutting-edge advances in microfluidics and data treatment that assure maximum accuracy and reliability of the biosensor devices as well as their regulatory approval and acceptance in clinics and environmental analysis.

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9. DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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