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Recent advances in nanoplasmonic biosensors: applications and lab-on-a-chip integration

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Abstract: Motivated by the recent progress in the nanofabrication field and the increasing demand for cost-effective, portable, and easy-to-use point-of-care platforms, localized surface plasmon resonance (LSPR) biosensors have been subjected to a great scientific interest in the last few years. The progress observed in the research of this nanoplasmonic technology is remarkable not only from a nanostructure fabrication point of view but also in the complete development and integration of operative devices and their application. The potential benefits that LSPR biosensors can offer, such as sensor miniaturization, multiplexing opportunities, and enhanced performances, have quickly positioned them as an interesting candidate in the design of lab-on-a-chip (LOC) optical biosensor platforms. This review covers specifically the most significant achievements that occurred in recent years towards the integration of this technology in compact devices, with views of obtaining LOC devices. We also discuss the most relevant examples of the use of the nanoplasmonic biosensors for real bioanalytical and clinical applications from assay development and validation to the identification of the implications, requirements, and challenges to be surpassed to achieve fully operative devices.

Keywords: nanoplasmonics biosensors; lab-on-a-chip; localized surface plasmon; bioanalytical applications; cellphone technology; integration; clinical diagnostics.

1 Introduction

The biosensor market is expected to reach $22.68 billion by 2020, and point-of-care (POC) diagnostics represents nowadays the main segment in the overall biosensor market (about 57%) [1]. The technological progress experienced by the nanotechnology field has significantly contributed to this expansion of the biosensor market. The increasing demand for cost-effective, portable, and easy-to-use POC platforms, which require low-sample consumption and provide sensitivity and real-time response, has considerably raised the innovation in the design of biosensors as complete lab-on-a-chip (LOC) platforms. Among biosensor devices, optical biosensors show unquestionable advantages compared to other biosensor technologies. They can deliver label-free quantitative analysis and show exceptional potential for multiplexing and miniaturization. Among them, those based on localized surface plasmon resonance (LSPR) have been subjected to a great scientific interest in the last few years as the novel counterpart of the well-established SPR sensor.

The working principle of plasmonics is well known and has been extensively reviewed [2]. Surface plasmons are surface charge waves generated when an electromagnetic (EM) wave is confined and propagates at the interface between a noble metal thin layer and a dielectric that own oppositely signed optical constants. SPR is limited to transverse magnetic (TM) modes (TM polarization indicates that the magnetic field vector is in the plane of the metal-dielectric interface), as transverse electric modes cannot excite surface plasmons. This EM phenomenon generates transversal field distributions with their maximum field intensity located at the metal-dielectric,

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while decaying evanescent waves penetrate into both adjacent media [3]. Surface plasmons can be excited by means of grating couplers or by a dielectric waveguide, although the most common way is using a coupling prism (see Figure 1A). However, this configuration considerably reduces the potential for multiplexing and miniaturization of plasmonic sensors [4].

The need for coupling elements can be overcome using metallic nanostructures in the subwavelength size range instead of thin metallic layers, generating the phenomenon of LSPR. In this case, particular electronic modes can be excited when light strikes the metallic nanostructures, so that free electrons oscillate collectively. As a result of these resonance oscillations (i.e. localized surface plasmons), the nanostructures strongly scatter light at a specific wavelength range. Analogous to SPR, LSPR can be exploited for biosensing applications, as the wavelength is highly dependent on the refractive index of the surrounding media. The binding on the surface of the nanostructures results in a refractive index change, causing a shift in the extinction peak wavelength, λ_{max}. This shift in λ_{max} is given by the following equation [3]:

$$\Delta\lambda_{max} \cong m\Delta n[1\text{-}\exp(-2d/l_d)]$$

where m is the sensitivity factor, Δn is the change in the refractive index, d is the effective adsorbate layer thickness, and l_d is the EM field decay length. The extinction can be maximized by optimizing the nanostructure characteristics (i.e. metal type, size, shape, and structure orientation).

LSPR has gained much interest as an alternative technique to the standard SPR, as metal nanoparticles offer unprecedented opportunities for multiplexing (the sensing area is limited by the size of the nanostructure, which virtually expands the throughput to the nanostructure level) and sensor miniaturization [as no bulky coupling methods are required and simple transmission or reflection configurations are sufficient to generate the LSPR effect [5] (see Figure 1B)]. Both aspects expand the possibilities of LSPR-based devices to become truly integrated LOC platforms [6].

However, the claimed enhanced sensitivity that nanoplasmonic sensor devices can reach has not been fully achieved yet, and there is in fact some controversy in the field regarding the real improvement that LSPR can provide compared to conventional SPR sensors [7–9]. Conventional SPR sensors have proven effectiveness in the monitoring and characterization of biomolecular interactions with a sensitivity that usually ranges between 10^{-5} and 10^{-7} refractive index units (RIU). In the case of LSPR sensors when using conventional nanostructures such as nanospheres, nanorods, or nanodisks, the sensitivity is in the same average range or even lower (usually between 10^{-4} and 10^{-6} RIU) [10]. Whereas, in terms of bulk sensitivity, SPR clearly outperforms LSPR [11], a significantly better surface sensitivity can be theoretically obtained in LSPR [11]. In general terms, although some publications have dealt with this controversy [7, 8, 11], there is still a lack of convincing studies that confirm whether nanoplasmonics is competitive enough with SPR in terms of surface sensing performance. However, a few recent works demonstrate that sensitivity levels are in the same order of magnitude, although the sensitivity seems to improve and be higher at low analyte concentration in the case of LSPR, both in a competitive assay [12] and in direct approaches [13, 14]. This could be partially due to the strong LSPR field confinement of the nanostructures compared to SPR, which becomes more evident at low target concentrations, especially in direct assays.

In evanescent wave-based biosensors and in traditional propagating plasmonics, the evanescent decay expands hundreds of nanometers into the sensing medium, resulting in high sensitivity for bulk refractive index changes. In contrast, in plasmonic nanostructures, the EM field is highly confined close to the surface, with an average sensitive area that expands a few nanometers

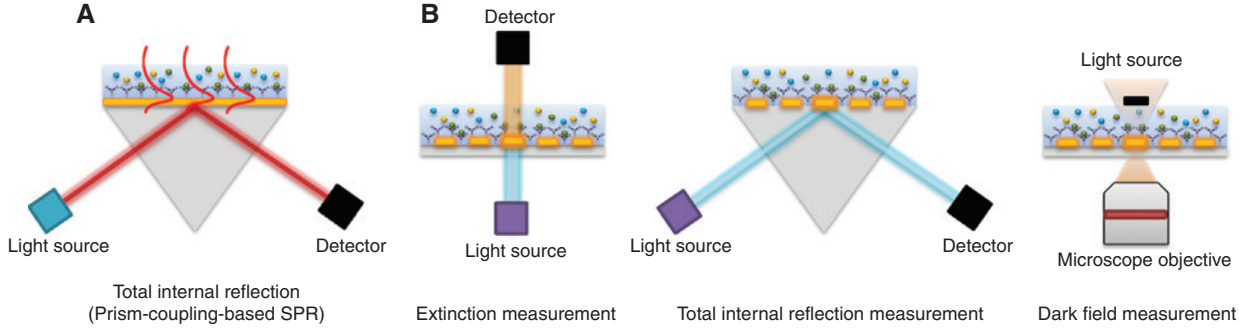


Figure 1: Schemes representing the most common configurations for (A) SPR and (B) LSPR sensors.

away from the surfaces [15]. The size of the biomolecules (receptors immobilized and target analyte), the thickness of the bioactive layer, and the decay length of the evanescent field of the nanostructure [16] are, therefore, important factors to tune the final surface sensitivity. Moreover, the EM field confinement makes LSPR less susceptible to bulk changes occurring in the media such as temperature fluctuations and more sensitive to smaller targets at lower concentrations. Sensitivity can be significantly enhanced if appropriate nanostructures are designed, exploiting geometries that promote different resonance modes (i.e. surface-enhanced Raman [17], fano-like [18], and cavity mode [19] resonances) and also promoting the interaction in those areas of the nanostructure with enhanced EM field (i.e. hot spots) [20]. Addressing detection in the hot spots, minimizing the influence of the substrate, and assuring efficient coverage of the nanostructure to get optimal detection are crucial aspects that must still be resolved, whereas the effect of mass transport, concentration, affinity constants, and microfluidics should also definitely be taken into account. In fact, we can assert that, compared to the limited effort perceived towards fostering highly controlled biofunctionalization strategies to maximize the inherent sensitivity of the nanostructure, nanofabrication has obviously become a fundamental field of research in constant growth.

Particularly interesting is the pursuit of new reproducible, precise, large area production, fast, and inexpensive processes that result in high-performance nanoplasmonic substrates with innovative designs, which can be incorporated in competitive cost-effective integrated biosensor platforms. Bottom-up methods based on the chemical synthesis of nanoparticles [21] or colloidal lithography [22, 23] fulfill some of these aspects, although top-down approaches [24] are also providing less expensive methods compared to traditional electron beam lithography (EBL) [25] or focus ion beam (FIB) [26, 27]. Nanostencil lithography (NSL) based on shadow-masked patterning of the nanostructure [28], nanoimprint lithography that creates nanopatterns by the mechanical deformation of imprint resist [29], and interference lithography where an interference pattern is recorded in a photoresist material [30] are processes that can achieve market significance by offering simple, scalable, and cost-effective fabrication methods and have also the possibility of using flexible substrates. Nanoplasmonic structures such as hybrid nanocavities [31], nanopillars [32], or nanoslits [33] are examples of recently fabricated nanostructures using some of these processes. They show extremely high sensitivity performance (8066, 1010, and 926 nm RIU^{-1} with figures of merit of 179, 108, and 252, respectively) that even exceeds the theoretically predicted upper limit for conventional SPR sensors, leading to LSPR wavelength shifts large enough to produce color differences noticeable by the naked eye at very small refractive index changes. Taking full advantage of their potential will ultimately depend on an appropriate surface biofunctionalization addressing exclusively the hot spots. This aspect still shows little progress as recently reviewed [10], although the number of examples applying LSPR-based biosensors for bioanalytical applications is steadily increasing.

Besides the certain evolution in nanostructure fabrication, in recent years, the improvement observed in the following aspects has also become substantial: (i) complete integration in a compact autonomous platform to achieve fully automated devices and (ii) technological transfer to the market, with validated applications in relevant clinical or environmental scenarios. These two parts are crucial in biosensor development. In this review, we do not intend to describe the general progress in the fundamentals of nanoplasmonics (i.e. nanostructure design, characterization, or sensitivity). On the contrary, we have especially focused on pointing out those prominent achievements reported in the last few years in the integration of LSPR in LOC platforms, the merger of nanoplasmonics with microfluidics and optical components with views of developing truly POC devices. We discuss the recent improvements in analytical performance and designs while identifying the implications, requirements, and challenges to achieve fully operative devices. We have also devoted special emphasis in examples showing a medium to high multiplexed sensing degree while demonstrating their capabilities and feasibility for real-life applications.

2 Breakthroughs in multiplexing and LOC platforms

Integration and high-throughput analysis are both essential requisites to succeed in developing POC devices for medical and clinical diagnostics. In nanoplasmonic biosensing, the merger of multiplexed analysis with an all-integrated platform is scarcely reported. Microfluidics is an indispensable module to provide simultaneous analysis and to assure low sample and reagent consumption. The appropriate dimensions of the microfluidic chambers can enhance the diffusive mixing and, as consequence, the speed and accuracy of reactions. Performance improvements such as reduced response times, improved sensitivity, higher selectivity, and parallelism can be obtained using an appropriate microfluidic system. We detail some

examples that, despite not solving all the challenges involved (optics and microfluidics, full biosensing demonstration at significant sensitivity levels and multiplexing), pave the way towards the full achievement of LOC platforms. In terms of reaching multiplexed measurements, the main progress is based on the use of imaging techniques, where a CMOS/CCD camera measures the intensity distribution in a wide area with multiple sensing spots. The intensity changes as biomolecular binding events are detected with digital image processing algorithms. These imaging systems are, however, limited in terms of sensitivity as a result of their narrow wavelength response and the low quantum efficiency of conventional CMOS/CCD cameras. Two recent examples have been reported, but both ended in bulky setups. Ruemmele et al. developed a proof-of-concept multiplexed LSPR imaging system in transmission configuration with simultaneous measurements in Au/Ag nanodisk sensors, by modifying a commercial SPR imaging system, using a CCD camera and a wavelength filtered light source through a liquid crystal tunable filter. A sequence of images is processed to correlate the intensity information with the illumination wavelength to construct a spectrum of each sensing area [34]. Yoshikawa et al. [35] developed a hyperspectral imaging system capable of performing parallelized detection (12–15 sensing spots can be measured in a single acquisition covering a sensing area of 2.5×4 cm) of a multiarray chip based on Au-capped nanopillar structures. The system employs a thin-film tunable band-pass filter and a cooled CCD camera to sequentially obtain optical images at different wavelengths. The setup, however, does not incorporate microfluidics, limiting somehow its applicability.

Acimovic et al. developed an Au nanorod LSPR multiplexed imaging chip with 32 sensing sites distributed across eight independent polydimethylsiloxane (PDMS) microfluidic channels [36]. Microfluidics incorporates micromechanical valves that can actively control the fluid flow. The design is versatile enough to allow two modes of operation depending on whether common reagents are flown to all the channels (common inlets that are then split in eight chambers to deliver in each channel) or individual samples (individual inlets dedicated to each channel). The readout configuration involves the use of a microscope in transmission configuration equipped with scanning detection and combined with spectrometric resonance shift measurements. This approach performs real-time parallel analysis and shows reproducible responses among the channels as demonstrated with biosensing experiments (direct and sandwich immunoassays). The design does not consider a complete integration yet and the experimental setup involves the use of an optical microscope in bright-field transmission coupled with a spectrometer and a visible/near-infrared (NIR) lamp. Soler et al. developed a simple and small nanoplasmonic device based on total internal reflection (TIR) using short-range ordered Au nanodisks. The excitation occurs at high angles of incidence, exploiting the enhanced sensitivity and improvement in S/N [37]. A simple miniaturized device was designed with fixed angles of light incidence resulting in a platform of <20×20 cm. A white light source and a small spectrophotometer are used, which somehow hinder complete integration, although both components can be substituted by LEDs and by commercially available miniaturized spectrophotometers, respectively. With a simple microfluidic and LabView-developed software, bioanalytical applications relevant in the clinical field have been developed reaching excellent levels of sensitivity, even when analyzing real clinical samples [13, 14]. Expanding the microfluidics to a multiplexed format would place this kind of platform closer to the LOC concept.

A more integrated design has been recently reported [38] based on a simultaneous multiplexed LSPR imaging system with a high level of integration, small size, and simple instrumentation using plasmonic arrays of periodic Au nanoholes as nanoplasmonic substrate. The simplicity and the portability of the system have a strong potential to become a POC platform (see Figure 2A). However, it has a limited sensitivity (in the range of microgram per milliliter) and it lacks microfluidics. The authors incorporated a flow cell (single channel) for sample and fluid handling in an upgraded version of the device [39]. Regarding sensing performance, the authors also combined two different LED sources spectrally located in the extremes of the plasmonic resonance mode, yielding two different diffraction patterns for each nanohole array. The dual-color biosensor platform and the microfluidics yield more than two-fold improvement in the detection limit compared to their previous lens-free detection design. With this device, protein-protein binding kinetics experiments demonstrated the viability of the approach. A similar system based on transmission configuration has been recently presented by Cappi et al. [40] (see Figure 2B). Au nanoislands created by the thermal annealing of Au films were employed. The use of a wide emission spectrum white light LED and a CMOS detector camera allows analyzing a large area by collected images in their different chromatic components in an RGB space color. The portable palm-sized setup incorporates a simple two-channel microfluidics module that performs real-time evaluations. In fact, the authors were capable of detecting in a direct assay low

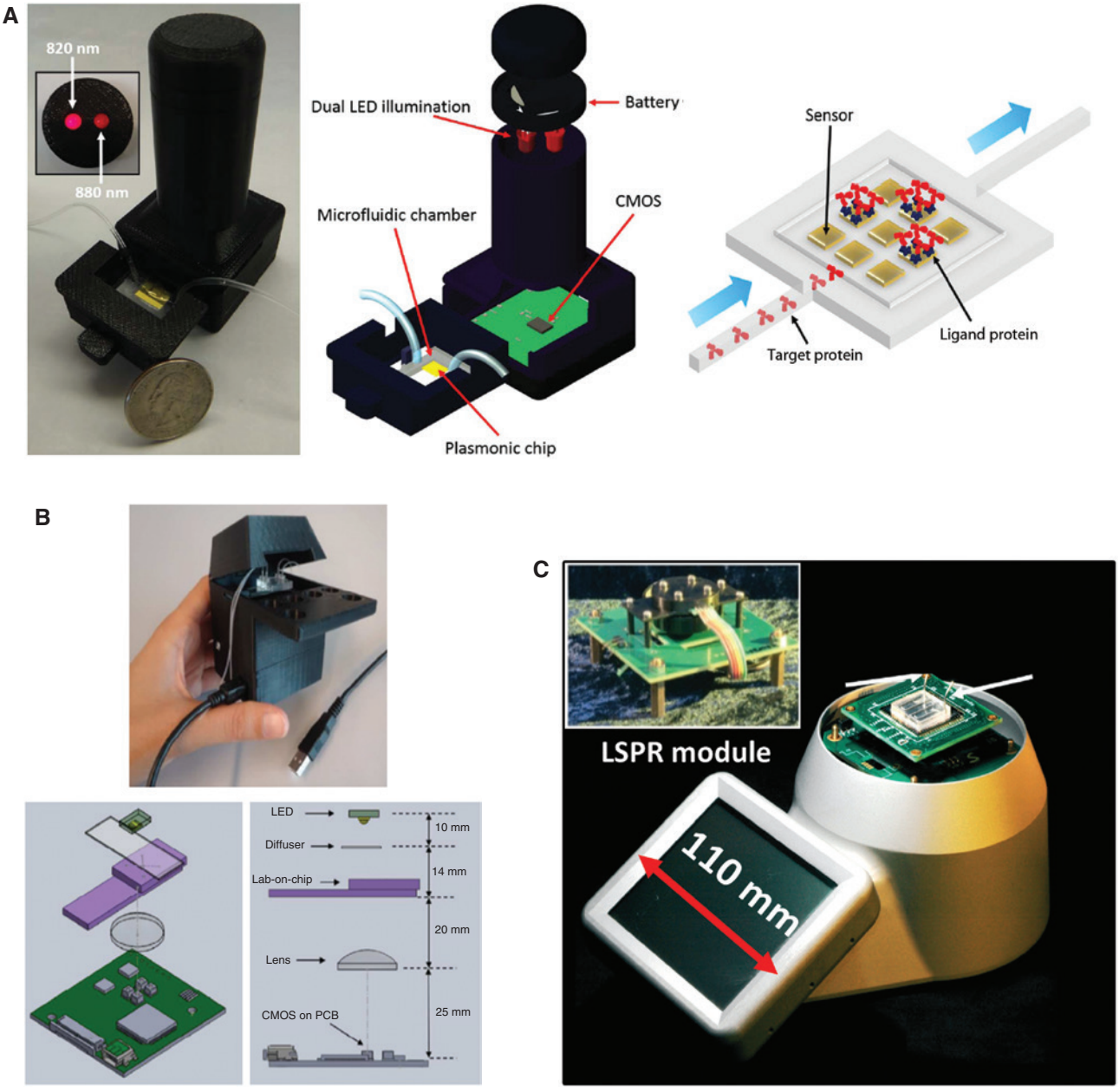


Figure 2: Examples of LSPR-based integrated devices.
(A) Plasmonic biosensor platform using dual-color lens-free on-chip imaging configuration. Two LEDs employed for the simultaneous illumination of the microfluidic chips, a scheme of all the components in the device, and a picture showing the microfluidics onto nanohole arrays. Reprinted with permission from Macmillan Publishers Ltd.: Scientific Reports [18]. © Copyright 2014. (B) Picture of a complete custom-made transmission LSPR (T-LSPR) setup, including the digital rendering of the components and their relative distances and dimensions. Reprinted with permission from [19]. © Copyright 2015 American Chemical Society. (C) Handheld, battery-operated LOC system. It contains four printed circuit boards (one for power management; a board for the central processor unit, power supply, stepper motor and touch-screen display controller, the USB communication, and the flash card memory; and two others for temperature controllers and for four lock-in amplifiers, respectively). The inset image shows the module for LSPR application based on the reflection using an optical head incorporating four LEDs with different wavelengths and a single photodiode as detector. Adapted and reproduced with permission from [20]. © Copyright Royal Society of Chemistry.

molecular weight compounds (tobramycin; MW=467 Da) using specific aptamers as bioreceptors, reaching a limit of detection (LOD) of 0.5 μM (0.2 μg ml^{-1}) in buffer. Filtered undiluted serum analysis was attempted at the expenses of sacrificing the LOD.

Both platforms described above are good examples, although still being at a proof-of-concept stage and showing moderate sensitivities, of cost-effective, integrated, simple platforms with potential to be employed as POC devices at decentralized settings.

A very attractive design that shows a relevant advancement in LOC platform has been the multiple sensor handheld battery-operated platform developed by Neuzil et al. [41] (see Figure 2C). The platform is fully portable and modular in such a way that several transducers can be exchanged according to the module board incorporated (electrochemical sensors, nanowires, or, for example, Au nanostructures for LSPR sensing; see Figure 2C). The instrumentation required for a real-time measurement is highly integrated, including a microfluidic chamber, a high-voltage source, a temperature controller, and a four-channel lock-in, all fully controlled by a single chip with a touch-screen LCD display [41]. The LSPR module is based on a previously described integrated device based on LED illumination (four different combined LEDs at different wavelengths) and reflection measurements [42]. The main purpose of the platform is so far educational and the demonstration made with the LSPR module is really preliminary (it does not incorporate microfluidics and does not include any biosensing). However, it represents a good example on how a potential platform for POC can be envisaged.

Finally, the migration of integrated versions of biosensor platforms to current cell phones, with their advanced imaging and communication capabilities, represents a powerful approach for the achievement of affordable LOC-based biosensors [43]. The high-performance cameras [44, 45], processing power, data acquisition, and constantly upgraded software of existing smartphones make them an attractive complement for the development of multiplexed biosensor devices. Either auxiliary disposable or reusable modules, which incorporate optics, transducer, and microfluidics where the biointeraction takes place, can be coupled to the camera and/or the phone screen [44, 46]. Using customized acquisition software, the monitoring of the signal can be extracted. Cell phone-based technology has been already applied for conventional SPR [47] using the screen as illumination light source and the front camera for reflected light collection. A disposable PDMS element that includes the optical component to direct the light and collect it and a simple microfluidics with the plasmonic surface is incorporated. The viability for biosensing is demonstrated by the detection of a protein, although the sensitivity is limited. Another recent example incorporates a fiber-optic SPR on the backside of a smartphone, the LED flash as light source, and the camera to capture the images that are processed by a specifically developed application to obtain relative intensity [48]. The design incorporates a reference sensing channel to compensate fluctuations coming from the LED source. All the functionalities are integrated in the platform: flow cell, optics, illumination system, detection system, and software. Based on proof-of-concept biosensing, the authors show that the system performance is comparable to a conventional SPR instrument, demonstrating that this cost-effective, palm-sized version can provide interesting features and represent an attractive and affordable alternative for many on-field applications. Moreover, although it is based in a Au-coated capillary, it would be possible to use nanostructures in the capillary for an LSPR version of the device.

Recently, an LSPR-based POC system has been implemented using Au nanohole arrays [49]. The detection configuration is based on surface plasmon-enhanced fluorescence spectroscopy rather than on detecting LSPR wavelength shift, which can significantly enhance sensitivity. The POC design contemplates a handheld-sized version using the mobile phone camera to detect the fluorescence and a desktop version using a high-resolution microscopic camera for enhanced performance. The design incorporates disposable fluidics with either four or nine chambers controlled by micropumps to deliver the fluid. Although it requires fluorescent labels and the sensitivity of the handheld version is limited, the design incorporates the necessary software to operate automatically and contemplates all the aspects to have an affordable portable POC to be used in remote areas. Another example more elaborate from the biosensor point of view implements a label-free detection experiment for pesticide detection using Ag nanoslits and transmission measurements with a smartphone [50]. Although, in principle, it is a qualitative estimation based on naked-eye colorimetric detection, it can be further optimized with image processing to get more precisely semiquantitative analysis by developing spectrometric software for the smartphone and a POC design with integrated optics.

3 Bioanalytical and clinical applications

Although many of the reported applications based on the use of LSPR-based biosensor still remain at a

proof-of-concept level, some interesting results have recently appeared in fields ranging from food monitoring to disease diagnostics. Besides sensitivity issues that may be a limiting factor in some applications especially in the clinical area, the main problems are often related to the specificity and selectivity of the assays and the application to the analysis of real complex samples without previous pretreatment. The advances in surface chemistry and biotechnology could help solve these challenges as addressed in some of the examples detailed below, which demonstrate real-life applications.

The use of LSPR-based biosensors for food safety and environmental and pharmaceutical analysis is highly attractive for day-to-day decentralized monitoring (for example, in the pharmaceutical field) to discriminate enantiomers from a racemic mixture [51]. This is a crucial aspect because usually only one of the enantiomers is responsible for the desired physiological effects, whereas the other one may be inactive or even responsible for adverse effects. Using accurately selected weak receptors individually immobilized on a dual-channel microfluidic biosensor, both (*R*)- and (*S*)-1,2,3,4-tetrahydro-1-naphthylamine (TNA) enantiomers were discriminated. With an LOD of 150 nM and the possibility to develop a full portable system, it is a potential alternative to conventional chromatographic methods [51]. In the context of food safety, Park et al. developed a complete assay for the detection of ochratoxin A (a mycotoxin produced by molds) using Au nanorod-based substrates and specific aptamers as receptors. However, it did not include setup development or microfluidics, as measurements were directly done with a conventional plate reader [52]. SadAbadi et al. proposed the monitoring of bovine growth hormone (bovine somatotropin) in milk. This hormone is used in dairy farming to increase milk production. A biosensor based on Au nanoparticles immobilized in PDMS microfluidic channels in transmission configuration using a conventional spectrometer was employed for this purpose. The microfluidic channel acts also as a reactor for the nanoparticle synthesis, leading to narrow size distribution and extinction spectra compared to those synthesized at macroscale [53]. However, the biosensor demonstration was rather limited, testing only biointeractions in buffer and omitting specificity and reproducibility studies. A more complete assay was developed by Ming et al., demonstrating the specific detection of *Bacillus thuringiensis* Cry1Ab protein in crop samples with a hybrid plasmonic biosensor platform consisting of a bimetallic Au/Ag nanoparticles operating in TIR [54]. Although the biosensor scheme requires sample pretreatment for protein extraction and the assay is performed with a bulky platform, a low detection limit is achieved (4.8 ng ml^{-1}) with good reproducibility and specificity.

An interesting example with on-site analysis has been recently provided by Lee at al., who developed a simple affordable imaging system coupled to a smartphone for the qualitative and semiquantitative detection of a pesticide (imidacloprid) [50]. The plasmonic chip is based on capped nanoslit arrays (Ag arrays coated with a 4 nm layer of Al_2O_3) and the detection format is based on an indirect competitive immunoassay that allows direct detection by the naked eye (with a detectability of 1 μg l^{-1}) or a more accurate analysis using image processing software in the smartphone. Finally, there is a rising interest in the development of sensor platforms for the detection of biological hazards for risk assessment in food and for the detection of chemical and biological warfare agents. For instance, Nagatsuka et al. developed an LSPR-based biosensor for the detection of biological toxins (ricin, Shiga toxin, and cholera toxin) [55]. Natural glycosyl ceramides were used as specific receptors and immobilized on Au nanoparticles, which were previously attached to glass surfaces. These carbohydrate molecules are known to bind microorganisms and biological toxins. The LSPR system based on transmission measurements can detect ricin, Shiga toxin, and cholera toxin with LODs of 30, 10, and 20 ng ml^{-1}, respectively, although the optimization of the assays is still limited.

It is in the biomedical field and in clinical diagnosis, in particular, where more efforts are concentrated. The development of efficient analytical tools for the fast and simple detection of disease-related biomarkers in human fluids or tissues can lead to earlier and more accurate diagnosis and prognosis as well as appropriate therapy monitoring. Early diagnosis commonly implies the detection of biomarkers at very low concentration; therefore, it requires high sensitivity, specificity, and fast analysis. Avoiding any treatment of the biological sample is also a must to push the analysis speed and the potential transfer to POC devices. For label-free optical configuration such as in plasmonic-based devices, false positives commonly come from the nonspecific binding of either the target biomarker on the surface or the additional components present on the complex sample (urine, saliva, blood, etc.). Besides using high-quality bioreceptors to push specificity, it is extremely necessary to tune the properties of the bioactive layer to minimize both types of undesired bindings [56]. This process can become extremely complex, because it strongly depends on the nature of the sample, and in many cases, it is also difficult to extrapolate conditions from bioassay to bioassay. In most occasions, it becomes inevitable either to

dilute the sample, reducing also the matrix interfering substance and subsequently worsening the detection capabilities, or to introduce a more elaborate pretreatment to extract the biomarker. Both solutions, however, go into the detriment of POC-based devices and constitute the main reason why POC biosensors have not successfully reached the market yet.

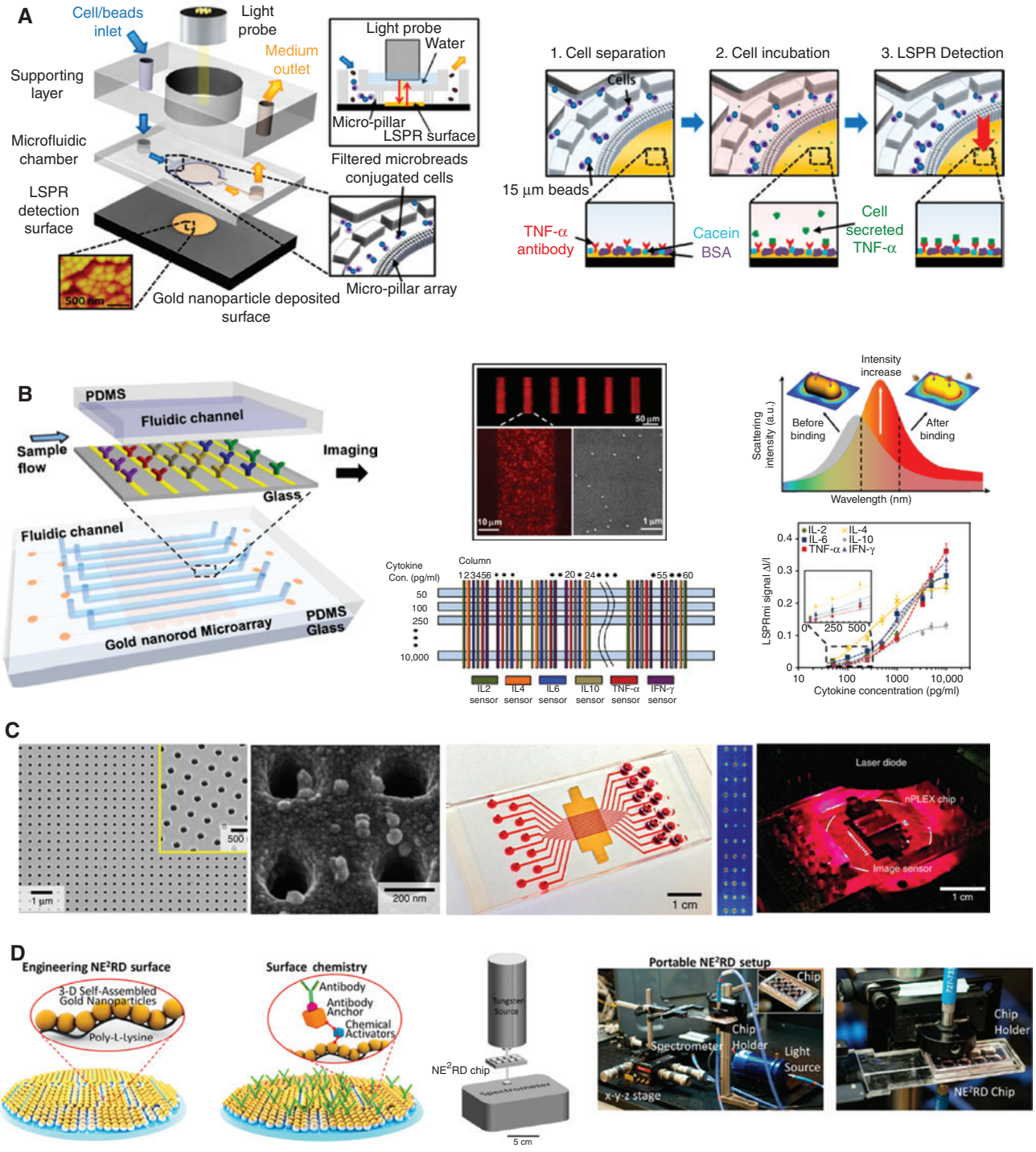
A work that deals with some of these aspects and attempts the detection in urine and serum is the one recently reported by Soler et al. In this work, they tested an oriented-based antibody immobilization strategy to bind proteins in a uniform and tight manner on Au nanodisks. The strategy was applied to the direct detection of protein biomarkers such as the human chorionic gonadotropin (hCG), C-reactive protein (CRP), and focal adhesion kinase (FAK) protein [14]. Direct detection in urine was demonstrated, keeping unaltered the LODs compared to buffer conditions, although for the analysis in serum it was necessary a 1:10 dilution factor. Using the same setup, a biosensor for amoxicillin allergy diagnosis has been recently developed [13]. The biosensor detects specific IgEs against the antibiotics that are produced by the immune system and appear in the patient's serum during an allergy outbreak. An extremely good LOD of 0.6 ng ml^{-1} was achieved, and more importantly, no sample pretreatment or dilution of serum before analysis was necessary after the optimization of the surface blocking. The samples from allergic patients were evaluated and validated with a conventional clinical immunofluorescence assay, confirming an excellent correlation between both techniques. Although multiplexing is not addressed yet in this configuration, these results represent an important step to fully functional biosensor platforms with high relevance for clinical diagnostics.

Using Ag nanoparticle arrays and extinction measurements, it was possible to detect a specific antigen related to cervical cancer (squamous cell carcinoma antigen) with excellent LODs (0.125 pM) comparable to conventional chemiluminescence immunoassays and with well-defined specificity and reproducibility. Preliminary data collected from few cervical cancer patients indicate good potential for real sample detection. Other works have attempted multiplexed detection. Acimovic et al. addressed the detection of two cancer biomarkers (human α-fetoprotein and prostate-specific antigen) using an Au nanorod-based chip in a multiplexed format incorporating an elaborated eight-channel microfluidics, as described in the previous section [36]. The detection is based on a sandwich immunoassay, where a secondary specific antibody is added to increase the signal and to improve detectability. With this approach, a minimum protein concentration of 500 pg ml^{-1} was achieved with 50% diluted human serum.

A remarkable application in the clinical field recently reported by Kurabayashi's group shows the detection of cell-secreted cytokines [in particular, tumor necrosis factor-α (TNF-α)] in lysed human blood samples using an LSPR-based optofluidic platform [57]. Cytokines are secreted by immune cells and are related to the dynamic regulation of the immune system [58]. Their secretion and accurate quantification can help in determining changes or alterations in the immunosystem, such as inflammatory disease conditions [59, 60]. The device incorporates a nanostructured surface (Au nanoparticles with specific antibody against TNF-α immobilized on the surface) and

Figure 3: Examples of microfluidic designs for LSPR biosensors.
(A) Scheme of an integrated LSPR optofluidic platform that includes an Au nanostructured surface, a microfluidic chamber with in and out channels (the chamber has integrated micropillar array to trap bead-bound target cells), and a top layer as structural support for light alignment and sample delivery. A scheme representing the different steps of the process (cell separation, incubation, and subsequent detection of secreted cytokines) is also shown. Adapted and reprinted with permission from [35]. © Copyright 2014 American Chemical Society. (B) Scheme showing the microfluidic design for the simultaneous detection of six cytokines with a nanorod microarray. It consists of eight parallel microfluidic channels (each one with independent inlet and outlet for sample delivery, which are perpendicularly located to the nanoparticle array). A nonpermanently bonded PDMS mask has been used for patterning the Au nanorod stripes (2 cm length and 50 μm pitch, as can be seen in the dark-field microscopy images). Subsequent antibody conjugation was done before removing the mask and incorporating the final one. The current chip design integrates 480 AuNR microarray sensor spots, as can be seen in the final microarray chip layout (60 antibody-functionalized AuNR stripes segmented by 8 microfluidic detection channels). The obtained calibration curves are also shown. Adapted and reprinted with permission from [36]. © Copyright 2015 American Chemical Society. (C) Label-free detection of exosomes with an nPLEX sensor. From left to right: Pictures of the Au nanoholes used as LSPR sensor, including an SEM image where the exosomes are captured by the antibody-coated surface, are shown; picture of the microfluidic cell with 12 independent channels and with three differentiated sensing areas; a photograph of the miniaturized imaging version of the setup, including the nPLEX chip. Reprinted with permission from Macmillan Publishers Ltd.: *Nature Biotechnology* [42]. © Copyright 2014. (D) Scheme of a nanoplasmonic electrical field-enhanced resonating device (NE^2RD). The 3D-oriented Au nanoparticle substrate and the portable setup based on a disposable microfluidic chip with eight individual incubation chambers are shown. Images reproduced with permission from [61]. © Copyright *Proceedings of the National Academy of Science of the United States of America*.

a microfluidic chamber (that includes arrays of micropillars that promote the trapping and spatial confinement of the immune cells) to increase the target protein concentration to be detected by LSPR of Au nanoparticles (see Figure 3A). The interference of blood components is minimized by extracting the cells using microbeads. This novel approach, which combines advance microfluidics and the sensitivity levels of LSPR-based reflection measurements, reduces up to 100 times the sample volume and up to three times the time required for the immunoassay compared to conventional enzyme-linked immunosorbent assay (ELISA). This represents a clear advantage of the microfluidic systems in which the evaluation times, the sample amount, and the pretreatment can be reduced. In



a more recent study, the same authors developed a multiarrayed Au nanorod biosensor with capability for parallel multiplex immunoassays with the goal of monitoring up to six different cytokines in neonate complex serum matrix [62]. Although the detection is based in dark-field imaging (which highly reduces the portability of the system), the potential of throughput is impressive: the multiarray has 480 nanoplasmonic sensing spots (60 stripe-like segments in each of the eight microfluidic channels incorporated in the design of the array; see Figure 3B). The device allowed parallel cytokine measurements at meaningful concentrations for these biomarkers (down to 5–20 pg ml^{-1}) with only 1 µl serum sample and a total assay time of 40 min (including loading, incubation, and evaluation of the whole array). Moreover, besides assay optimization, results were validated with ELISA assays, showing excellent correlation. Even real serum samples from two neonate patients were tested to monitor the inflammatory response of infants before and after surgery. Serum for both patients was collected before surgery and afterwards for 4 consecutive days. Although different degrees of response were observed in the two patients, the pattern was similar in both: increased levels on the first 2 days after surgery and a return to preoperative levels after day 3. This proof-of-concept experiment demonstrates the capabilities of the LSPR platform to detect [62].

Scarce examples are found applying LSPR configuration to detect nucleic acids. Dodson et al. developed a periodic Au nanorod array for the detection of single nucleotide polymorphisms (SNP) in a gene that is frequently occurring in early stages of colon cancer with an LOD under 10 nM [63]. However, the study shows preliminary results, as it did not include any type of microfluidic cell, it involves static measurements, and no real samples (or spiked complex samples) have been assessed so far. A more elaborate work shows the detection of microRNAs (miRNAs), which have become a very attractive family of biomarkers. The authors use Au nanoprisms [64] and extinction evaluation, monitoring and quantifying the binding of an miRNA with a specific role in pancreatic cancer over complementary DNA sequences immobilized on the surface of the nanostructures. The authors use these nanoprisms whose structural properties provide extremely good sensitivity due to its strong EM field enhancement in the sharp tips of the triangle. Diluted plasma (40%) can be measured achieving impressive LODs in a direct label-free approach (between 23 and 35 fM either in buffer or plasma). A proper surface functionalization using pegylated compounds as lateral spacers to both improve hybridization efficiency and minimize interferences from plasma led to these levels of detectability that otherwise were in the low picomolar range. Serum samples were directly diluted to 40% and measured with the biosensor, and results were correlated with quantitative reverse transcription-polymerase chain reaction (qRT-PCR), a technique commonly used for miRNA detection. By optimizing nanoprism structure, even better LOD can be reached (~91 aM in 1/25 diluted plasma) [65], which may represent one of the lowest LODs reported for LSPR-based biosensors. Even miRNA levels in exosomes (i.e. phospholipid nanovesicles that are bound to membrane and carry biological material whose composition can be relevant for diagnosis purposes) were quantified after extraction and isolation. Cancer patients' samples were analyzed and miRNA expression levels were compared to normal controls, exemplifying the usefulness of the strategy. The design does not include real-time analysis, and the measurements were performed using conventional UV-visible spectrophotometer and the substrate immersed in a quartz cuvette. Although not translated yet to a compact device with a POC concept, these works demonstrate the importance of both the type of nanostructure and the proper design of the surface biofunctionalization to obtain a successful detection at meaningful sensitivities and in complex media conditions.

Other examples for clinical diagnosis with novel cancer markers such as human telomerase and DNA mismatch repair protein (MutS) have been recently developed using an LSPR sensor based on Au nanoparticle immobilized on glass substrates and using DNA fragments, where the biomolecules selectively bind, as affinity ligand [66]. Dark-field microscopy combined with Rayleigh scattering measurements at single particle level limits its potential portability, but it confers high sensitivity, for instance, to detect mutant DNA by detecting MutS even in human serum or telomerase in cell extracts (as low as 10 cells), which is better than in conventional PCR assays. Based on the same configuration, circulating tumor DNA (ctDNA) and its epigenetic modifications (DNA methylation) using DNA molecules as affinity receptors have also been detected [67]. In a first step, a double-stranded ctDNA of two mutant forms binds to complementary DNA, being able to detect down to 50 fM. Subsequently, Au colloids bearing monoclonal antibodies specific for methylated cytosine were added, amplifying the signal in case of methylation. In this way, both mutations and epigenetic modifications can be detected with a single platform even in serum.

Two recent works illustrate the potential to detect other species such as virus-like particles (VLPs) for HIV diagnosis [68] and exosomes for cancer diagnosis [69]. In the first example, Lee et al. developed an LSPR biosensor

based on an Au nanopatterned substrates with specific antibody fragments (gp120 monoclonal antibody fragments) immobilized on the surface. Transmission measurements were performed and the detection of HIV-1 VLPs (noninfectious viruses without the genetic load inside) reached detection limits of 200 fg ml^{-1}, considerably lower than those of conventional SPR analysis using Au substrates (25 pg ml^{-1}). However, the biosensor does not employ microfluidics and the approach still requires an evaluation of complex and real samples to confirm its feasibility as a suitable methodology for virus detection. Im et al. successfully attempted the challenging detection of exosomes with a complete procedure including setup design, microfluidics, assay optimization for 12 targets, and real patient sample analysis [69]. A plasmonic chip based on plasmonic nanoholes (nPLEX) was specially designed (both nanohole size and periodicity) to maximize sensitivity to the size of the target exosomes. Different antibodies were immobilized on their surface to target different exosomal protein markers. Microfluidics with 12 independent channels (0.3 µl volume) were also incorporated, making possible a high level of throughput with 36 sensing regions (three sites in each fluidic channel; see Figure 3C). According to the authors, the system is scalable to 10^5 sensing elements. All the steps from biofunctionalization to exosome detection were performed within the fluidic cell. In particular, the nanoplasmonic sensor was applied to the detection of exosomes secreted by ovarian cancer cells. Transmission measurements were done with a conventional microscope, although the authors have designed a portable imaging setup with a laser diode as light source and a CMOS imager, considerably decreasing the size of the whole device ($10\times5\times5$ cm^3; see Figure3C). Specificity and sensitivity studies, evaluation of nonspecific binding, and reproducibility were addressed and finally filtered ascites containing exosomes were directly measured. Quantification and normalization of the data to precisely extract information about the protein level in the exosomes were also done in such a way that it was possible to profile the protein levels from ovarian patients and compare them to control subjects and even to evaluate disease progression after treatment. Overall, this work represents an excellent and impressive example of a POC biosensor with a real-life application.

Other approaches deal with the combination of patterned nanoplasmonic substrates using microfluidics, which define the sensing areas to provide multiplexed analysis. Patterned arrays of nanostructures can be easily obtained, combining photolithography with fabrication processes such as colloidal lithography and obtaining any desired design (channel-based or 96-spot plate-based) [70] or simple nanoparticle assembly (8- or 96-spot-based plates) [61]. Taking advantage of this, a device has been reported for the multiplexed detection of several targets of different nature: proteins, drugs, cells, bacteria, and viruses [61]. Nanoplasmonic 3D-oriented substrates based on assembled Au nanoparticles (with highly reduced interparticle distance; see Figure 3D) enable a strong surface electric field enhancement and provide extremely good levels of sensitivity for the targets. Disposable microfluidics has been designed to integrate the initial 96-microwell-based concept into a more portable POC prototype (using a chip design with eight chambers), which only requires a customized sensing platform (a spectrometer and a light source to measure wavelength and extinction intensity). The device does not operate in real-time and requires an incubation-washing step, but the resultant LODs for the 96-well format under label-free conditions outperform gold standard ELISA. The multiplexed potential of this approach, the feasibility of measuring whole blood, serum, or saliva, and the validation with clinical samples have essentially been demonstrated with the more robust 96-well plate format. A partial analysis of some of the targets with the small portable chip-integrated prototype showed slightly worst performance in terms of repeatability and background levels, but in general terms it can provide an affordable, easy-to-use, convenient out-of-laboratory setup with broad applicability in clinical settings.

4 Conclusions and future perspectives

POC diagnostics represents an enormous research field that still has to face many unmet challenges necessary for the development and commercialization of portable devices. Microfluidics for providing multiplexed capability, adequate miniaturization and robustness, handling simplicity, reliability, and competitive affordability are desirable requisites that should be included in the design without negatively affecting the sensitivity and the reproducibility standards required in the clinical analysis. Nanoplasmonic technology can play a prominent role in the POC area, as the sensitivity levels and miniaturization are realistically achievable goals. The fabrication of novel structures that can offer enhanced performance is continuously evolving, and there is an increasing trend of using scalable, low-cost fabrication methods that are compatible with the use of flexible materials such as polymeric or paper supports as supporting substrates that may reduce the final cost. Biosensor feasibility seems to start

leaving the proof-of-concept stage and a growing number of analytes has already been detected — from proteins, small molecules, and nucleic acids to more complex entities, such as exosomes, bacteria, viruses, or cells which demonstrate the versatility of the technology. In some particular cases, the validation with real samples in diverse clinical scenarios reinforces the suitability of LSPR-based biosensor devices.

However, there are still several challenges to overcome, which limit the progress of technology transfer and commercialization, mainly related to the difficulties in the integration of all the components into a single portable platform. A few designs have recently appeared at the research level with a certain level of compactness, incorporating also microfluidics to facilitate the sample delivery and expand the throughput, although the development has not reached complete integration yet, limiting also their operability and market significance. However, a noteworthy example has already hit the market. The device operates under label-free configuration, although it is a bench-top instrument with four independent channels (LightPath™ S4 from LamdaGen). Another version more suitable for POC diagnostics is also commercialized, although it requires labeled reagents as it involves an ELISA-type amplification step. Besides this example, the progression is rather slow, although we can envisage a significant advancement in cell phone technology as POC and mobile health diagnostics, in particular, for deployment at developing countries and low-resource decentralized settings. The exponential growth in the development of mobile applications and the affordability of these devices are called to revolutionize health delivery and open the door to a new stage in the global health access. Yet, there is still a long road ahead for this emerging technology to be fully adapted to nanoplasmonics, especially to adjust and improve microfluidics and analytical performance, and probably at this stage, there may be necessary a trade-off between cost-efficiency and optimum sensing performance before reaching strict clinical requirements. LSPR biosensors possess, however, all the features necessary to become an outstanding option to succeed.

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