Label-free Plasmonic Biosensors for Point-of-Care Diagnostics: a review

Maria Soler[1]*, Cesar S. Huertas[1,2], Laura M. Lechuga[1]

[1] *Nanobiosensors and Bioanalytical Applications Group, Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC, BIST and CIBER-BBN, Campus UAB, Ed-ICN2, 08193 Bellaterra, Barcelona, Spain.*

[2] *Present address: School of Engineering, RMIT University, Melbourne 3001, Australia*

* Corresponding author: Maria Soler; maria.soler@icn2.cat

Authors Biographies:

Maria Soler is a senior scientist at ICN2 in Barcelona (Spain). She obtained her PhD in 2015 working in the same Institute, specializing in nanoplasmonic biosensors for point-of-care diagnostics. After a postdoctoral stage in the Ecole Polytechnique Federale de Lausanne (EPFL, Switzerland), she is now leading the research line for the development of new optical biosensors for therapy and diagnostics applications.

Cesar S. Huertas obtained his PhD at the ICN2, where he introduced and established a novel research line for the analysis of genomic and epigenomic markers using nanophotonic biosensors. Now, he works as a postdoctoral fellow at the RMIT in Melbourne (Australia), where he has a leading position in the development of diagnostics and prognostics applications for microfluidics-integrated optical biosensors.

Laura M. Lechuga is full professor of the Spanish National Research Council (CSIC) and head of the Nanobiosensors and Bioanalytical Applications Group at ICN2. The principal focus of her research is the technological development of photonic (nanoplasmonic and silicon-based) biosensors, their integration in portable lab-on-a-chip platforms and their application for clinical and environmental diagnostics.

Abstract

Introduction: Optical biosensors and particularly those based on nanoplasmonics technology have emerged in the last decades as a potential solution for disease diagnostics and therapy follow-up at the point-of-care. These biosensor platforms could overcome some of the challenges faced in conventional diagnosis techniques offering label-free assays with immediate results and employing small and user-friendly devices.

Areas covered: In this review, we will provide a critical overview of the recent advances in the development of nanoplasmonic biosensors for point-of-care diagnostics. We focus on those systems with demonstrated capabilities for integration in portable platforms, highlighting some of the most relevant diagnostics applications targeting proteins, nucleic acids, and cells as disease biomarkers.

Expert Commentary: Despite the attractive features of label-free nanoplasmonic sensors in terms of miniaturization and analytical robustness, the route towards an effective clinical implementation necessarily involve the integration of fully automated microfluidic systems for sample processing and analysis, and the optimization of surface biofunctionalization procedures. Along with that, the development of multiplexed sensors for high-throughput analysis and including specific neoantigens and novel biomarkers in detection panels, will provide the means for delivering a powerful analytical technology for an accurate and improved medical diagnosis.

Keywords:

optical biosensors; point of care diagnostics; nanoplasmonics; clinical applications; lab-on-a-chip integration; portable devices.

1. Exploiting light for better diagnostics

Photonic biosensors are systems that seize different light-based phenomena for the fast detection and quantification of clinical biomarkers (i.e. molecules or pathogens which presence or quantity is an indicator of the onset of a disease). Fundamentally, an optical biosensor consists of specific bioreceptor in close contact with a physical transducer, which translates the capture of an analyte in a measurable variation of a light property, e.g. refractive index, wavelength, resonance, or intensity. Optical sensing can employ various physical transduction methods, such as interferometers [1], resonators [2], gratings [3], or plasmonic [4]. The plasmonic based sensors are probably the best known and most widely employed. The Surface Plasmon Resonance (SPR) biosensor is considered the landmark in optical and plasmonic biosensors. Since the introduction of the SPR biosensing principle more than three decades ago, these optical biosensors have spread astonishingly, being commercialized by a high number of companies worldwide and routinely used in the pharmaceutical industry and research laboratories for the study of any type of biomolecular interactions [5]. SPR biosensors are able to detect, monitor, and quantify molecules attaching to the sensor surface by measuring the change of the refractive index (RI) produced at its immediate vicinity, thus skipping the need of amplification steps or molecular labeling. Note that the detection principle and operation modalities of SPR biosensors are described in Section 2.1.

Certainly, the capability for label-free and real-time molecular analysis is the major strength of SPR biosensors. They can provide direct quantification of a diversity of analytes in a few minutes, in a non-invasive manner and without interferences from tags and labels, extremely reducing the consumption of reagents, and even offering to retrieve kinetic information from the biomolecular interaction under study. These features could overcome some of the challenges faced by the traditional diagnosis methods currently performed at hospitals, such as microbiology culture, enzyme-linked immunosorbent assays (ELISA), or quantitative polymerase chain reaction (qPCR) tests. In addition, plasmonic biosensors offer advantages over other biosensing methods as the predominant electrochemical ones such as a high robustness to external electromagnetic interferences and stability in aggressive environments. This has been vastly demonstrated with the number of exponential publications reporting new and valuable applications for SPR biosensors, including not only early disease diagnosis, but also therapy monitoring, drug discovery, or food and environmental control [5,6]. However, despite its long-term presence in the market and its demonstrated applicability, the conventional SPR biosensor has not yet reached the clinical field expectations. According to the World Health Organization, the ideal diagnostic system should be Affordable, Sensitive and Specific to biological agents, User-friendly, Equipment-free, and Deployable to the point of care (i.e. ASSURED criteria) [7]. The actual research in plasmonics, nanotechnology, and bioengineering are upgrading the SPR-based sensors in order to achieve the envisioned ultra-sensitive point-of-care optical biosensor able to accomplish the ASSURED criteria

In this article, we review the lastest advances in plasmonic sensor platforms and their implementation as medical instruments. In particular, we will discuss how the incorporation of the nanotechnology, or the integration in today's devices like smartphones, can provide new opportunities for building miniaturized and portable biosensors, easy to use, and with outstanding sensitivities. The main challenges and limitations of plasmonic biosensors are also highlighted, as well as emerging strategies and the near-future perspectives. Finally, a revision of some of the more interesting

biomedical applications will be provided, focusing on novel strategies offering timely and highly precise diagnosis of prevailing diseases, such as cancer, immunological disorders, or pathogenic infections.

2. Overview of nanoplasmonic technologies for label-free biosensing

Driven by the need of point-of-care (POC) biosensors to improve and promote healthcare worldwide, research in plasmonics has mainly focused on the automation and integration of SPR biosensors as well as the development of sophisticated optical transducers based on metallic nanostructures (i.e. nanoplasmonics) that enhance the sensing capabilities and facilitate its miniaturization. Likewise, the study and optimization of surface biofunctionalization strategies has been a key factor for their real clinical application, providing the necessary sensitivity and selectivity for an accurate label-free analysis. In this section, we will briefly describe the most employed detection methods in refractometric nanoplasmonic sensing, and the surface chemistry procedures for correctly attaching specific biorecognition elements (e.g. antibodies, proteins, DNA strands, aptamers, or molecularly imprinted polymers (MIPs) to the plasmonic sensor surface.

2.1 Nanoplasmonic-based detection methods

SPR refers to the collective oscillation of free electrons of a metal (e.g. gold, silver in visible frequencies) at the interface with a dielectric, which propagates along the surface as an electromagnetic resonance. This resonance exhibits an electromagnetic field that evanescently penetrates into the adjacent dielectric medium and serves as a sensing probe, extremely sensitive to changes in the RI like those caused by biomolecular interactions. For SPR excitation, an incident light needs to be coupled to a thin layer of metal – typically 50 nm of gold – obeying certain conditions, such as polarization, angle, and wavelength. For efficient light coupling, usually a prism-based scheme is employed (i.e. Kretschmann configuration) although other methods such as waveguide coupling, diffraction grating, or optical fibers can also be used (see Figure 1a) [4,8].

In prism-coupled systems, the SPR phenomenon is characterized by the appearance of an intensity dip in the reflected light, which is monitored to track biomolecular interactions occurring at the sensor surface. For that, three operation modes are commonly employed: angle, wavelength, or intensity interrogation. For angular interrogation, the SPR is excited with a monochromatic light and the incident angle is continuously scanned over a certain range. The reflected light shows the SPR dip that will shift upon a change of the RI, providing real-time sensorgrams with a signal increase for the analyte capture and signal decrease for detachment or dissociation. On the other hand, in wavelength interrogation, the SPR system employs a polarized broadband light source and a spectrometer to analyze the reflected light (i.e. SPR spectroscopy). The spectrum shows the dip located at the specific SPR wavelength (λ_{SPR}), which will also vary directly proportional to the number of molecules attaching to the surface. Both techniques are widely employed, and offer high sensitivities (limit of detection of 10^{-6} – 10^{-5} refractive index units, RIU) [5]. They can also be fully automatized and integrated in relatively compact systems as bench-top instruments, so a number of commercial devices are already available. Finally, intensity measurements are performed at a fixed incident angle and wavelength of the light source, with the RI

variations being monitored as changes of the SPR dip intensity, for example with a CCD camera. This is the general principle employed for SPR imaging (SPRi) [9]. The main advantage of such plasmonic imaging systems is the possibility to visualize the whole SPR chip, therefore it allows for real-time detection in a multiplexed array format. However, it also suffers from important limitations in terms of noise background and resolution. Overall, the robustness and large versatility of SPR biosensor keeps motivating researchers to miniaturize and integrate it in small and portable platforms for POC applications. Some examples are underlined in Section 3.

In a parallel effort, with the progress of nanotechnology in the last decade, the SPR biosensor has evolved by incorporating novel metallic nanostructures. Nanoplasmonic structures can be precisely fabricated with an excellent control of size and shape, including nanodisks, nanorods, nanopillars, nanoholes, nanoslits, nanostars, nanopyramids, etc. The coupling of light to plasmonic nanostructures smaller than the wavelength generates a non-propagating collective oscillation of the free electrons that results in a significantly confined electromagnetic field (i.e. localized surface plasmon resonance, LSPR) [10]. The LSPR resonance is characterized by its extinction wavelength peak (maximum light absorption and scattering), which can be spectrally monitored to detect RI changes occurring at the surface of the nanoparticles. The superiority of LSPR sensing is primarily explained as a consequence of both a simpler coupling of the light and the new operation modalities that facilitate device miniaturization or enable a high-resolution analysis (Figure 1b) [11]. For high nanostructure densities, extinction measurements are the easiest way. In this case, light is normally shed on the nanoplasmonic sensor and the transmitted light is analyzed with a spectrometer, a CCD camera or a CMOS sensor. The acquired LSPR peak can therefore be monitored through wavelength displacements or changes in the peak intensity. This modality offers advantages for POC biosensors, such as the elimination of optical components for light coupling and the use of low-cost and tiny light sources (e.g. light-emitting diodes, LEDs), which maximize its capabilities for multiplexing and high-throughput analysis. On the other hand, the LSPR principle has also demonstrated a significant enhancement of the analytical sensitivity, even achieving single-molecule detection. For that, either dark-field (DF) or total internal reflection (TIR) microscopies are employed. However, both of them are difficult of being integrated in portable devices for clinical applications. Finally, nanoplasmonic sensors can also be incorporated into traditional prism-coupled systems working in wavelength interrogation. This approach not only offers benefits in terms of robustness and versatility, but also its nanostructured surface provides interesting opportunities for selective functionalization and sensitivity improvement.

2.2 Surface functionalization strategies for sensitive and specific detection of biomarkers

One of the main challenges in label-free nanoplasmonic biosensing is to assure the high sensitivity and specificity for the detection of the biomarker of interest directly in a real sample. Clinical samples are usually body fluids like blood, serum or plasma, urine, or saliva that contain large amounts of different compounds and with a large variability among individuals. The selective capture and quantification of minute amounts of the target molecule contained in such complex matrices, without any amplification or secondary step, can become an arduous task in the development of a functional plasmonic biosensor.

The surface of the sensor needs to be previously functionalized to attach the specific bioreceptor for selective analyte capture while preventing non-specific adsorptions of other molecules present in the complex sample matrix [12]. The most employed biorecognition elements are antibodies, nucleic acids, or cell membrane receptors. These biomolecules show an extraordinary affinity and specificity towards their corresponding antigen, ligand, or complementary oligonucleotide strand, and most of them are commercially available. Alternatively, the use of aptamers – single-stranded nucleotide chains that specifically bind proteins via secondary-structure formation – has emerged in the recent years as an attractive strategy, showing affinities comparable to antibodies, although they are still not available for most of the biomarkers [13–15]. Another approach employs the so-called molecularly imprinted polymers (MIPs). This methodology is based on preparing affinity polymers with specific binding sites modeled with the proper size, shape, and orientation of functional groups for selective capture of the target molecules. Although still not widely employed, the MIPs could offer interesting advantages in terms of robustness and affordability [16].

The immobilization of the bioreceptor onto the metal transducer is not advised to be done by simple physical adsorption as in the case of ELISA plates. This strategy implies some drawbacks for label-free detection, such as low reproducibility, false positive signals due to non-specific binding, or even denaturation or unfolding of the biological receptors. An optimum immobilization must consider the packing density and orientation, the activity and stability during the analysis time, and, in the case of nanostructured substrates, the selective tethering solely onto the active sensing areas. In addition, since the sensing field of nanoplasmonic devices rapidly decays into the dielectric medium, it is important to immobilize the receptors relatively close to the surface (< 100 nm). The basic methodology for surface functionalization is to chemically modify the substrate with certain organic molecules carrying one or more reactive groups. For gold surfaces, the thiol (-SH) chemistry is the most popular and efficient procedure. Alkane chain molecules with a thiol group at one end are known to firmly attach to gold by chemisorption, and due to hydrophobic and electrostatic interactions between the carbon chains, they spontaneously assemble forming a well-ordered chemical matrix (i.e. self-assembled monolayer, SAM) (Figure 2a) [17]. The other end of the molecules is available to covalently bind proteins, peptides, or oligonucleotides through different functional groups (e.g. COOH, NH_2, etc.). Detailed examples of these procedures are explained below. An improved version of the conventional SAM strategy incorporates polyethylene glycol (PEG) monomers or oligomers within the carbon chain. Such molecules are highly hydrophilic, so that they attract water molecules to the chemical matrix that will help repealing proteins or other compounds present in the sample [18]. The antifouling character of these PEGylated SAMs has demonstrated to be very useful for minimizing nonspecific adsorptions. Nanoplasmonic substrates offer further benefits in this regard, allowing for site-selective surface modification (Figure 2b). Due to the combination of different materials (e.g. gold particles on a glass substrate), it is possible to functionalize specifically the active areas via thiol chemistry and coat the substrate with an inert blocking agent (e.g. polymers, silanes). This strategy assures that target biointeractions occur only at the sensing spots. Another advantage of the nanostructured surfaces has been the easy implementation of more sophisticated functionalization methodologies, like the supported lipid bilayers (SLB). The formation of planar lipid bilayers on solid substrates (e.g. glass) has been exploited in bioengineering as artificial cell membranes,

for the study of cell proteins, interactions and signaling, mainly using fluorescent techniques. The transfer to label-free plasmonic sensors has not been straightforward, since these lipid membranes are not stable on metals like gold. However, the use of low-density nanoparticle arrays made on glass substrates has demonstrated to mimic the conventional surfaces and provide enough stability for the formation of SLB (Figure 2c). This approach has demonstrated to be very useful for the analysis of membrane proteins in a biomimetic environment, and it could boost the development of new therapies and diagnosis [19].

Once the chemical matrix is formed on the sensor substrate, the biorecognition elements are to be immobilized. In the case of nucleic acids (i.e., DNA probes or aptamers), the versatility of DNA and RNA artificial synthesis allows the direct incorporation of the desired functional groups at the end of the sequence. Therefore, capture probes can be designed for any particular surface chemistry. Yet, smart considerations need to be taken, such as controlling the pH and ionic strength of the buffer, or adding a vertical spacer to the bottom-end of the probe to facilitate verticality and target accessibility (Figure 2d) [20]. Far more complex can result the immobilization of proteins, and especially antibodies. The particular structure of antibodies, with the antigen binding sites exclusively located on the Fab regions, makes the orientation control essential to maximize capture efficiency and sensitivity. Besides, since these molecules are biologically produced, they are relatively weak and can lose their recognition activity under aggressive conditions (e.g., heat, pH, etc.). Most commonly employed strategies for antibody immobilization consist in either covalent binding to a SAM through a crosslinker or using affinity molecules as intermediates. Covalent binding usually exploits functional groups in the antibodies, like amine ($-NH_2$) groups of terminal lysine residues or the carbohydrate moieties in the Fc region. Amine groups are easily accessible and can readily react with carboxylic-functional SAM via carbodiimide chemistry (i.e. EDC/NHS), but this strategy results in random orientation of the antibodies (Figure 2e). Instead, carbohydrate chains can provide a better control of the orientation, although it requires a partial oxidation process to activate them and it might risk antibodies integrity and activity. On the other hand, the prime example of affinity-mediated immobilization employs the biotin/streptavidin system. Biotinylated antibodies –with the biotin tag ideally conjugated to the carbohydrate groups – bind with an extreme affinity to streptavidin molecules, which have been previously attached onto the sensor surface (Figure 2f). This method provides a highly stable and oriented layer of antibodies. Another approach makes use of affinity proteins like Protein A or G, which are produced in bacteria and naturally capture antibodies through their Fc region, therefore in an oriented manner (Figure 2g). With the advances in bioengineering and molecular chemistry, other immobilization strategies have been proposed (e.g. recombinant antibody fragments with histidine or cysteine tags, calixarenes, DNA-mediated coupling, etc.). As this is out of the scope of this article, we refer to other specialized reviews for more details [21–23].

Finally, it is worth mentioning that the surface functionalization procedure must optimize the receptor density to minimize possible steric hindrance issues, for example when capturing large analytes. Additional blocking steps with proteins or hydrophilic polymers should also be considered to avoid non-specific adsorptions. Also, it must ensure stability and reproducibility over long periods, and the biosensor chip packaging and transport. Altogether, the sensor biofunctionalization is a key factor and crucial challenge for the development of label-free plasmonic biosensors and its application to

the biomedical field. Despite the extensive research and the myriad of strategies developed over the years, it is undoubtedly a main limitation to be solved for the final implementation of optical POC biosensors as medical instruments.

3. Integration in portable devices for user-friendly, equipment-free, and deployable POC diagnostics

In order to integrate plasmonic sensors into user-friendly, automated, and portable instruments for POC applications, the engineering of two main modules are critical: microfluidics and optical components. Here, we will provide a brief overview of the current state-of-the-art in terms of integration, showing some examples of the latest advances in the field.

Microfluidic systems intended for point-of-care plasmonic devices must employ simple and ideally automated operational principles, be compatible with light pathways (i.e. optically transparent), be fabricated with low-cost and scalable techniques, and should enhance the biosensing performance. The latter can be attempted by ensuring an efficient sample delivery, minimizing reagent and sample consumption, and enabling high-throughput and multiplexed analyses. Conventional microfluidics are usually fabricated as multilayered polymeric devices with input and transport channels – of several micrometers of size – and an output to a waste reservoir [24]. These systems generally are operated with the help of syringe or peristaltic pumps that provide a continuous and regular flow of the sample over the sensor. The simplicity of such design allows for including multiple channels, which can be further controlled with pneumatic or mechanic valves, for parallel multiplexed analysis. In this regard, Chen *et al.* developed a microfluidic patterning technique with 10 segments of 6 collocating parallel detection spots for the detection of inflammatory cytokines in serum (Figure 3a) [25]. Acimovic *et al.* reported an LSPR-based multiplexed detection platform with up to 32 sensing sites on a single sensor [26]. In their latest article, this system has been employed for the direct detection of different cancer biomarkers in human serum, proving the potential for disease diagnostics [27]. However, these biosensors still require bulky equipment (e.g. microscopes, spectrometers, etc.) not appropriate for POC settings. Another microfluidic approach to improve the biosensing performance is to exploit the nanoplasmonic structures for fluid manipulation. It is the case of flow-through schemes utilizing plasmonic nanoapertures as nanochannels, which has been employed for capturing pathogens specifically around the detection hot spots [28]. Finally, on the road towards full automation of microfluidics, numerous strategies are continuously developing including microreactors, droplet-based techniques, digital microfluidics, etc [29–31]. Although the integration of these advanced fluid-control methodologies with plasmonic biosensors does not seem to be easy, on-going research and future perspectives can anticipate an enormous boost of lab-on-a-chip POC diagnostics with the synergy of both technologies.

On the other hand, the miniaturization and integration of all optical components is essential for building compact and portable sensing devices. The use of light emitting diodes (LEDs) for illumination and complementary metal-oxide semiconductors (CMOS) detectors have allowed the development of small footprint devices and even handheld biosensors that could be deployed to the point of care. Tokel et al. have fabricated a portable SPR platform by integrating the plasmonic sensor with microfluidics, LEDs and CMOS detector that was able to detect different bacteria (*E.*

coli and *S. aureous*) with sensitivities in the order of 10^5 cells/mL [32]. Cetin *et al.* presented a handheld device based on plasmonic nanohole arrays, also using dual-LED illumination and a CMOS detector in transmission configuration[33]. Later, Coskun *et al.* demonstrated the applicability of the device for label-free detection of proteins with an integrated microfluidic system (Figure 3b) [34]. A similar nanoplasmonic device has been recently employed by Gomez-Cruz *et al.* for bacteria detection, achieving a limit of detection of 100 cells/mL [28]. Current steps in this field are seeking further integration taking advantage of our daily optical devices, like smartphones. Guner *et al.* mounted a SPRi platform by attaching an accessory that includes LED illumination and the nanoplasmonic sensor chip to the camera of a smartphone, which was used for intensity interrogation [35]. The plasmonic surface was fabricated by coating a Blu-ray storage disk with metals (silver and gold), resulting in a grating-coupling SPR sensor thanks to the periodic corrugations of the disk. Wang *et al.* developed a standalone smartphone-based system for LSPR sensing. In this case, they employed the LED source from the smartphone flashlight and the CMOS detector from the camera [36]. The plasmonic sensor chip was fabricated also taking advantage of the gratings of a compact disk. This platform was tested for the detection of human cardiac troponin I (cTnI), a biomarker for myocardial infarction, achieving limits of detection comparable to conventional benchtop SPR systems (approximately 50 ng/mL).

With no doubts, nanoplasmonic biosensors demonstrate remarkable capabilities for miniaturization and integration in compact lab-on-a-chip systems. Nevertheless, the real implementation of such devices for POC diagnostics critically requires the development and optimization of clinically relevant biomedical applications that move beyond the current proof-of-concept tests.

4. Bioanalytical applications for improved medical diagnostics

The simplicity, robustness, and versatility of SPR and LSPR biosensors have encouraged their use for novel biomedical assays that enable a more accurate, early, and informative diagnosis of human diseases in a non-invasive manner (e.g. without surgery). Plasmonic-based analysis can target almost any type of biomolecular marker, including proteins and peptides, nucleic acids, and cells, covering therefore a vast range of applications. In this section, we will describe some of the most relevant and recent studies with clinical prospective performed with nanoplasmonic biosensors. Figures 4 and 5 illustrate some of these applications.

4.1 Analysis of Proteins and Peptides

Circulating proteins are the gold standard biomarkers for disease detection and identification in most *in vitro* diagnosis techniques. The overexpression, deregulation, or simply the appearance of certain proteins in human tissues and fluids is closely related to a malfunctioning of cells, organs, or inflammation processes. Therefore, the rapid and precise quantification of these biomolecules is a key factor not only for detecting a particular disorder but also for determining the stage and prognosis of a disease. Furthermore, a POC biosensor able to easily monitor the levels of proteins can be extremely effective for the evaluation of therapies and monitoring the post-treatment progress. Nonetheless, plasmonic biosensors still face important challenges, such as the

high sensitivity required for detecting minute amounts of proteins and to quantify them directly in complex clinical samples.

As the paramount disease in our days, the majority of the applications focus on the early diagnosis of cancer, and some works have already demonstrated feasibility for clinical studies. Ertuk *et al.* have developed a SPR biosensor able to detect the prostate specific antigen (PSA) – a biomarker for prostate cancer – in human serum, achieving an outstanding limit of detection (91 pg/mL) [37]. The platform was further tested with clinical samples from prostate cancer patients showing an excellent accuracy. Sahu *et al.* employed a SPR biosensor for quantification of specific proteins involved in tumor genesis – Rac1 and Rac1b –. By analyzing clinical samples from different healthy individuals and cancer patients before and after treatment, they demonstrated that the monitoring of these proteins could be validated as a biomarker for non-small cell lung cancer diagnosis [38]. In another work, Soler *et al.* proposed a nanoplasmonic biosensor for the detection of novel tumor autoantibodies in serum for diagnosis of colorectal cancer at early stages, which could reduce the necessity of colonoscopies and be implemented as POC testing for population screening [39] (Figure 4a). Inflammatory processes are also a major disorder that affects most of the population and might be caused by numerous malignancies. Here, determining the deregulation of different cytokines in blood can be utilized for diagnosis. Chen *et al.* demonstrated a multiplexed detection and quantification of cytokines in serum using a microfluidics-integrated LSPR biosensor that employs less than 1 μL of sample and completes the assay in 40 minutes [25]. Chronic conditions, autoimmune disorders, or neurodegenerative diseases could also benefit from nanoplasmonic POC devices. For example, a plasmonic sensor was developed for quantifying gluten peptides in the urine of celiac patients as therapy follow-up test [40]. In recent works, SPR-based biosensors have also been used for diagnosis of Alzheimer disease, targeting fibrinogen or Tau protein [41,42]. In addition, these studies have further improved the understanding of this neurodegenerative disease, enabling simpler and clear comparison of analysis results.

4.2 Analysis of Nucleic acids

New molecular insights in biology research have placed nucleic acids (NA) in the front line as competitive biomarkers for early diagnosis, prognosis and therapy efficacy assessment for complex diseases [43,44]. The origin of many diseases and, especially cancer, has been primarily linked to genetic mutations that accumulate stepwise, and trigger a network of processes responsible for carcinogenesis [45]. However, in recent years, epigenetics has also attracted the field of diagnosis, being highlighted as a promising alternative for early cancer prediction. The study of epigenetic mechanisms, such as DNA methylation, microRNAs or the regulation of mRNAs, has contributed to gain a comprehensive knowledge of the different pathways taken by cancer cells for their outliving and proliferation over normal cells [46]. Most epigenetic changes occur in early stages and prior to histopathological changes, constituting outstanding biomarkers for cancer diagnosis and risk assessment [47]. In addition, the specific reversion of these routes represents a promising solution for cancer therapy and patient follow-up, promoting the development of personalized medicine. Frequent monitoring of genetic and epigenetics alterations is thus requested for an effective patient treatment plan.

Plasmonic and nanoplasmonic biosensors have emerged as promising platforms for

advanced nucleic acids detection [48]. However, challenges arise from the employment of NA as biomarkers, such as low concentration and relatively small size in most of the cases, as well as sequence similarities, which in some cases are close to the mismatch level [49,50]. SPR biosensors have been developed for the detection of single point-mutations in non-amplified human genomic DNA, reaching sometimes the attomolar concentrations [51]. Also, an LSPR biosensor for single nucleotide mismatch detection relevant to KRAS-related pathologies has been developed based on the rapid DNA hybridization process in binary solution [52]. They identified single-point mutations by the different kinetics between perfect matching sequences compared to mismatched ones. Other methodology benefits of the use of surface immobilized peptide nucleic acid (PNA) probes to improve the selectivity of the hybridization reaction with the target complementary sequence [53]. Additionally, a PNA-based nanoplasmonic biosensor has been also employed for the detection of not only tumor-specific mutations, but also epigenetic marks of circulating DNA of PIK3CA gene [54]. Several plasmonic biosensors have been developed for the accurate detection of DNA-methyl groups, involving different approaches for the specific detection of these particular epigenetic marks, such as bisulfite conversion [55], or DNA methyl-specific antibodies [56,57] (Figure 4b). The study of mRNA has been barely exploited through plasmonic biosensors for diagnostic purposes, probably due to the long RNA sequences and the similarity between mRNA isoforms that critically complicate the differentiation between the isoforms [49]. In order to solve this problem, Huertas *et al.* incorporated a fragmentation process to adapt the mRNA length to the biosensor convenience and standardize the detection procedure [20]. The amplification-free methodology performed an isoform-specific, accurate and efficient analysis of the alternative splicing alterations in HeLa cells for different genes.

Other epigenetic biomarkers extensively studied by plasmonic sensors are microRNAs. These short and single-stranded RNAs constitute a complex network of cellular regulation and an excellent source of valuable information regarding cancer diagnosis. Expression levels of specific miRNAs have been correlated with the outcome of serious diseases, such as heart diseases and various types of cancers [58]. Due to their small size, they are difficult to amplify through conventional methods and their homologous sequences can distort the analysis with false positive signals. In order to achieve wider dynamic ranges and appropriate sensitivity levels, some recent plasmonic approaches have made use of amplification steps by employing different strategies such as gold-nanorods [59] and gold nanoparticles [60], or specially designed probes to promote a better target capture [61]. They have shown fast time to results and, in most cases, LODs in the low pM and fM concentrations. In contrast, Joshi *et al.* quantified miRNAs at the attomolar concentration without the need of signal amplification by a LSPR biosensor based on highly sensitive gold nanoprisms [62]. They demonstrated an ultrasensitive detection of miRNA-10b in purified exosomes isolated from patients with pancreatic cancer or chronic pancreatitis in complex media.

4.3 Analysis of Cells and Pathogens

Using plasmonic biosensors for the direct capture and detection of whole cells and pathogens in human fluids is inherently a challenge due to the large size of such analytes and the issues related to their fluidic mass transport, but it is also a must for the implementation of POC biosensors in infections diagnosis. Infections are usually caused by the invasion of a pathogenic organism (e.g. bacteria, virus), that rapidly

multiply and produce toxins, triggering the immune system reaction. The consequences can vary from a simple fever, stomachache or headache, to fatal outputs, as in the case of sepsis. Moreover, pathogen infections can be easily transmitted among individuals, spreading to whole populations and becoming epidemics. Therefore, the sensitive, selective, and early detection of pathogens is crucial to defeat the significant burden of infectious diseases worldwide. Numerous articles in the literature report the application of plasmonic biosensors for detection of virus or bacteria [63]. For example, Inci *et al.* demonstrated the direct detection of intact viruses (HIV) from unprocessed blood with a nanoplasmonic biosensor [64] (Figure 5a). A multiplexed nanoplasmonic biosensor has been developed for the rapid diagnosis of two common sexually transmitted infections (*C. trachomatis and N. gonorrhoeae*) in urine samples [65]. And Yoo *et al.* also developed a LSPR biosensor for multiplexed bacteria detection that could identify up to four different species (*L. acidophilus, S. typhimurium, P. aeruginosa,* and *E. coli*) in a single assay [66].

Cell detection can also be utilized for cancer diagnostics. Quantification and analysis of circulating tumor cells (CTCs) is a new type of liquid biopsy that can be employed for metastasis diagnostic (Figure 5b). As an example, Mousavi *et al.* used a gold nanoslit SPR biosensor for the detection of CTCs from whole blood [67]. However, they required a pre-concentration and separation step with magnetic nanoparticles in order to be able to achieve 13 cells/mL of detection limit. To improve the biosensing application for rare cell detection, nanoplasmonics definitely needs to be combined with advanced and more sophisticated microfluidic systems, which could enable control and manipulation of cells, separating and trapping them according to the size, shape, or other physiological features. Finally, another interesting application for cancer diagnostics addresses the detection and analysis of cell exosomes. Exosomes are extracellular vesicles that the cells shed to body fluids for communication and signaling purposes. Since they carry the same proteomic and genomic information than the cell source, tumor exosomes can be a valuable biomarker for early diagnosis while providing accurate insights into cancer characteristics without the need of surgery. Im *et al.* reported a microfluidics-integrated nanohole-based biosensor for detecting and profiling exosomes in ovarian cancer samples [68]. Recently, Yang *et al.* have employed a similar nanoplasmonic system for profiling specific pancreatic cancer exosomes over 100 clinical samples [69]. This study showed the importance and significance of exosomes detection for the early cancer diagnosis.

5. Conclusions

As this review reflects, optical biosensors and especially those based on plasmonics nanotechnology demonstrate a strong potential to become the next-generation diagnostic tools. By exploiting the ultimate light-matter interactions, we can fabricate highly sensitive detection platforms that enable real-time and label-free analysis of almost any type of molecule. Furthermore, thanks to the progress of nanotechnology, the miniaturization and integration of plasmonic biosensors is now a reality, illustrated with numerous portable devices or sensor accessories that directly work with the common smartphone components. The exceptional versatility of nanoplasmonics has also motivated the development of a myriad of biomedical applications. Nanoplasmonic biosensors can be used for a simple and rapid quantification of circulating protein and nucleic acid biomarkers, for the evaluation and follow-up of therapies and treatments, for the discovery and establishment of new and more accurate

disease indicators, and for the rapid detection of pathogens in human fluids. The implementation of such assays in small and user-friendly platforms for point-of-care analysis will significantly improve healthcare and life quality of the population around the world.

5. Expert commentary

Plasmonic and nanoplasmonic biosensors are today a relatively mature technology, with demonstrated applicability in diagnostics and potential for integration into small and portable devices. But the definitive boost and admission in the clinical field seems to be more complicated than expected. The main challenges and limitations in this regard are closely related to the automation of the whole testing procedure - including sample preparation and analysis -, and the quality assurance. Medical instruments for point-of-care diagnostics are employed by clinical staff or even directly by the patients rather than trained laboratory personnel, therefore they need to be extremely simple to use, rapid, with a high degree of automation, and not requiring complex sample manipulation procedures. The analysis must be highly accurate, without false-positives or false-negatives, and sensitive enough to detect and identify a disease at the early stages. To meet these demands, the full development of optical POC biosensors urgently requires a multidisciplinary vision and synergy between different areas.

We are almost reaching out the limits for optical detection sensitivity in terms of plasmonic transducers. A myriad of different nanostructures, composites, and arrangements can be manufactured nowadays with the highest precision and outstanding sensitivities. Thereby, the focus is to be placed in combining this innovative photonics nanotechnology with advanced microfluidic systems – already widely employed in other fields – and further focused in bioanalytical applications that truly defeat conventional techniques, enabling multiplexed, label-free, and real-time assays. Introducing new bioreceptors and optimal surface functionalization strategies could enhance the biosensor performance and maximize sensitivity, selectivity, and reproducibility of the assays. Importantly, sample preparation and processing is nowadays one of the limiting factors for achieving functional POC biosensors. Employing automated microfluidics components that include separation membranes, pre-concentration chambers, or micro-reactors might facilitate the direct analysis of crude samples (e.g. blood). On the other hand, the miniaturization and integration of nanoplasmonic transducers with low cost and common optical components, like LEDs and CMOS detectors, has proven to be feasible, even working directly with the smartphone flashlight and camera. Unfortunately, most of the publications only demonstrate the feasibility as a proof-of-concept at laboratory level. A more comprehensive use of this technology for biomedical applications extending further to relevant clinical problems may be the imminent steps for the fully implementation of the so-called next-generation POC biosensors.

Fortunately, though, nanoplasmonic biosensors are already filling the biomedical field with new insights and prospects for an improved disease diagnosis. The versatility, simplicity, and robustness of plasmonic sensing together with their label-free and real-time capabilities have motivated the investigation of new bioanalytical strategies to provide a more accurate, informative, and timely diagnosis. Novel protein biomarkers are tested with SPR or LSPR biosensors for both determining molecular affinities and

evaluating their relevance as disease indicators in clinical studies. Others take advantage of the potential of plasmonic sensors for POC testing and suggest new strategies detecting peptides or proteins directly in urine or saliva for therapy follow-up. In the field of genomics, the innovation can be groundbreaking. The direct and label-free detection of circulating DNA or RNA markers without pre-amplification steps or even the analysis of complex genomic and epigenomic pathways in a simple and rapid manner are pushing forward new diagnosis routes able to identify the disease onset before the appearance of physiological disorders. Furthermore, a clearer understanding of the cause (e.g. mutations, deregulations in gene translation pathways, etc.) can notably facilitate the development of new and more personalized therapies against malignant diseases. Finally, plasmonic biosensor capabilities also enable the direct detection and quantification of whole cell entities. This is of great importance for offering rapid and multiplexed biosensors that detect and identify a pathogenic infection in a few minutes, without the need of long time-consuming microbiology cultures or specialized genomic extraction and detection tests. One can imagine the breakthrough and healthcare promotion worldwide if being able to rapidly detect and stop transmission of infections like HIV and other sexually transmitted diseases, Ebola or Zika viruses, tuberculosis, malaria, etc.

To our opinion, these ambitious goals are not that far. Optical biosensors have emerged as a powerful tool with the intrinsic benefits of light-based technologies: an extreme speed, robustness, tunability, and integration in miniaturized devices. The intensive research in the area will soon accomplish the strict demands for clinical diagnostics, and start delivering small and simple devices able to detect diseases in a few minutes, providing accurate prognosis or treatment evaluation, and all of it at the point of care.

6. Five-years view

Given the accelerated progress of nanophotonics in the last years, it is adventurous to predict the state-of-the-art in optical biosensors at five-year view. With the existent technologies, the next steps may be directed to demonstrate the multiplexing and high-throughput potential of nanoplasmonic sensors. The label-free and real-time analysis of numerous biomarkers in several samples simultaneously will be a key breakthrough for POC diagnostics. Along with that, including more specific biomarkers and novel diagnosis strategies based on genomic or cell analysis, could provide the means for detecting a disease at early stages and facilitate the administration of more personalized and efficient therapies, aiming in the route to a real precision medicine.

On the other hand, the new trends investigating innovative nanostructured materials (e.g. dielectric semiconductors like Si or Ge) with electromagnetic features that mimic those of conventional plasmonic metals could afford better performances. These dielectric nanostructures could offer important advantages for POC testing, such as direct integration in CMOS detectors, and even improve the biosensing performance with narrower resonant peaks that enhance the signal-to-noise ratio. One other aspect that could invade the biosensor field is the machine learning methodology. Implementing smarter algorithms that learn from the acquired data and that are able to make accurate decisions, could greatly help in the diagnosis process and motivate the development of novel systems that enable an *in situ* evaluation and regulation of treatments and therapies.

Key issues:

- Plasmonic and nanoplasmonic biosensors offer label-free and real-time detection of clinical biomarkers with high sensitivity and reliability.
- Optical transducers based on metallic nanostructures enable simple and low-cost detection methods and allow for sensor miniaturization.
- Plasmonic biosensors can be implemented in handheld portable systems or directly employ common smartphone components.
- The versatility of nanoplasmonic sensing has motivated the development of numerous bioanalytical applications targeting proteins, nucleic acids, and cells directly in body fluids.
- Point-of-care biosensors could facilitate an early, accurate and more informative disease diagnosis.
- Next-generation plasmonic biosensors involve full automation and multiplexing for high-throughput analysis in real time.

Abbreviations:

CCD: charge-coupled device
CMOS: complementary metal-oxide sensor
CTC: circulating tumor cells
DF: dark field
ELISA: enzyme-linked immunosorbent assay
LED: light emitting diode
LOD: limit of detection
LSPR: localized surface plasmon resonance
MIP: molecularly imprinted polymer
PEG: polyethylene glycol
POC: point of care
qPCR: quantitative polymerase chain reaction
RI: refractive index
RIU: refractive index unit
SAM: self-assembled monolayer
SLB: supported lipid bilayer
SPR: surface plasmon resonance
TIR: total internal reflection

Acknowledgements:

The ICN2 is funded by the CERCA programme / Generalitat de Catalunya. The ICN2 is supported by the Severo Ochoa programme of the Spanish Ministry of Economy, Industry and Competitiveness (MINECO, grant no. SEV-2013-0295).

Declaration of Interest:

The authors declare no conflict of interest.

References

[1] Kozma P, Kehl F, Ehrentreich-Förster E, et al. Integrated planar optical waveguide interferometer biosensors: A comparative review. Biosens. Bioelectron. 2014;58:287–307.

[2] Wade JH, Bailey RC. Applications of Optical Microcavity Resonators in Analytical Chemistry. Annu. Rev. Anal. Chem. 2016;9:1–25.

[3] Chiavaioli F, Baldini F, Tombelli S, et al. Biosensing with optical fiber gratings. Nanophotonics. 2017;6:663–679.

[4] Hill RT. Plasmonic biosensors. Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology. 2015;7:152–168.

[5] Nguyen H, Park J, Kang S, et al. Surface Plasmon Resonance: A Versatile Technique for Biosensor Applications. Sensors. 2015;15:10481–10510.

[6] Masson J-F. Surface Plasmon Resonance Clinical Biosensors for Medical Diagnostics. ACS Sensors. 2017;2:16–30.

[7] St John A, Price CP. Existing and Emerging Technologies for Point-of-Care Testing. Clin. Biochem. Rev. 2014;35:155–167.

[8] Homola J, Yee SS, Gauglitz G. Surface plasmon resonance sensors: review. Sensors Actuators B Chem. 1999;54:3–15.

[9] Wong CL, Olivo M. Surface Plasmon Resonance Imaging Sensors: A Review. Plasmonics. 2014;9:809–824.

[10] Unser S, Bruzas I, He J, et al. Localized Surface Plasmon Resonance Biosensing: Current Challenges and Approaches. Sensors. 2015;15:15684–15716.

[11] Lopez GA, Estevez M-C, Soler M, et al. Recent advances in nanoplasmonic biosensors: applications and lab-on-a-chip integration. Nanobiosensors Bioanal. Appl. Gr. 2017;6:8193.

[12] Oliverio M, Perotto S, Messina GC, et al. Chemical Functionalization of Plasmonic Surface Biosensors: A Tutorial Review on Issues, Strategies, and Costs. ACS Appl. Mater. Interfaces. 2017;9:29394–29411.

[13] Zhou W, Jimmy Huang P-J, Ding J, et al. Aptamer-based biosensors for biomedical diagnostics. Analyst. 2014;139:2627.

[14] Wang J, Zhou HS. Aptamer-Based Au Nanoparticles-Enhanced Surface Plasmon Resonance Detection of Small Molecules. Anal. Chem. 2008;80:7174–7178.

[15] Yang X, Wang Y, Wang K, et al. DNA aptamer-based surface plasmon resonance sensing of human C-reactive protein. RSC Adv. 2014;4:30934–30937.

[16] Selvolini G, Marrazza G. MIP-Based Sensors: Promising New Tools for Cancer Biomarker Determination. Sensors (Basel). 2017;17.

[17] Ulman A. Formation and Structure of Self-Assembled Monolayers. 1996;

[18] Goddard JM, Hotchkiss JH. Polymer surface modification for the attachment of bioactive compounds. Prog. Polym. Sci. 2007;32:698–725.

[19] Jonsson MP, Dahlin AB, Höök F. Nanoplasmonic Sensing Combined with Artificial Cell Membranes. Nanoplasmonic Sensors. New York, NY: Springer New York; 2012. p. 59–82.

[20] Huertas CS, Carrascosa LG, Bonnal S, et al. Quantitative evaluation of alternatively spliced mRNA isoforms by label-free real-time plasmonic sensing. Biosens. Bioelectron. 2016;78:118–125.

[21] Moran KLM, Lemass D, O'Kennedy R. Surface Plasmon Resonance–Based Immunoassays: Approaches, Performance, and Applications. Handb. Immunoass. Technol. 2018;129–156.
[22] Vashist SK, Luong JHT. Antibody Immobilization and Surface Functionalization Chemistries for Immunodiagnostics. Handb. Immunoass. Technol. 2018;19–46.
[23] Welch NG, Scoble JA, Muir BW, et al. Orientation and characterization of immobilized antibodies for improved immunoassays (Review). Biointerphases. 2017;12:02D301.
[24] Wang D-S, Fan S-K. Microfluidic Surface Plasmon Resonance Sensors: From Principles to Point-of-Care Applications. Sensors. 2016;16:1175.
[25] Chen P, Chung MT, McHugh W, et al. Multiplex Serum Cytokine Immunoassay Using Nanoplasmonic Biosensor Microarrays. ACS Nano. 2015;9:4173–4181.
[26] Acímović SS, Ortega MA, Sanz V, et al. LSPR Chip for Parallel, Rapid, and Sensitive Detection of Cancer Markers in Serum. J. Am. Chem. Soc. 2004;126:9.
[27] Yavas O, Acimovic SS, Garcia-Guirado J, et al. Self-calibrating on-a-chip LSPR sensing for quantitative and multiplexed detection of cancer markers in human serum. 2018;3:1376–1384.
[28] Gomez-Cruz J, Nair S, Manjarrez-Hernandez A, et al. Cost-effective flow-through nanohole array-based biosensing platform for the label-free detection of uropathogenic E. coli in real time. Biosens. Bioelectron. 2018;106:105–110.
[29] Becker H, Gärtner C. Microfluidics-Enabled Diagnostic Systems: Markets, Challenges, and Examples. Humana Press, New York, NY; 2017. p. 3–21.
[30] Millington D, Norton S, Singh R, et al. Digital microfluidics comes of age: high-throughput screening to bedside diagnostic testing for genetic disorders in newborns. Expert Rev. Mol. Diagn. 2018;1–12.
[31] Zhang Y, Nguyen N-T. Magnetic digital microfluidics – a review. Lab Chip. 2017;17:994–1008.
[32] Tokel O, Yildiz UH, Inci F, et al. Portable Microfluidic Integrated Plasmonic Platform for Pathogen Detection.
[33] Cetin AE, Coskun AF, Galarreta BC, et al. Handheld high-throughput plasmonic biosensor using computational on-chip imaging. Light Sci. Appl. 2014;3:e122–e122.
[34] Coskun AF, Cetin AE, Galarreta BC, et al. Lensfree optofluidic plasmonic sensor for real-time and label-free monitoring of molecular binding events over a wide field-of-view. Sci. Rep. 2015;4:6789.
[35] Guner H, Ozgur E, Kokturk G, et al. A smartphone based surface plasmon resonance imaging (SPRi) platform for on-site biodetection. Sensors Actuators B Chem. 2017;239:571–577.
[36] Wang X, Chang T-W, Lin G, et al. Self-Referenced Smartphone-Based Nanoplasmonic Imaging Platform for Colorimetric Biochemical Sensing.
[37] Ertürk G, Özen H, Tümer MA, et al. Microcontact imprinting based surface plasmon resonance (SPR) biosensor for real-time and ultrasensitive detection of prostate specific antigen (PSA) from clinical samples. Sensors Actuators B Chem. 2016;224:823–832.
[38] Sahu V, Gupta A, Kumar R, et al. Quantification of Rac1 and Rac1b in serum of non small cell lung cancer by label free real time assay. Clin. Chim. Acta. 2016;460:231–235.

[39] Soler M, Estevez M-C, Villar-Vazquez R, et al. Label-free nanoplasmonic sensing of tumor-associate autoantibodies for early diagnosis of colorectal cancer. Anal. Chim. Acta. 2016;930.
[40] Soler M, Estevez M-C, Moreno MDL, et al. Label-free SPR detection of gluten peptides in urine for non-invasive celiac disease follow-up. Biosens. Bioelectron. 2016;79.
[41] Kim J, Kim S, Tai Nguyen T, et al. Label-Free Quantitative Immunoassay of Fibrinogen in Alzheimer Disease Patient Plasma Using Fiber Optical Surface Plasmon Resonance.
[42] Shekhar S, Kumar R, Rai N, et al. Estimation of Tau and Phosphorylated Tau181 in Serum of Alzheimer's Disease and Mild Cognitive Impairment Patients. Garg P, editor. PLoS One. 2016;11:e0159099.
[43] Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11:426–437.
[44] del Sol A, Balling R, Hood L, et al. Diseases as network perturbations. Curr. Opin. Biotechnol. 2010;21:566–571.
[45] Ortmann CA, Kent DG, Nangalia J, et al. Effect of Mutation Order on Myeloproliferative Neoplasms. N. Engl. J. Med. 2015;372:601–612.
[46] Chatterjee SK, Zetter BR. Cancer biomarkers: knowing the present and predicting the future. Futur. Oncol. 2005;1:37–50.
[47] Veenstra TD, Conrads TP, Hood BL, et al. Biomarkers: mining the biofluid proteome. Mol. Cell. Proteomics. 2005;4:409–418.
[48] Bellassai N, Spoto G. Biosensors for liquid biopsy: circulating nucleic acids to diagnose and treat cancer. Anal. Bioanal. Chem. 2016;408:7255–7264.
[49] Carrascosa LG, Huertas CS, Lechuga LM. Prospects of optical biosensors for emerging label-free RNA analysis. TrAC - Trends Anal. Chem. Elsevier; 2016. p. 177–189.
[50] Chang K, Deng S, Chen M. Novel biosensing methodologies for improving the detection of single nucleotide polymorphism. Biosens. Bioelectron. 2015;66:297–307.
[51] D'Agata R, Breveglieri G, Zanoli LM, et al. Direct Detection of Point Mutations in Nonamplified Human Genomic DNA. Anal. Chem. 2011;83:8711–8717.
[52] Rapisarda A, Giamblanco N, Marletta G. Kinetic discrimination of DNA single-base mutations by localized surface plasmon resonance. J. Colloid Interface Sci. 2017;487:141–148.
[53] Bertucci A, Manicardi A, Candiani A, et al. Detection of unamplified genomic DNA by a PNA-based microstructured optical fiber (MOF) Bragg-grating optofluidic system. Biosens. Bioelectron. 2015;63:248–254.
[54] Nguyen AH, Sim SJ. Nanoplasmonic biosensor: Detection and amplification of dual bio-signatures of circulating tumor DNA. Biosens. Bioelectron. 2015;67:443–449.
[55] Shiddiky MJA, Sina AAI, Carrascosa LG, et al. Methylsorb: A simple method for quantifying DNA methylation using DNA-gold affinity interactions. 8th Int. Conf. Electr. Comput. Eng. Adv. Technol. a Better Tomorrow, ICECE 2014. 2015. p. 17–20.
[56] Kurita R, Yanagisawa H, Yoshioka K, et al. On-Chip Sequence-Specific Immunochemical Epigenomic Analysis Utilizing Outward-Turned Cytosine in a DNA Bulge with Handheld Surface Plasmon Resonance Equipment. Anal. Chem. 2015;87:11581–11586.

[57] Huertas CS, Aviñó A, Kurachi C, et al. Label-free DNA-methylation detection by direct ds-DNA fragment screening using poly-purine hairpins. Biosens. Bioelectron. 2018;120:47–54.
[58] Šípová H, Zhang S, Dudley AM, et al. Surface plasmon resonance biosensor for rapid label-free detection of microribonucleic acid at subfemtomole level. Anal. Chem. 2010;82:10110–10115.
[59] Hao K, He Y, Lu H, et al. High-sensitive surface plasmon resonance microRNA biosensor based on streptavidin functionalized gold nanorods-assisted signal amplification. Anal. Chim. Acta. 2017;954:114–120.
[60] Wang Q, Li Q, Yang X, et al. Graphene oxide-gold nanoparticles hybrids-based surface plasmon resonance for sensitive detection of microRNA. Biosens. Bioelectron. 2016;77:1001–1007.
[61] Aviñó A, Huertas CS, Lechuga LM, et al. Sensitive and label-free detection of miRNA-145 by triplex formation. Anal. Bioanal. Chem. 2016;408:885–893.
[62] Joshi GK, Deitz-McElyea S, Liyanage T, et al. Label-Free Nanoplasmonic-Based Short Noncoding RNA Sensing at Attomolar Concentrations Allows for Quantitative and Highly Specific Assay of MicroRNA-10b in Biological Fluids and Circulating Exosomes. ACS Nano. 2015;9:11075–11089.
[63] Yoo SM, Lee SY. Optical Biosensors for the Detection of Pathogenic Microorganisms. Trends Biotechnol. 2016;34:7–25.
[64] Inci F, Tokel O, Wang S, et al. Nanoplasmonic Quantitative Detection of Intact Viruses from Unprocessed Whole Blood. ACS Nano. 2013;7:4733–4745.
[65] Soler M, Belushkin A, Cavallini A, et al. Multiplexed nanoplasmonic biosensor for one-step simultaneous detection of Chlamydia trachomatis and Neisseria gonorrhoeae in urine. Biosens. Bioelectron. 2017;94.
[66] Yoo SM, Kim D-K, Lee SY. Aptamer-functionalized localized surface plasmon resonance sensor for the multiplexed detection of different bacterial species. Talanta. 2015;132:112–117.
[67] Mousavi M, Chen H-Y, Hou H-S, et al. Label-Free Detection of Rare Cell in Human Blood Using Gold Nano Slit Surface Plasmon Resonance. Biosensors. 2015;5:98–117.
[68] Im H, Shao H, Il Park Y, et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. 2014;
[69] Yang KS, Im H, Hong S, et al. Multiparametric plasma EV profiling facilitates diagnosis of pancreatic malignancy. Sci. Transl. Med. 2017;9.

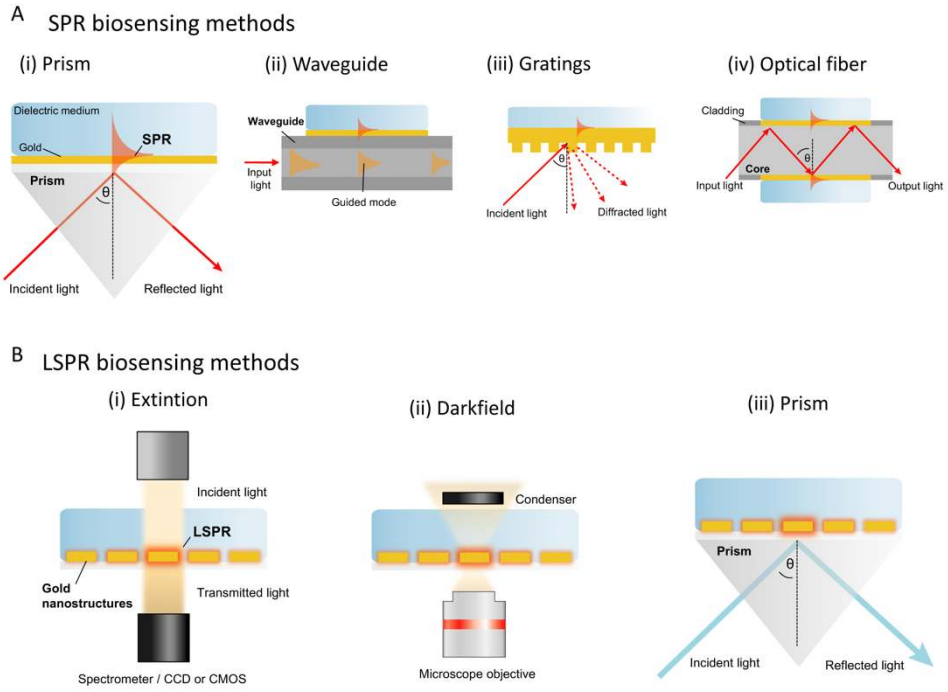

Figure 1. Illustrations of the different plasmonic and nanoplasmonic biosensor schemes: **(A)** Surface Plasmon Resonance (SPR) biosensor in prism-coupling configuration, waveguide, grating, and optical fiber, respectively and **(B)** localized SPR (LSPR) biosensor through extinction measurement, darkfield microscopy and prism-coupling scheme, respectively. All schemes included in the figure are original work made by the authors.

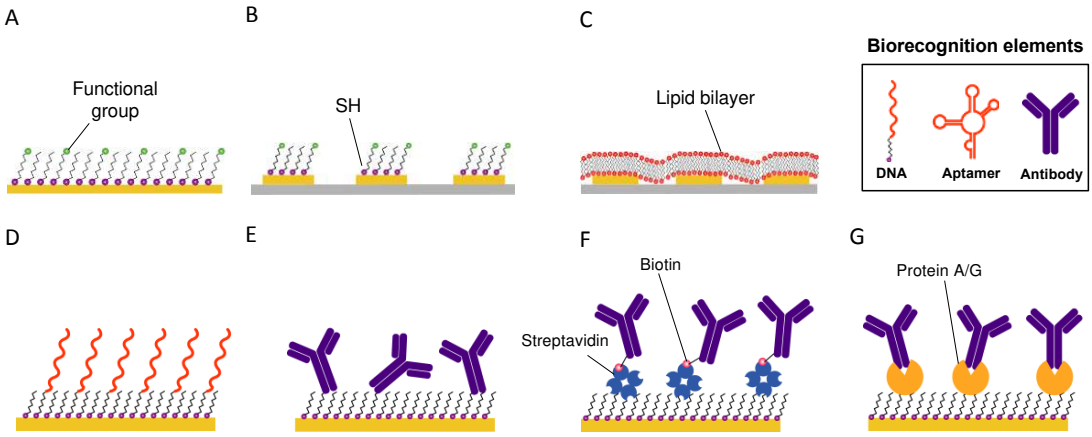

Figure 2. Schematics of different surface functionalization strategies: (**A**) Functional alkanethiol self-assembled monolayer (SAM) on gold; (**B**) Site-selective SAM formation on gold nanostructured surface; (**C**) Supported lipid bilayer (SLB) on gold nanostructured surface; (**D**) DNA probe immobilized on a SAM; (**E**) antibodies immobilized on a SAM by covalent binding; (**F**) antibodies immobilized on a SAM by biotin-streptavidin interaction; (**G**) antibodies immobilized on a SAM by Protein A/G interaction. Inset illustrates the structure of common biorecognition elements: DNA probe, aptamer, and antibody. All schemes included in the figure are original work made by the authors.

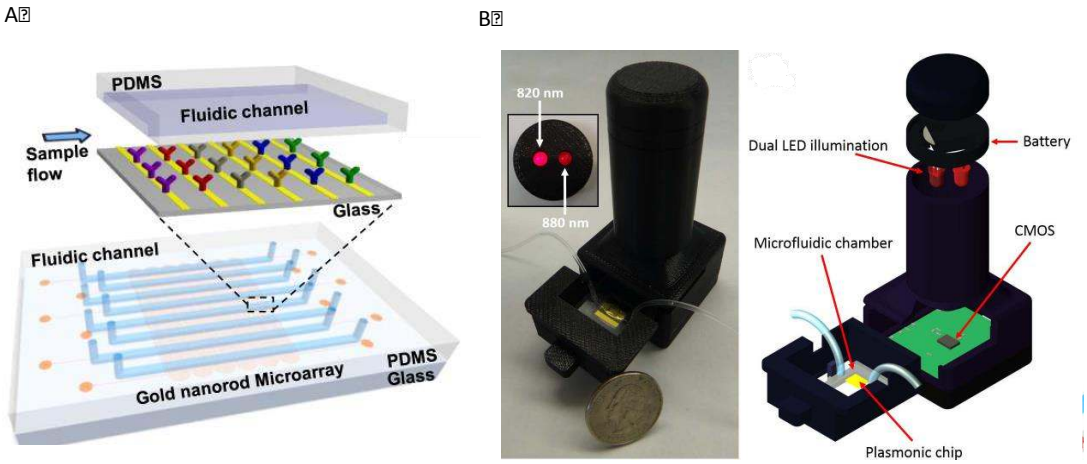

Figure 3. Examples of nanoplasmonic biosensors integrated in lab-on-a-chip and portable devices: (**A**) Multichannel microfluidics for multiplexed analysis (adapted with permission from [25] – Copyright 2015, American Chemical Society). (**B**) Handheld nanohole array biosensor for protein detection (adapted with permission from [34] – Creative Commons License Deed).

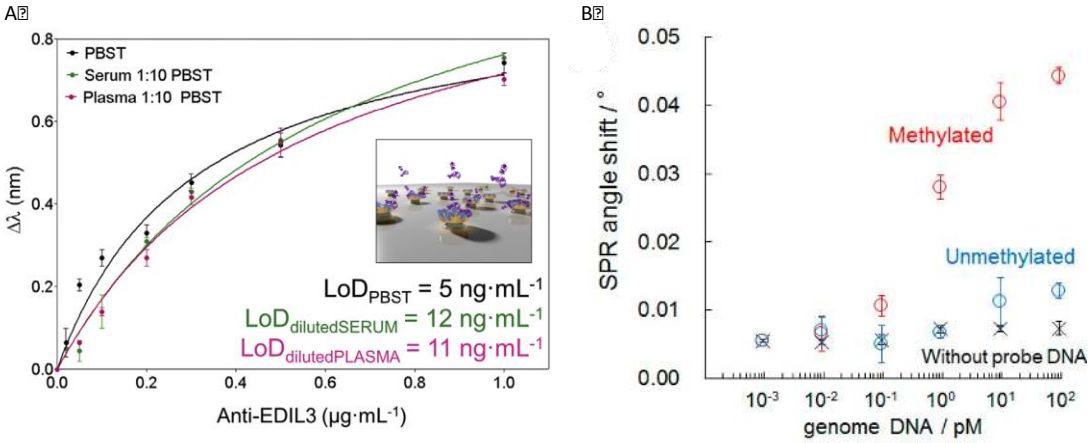

Figure 4. Examples of biomedical applications of nanoplasmonic biosensors: (**A**) Detection of tumor-associated autoantibodies for colorectal cancer diagnosis (adapted from [41], Copyright (2016), with permission from Elsevier). (**B**) Analysis of DNA methylation (adapted with permission from [56] – Copyright 2015 American Chemical Society).

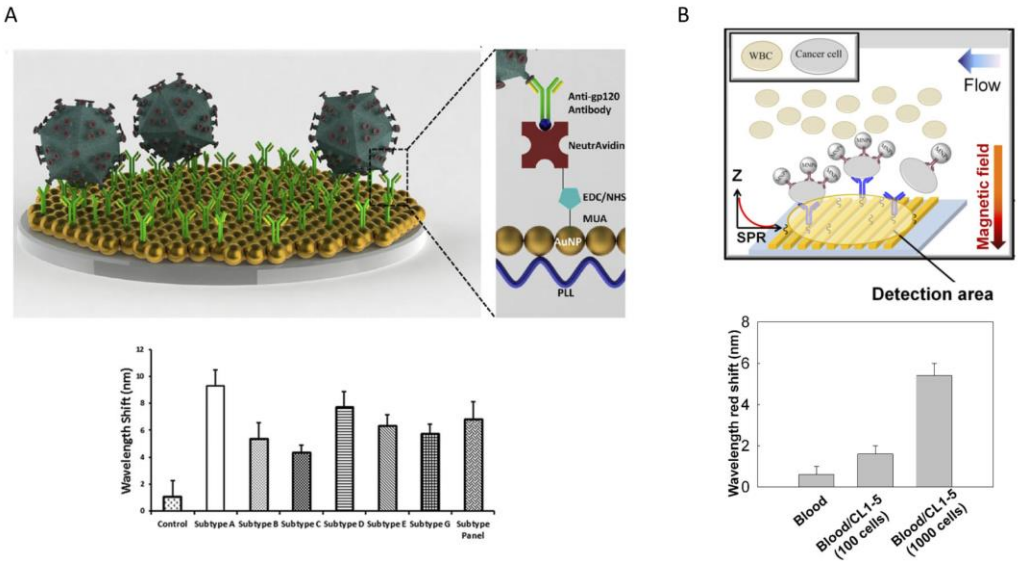

Figure 5. Examples of biomedical applications of nanoplasmonic biosensors: (**A**) Direct detection of intact viruses from blood (adapted with permission from [64] – Copyright 2013 American Chemical Society). (**B**) Detection of circulating tumor cells (CTCs) from blood (adapted with permission from [67] – Creative Commons License).