Analytica Chimica Acta 1104 (2020) 10–27

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Review

Advancements in SPR biosensing technology: An overview of recent trends in smart layers design, multiplexing concepts, continuous monitoring and *in vivo* sensing

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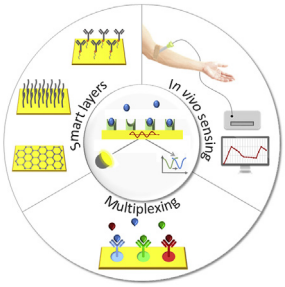
HIGHLIGHTS

- Continuous progress in material sciences promotes SPR biosensors development.
- Smart layers improve receptor orientation, fouling resistance and sensitivity.
- Challenging multiplexing is complemented by parallel analysis on SPR biosensors.
- Continuous and *in vivo* sensing with SPR biosensors remains yet to be attained.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 13 September 2019
Received in revised form
4 December 2019
Accepted 24 December 2019
Available online 27 December 2019

Keywords:
Surface plasmon resonance
Smart layers
Multiplexing
Continuous monitoring
In vivo sensing
Biosensor

ABSTRACT

Inspired by the rapid progress and existing limitations in surface plasmon resonance (SPR) biosensing technology, we have summarized the recent trends in the fields of both chip-SPR and fiber optic (FO)-SPR biosensors during the past five years, primarily regarding smart layers design, multiplexing, continuous monitoring and *in vivo* sensing. Versatile surface chemistries, biomaterials and nanomaterials have been utilized thus far to generate smart layers on SPR platforms and as such achieve oriented immobilization of bioreceptors, improved fouling resistance and sensitivity enhancement, collectively aiming to improve the biosensing performance. Furthermore, often driven by the desires for time- and cost-effective quantification of multiple targets in a single measurement, efforts have been made to implement multiplex bioassays on SPR platforms. While this aspect largely remains difficult to attain, numerous alternative strategies arose for obtaining parallel analysis of multiple analytes in one single device. Additionally, one of the upcoming challenges in this field will be to succeed in using SPR platforms for continuous measurements and *in vivo* sensing, and as such match up other biosensing platforms where these goals have been already conquered. Overall, this review will give insight into multiple possibilities that have become available over the years for boosting the performance of SPR biosensors. However, because combining them all into one optimal sensor is practically not feasible, the final application needs to be considered while designing an SPR biosensor, as this will determine the requirements of the

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https://doi.org/10.1016/j.aca.2019.12.067

bioassay and will thus help in selecting the essential elements from the recent progress made in SPR sensing.

Contents

1. Introduction

Over the past thirty years of extensive studying, surface plasmon resonance (SPR)-based platforms have turned out to be one of the most powerful technologies for real-time monitoring of molecular interactions [1,2] next to quantification of various biomarkers such as proteins [3–5], DNA [6–9], whole cells [10,11], etc. These applications have been widely investigated in the area of clinical diagnostics [12], biological and pharmaceutical analysis [13], food quality and safety evaluation [14], among others. Up to now, efforts have been mainly devoted towards improving the sensitivity and specificity of biosensing performance [15], miniaturization of conventional SPR setups [17], simplification of the workflow [16], detection in complex matrices [17], and paving the way for commercialization [18]. Particularly, one of the most crucial parts of SPR biosensors is the sensor surface as it plays a vital role in the overall sensing performance. Therefore, many studies have focused on the exploitation of smart sensing layers to realize more versatile applications [9,19–22]. Additionally, the increasing demand in the field to achieve multiplex detection, continuous measurements and *in vivo* sensing has provoked plenty of research on the SPR devices tackling these challenges.

However, some limitations of conventional SPR biosensors remain to date, thereby hindering the progress of the above-mentioned aspects. Currently, the bottlenecks of SPR biosensor development endure in the low sensitivity of direct label-free assays (especially for small molecules), the limitations in multiplex analysis, fouling or instability of the SPR sensing surface when subjected to complex or harsh environments, and the inability to perform continuous monitoring. To overcome these difficulties, several approaches have proven to be effective, such as combining SPR biosensors with materials science [23–25], nanotechnology [5,26], microfluidics or other related technologies [27–29], as well as to refer to the smart approaches used in the development of other biosensors [30–32]. Therefore, in this review, we will focus on the progress related to these aspects made over the past five years, and highlight some of the most promising trends, starting from a brief introduction of fundamentals of SPR biosensors. Firstly, a comprehensive overview of smart layers will be given in the context of oriented immobilization of bioreceptors, fouling resistance and sensitivity enhancement. Secondly, we will discuss the feasibility of multiplex bioassays in terms of SPR imaging (SPRi) and sensor surface functionalization, as well as the progress made in parallel analysis, enabled by the integration of chip-SPR setups with microfluidics and physical modification of optical fibers. Lastly, we will envisage the current problems hindering the achievement of continuous measurement and *in vivo* sensing as well as propose promising strategies regarding these aspects. An overview summarizing the main contents to be discussed is presented in Fig. 1. As continuous progress regarding the aforementioned trends has been made on chip-SPR and FO-SPR platforms, we will discuss both of them in this review, specifying the respective platform when we introduce different examples.

2. Technical aspects of SPR sensing

2.1. SPR fundamentals

SPR is induced by the light excitation of surface plasmons (SPs) at the interface between a dielectric and metal, such as silver (Ag) or gold (Au). Although the SPR effect is most prominent with the former, Au is mostly used as it is more resistant against oxidation. The SPs can only be excited when the wave vector of the incident light (k) is equal to the wave vector of the SPs (k_{sp}) in both direction and magnitude, as presented in Fig. 2A. More specifically, since SP waves are generated in the metal and their wave vector propagates

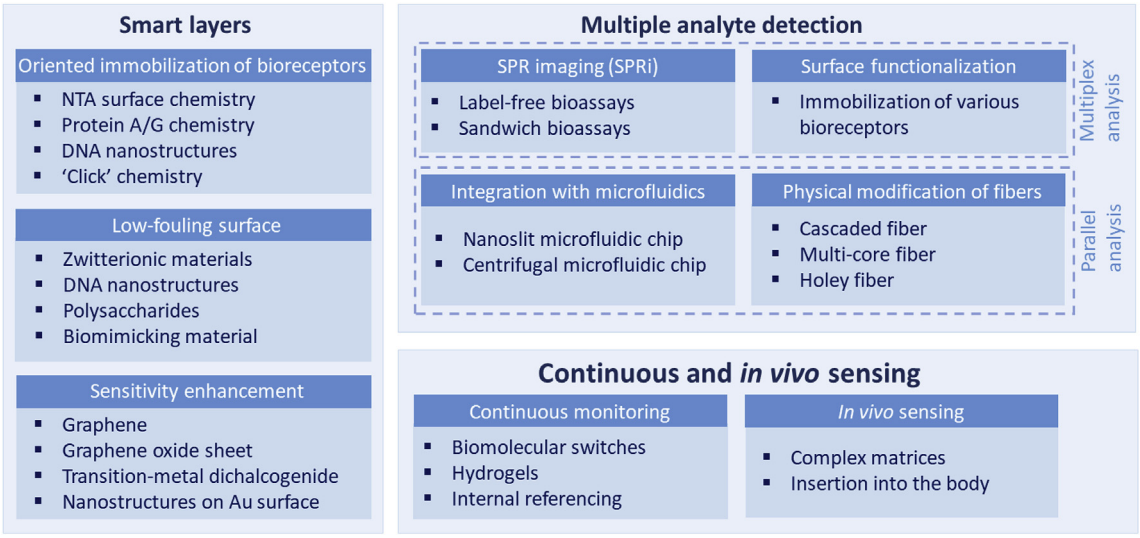

Fig. 1. Overview of the new trends to be discussed in this review.

parallel to the metal surface, the parallel component of the wave vector of the incident light (k_x) should match the wave vector of the SPs, which is expressed as follows:

$$k_{sp} = k_0 \sqrt{\frac{\varepsilon_m(\lambda) * \varepsilon_s(\lambda)}{\varepsilon_m(\lambda) + \varepsilon_s(\lambda)}} = \frac{2\pi}{\lambda} \sqrt{\frac{\varepsilon_m(\lambda) * \varepsilon_s(\lambda)}{\varepsilon_m(\lambda) + \varepsilon_s(\lambda)}} \quad (1)$$

Where k_0 is the wave vector of the incident light in vacuum, ε_m and ε_s are the dielectric constants of the metal and the surrounding medium, respectively, and λ is the wavelength of the incident light. This relation, however, cannot be fulfilled when direct light is sent through vacuum to the metal dielectric interface [2,33–35]. To increase k_x, total internal reflection (TIR) of p-polarized light can be exploited, by sending the light through a dielectric material at an angle above the critical value. Hereby, evanescent field waves are generated, which exponentially decay with distance from the surface (over the distance of approximately one λ) and penetrate the metal/dielectric interface. With this approach, the wave vector of the incident light is increased and can be expressed as follows:

$$k_x = k_0 \sqrt{\varepsilon_d} \sin(\theta) = \frac{2\pi}{\lambda} \sqrt{\varepsilon_d} \sin(\theta) \quad (2)$$

with ε_d and θ being the dielectric constant of the dielectric material and the angle of incidence, respectively. Using this strategy, k_x may be equal to k_{sp} and thus SPs can be excited. As k_x is dependent on both θ and λ, either can be used to tune k_x [2,34,36].

Another method to couple k_x and k_{sp} is by applying a grating to the metal layer. Hereby, the incident light will be diffracted, of which a fraction will obtain a wave vector parallel to the surface. Using this approach, the incident light illuminates the metal layer from the side of the surrounding medium, limiting its use to optically transparent samples [37].

The excitation of SPs can be seen as a dip in the reflectance spectrum as depicted in Fig. 2B. Moreover, when the RI of the surrounding medium changes, k_{sp} is altered and the excitation of SPs will occur at another λ or θ. This will be manifested as a shift of the dip in the reflectance spectrum towards other λ or θ, which is the detection principle of SPR sensors. Due to the exponential decay of the evanescent field wave, the sensitivity to the changes of RI also decreases with increasing distance from the surface [2,34,36]. In order to increase the penetration depth, the metal layer can be sandwiched between two dielectric materials having a similar RI. Hereby, the SP waves of both metal/dielectric interfaces can overlap, leading to an increased sensitivity further from the surface. This principle is applied in long range SPR (LRSPR) [38].

For detecting specific target molecules, bioreceptors can be immobilized on the SPR surface. Upon binding of target molecules to these bioreceptors, the RI increases, causing the dip in the reflectance spectrum to shift. When the position of the λ or θ is

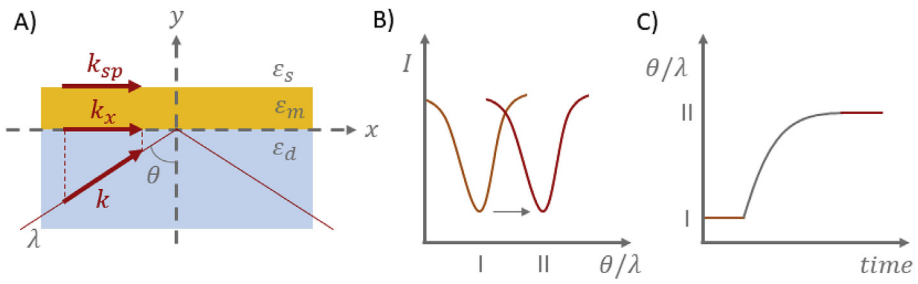

Fig. 2. Working principle of SPR sensors. A) When p-polarized light is sent to a metal/dielectric interface, SPs can be excited when k_x is equal to k_{sp}. This can be obtained by sending the light through a dielectric at an angle above the critical angle, so TIR occurs at the metal/dielectric interface. As k_x depends on λ and θ, it can be adjusted to be equal to k_{sp} by altering λ or θ, and SPs can be excited. B) This excitation can be seen as a dip in the reflectance spectrum. When the refractive index (RI) of the surrounding medium (and thus ε_s) changes, the magnitude of k_{sp} will change and the dip will shift to another λ or θ, depending on which of both is altered. C) In SPR sensors, typically the position of the dip is monitored over time, which is presented in a sensorgram, giving real-time information about RI changes and thereby (un)binding interactions.

followed over time, a sensorgram can be obtained, providing label-free and real-time information about molecular binding events, as shown in Fig. 2C [2,34,36].

As an alternative to a metal film, confined conductive nanostructures can also be applied to the surface, with dimensions smaller than λ. Hereby, coherent localized SPs are generated, with a frequency depending on the size, geometry and composition of the nanostructures. With this approach, known as localized SPR (LSPR), the surface area is increased and more bioreceptors can be immobilized, providing more opportunities for target binding and thereby increasing the sensitivity. However, as the penetration depth of the evanescent field wave is reduced compared to the SPR effect on metal films (due to the highly localized SPs), the sensitivity for larger targets can be compromised [35,39].

2.2. Chip-SPR

The most commonly used chip-SPR devices are prism-based setups, wherein a high RI prism, covered with a metal film, is employed to achieve TIR of the incident light and excite SPs. Within this category, three strategies can be employed, being θ, λ and intensity modulation.

In angle-based systems, monochromatic light is sent through the prism and the reflectance spectrum is measured as a function of θ. This principle has been used in the Biacore instruments, which are up to now the most successful commercial chip-SPR devices, known as benchmark in the field of SPR biosensing. Although they are suitable for sensitive and label-free detection of biomolecular interactions, due to their bulky size and necessity of expensive optical components, their application outside research centers remains limited [36,40,41]. Other companies that commercialized angle-based chip-SPR setups include BioNavis, Reichert Technologies and Biosensing Instruments [42].

Next to θ, λ can also be altered to tune k_x, which is done in λ-based setups. Specifically, θ is kept constant and a polychromatic light source is employed, thus obtaining the reflectance spectrum as a function of λ. This strategy is mostly used in FO-SPR setups which will be explained in Section 2.3 [36].

Another strategy is intensity-based SPR (i.e. SPRi), where, unlike θ- or λ -based setups, both θ and λ are kept constant and the intensity of the reflectance spectrum is recorded by a CCD camera. Although sensitivities are generally lower than for angle-based systems, many (up to several hundreds) spots with distinct bioreceptors can be structured on a single chip, enabling multiplex measurements, which will be additionally discussed in Section 4.1. This has been applied in commercially available chip-SPR instruments from companies like Horiba, IBISWorld and Sierra Sensors [36,42].

2.3. FO-SPR

FO-SPR represents a simpler and more cost-effective alternative to chip-SPR platforms where polychromatic light is sent through a metal-coated optical fiber. As θ cannot be fixed, FO-SPR sensors are typically λ- or intensity-based. As depicted in Fig. 3, two different configurations have been proposed: (i) the sensing zone in the middle of the optical fiber where the transmitted light is being measured (forward scattering mode, Fig. 3A) and (ii) the sensing zone at the end where the reflected light is measured (backward scattering mode, Fig. 3B) [36,41]. Next to the low cost, high flexibility and possibility for miniaturization, which are associated with FO-SPR sensors in general [41] -and as such represent interesting alternative to the conventional prism-based devices for application both in and outside research centers-the use of backward scattering mode configurations has other interesting advantages. Because of its 'dipstick' shape, this setup can be used directly with various sample matrices, such as milk [43], human serum/plasma [3,44], whole blood [17] or even soft matter [45]. Furthermore, the use of microfluidics can be circumvented, allowing the application of nanoparticles (NPs) to enhance the sensitivity [17,43,46–49]. Additionally, this configuration is compatible with thermocycling, which opens up possibilities for new applications, such as real-time monitoring of DNA amplification [6–8] and melting assays [46,50,51]. This configuration was recently commercialized by FOx Biosystems as a benchtop device.

It is interesting to note that both abovementioned configurations are included in so-called Fiber Bragg gratings (FBGs) with periodic variations in the RI of the fiber core. Through FBGs, part of the λ is reflected, while the rest is transmitted. These configurations are highly sensitive to physical parameters like temperature, pressure, vibration, and strain, thereby being commonly used for these applications [52–54] and commercialized by Infibra Technologies. By tilting the FBGs, the coupling of light into SPs can be greatly enhanced compared to regular (straight) FBGs, which renders tilted FBG (TFBG) FO-SPR sensors suitable for biosensing purposes. Although this increased sensitivity is generally still lower than the sensitivities obtained for other SPR configurations, a higher accuracy can be obtained thanks to the narrow SPR dips [10,41,52,55].

3. Smart layers

The interface layers between the metal coating of SPR sensors and the bioreceptors determine the bioreceptor immobilization, surface resilience to complex matrices and sensitivity, and as such the overall sensing performance. Along with the prompt advances in materials science, plenty of emerging materials have been tested to fabricate SPR sensing layers for specific goals, involving nanomaterials or nanostructures [4,22,56–58], polymeric materials [59,60], biomaterials [24,25,32,61], and hybrid or three-dimensional (3D) structures [5,22], among others. These interface layers between the metal coating of SPR sensors and the bioreceptors will be referred to in this review as smart layers. In this context, although there might be other strategies to achieve the same goals [62–66], we will review here only the efforts that have been made regarding the development of smart layers to improve the performance of SPR biosensors regarding oriented immobilization of bioreceptors [5,9,19,20,26,67–77], low-fouling sensing surfaces [9,21,24,25,32,59,61,78–85] and sensitivity enhancement [4,15,22,26,56–58,86–92].

3.1. Oriented immobilization of bioreceptors

Over the years, several surface chemistries have been used for attachment of molecules to the Au-coated surfaces. For instance, thiol chemistry is often employed to immobilize thiolated nucleic acids (NA) or protein bioreceptors directly to the Au surface or to attach thiolated self-assembled monolayers (SAMs) as well as polymer films that subsequently serve as scaffolds for further immobilization of bioreceptors. Another strategy to immobilize biomolecules is silane chemistry. Here, a silane coupling agent is used with one end containing silane-reactive groups (e.g. alkoxy) that enables attachment to a silicon layer (deposited on the Au-coated surface) and the other end containing functional groups to facilitate the covalent binding of biomolecules [93]. Although frequently used, none of these approaches ensures controlled orientation or distribution of bioreceptors. Therefore, numerous efforts have been devoted to the exploitation of smart layers for obtaining uniform orientation, high surface coverage, maximal maintenance of bioactivity and reduced steric hindrance, rendering

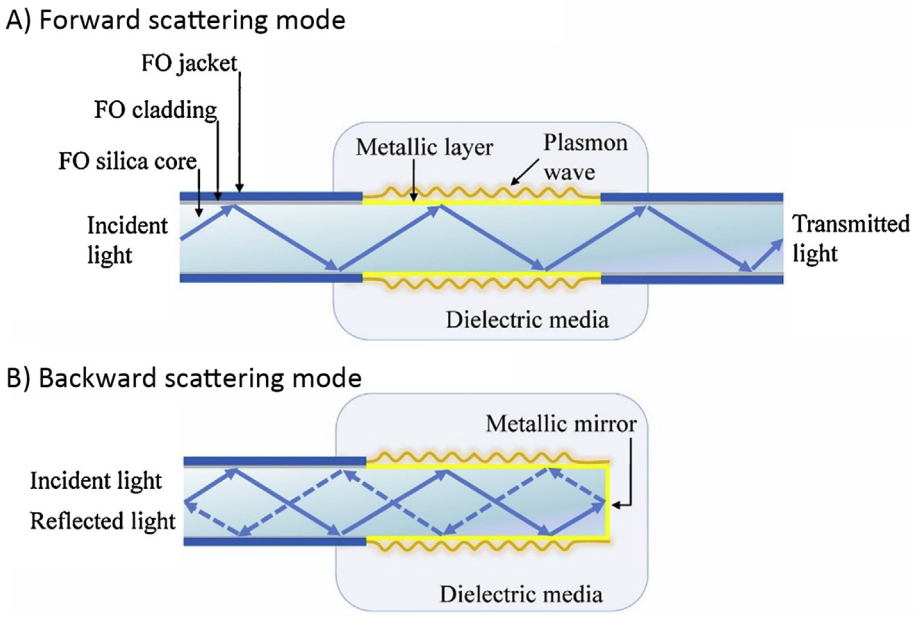

Fig. 3. Two FO-SPR configurations. A) For forward scattering mode, the sensing zone is located in the middle of the optical fiber. The incident light undergoes TIR in the fiber and reacts with the metallic layer, exciting SPs. The transmitted light is then measured at the other end of the optical fiber. B) For backward scattering mode, the sensing zone is located at the end of the fiber. The incident light is reflected back and measured at the same side where it enters the fiber. Adapted with permission from Ref. [41]. Copyright (2015) Elsevier.

high accessibility for analyte binding and paving the way for multiplex bioassays, which will be discussed in the following sections.

Nitrilotriacetic acid (NTA) SAMs have been employed for oriented protein immobilization, inspired by its extensive use for purification of recombinant proteins with a hexa-histidine (His_6) tag. After chelate formation between NTA molecules and metal ions (*e.g.* Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}), two free binding sites in each NTA chelate can be replaced by two consecutive histidines in the His_6-tag of the proteins (Fig. 4A) [69]. Because the His_6-tag can be incorporated at specific locations in the protein, a uniform and oriented distribution of protein bioreceptors on the surface of SPR biosensors can be easily achieved [71,75]. Due to the complex 3D structure and large size of proteins, one NTA chelate with His_6-tag as anchor may not be stable enough to ensure the proteins erect on the surface. Therefore, multiple His_6-tags have been introduced into the proteins to position them more strategically on a chip-SPR biosensor and ensure that they could be immobilized in an 'end-on' (Fig. 4B) instead of 'side on' (Fig. 4C) mode, leaving the specific binding sites accessible. However, as several His_6-tags have to be integrated at specific locations, engineering of the protein bioreceptors becomes more demanding [19]. Importantly, chelate-based immobilization has enabled regeneration of His_6-tagged proteins, by easy disruption of the chelate entity, and thereby provided potential for sensor reusability. A drawback of this easy disruption, however, is the instability of the bioreceptor layer, which limits its biosensing use in complex matrices. Therefore, to avoid gradual dissociation of proteins from the coordination complex and thereby enable kinetic studies of target interactions during long-term measurements or in harsh environments, an amine-coupling reaction was used immediately after the chelation. In this way, the His_6-tagged proteins were further attached to the carboxyl (COOH) groups present in the underlying carboxymethylated dextran layer, which serve as cross linkers to enhance the stability of the immobilization. Using this strategy, a high density of oriented bioreceptors was obtained on a chip-SPR platform, allowing accurate kinetic measurements and sensitive quantification of 4-carboxybenzene-sulfonamide with a limit of detection

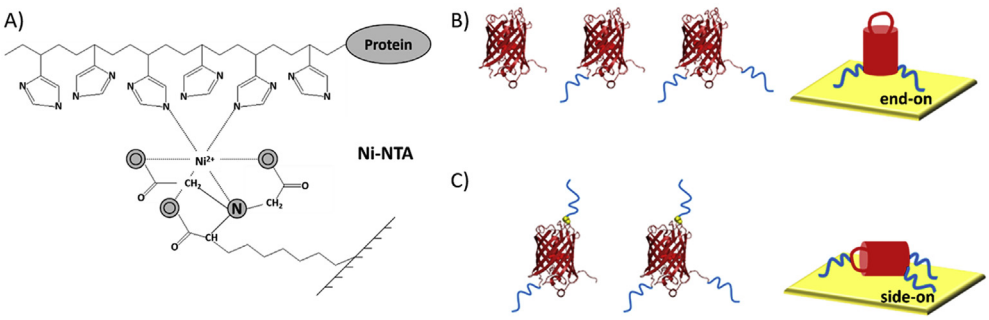

Fig. 4. NTA strategy for oriented immobilization of bioreceptors. A) Illustration of the immobilization of His_6-tagged proteins using NTA SAM. NTA molecules form chelates with metal ions (e.g. Ni^{2+}). Afterwards, two free binding sites in each NTA chelate are left which will capture the imidazole groups in the His_6-tag, thereby immobilizing His_6-tagged proteins [69]. Controlled immobilization of His_6-tagged proteins via NTA SAM in B) end-on and C) side-on modes, by binding various numbers of His_6-tags located at several sites in the protein bioreceptors [19]. Adapted with permission from Refs. [19,69]. Copyright (2009, 2017) Elsevier and American Chemical Society.

(LOD) of 14 nM [72]. For uniform immobilization of His_6-tagged proteins on SPR biosensing platforms, other NTA complexes have also been studied, such as those containing copper (II) and thiol derivatives of dipyrromethene or pentetic acid thiol ligands [76]. Especially interesting is the fact that biotin can also be attached to NTA chelates with Zn^{2+}, Cu^{2+} and Mn^{2+} via the thioether group [94]. For instance, this strategy was employed to immobilize biotin-tagged bioreceptors by Cu^{2+}-NTA chelates on functional graphene layers in a chip-SPR sensor, which will be elaborated in Section 3.3 [4]. Although the use of NTA SAMs is suitable to obtain oriented immobilization of protein bioreceptors, this approach is limited to proteins in which His_6-or biotin-tags need to be incorporated, thus restricting its extensive use.

Another oriented immobilization strategy is the use of protein A/G as mediating layer to specifically bind the Fc domain of immunoglobulins G (IgG), thereby exposing the Fab regions for interaction with analytes. The feasibility of this strategy has been investigated by exploring various linkages of protein A/G onto the sensor surface including parylene-H [77], graphene oxide (GO) [26] and carbon disulfide [74]. Compared to the NTA-His_6-tag approach, this protein A/G linking methodology has a wider application range thanks to its specific binding to IgG, which are broadly employed as bioreceptors. However, as this approach is based on affinity interactions, dissociation of the bioreceptors may occur, limiting its potential for the application in complex matrices and the reusability of sensor surfaces. Therefore, to further improve this strategy, bioreceptor immobilization was stabilized via crossing-linking. Particularly, for label-free detection of human growth hormone in serum, the sensitivity was increased 2.6 times using protein G mediated immobilization of antibodies on a chip-SPR surface compared to random covalent immobilization by COOH SAM. Moreover, an enhanced sensitivity (3.5 times) was obtained with an LOD of 3.31 nM when chemical cross-linking was added after antibody immobilization [67].

Additionally, various 3D DNA nanostructures, such as DNA origami (on an FO-SPR platform) [5] (Fig. 5A and B) and tetrahedron structures (on both chip-SPR and FO-SPR platforms) [5,20] (Fig. 5C), have been developed and used as linking layers for oriented immobilization of bioreceptors. In contrast to DNA tetrahedrons, DNA origami can realize not only oriented immobilization, but also pattern the bioreceptors in a predefined order. Specifically, the spacing between bioreceptors can be preset in DNA origami structures to achieve highly designed and accessible sensing surfaces according to diverse demands. However, the proposed configurations can still be improved by decreasing the distance between the bioreceptors and the Au surface, which has a major influence on the sensitivity of SPR sensors [5], as already mentioned in Section 2.1.

Site-specific coupling can also be achieved by copper(I)-catalyzed azide-alkyne cycloaddition 'click' chemistry, which occurs between bioreceptors engineered with alkyne/azide groups and an azide/alkyne-functionalized solid surface, forming a covalent bond under copper(I) catalysis and mild reactions. However, the requirement to have bioreceptors with an incorporated alkyne group restricts its extensive use [68,73]. Furthermore, to eliminate the cytotoxic copper catalyst, the concept of copper-free 'click' chemistry was proposed to improve biocompatibility for *in vivo* applications. Using this strategy, azide-containing penicillin-binding proteins (PBPs) were homogeneously immobilized on a dibenzylcyclooctyne-coated surface. To demonstrate the potential of this strategy, the activity of PBPs was evaluated by an *in vitro* peptidoglycan synthesis assay on a chip-SPR biosensors where PBPs showed to be fully active after being immobilized [70]. Because this strategy seems to be promising, future work is needed to explore its impact on the sensing performance.

3.2. Low-fouling surface

One of the major challenges for any biosensing platform, including the SPR biosensors, is surface fouling in complex matrices, which can result in substantial non-specific binding, thereby impeding the specific signal. Hence, many recent studies have focused on the development of a low-fouling surface to resist the adhesion of foulants from complex matrices (such as whole blood [85], plasma or serum [9], cell lysates [78] and crude food samples [60]) while still allowing the attachment of bioreceptors.

In this context, various polymers and copolymers have been designed to replace the commonly used poly(ethylene glycol), *i.e.* PEG-based low-fouling materials, which function by forming a strong hydration layer, thereby repelling nonspecific adsorption of proteins, cells and other foulants [59,79,80]. The most widely studied strategy is the use of zwitterionic materials, which exhibit superior low-fouling capability due to their super-hydrophilicity caused by electrostatic interactions [82]. Therefore, numerous studies have investigated zwitterionic polymers or copolymers as an SPR sensing layer by evaluating the amount of foulant

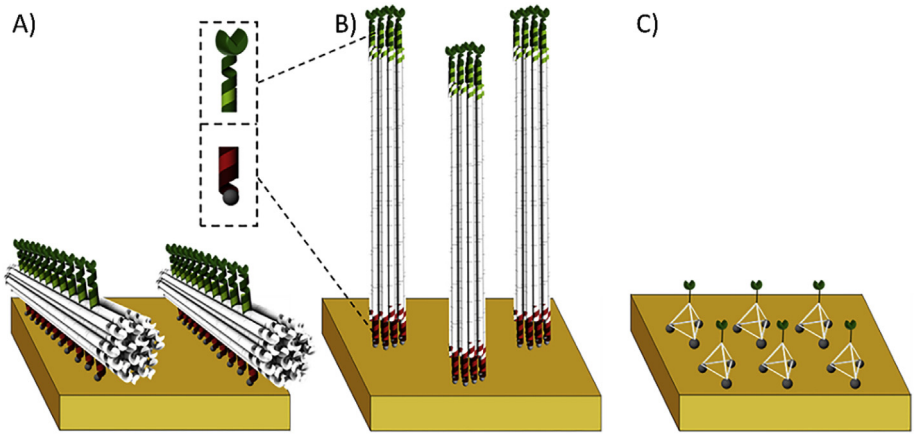

Fig. 5. Strategies for oriented immobilization of aptamers based on DNA nanotechnology. A) 3D DNA origami with aptamers (green) on their lateral side (LS), linked via thiolated linker groups (red) to the Au surface, B) 3D DNA origami with aptamers at their distal ends (DE), linked via thiolated linker groups (red) to the Au surface, and C) 3D DNA tetrahedrons with aptamers on top. Adapted with permission from Ref. [5]. Copyright (2018) American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

adsorption [60,61,85]. More importantly, the low-fouling function of the zwitterionic materials should be maintained after functionalization with bioreceptors. Therefore, the fouling resistance of two different polymer brushes, being hydroxy-functional poly(2-hydroxyethyl methacrylate) (pHEMA) and carboxy-functional poly(carboxybetaine acrylamide) (pCBAA), were compared after antibody immobilization on a chip-SPR biosensor. Strikingly, due to the zwitterionic groups, pCBAA showed great capacity in recovering the COOH groups, which are advantageous to obtain a hydration layer, and could thereby obtain highly sensitive detection of bacteria in complex food samples. In contrast to pCBAA, pHEMA almost lost all of its resistance to fouling after the activation of hydroxyl (OH) groups due to the loss of surface hydrophilicity [60,81,84]. Moreover, a 3D structured zwitterionic hydrogel thin film was developed composed of pCBAA with carboxybetaine diacrylamide crosslinkers, as illustrated in Fig. 6. An ultra-low fouling performance (less than 5 ng mL^{-1} of foulants in undiluted serum) was achieved with long-term stability and a high load of bioreceptors at the optimized crosslinker ratio and film thickness [21]. Most recently, a bottlebrush shaped polymer was created on a chip-SPR surface, composed of zwitterionic polymers, *i.e.*, poly(2-methacryloyloxyethyl phosphorylcholine), to form a strong hydration layer, which were all grafted on a hydrophobic copolymer core to acquire a durable support. Amazingly, a highly low-fouling surface was obtained with less than 0.2 ng cm^{-2} of protein adsorption [24].

In addition to zwitterionic low-fouling layers, many other novel strategies have been proposed, as it is challenging to fulfill all the aforementioned requirements. Indeed, by covalently attaching DNA tetrahedron probes to the Au-coated chip-SPR surface, sensitive detection of microRNA in undiluted human serum and cell lysate was enabled. Ultralow adsorption (less than 8.0 ng cm^{-2}) was achieved, mainly due to the strong hydration ability of the tetrahedrons and the steric hindrance caused by its special conformation, as shown in Fig. 5C [9]. Interestingly, it was proven that when immobilizing bioreceptors on DNA origami structures (Fig. 5A and B), label-free detection of thrombin was achieved with LODs of 11.2 nM and 6.1 nM for LS and DE structures respectively, without the need for backfilling with PEG to prevent non-specific binding. This indicated the feasibility and potential of using DNA origami structures in complex matrices to prevent fouling [5]. Furthermore, polysaccharides also show to be promising low-fouling materials thanks to their high hydrophilic capability. Therefore, as an inert and functional polysaccharide, hyaluronic acid was covalently attached to the chip-SPR surface. Consequently, ultralow protein adsorptions (as low as 3 ng cm^{-2} for lysozyme and soybean milk next to 17 ng cm^{-2} for cow milk and 10% blood serum) were observed as well as a high immobilization capacity and biocompatibility for bioreceptors [83].

Regarding the strategies used on other biosensor platforms, a phosphatidylcholine (PC)-terminated monolayer mimicking cell-membrane was applied as the sensing layer in an electrochemical sensor. The problem of baseline drift in whole blood was greatly overcome, thus enabling both *in vitro* and *in vivo* detections. The innovation lies in the formation of fouling resistance by the PC end, inspired by the hydration ability of eukaryotic cellular membranes [32]. Therefore, the use of biomimetic materials is quite promising and could also be applied for the improvement of multi-functional SPR sensing layers.

3.3. Sensitivity enhancement

LSPR and LRSPR biosensors have been popularly studied as approaches for increasing the sensitivity of SPR platforms [36,40,95]. In LRSPR sensors, a dielectric buffer layer (DBL) is added between the dielectric material and metal film, which can be complemented by a second DBL on the metal film (symmetrical LRSPR). In this way, the penetration depth of the evanescent field waves is increased, allowing more sensitive detection further from the surface [38]. In LSPR sensors, conductive nanostructures are assembled on the surface of the dielectric material instead of a metal film. Hereby, the sensitivity is increased at smaller distances from the surface due to the increased surface area, allowing a higher capacity for molecular binding. At larger distances, however, the sensitivity is compromised due to the decreased penetration depth of the evanescent field waves [40,95]. Although both approaches have generated significant sensitivity improvements, in this review we will focus on the smart layers as a linking material between the metal film and bioreceptors. Numerous potential nanomaterials have been investigated to date in this context, involving graphene [4,90] and its derivatives [15,26,56,92], transition-metal dichalcogenide (TMDC) nanomaterials as well as their combination with graphene [22,57,58,88,91].

Among all the promising materials, graphene has certainly attracted the most attention, attributed to its superior function, including high electron and charge carrier mobility, broad wavelength adsorption, large surface area and rich π conjugation structure. For instance, a single graphene layer was grown on an Au-coated chip-SPR surface by chemical vapor deposition. Subsequently, this graphene layer was functionalized with polypyrrole-

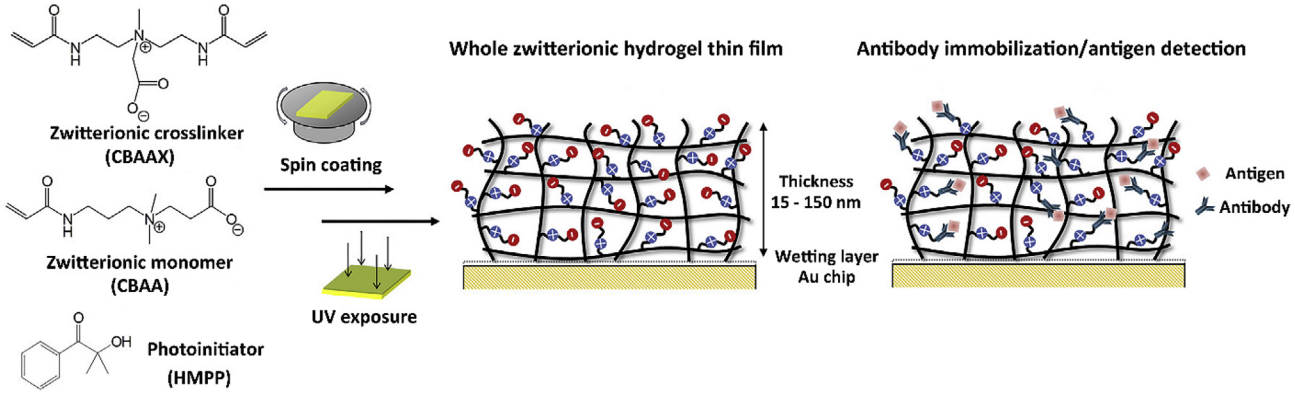

Fig. 6. Production of a low-fouling zwitterionic carboxybetaine thin film hydrogel on a chip-SPR surface. This process involves the mixing of monomer, crosslinker and photoinitiator, spin coating of the mixture on the chip, cross-linking by UV exposure, formation of hydrogel-coated substrate as well as antibody immobilization and antigen detection. Adapted with permission from Ref. [21]. Copyright (2016) Elsevier.

NTA to immobilize biotin-tagged bioreceptors in an oriented manner. Using this strategy, a highly sensitive label-free detection of anti-cholera toxin antibody was obtained with an LOD of 4 pg mL^{-1} [4]. To allow a more versatile biomolecule immobilization, GO sheets (GOSs), known as the oxidized counterparts of graphene, were deposited on SPR sensors. GOSs possess high flexibility in surface modification as well as a tunable dielectric constant, resulting from the controlled adsorption of oxygen-containing groups such as epoxy (-O), OH, and COOH groups. Therefore, GOSs were placed on the Au-film of an SPR biosensor as a linking layer to attach biomolecules [15,26,56,92]. Higher sensitivity was achieved compared to conventional Au-film based sensors such as carboxymethylated dextran (2.5 times higher) and graphene layers (3.25 times higher) for detection of streptavidin [26], owing to the large surface area and abundant functional groups of GOSs, as elaborated in Fig. 7. More recently, TMDC emerged as a promising candidate or supplement of graphene for smart layers, thanks to its remarkable optical parameters. Many numerical studies have been performed involving monolayer molybdenum disulfide (MoS_2) [22], a hybrid structure of graphene and MoS_2 [22,91], and graphene in combination with tungsten disulfide (WS_2) [58], barium titanate ($BaTiO_3$) [88] or multilayer $Ti_3C_2T_x$ MXene [57]. However, thus far, the investigations of the aforementioned TMDC materials are still limited to theoretical analysis awaiting therefore validation in experimental studies.

In addition, nanostructured materials have been introduced as smart layers to immobilize bioreceptors on the Au surface, thereby increasing the sensitivity. Specifically, 'double click' chemistry was used to assemble a nanostructured layer of azide-terminated iron oxide (Fe3O4@N3) NPs on an alkyne-terminated Au surface and functionalize the NPs with biotin bioreceptors containing alkyne groups. Hereby, both the advantages of SPR and LSPR were combined. The sensitivity of the sensor was greatly enhanced (from 1300 nm RIU^{-1} with 0% NPs to 5200 nm RIU^{-1} with 100% NPs) due to the high RI of NPs. Furthermore, due to the nanostructured surface, the accessibility of the bioreceptors was improved, which increased the SPR shift of streptavidin binding from 3 nm (0% NPs) to 41 nm (100% NPs) [96].

4. Feasibility of multiplex bioassays

Development of multiplex bioassays has drawn a lot of attention, because quantification of a panel of targets in one single measurement is more time- and cost-effective, next to being beneficial when samples are available in low volumes. Strictly speaking, multiplexing in bioassays refers to the simultaneous analysis of multiple analytes in a single sample by generating separate detectable signals in one single measurement [97]. SPRi is currently the most feasible SPR technology for multiplex detection, as it provides simultaneous readout of various spots (ranging from a few to hundreds) on a microarray chip, functionalized with different bioreceptors. However, as SPRi generally has a lower sensitivity than θ- and λ-based SPR platforms and the achievement of multiplexing on the latter remains challenging, many efforts have been devoted to obtain parallel analysis of multiple analytes in one single device. In particular, the sample is distributed into multiple parallel compartments, where for each analyte, a measurement is performed individually, generating a comparable performance as multiplexing. Based on the distinction between multiplexing and parallelization, in this section, we will first discuss the efforts that have been made to realize multiplex bioassays in terms of SPRi [98–101] and sensor surface functionalization [102,103]. Additionally, we will discuss the progress in parallel quantification of multiple analytes in one single device, enabled by the combination of chip-SPR with microfluidics [27–29,104–109] and physical modifications of FO-SPR sensors [110–117].

4.1. Multiplex bioassays using SPRi

SPRi permits simultaneous quantification of multiple analytes, through immobilization of different bioreceptors on a single microarray chip. Using this technique, label-free bioassays were performed to detect three islets hormones, namely insulin, glucagon and somatostatin. Since the analytes were small peptides, sensitivity was increased with the format of competitive bioassays by immobilizing different analytes on respective spots, followed by a mixture of different antibodies. Hereby, multiplex detection was realized with LODs of 1 nM, 4 nM and 246 nM, respectively [99]. Moreover, sandwich bioassays were developed to enhance the sensitivity of SPRi platforms, by using AuNPs functionalized with detection probes. For instance, four cytokines spiked in buffer and equine synovial fluid were detected reaching an LOD as low as 50 fg mL^{-1}, by sequential labeling with a mixture of biotinylated detection antibodies, neutravidin and biotinylated AuNPs [100]. In another study, a universal detection probe was developed, which

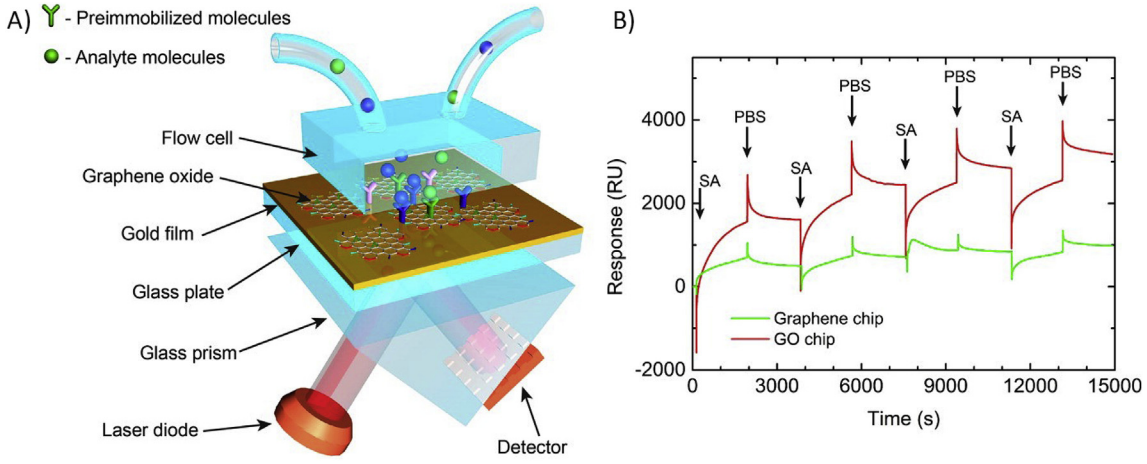

Fig. 7. Detection of streptavidin using a GOSs-modified chip-SPR biosensor. A) Diagram of a chip-SPR biosensor covered with GOSs in conjunction with bioreceptors. B) Sensorgram obtained after adding 100 μg mL^{-1} of streptavidin for the sensor chip coated by monolayer graphene (green) or airbrushed GOSs (red). Adapted with permission from Ref. [26]. Copyright (2015) American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

could achieve signal enhancement for all spots simultaneously, thus greatly simplifying the whole process. Specifically, as illustrated in Fig. 8, various DNA probes were immobilized on the SPRi microarray to capture microRNA targets, and subsequently labeled by AuNPs functionalized with anti-DNA/RNA detection antibodies, which bind to all DNA/RNA hybrids. This strategy promoted multiplex detection of four microRNAs with an LOD as low as 0.5 pM [101]. In addition to the above discussed latest studies, SPRi has been comprehensively reviewed elsewhere [118–120].

4.2. Multiplex bioassays enabled by surface functionalization

As mentioned above in Section 3.1, a surface with a high immobilization capacity, which also maintains the bioactivity of immobilized bioreceptors, is required as a technical basis for multiplexing. However, most of the studies discussed above still focus on the improvement of singleplex sensing without exploring to date the potential of these smart layers for multiplexing purposes. Nevertheless, two examples of chip-SPR platforms (other than SPRi) have been described where two different antibodies were immobilized by standard amine coupling chemistry between COOH-SAM and proteins [102,103]. Specifically, a multiplex detection of two protein biomarkers (alpha-1 antitrypsin and Tau 381) in plasma was achieved in a single chip-SPR channel, where two types of capture antibodies were immobilized at specific ratios to control the surface density and thereby obtain detection ranges of micromolar and femtomolar concentrations for each analyte, respectively. However, with this strategy multiplexing had to be achieved by subsequent labeling of both target molecules. Otherwise, the signal generated by each target could not be distinguished. For instance, DNA aptamers, used here as labels, had to be flowed over the chip in tandem for the subsequent recognition of each target. Thereby, the specific signal generated by labeling of each target could be distinguished in time and multiplex detection could be obtained [102]. Apparently, the dependency on labeling procedures to acquire specificity has restricted the extensive use of this strategy for multiplex bioassays.

4.3. Integration of chip-SPR and microfluidics for parallelization

Multi-channel chip-SPR sensors have been constructed to realize parallel screening of multiple analytes in a single device [104,105]. However, as this can be a tedious and time-consuming process requiring many manipulation steps of liquid samples, numerous efforts have been made for integrating chip-SPR with microfluidics in order to achieve automated parallel analysis of multiple analytes [27–29,107–109]. In fact, parallel detection of microRNA was achieved in an automated way, by combining microfluidics with transmission chip-SPR, where the transmitted light is measured instead of the reflected light, as in conventional SPR sensing. Therefore, the surface was structured with nanoslit arrays for the immobilization of four monitoring probes (three capture and one reference probe) onto different regions of this surface. Furthermore, functionalized AuNPs were used for signal enhancement and a high sensitivity was achieved, with an LOD of 30 fM in urine samples [27]. Additionally, a highly integrated centrifugal microfluidic chip was reported composed of eight layers, as shown in Fig. 9A. In the chip, multiple processing functions were integrated, involving plasma extraction from blood, division of the plasma into five parallel channels (functionalized with five different anti-IgG antibodies via COOH-SAM), incubation and washing steps, and parallel detection as indicated in Fig. 9B. The chip was successfully employed to detect five different IgG antibodies in whole blood in an automated way. Furthermore, the whole process was completed within less than 1 h, obtaining a high sensing performance, with a detection range of 0–100 μg mL^{-1} and an LOD of 19.8 μg mL^{-1} [28]. This integration of chip-SPR biosensors with microfluidics can be a substantial step towards development of point-of-care (POC) devices. Moreover, it is expected that the rapid progress in the microfluidics field will further boost the development of chip-SPR biosensors for parallel detection.

4.4. Physical modification of optical fibers for parallel analyte detection

FO-SPR has attracted plenty of research interest over the years as a more cost-effective alternative to chip-SPR devices. Many strategies have been described for physical modifications of optical fibers, such as the use of cascaded fibers [116,121], multi-core fibers [111,113] or holey fibers [114,115,117] that can be advantageous for parallel detection as well as for self-referencing to compensate for interference from background RI, physical adsorption and other

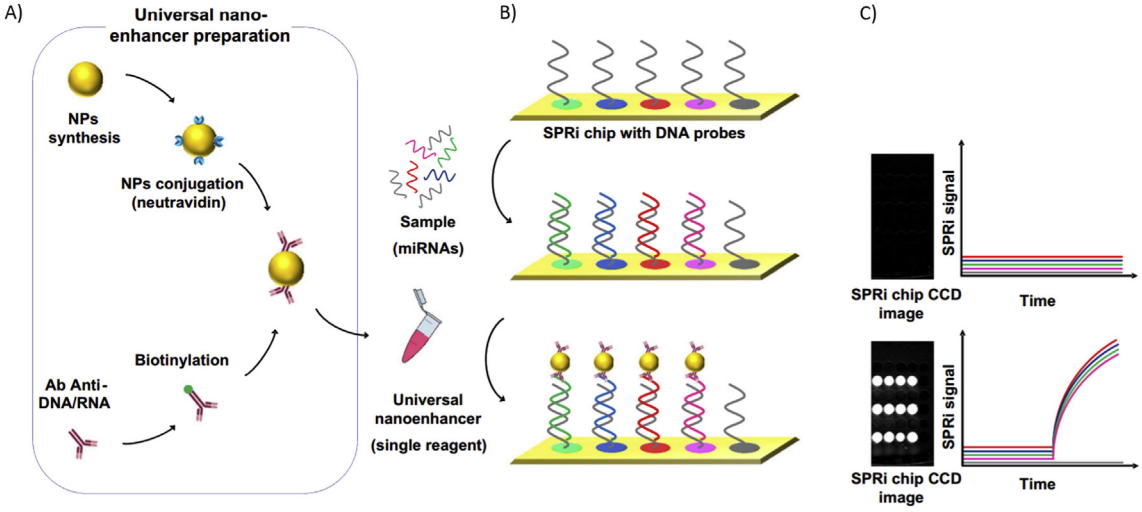

Fig. 8. Multiplex detection of four microRNA strands by SPRi. A) AuNPs were synthesized and functionalized with neutravidin to bind biotinylated detection antibodies, which can recognize all DNA/RNA hybrids. B) Four DNA capture probes were immobilized on the microarray together with a negative control. Different target RNA strands were specifically captured by respective probes and subsequently labeled by the functionalized AuNPs. As the detection antibodies bind to all DNA/RNA hybrids, the labeling of all different targets could be performed simultaneously, which greatly simplifies conventional labeling procedures for which many different detection probes are required. C) SPR sensorgrams obtained after target binding (top) and labeling with AuNPs (bottom) with the respective CCD images. Adapted with permission from Ref. [101]. Copyright (2019) Springer.

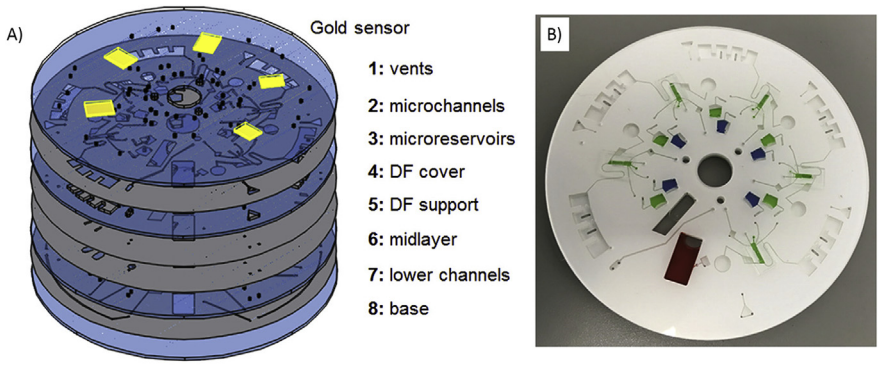

Fig. 9. SPR sensors integrated in a centrifugal microfluidic chip for parallel analysis. A) The vertical profile of the chip-SPR sensor integrated in an eight-layered centrifugal microfluidic chip, composed of a top PMMA layer for loading and venting, microchannels for liquid transport and pneumatic venting, microreservoirs for reagents, dissolvable films (DF) cover and support, midlayer providing interconnecting vertical vials, lower channels and a PMMA base. Five different IgG antibodies were finally detected in parallel channels extracted from one single sample. B) Functional visualization of different chambers within the disk by using red, blue and green dyes, which indicates sampling, washing and measuring, respectively. Adapted with permission from Ref. [28]. Copyright (2018) Elsevier. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

factors [111,121]. Time division multiplexing (TDM) and wavelength division multiplexing (WDM) are commonly used approaches to achieve multi-channel chip-SPR sensing. In terms of multi-channel FO-SPR sensors, WDM can be realized by fabricating multiple sensing positions on the optical fiber to obtain multiple dips in the reflectance spectrum [110,116,121]. In particular, a two-cascaded FO-SPR sensor was sputtered with different thicknesses of Au (30 and 60 nm) on two sensing zones, which were separately functionalized with two distinct molecularly imprinted polymers (MIPs) (Fig. 10A) and as a consequence, two independent SPR dips were obtained. In this way, parallel detection of dibenzyl disulfide and furfural in power transformer oil was realized, with a linear detection range of up to 0.1 mg L^{-1} and 0.02 mg L^{-1}, respectively. Although it was difficult to employ TDM on single-core FO-SPR sensors due to their thin core, TDM was successfully implemented on multi-core fibers, by generating parallel sensing zones through micro-structuring [112,113]. As depicted in Fig. 10B, a twin-core fiber (TCF) provides two independent sensing channels for parallel detection by TDM technology. Furthermore, by adding a traditional multimode fiber (MMF) sensing zone to the TCF, TDM and WDM can be combined. Hereby, the sensing zones can be doubled, enabling more targets to be simultaneously detected [111,113]. This novel design shows great potential for parallel analysis, but no experimental validation regarding this application has been performed yet. Alternatively, theoretical studies have been performed on holey fibers, which can be fabricated by PCF technology [117]. Fig. 10C illustrates the cross section of a proposed PCF sensor design, containing six similar air holes. Outside the Au layer, four microfluidic slots were structured to contain analytes and enable the excitation of the SPR modes. This design can enable both

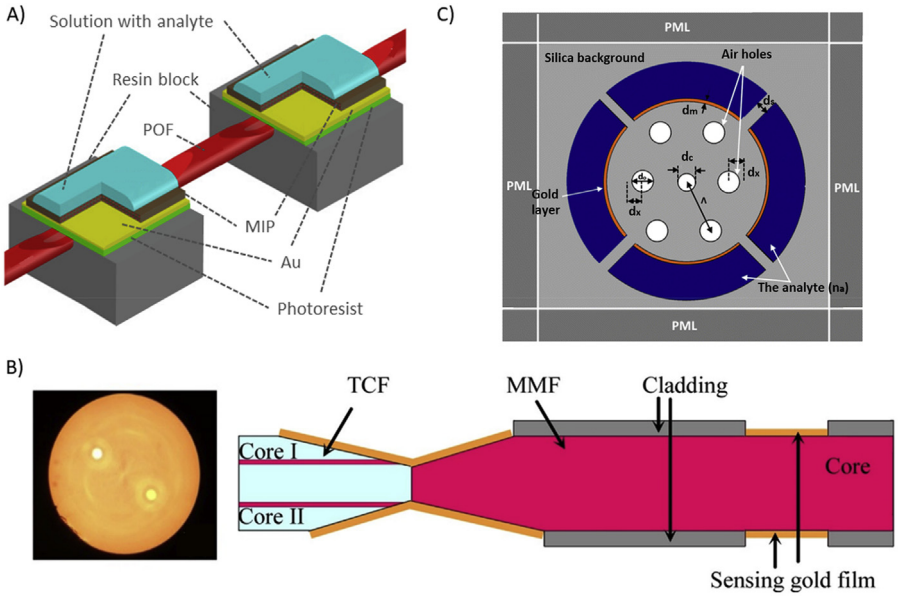

Fig. 10. Different structural profiles of FO-SPR sensors for parallel analysis. A) A two-cascaded FO-SPR sensor, containing two sensing zones, covered with Au films of different thickness, immobilized each with different MIPs. Hereby, the signals of both targets can be distinguished by two independent SPR dips [116]. B) A cascaded sensor consisting of the sections of TCF and MMF, the latter being coated with 50 nm Au sensing film and constructing the second-cascaded sensing zone to combine WDM with TDM [113]. C) Cross-section of a PCF sensor design, consisting of a core with six similar air holes for light guidance. The analytes are contained in four microfluidics slots on the Au layer to excite the SPR modes. Anisotropic perfectly matched layers (PML) are applied as boundary conditions in numerical analysis by full vectorial finite element method (FV-FEM) [114]. Adapted with permission from Refs. [113,114,116]. Copyright (2017, 2016, 2015) Elsevier and Optical Society.

parallel sensing and self-referencing, by using different fundamental modes supported by the sensor structure [114,115]. Although this design seems promising in simulation studies, experimental validation is essential to verify the feasibility of this strategy for parallel detection.

5. Continuous and *in vivo* sensing

In recent years, real-time and continuous monitoring is gaining a lot of attention in industries related to healthcare [122], biotechnology [123,124], nutrition [125–127], and environment [128], among others. Enormous efforts and progress have been made to close the gap between batch measurement and continuous sensing, resulting in numerous sensors being developed for continuous measurements, some of which have even become commercially available [127,129–131]. The process of batch measurements consists of sampling, sample transportation to the sensing setup, measurement, and data output. As this whole process requires tedious preparation and measuring steps, a time lag between the sampling and output may occur, limiting the possibilities for rapid intervention, which might be crucial in certain situations (*e.g.* the intensive care unit). Furthermore, by infrequent sampling, certain important events can be overlooked, restricting the potential for real-time intervention. In continuous monitoring on the other hand, sensors are placed directly into the environment of interest, where they can continuously measure the respective parameters, thus offering instant feedback and intervention. However, most of the developed continuous monitoring sensors are used to monitor physical parameters, *e.g.* temperature and pressure, whereas the possibility for continuously monitoring underlying biochemical processes remains limited. For instance, the detection of proteins, NA, drugs, and other molecules still relies on acquiring samples (*e.g.* blood, urine, cell culture and fermentation products) from a biosystem or bioprocess [132–134], which is therefore subjected to the abovementioned challenges.

5.1. Advancements and remaining hurdles in the field of continuous monitoring

In order to realize continuous measurements of specific analytes, the following strict requirements must be fulfilled: the biosensor (i) may not consume reagents or generate any products, (ii) cannot rely on multistep processes that involve washing, incubation, amplification or regeneration steps, (iii) should be able to dynamically respond to the changing analyte concentrations, (iv) must be stable for a long time period and (v) should contain internal referencing to compensate for the continuously changing environment [135,136]. Different challenges which still have to be resolved before these requirements are fulfilled will be discussed in detail.

The best-known examples of continuous sensors for specific detection of analytes are glucose sensors. However, as the detection strategy used in these sensors depends on the reactivity of the target itself, *i.e.* the oxidation of glucose by the glucose oxidase enzyme, it cannot be easily transferred to the detection of other analytes [137]. Besides enzymes, numerous other bioreceptors, such as antibodies, nanobodies, NA (*e.g.* single stranded DNA (ssDNA) and aptamers), MIPs, *etc.* have been employed to develop bioassays. In contrast to enzymes, however, the binding of targets to these bioreceptors typically does not directly generate a detectable output. For instance, antibodies do not generate electrons, emit light or cleave a substrate upon target binding. Therefore, additional entities such as enzymes, fluorophores or electron donors are often included to create a measurable signal. Many washing and incubation steps, and extra reagents are thus required, restricting their use in continuous measurement applications. Furthermore, although a strong and high affinity capture is often considered advantageous to achieve low detection limits, it might lead to an irreversible interaction, restricting the dynamic response of the biosensor.

SPR is a favorable technique to realize continuous monitoring, as it provides real-time information about target binding, without requiring extra labeling steps or reagents. Nevertheless, bioassays are mostly performed in batch processes rather than in a continuous manner, as in many cases, when low molecular weight targets have to be detected or when high sensitivity is required, signal amplification strategies have to be applied. Even for label-free assays, regeneration steps have to be frequently applied in-between measurements, because without regeneration the binding between the target and the biorecognition element is often irreversible, limiting the dynamic response of the biosensor to the changing conditions. Although reusable surfaces have been regularly reported, which can be implemented for consecutive batch measurements [138–140], the execution of consecutive measurements cannot be considered as continuous monitoring, because it relies on sampling and therefore suffers from the aforementioned disadvantages.

Despite the fact that these challenges have not been overcome on SPR sensors yet, various approaches have been reported and implemented on other biosensing platforms to eliminate the need for multistep bioassays and thereby realize continuous measurements. Therefore, we will shortly discuss these platforms along with the feasibility to implement similar strategies on SPR sensors in the future. For instance, biomolecular switches are bioreceptors (*e.g.* proteins and NA) that are able to couple target binding directly into a detectable output, by undergoing a reversible conformational or other reportable change. They have been frequently applied on electrochemical sensors, as presented in Fig. 11, where the conformational change after target binding enables (Fig. 11A) or disables (Fig. 11B) electron transfer [141–145]. In fact, by using biomolecular switches, the concentration profiles of analytes (*e.g.* drugs, DNA and proteins) could be monitored over time without requiring regeneration, washing, incubation or amplification steps [30,145]. Biomolecular switches, which have been extensively reviewed elsewhere [135,146,147], might be a promising strategy for implementation on the SPR platform as well. In particular, as they can undergo reversible conformational changes upon target binding, they could be engineered to bring mass closer to the sensor surface during this conformational change, thereby causing RI changes and

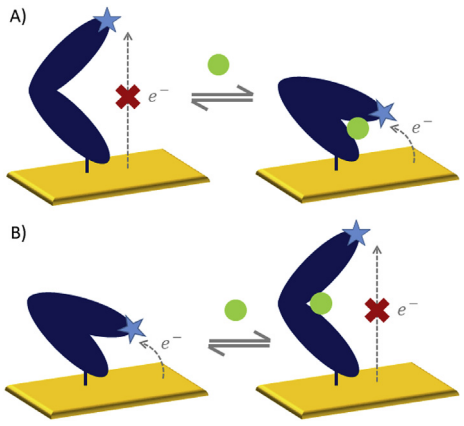

Fig. 11. Working principle of biomolecular switches, labeled with a redox reporter on an electrochemical platform. After target binding, the biomolecular switches undergo a conformational change, which A) enables or B) disables electron transfer, thereby directly creating a detectable output.

thus directly creating a detectable signal. Moreover, when NPs are applied as part of the biomolecular switch, the signal response could result from the coupling of the electromagnetic fields of the NPs and metal film, additionally to the RI changes. As this coupling effect leads to significant SPR shifts and is highly dependent on the distance between the NP and metal film, the sensitivity to the conformational change of the biomolecular switch could be greatly enhanced [148]. Using this strategy, signal amplification can be immediately obtained without requiring extra washing and incubation steps. Furthermore, as the conformational change is reversible, no regeneration steps need to be applied. In consequence, batch measurements can be avoided, opening up possibilities for continuous monitoring of specific analytes with SPR sensors. However, as the dynamic response of biomolecular switches is related to the kinetic parameters of the applied recognition elements, particular care should be given to the switch design, especially on the compromise between affinity and dynamic response depending on different applications.

As an alternative to biomolecular switches, the use of hydrogels, consisting of cross-linked polymer networks, shows great prospect in SPR-based continuous measurements [149,150]. Depending on their composition, the hydrogels can swell or shrink due to adsorption of water, binding of specific analytes, cross-linker degradation or chemical reactions [149–152]. Furthermore, as their volume changes are accompanied by RI changes, SPR sensors can record the respective causes after implementation of these hydrogels on the sensing surface. This strategy has already been applied on an FO-SPR sensor for continuous monitoring of pH, where the volume and RI of the hydrogel are dependent on the amount of dissociated carboxylic ions [150]. Similarly, an FO-SPR sensor for the detection of trichloroacetic acid (TCA) was equipped with a hydrogel, containing chitosan stabilized AgNPs, where RI changes were observed due to the interaction between TCA and AgNPs [149]. Moreover, as recently numerous target responsive switchable hydrogels have been reported and reviewed [151,152], this approach could also be a promising strategy to continuously monitor specific analytes, on account of their dynamic response to the bioreceptor-analyte interaction.

On the road towards achieving successful continuous SPR biosensing, an important aspect that has been tackled so far is internal referencing. More specifically, an extra reference zone was created on FO-SPR sensors and covered with a polymer exhibiting a high thermo-optic coefficient (highly temperature dependent RI) as shown in Fig. 12, where extra (A) silica or (B) polydimethylsiloxane (PDMS) was added on the Au-coating. In this way, two distinguishable dips were obtained in the SPR spectrum, one from the 'sensing stage' responsible for measuring the overall RI changes, and the other one from the 'reference stage', which is in charge of referencing due to its high sensitivity to temperature changes. As both dips are affected by temperature changes (Fig. 12C) and only the 'sensing stage' dip is affected by changing RI (Fig. 12D), the extra 'reference stage' dip was used to correct the 'sensing stage' signal for temperature changes [153,154]. The approach to create an extra reference zone and thereby generate an extra reference dip can also be implemented to correct the SPR signal for bulk RI changes. More specifically, as the reference dip should be distinguishable from the dip of the sensing zone, and the SPR effect is dependent on the thickness and the dielectric constant of the metal, the two distinct zones can be equipped with different metals or film thicknesses. Thereby, two unique dips can be created of which one can be used to monitor target binding and the other can be used to monitor bulk RI changes. The application of different metal film thicknesses on separate sensing zones was already successfully used for parallel detection [116] and could therefore also be implemented for self-referencing. Furthermore, other strategies explained in Section 4 can also be applied for self-referencing purposes rather than only for parallel detection, such as the use of multi-channel chip-SPR sensors, TDM and the use of a multi-channel holey fiber.

5.2. Trends towards in vivo SPR biosensing

In vivo biosensors are promising tools in the fields of diagnostics and biomedical research, as they can provide real-time information about the biochemical processes occurring inside the body. For instance, continuous monitoring of biomarker or drug concentrations inside patients' body would improve our knowledge about their health as well as advance medical diagnosis and treatment. Although symptom-based diagnosis is often satisfactory, in certain settings, such as the intensive care unit, the health status of certain patients is highly variable and should therefore be more intensively monitored. Continuous monitoring of biomarker concentrations (*e.g.* creatinine or inflammatory markers) would provide instantaneous information about the changing health status (*e.g.* kidney failure or inflammation), thereby allowing rapid intervention and increasing chances for survival. This is much more informative than the body indices that are normally used for rapid diagnosis and intervention, such as heart rate, blood pressure or temperature, as it provides information about the cause of the changing health status (*e.g.* inflammation) rather than the consequence (*e.g.* increased temperature). Furthermore, alterations in biomarker concentrations will manifest before visible symptoms appear, which is why detection and intervention can be performed earlier [30,135,136]. Importantly, *in vivo* biosensors can be used to continuously monitor the therapeutic concentrations of drugs with a narrow therapeutic range. When the concentrations fall beyond or below this range, the therapeutic effect will be seriously affected, leading to toxic side effects or the absence of therapeutic effects, respectively. By continuously monitoring *in vivo* drug concentrations, dosing can be tailored to each individual patient rather than administrating estimated dosages, thereby enhancing the therapeutic effect and avoiding toxic side effects of excess medicine [132]. Furthermore, through *in vivo* biosensing, tedious blood sampling could be avoided, which is currently a major obstacle in therapeutic drug monitoring.

A lot of research has been done towards the development of *in vivo* biosensors. More specifically, in the field of SPR biosensing, bioassays for a number of clinically relevant biomarkers and drugs have been developed [3,9,17,155,156]. Moreover, efforts were made to apply SPR bioassays to clinical samples [3,9,107,155,156] and standard SPR setups were miniaturized [107,113,157]. Although SPR biosensing is ready for the application in clinical settings [18], *in vivo* SPR biosensing has not yet been demonstrated. To reach this goal, measurements in undiluted biological fluids, long-term stability in complex matrices and insertion into the body are crucial. These aspects will be discussed together with strategies applied for other biosensors that could be relevant to attain these goals.

For achieving *in vivo* biosensing, one of the main challenges to overcome is the interference from complex biological matrices. Despite the efforts that have been made to perform SPR bioassays in clinical samples, most of them are still performed in serum, plasma or diluted blood [3,9,17,107,155,156]. Although these samples are adequate for the application in clinical settings, bioassays must be effective in whole blood or other undiluted biological matrices to enable *in vivo* measurements, without prior sample preparation. In theory, SPR measurements in opaque samples, such as whole blood, should be feasible as the reflected light is measured, which is not in direct contact with the sample. Therefore, the signal does not suffer from light scattering by the highly dense matrix. A serious drawback, however, is the aspecific binding of components other than the target molecules (*e.g.* cells and proteins), present in whole

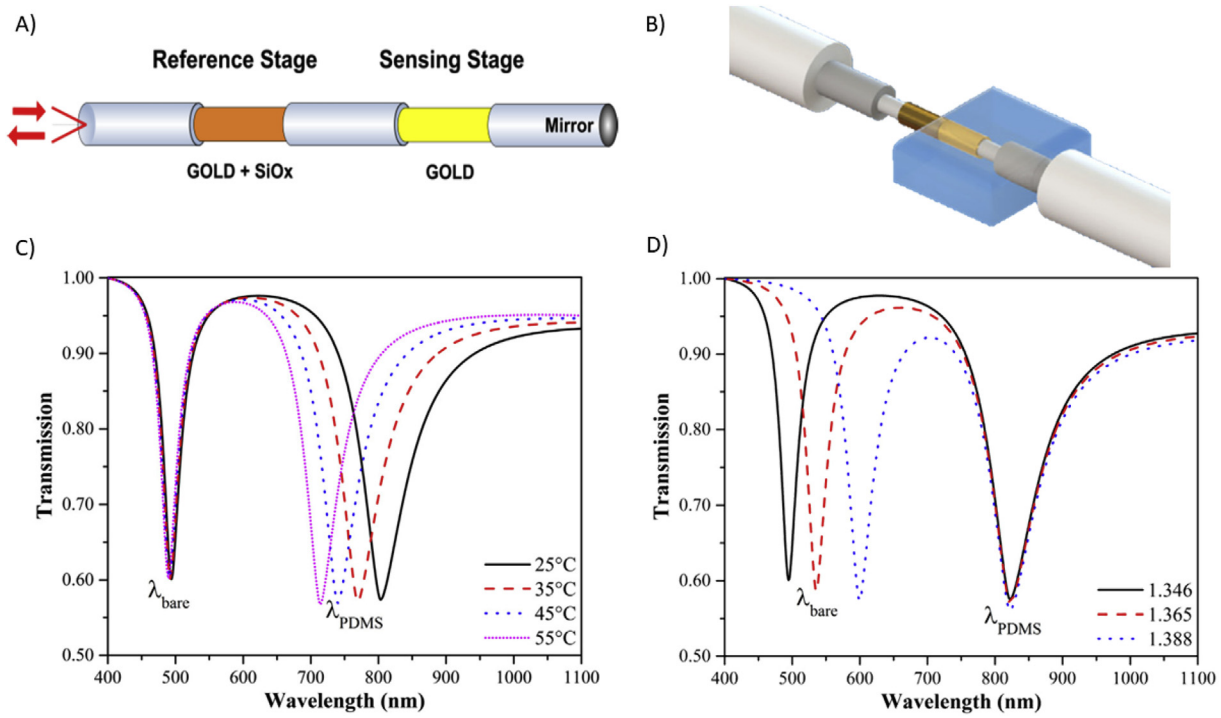

Fig. 12. FO-SPR sensors configured with an internal reference for temperature changes. The sensors are equipped with a reference zone covered by a polymer with a high thermo-optic coefficient being A) silica [154] or B) PDMS [153], to compensate for temperature changes. C) 2 distinguishable dips obtained in the SPR spectrum, measured at various temperatures. Changes in temperature produce shifts for both dips, therefore, the dip obtained by the reference stage, covered with PDMS (highly sensitive to temperature changes) was used to correct the signal of the sensing stage (bare). D) 2 distinguishable dips obtained in the SPR spectrum, measured at various RI. Only the dip from the sensing stage is affected by RI changes. Adapted with permission from Refs. [153,154]. Copyright (2017, 2014) Elsevier and Institute of Electrical and Electronics Engineers.

blood, which can cause signal interference due to the mass-based sensing principle. This effect can be counteracted using the strategies reviewed in Section 3.2. In addition, another valuable approach to reduce fouling would be the combination of SPR with dialysis or with size exclusion. By using a microdialysis membrane, large components such as blood cells cannot reach the SPR surface, whereas small molecules can easily diffuse through the membrane. In this way, fouling effects were significantly reduced and a small peptide with anti-atherosclerotic activity (DBG178) was detected in whole blood at micromolar concentrations using a chip-SPR platform. Nevertheless, small proteins and peptides, which are the major foulants in whole blood, still contributed to a large aspecific shift observed after long enough diffusion time, as they diffused through the membrane [158]. In a size-exclusion approach, a microslit array was fabricated, blocking blood cells and other large components from binding to the chip-SPR surface. Using this strategy, IgG was detected in whole blood samples as a proof-of-concept [159]. However, the sensor surface was only in contact with the complex matrix for a limited period of time (100 s), which probably helped in avoiding aspecific binding of smaller foulants, as noticed for the microdialysis membrane. Moreover, a drawback of both approaches is that as they are size-based, large components such as cells, extracellular vesicles, and large proteins cannot reach the sensor surface and thereby cannot be detected.

Despite most research focuses on biomarker detection in liquid samples, recently, an FO-SPR sensor with TFBGs was employed for the detection of cytokeratin 17 (CK17, a lung cancer biomarker) in soft matter. More specifically, the dip probe was encapsulated by a biocompatible packaging (Fig. 13A) and inserted into human lung tissue (*ex vivo*) (Fig. 13B). A clear distinction between the signal response of tumoral and healthy tissues was observed, as shown in Fig. 13C [45].

To translate the assays towards the *in vivo* environment, the long term stability of the SPR sensor surface and immobilized bioreceptors should be validated in complex matrices. In this context, several strategies have been described to obtain more stable bioreceptors [160–166], including protein engineering to increase the stability of antibodies. However, as this strategy is limited to the antibodies with known structural data, it cannot be generally applied [160]. On the other hand, NA (ssDNA or aptamers) were easily modified to resist nuclease degradation, which is the main factor limiting their use in complex matrices, and are therefore suitable bioreceptors to obtain long-term stability in biologicals fluids [161–164]. Additionally, MIPs were successfully applied for long-term measurements in complex media thanks to their inherent stability, even under harsh conditions [165,166]. More importantly, the final application should be taken into account when designing the biosensor and choosing the bioreceptors, as this will ultimately determine the conditions in which the sensor should be stable. Besides the stability and performance of SPR biosensors in complex matrices, another key aspect is the ability to instantly respond to changing target concentrations, as already elaborated in Section 5.1.

A more practical difficulty in developing *in vivo* SPR biosensors is their insertion into the body. Although this cannot yet be achieved with SPR sensors, possible strategies to reach this goal will be discussed step-by-step. Inspiration was obtained from non-enzymatic electrochemical biosensors that have already reached the *in vivo* stage [167,168]. More specifically, a microfluidic chip containing an electrochemical sensor was reported, which could monitor real-time concentrations of therapeutic drugs over multiple hours. This was first achieved *ex vivo* by continuously drawing a small amount of blood from rats and directly sending it through the microfluidic chip (Fig. 14A) [145] followed by an improved

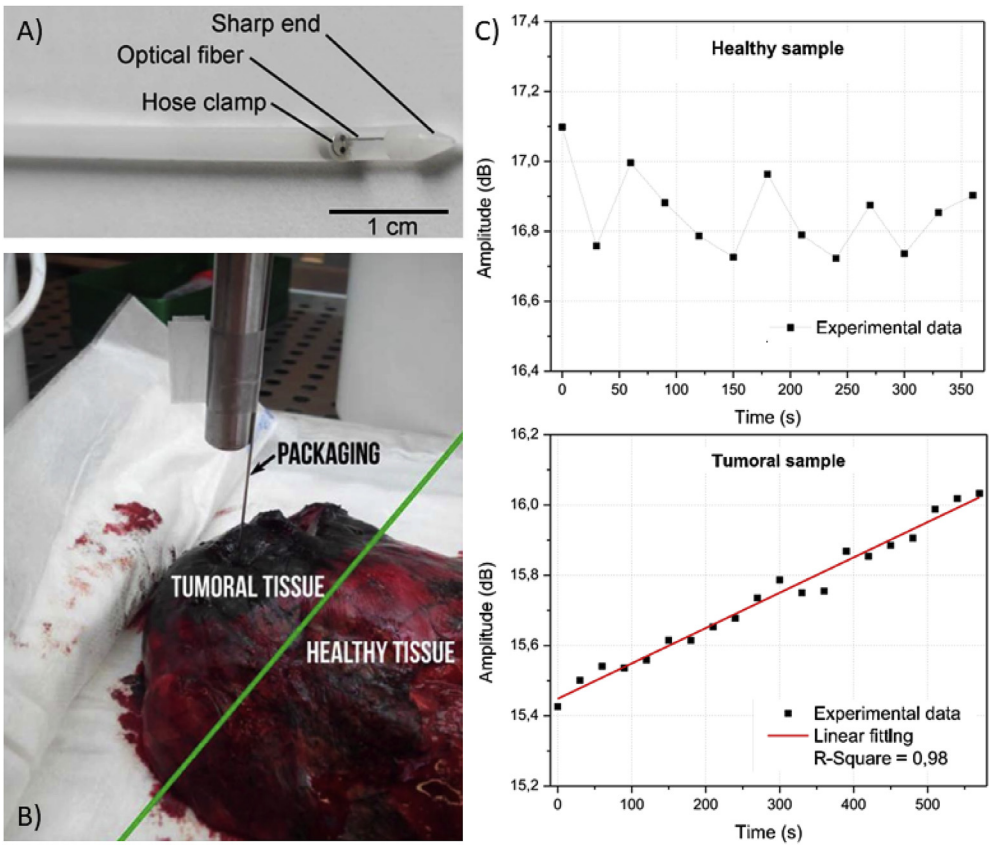

Fig. 13. Detection of CK17 in human lung tissue. A) The FO-SPR sensor probe encapsulated by a biocompatible packaging. B) Insertion of the packaged FO-SPR sensor probe into human tumoral lung tissue. C) Experimental data obtained from both healthy and tumoral tissue samples, the latter being an increasing linear signal due to the presence of CK17 (a lung cancer biomarker). Adapted with permission from Ref. [45]. Copyright (2017) Elsevier.

setup with the sensor directly inserted in a rat's bloodstream via an intravascular catheter (Fig. 14B) [167]. Another reported approach for electrochemical sensors involved a microdialysis probe. In particular, a probe was inserted into a mouse brain and the target analytes were exchanged between the extracellular matrix and the dialysate, where the bioassay was carried out *ex vivo* [169].

An important consideration when inserting biosensors into the body is biocompatibility, as implantation of foreign material can result in undesired reactions, potentially harmful to the patient. Therefore, much research regarding this aspect has been performed and many biocompatible materials and packaging techniques have been reported [136,170–173]. Concerning biocompatibility, in the aforementioned *in vivo* biosensor examples, aptamers were used as bioreceptors, which are non-toxic and non-immunogenic and exhibit great biocompatibility. Furthermore, a biocompatible polysulfone membrane was used to encapsulate the sensor, thereby reducing the fouling effects [167].

Similarly to electrochemical sensors, microfluidic chip-SPR sensors could be linked directly to patients' bloodstream or indirectly to the extracellular matrix via a microdialysis probe, as an intermediate step towards *in vivo* biosensing. Later, SPR and more specifically FO-SPR sensors could also be integrated into an (intravascular) catheter to enable *in vivo* measurements for several hours. In the setup we propose, the FO-SPR sensor probe would be placed inside the body whilst still being attached to the device containing the light source and spectrometer, as depicted in Fig. 14C. FO-SPR sensors seem suited for *in vivo* measurements thanks to their simplicity, miniaturized sensor system, low cost and capability for remote sensing. Importantly, particular attention should be paid to the materials used for the *in vivo* FO-SPR biosensor regarding biocompatibility and stability.

6. Conclusions and future perspectives

Over the last five years, numerous efforts have been made to obtain more versatile and flexible SPR biosensors to increase their market potential. However, current applications of SPR biosensors are still mostly limited to assays in diluted biofluids and involve complicated manual operation. The unavailability for POC testing and continuous measurements, and low sensitivity for small molecules in label-free assays are other limitations. In order to overcome the aforementioned barriers, we have mainly reviewed the latest trends in designing smart layers to better control bioreceptor immobilization, achieve low-fouling sensor surfaces and enhance sensitivity, feasibility of multiplex bioassays, as well as the envisions towards continuous measurements and *in vivo* sensing.

In order to improve sensing performance, oriented immobilization of bioreceptors on the sensor surface can be achieved by using specific layers, based on different surface chemistries, featured biomolecules, or nanotechnology. However, in many cases, bioreceptors have to be engineered to include specific functional groups to fit the smart layers, which limits the extensive use of the proposed strategies for oriented immobilization. Therefore, in the future, efforts should be made to develop more versatile approaches for oriented immobilization that could be widely applied to different SPR sensors. Moreover, to reduce the fouling problem, which is currently one of the major challenges in SPR sensing, and realize bioassays in complex matrices, various materials (*e.g.*

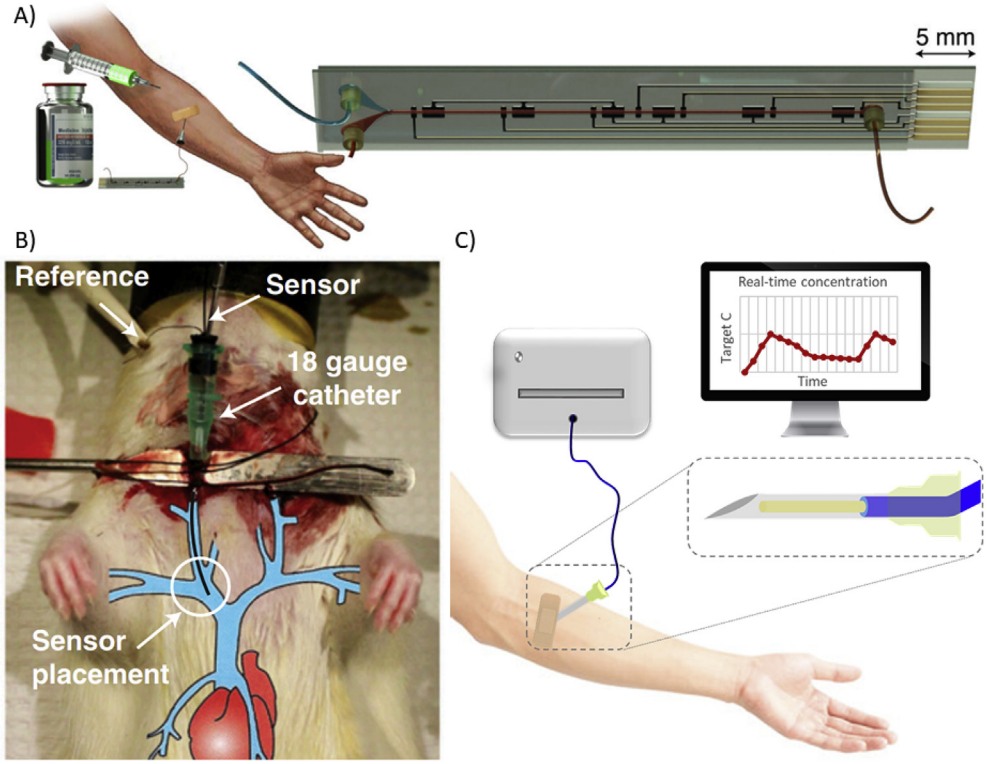

Fig. 14. Steps towards *in vivo* sensing. A) Representation of an electrochemical chip for continuous monitoring of therapeutic agents, attached to the patient's bloodstream [145]. B) Electrochemical, dip-shaped sensor, included in an 18 gauge catheter together with a reference electrode for insertion into a rat's vein [167]. C) A prototype of the proposed setup for *in vivo* SPR biosensing. An FO-SPR biosensor with the fiber probe integrated into an (intravascular) catheter can be inserted into the body. Target concentrations can be monitored in real-time over a certain time period. Adapted with permission from Refs. [145,167]. Copyright (2013, 2017) American Association for the Advancement of Science and Proceedings of the National Academy of Sciences.

zwitterionic materials, (co)polymer brushes, DNA origami and tetrahedrons, and polysaccharides) have been designed to form a hydration layer, thereby preventing or reducing foulant adsorption. The main remaining obstacle here is the preservation of the low-fouling property or recoverability of hydrophilicity after surface functionalization with bioreceptors. Furthermore, due to nano-material advancement, diverse 2D nanomaterials have been evaluated as sensing layers for sensitivity enhancement. However, as many of these materials have only been evaluated by theoretical studies, in the future, experimental validation of the respective approaches should be reported. Although new strategies for developing smart layers are being continuously described, most of them focus on only one specific aspect to improve the sensing performance. Future studies should try to combine the advantages of various smart layers to obtain an optimal sensing surface.

To boost the time- and cost-effectiveness of SPR bioassays, efforts have been made to achieve multiplex measurements as well as parallel quantification of multiple analytes in various aspects. Multiplexing could be obtained by SPRi or by functionalization of a single sensor surface with several distinct bioreceptors. However, subsequent labeling steps for each analyte have to be included for the latter to distinguish the signals originating from different analytes. Meanwhile, chip-SPR biosensors have been incorporated in microfluidic systems to realize parallel measurements in an automated way, which is highly preferable and advantageous for the development of POC devices in diagnostics. Furthermore, physical modifications of optical fibers including cascaded design, multi-core fibers and PCFs have been demonstrated as alternative solutions to enable parallel biosensing on FO-SPR platforms. More specifically, by creating various sensing zones and by dividing the fibers into different sensing channels, WDM and TDM can be combined. However, this requires a more cost- and time-intensive manufacturing process. Although promising, the feasibility of multi-core fiber and PCF technology for parallel analysis still has to be validated experimentally. Therefore, despite the fact that the possibilities for multiplex or parallel analysis on SPR sensors exist, there is still a long way to go before we can really eliminate the necessity of labor-intensive manipulations and realize fast screening of numerous analytes. In the future, more studies can be conducted to reduce handling steps, simplify the complex physical manufacturing, as well as combine SPR with other ever-improving technologies such as microfluidic systems.

SPR biosensors have huge potential for continuous measurements of specific analytes, as they provide real-time information about target binding and unbinding. Despite their potential, continuous measurements are not possible yet, and many future efforts are still required to circumvent the multistep processes and exclude exogenous reagents and product generation, which are still needed for signal amplification and surface regeneration. These requirements can be fulfilled by implementing strategies used in other biosensors, such as the application of biomolecular switches or hydrogels. On the other hand, to compensate for the continuously changing environment, various approaches for internal referencing have been reported that could also be beneficial for more accurate long-term measurements. These are however not yet standardly integrated in SPR sensors, so additional work is needed to enable a more widespread application, which should allow correction of the obtained SPR signal irrespective of unstable environmental factors.

Although SPR sensors have reached a mature stage and are

ready for the applications in clinical settings, they have not yet been applied for *in vivo* measurements. Efforts have been made to reduce the effect of fouling and enable measurements in whole blood and soft matter. However, the stability of SPR sensors in undiluted biofluids should be evaluated over a longer time scale, as for now, measurements were only performed in batch. Furthermore, strategies and examples on how to perform *in vivo* measurements of biomarkers and drugs could be adopted from other biosensors, such as electrochemical sensors with attention to the use of biocompatible materials.

Altogether, a large variety of strategies to improve SPR sensing in various aspects have been proposed in literature over the past years. Due to the particular improving focus from respective approaches, it is challenging to combine several improvements into one optimal sensor surface. Therefore, the final application should be taken into account while designing an SPR sensor. This will ultimately determine the requirements and conditions of the bioassay, and thereby help in selecting different elements to be integrated into the biosensor development.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Professor Jeroen Lammertyn is a board member of FOx Biosystems, a spin-off company of KU Leuven commercializing FO-SPR platforms, next to the principal investigator of the Biosensors group.

Acknowledgments

This work was supported by Research Foundation Flanders (FWO SB/1SC8519N and FWO SBO/S006319N), Guangzhou Municipal Human Resources and Social Security Bureau, China, Oversea Study Program of Guangzhou Elite Project, Vlaamse Liga Tegen Kanker, Belgium, VLK (C8744) and Kom op tegen Kanker (Stand up to Cancer, the Flemish cancer society). This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 764281 (H2020-MSCA-ITN-AiPBAND).

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