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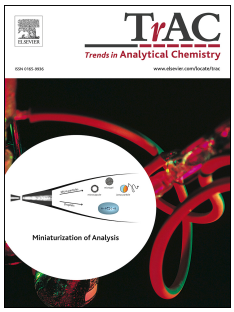

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Electrochemistry Combined-Surface Plasmon Resonance Biosensors: A Review

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Abstract

Over the years, most of the literature reported applications of electrochemical and surface plasmon resonance (SPR) immunoassays for biosensing but, so far, the combination of the two methods in the same sensing spot for analytical purposes is much less explored and discussed. The aim of this Review is to highlight the great potential of electrochemistry combined-SPR (eSPR) as analytical tool for screening chemically and biologically relevant (bio)molecules by combining the unique features of SPR integrated with electrochemical readout.

In the first part of the Review, we describe the urgent need of innovative methods for screening clinical biological markers (*General Introduction*), briefly discuss general concepts of SPR and electrochemical sensing (*Concepts behind eSPR biosensors*) and highlight the hyphenation of two methods to developed combined biosensing systems (*Set-up configuration and eSPR principles*). Firstly, we briefly give an overview of the setup for implementation of eSPR technique and discuss some relevant experimental conditions to perform the combined optical and electrochemical measurements. Then, the principles and fundamentals of eSPR biosensors are presented and described. We also present representative examples of eSPR biosensors in the literature (*Applications of eSPR biosensors*).

In the second part, we review studies on how combined electrical and plasmonic detection contributed to the biosensing field, in particular, for the successful screening of clinically relevant biomolecules, namely proteins (*Detection of proteins*), nucleic acids (*Detection of nucleic acids)*, small size chemical species (*Detection of small molecules*) and cells (*Living-cell Analysis*).

Finally, we discuss the current limitations of eSPR biosensors performance and suggest possible ways to overcome these limitations (*Limitations and optimization*) and then we explore aspects about the development of the method and its applications and discuss areas of likely future growth (*Conclusions and perspectives*).

Keywords: electrochemistry; SPR; electrochemistry combined-surface plasmon resonance (eSPR); detection; nucleic acids; protein; biomarkers;

Abbreviations

AA: Ascorbic acid
ACV: Alternating current voltammetry
ALP: Alkaline phosphatase
CV: Cyclic voltammetry
CE: Counter (or auxiliary) electrode
CNTs: carbon nanotubes
EC: Electrochemical
EIS: Electrochemical impedance spectroscopy
eSPR/ESPR/EC-SPR: Electrochemical surface plasmon resonance (or electrochemistry combined-SPR)
ELISA: Enzyme-linked immunosorbent assay
Fc: Ferrocene
GOx: Glucose oxidase
HPR: Horseradish peroxidase
MIP: Molecularly imprinted polymer
MB: Methylene blue
MUA: 11-mercaptoundecanoic acid
NPs: nanoparticles
NSB: nonspecific binding
PCR: Polymerase chain reaction
PoC: Point-of-care
PNA: Peptide nucleic acid
PEG: Poly(ethylene glycol)
RE: Reference electrode
RI: Refractive index
SCE: saturated calomel electrode
SPR: Surface plasmon resonance
SPRI: Surface plasmon resonance imaging
SWV: Square wave voltammetry
SAM: Self-assembled monolayer
SPFS: Surface plasmon fluorescence spectroscopy
SPP: Surface plasmon polariton
TSPR: transmission surface plasmon resonance
UA: uric acid
WE: working electrode

1. General Introduction

Biomarkers currently play a pivotal role in clinical practice for the early disease diagnosis (and prognosis), to monitor individuals medical condition and/or response to therapeutic intervention or drug treatment [1-3]. Common examples ranges from circulating cells, proteins and nucleic acids (cell-free DNA, cell-free RNAs, etc.) to metabolites and smalls molecules, being objectively measured in several human bodily fluids (blood, urine, saliva, respiratory tract, etc.), using established standard techniques, namely ELISA, PCR and chromatography (LC-MS) [1-3]. However, these methods are laborious, time-consuming, have limited automation of procedures and are rather expensive. Thus, nowadays, the development of innovative sensing solutions is currently a demand in clinical setting for simple, cost-effective, accurate and timely screening of endogenous biomarkers to support rapid medical decision and action.

In this review we focus on applications of electrochemical surface plasmon resonance (eSPR) approach for detection of clinically relevant biomolecules, such as drugs and disease-associated proteins and nucleic acids, highlighting some practical aspects for the successful development of eSPR biosensors.

Search results from Scopus database within the past two decades in the "electrochemical sensing" and "SPR sensing" topics returned an impressive number of publications of ~20,000 and ~3,000, respectively (see search details in Fig. S1, Supporting File). The exponential rate growth over time of papers reporting electrochemical detection can be explained by the mass fabrication of miniaturized chips that brought in significant developments to electrochemical biosensing field due to the development of portable electrochemical chips (lab-on-a-chip) and wearable devices for PoC testing and *in situ* monitoring. Moreover, the increasing number of publications over recent years related to plasmonic biosensors is probably related to increasing availability of commercial SPR devices worldwide which attracted the attention of many research groups for this powerful technique.

The combined plasmonic and electrochemical approach is usually referred in the literature as "electrochemical surface plasmon resonance" and common research abbreviations of this technique are eSPR (or ESPR) and EC-SPR. Works reporting hyphenation of the two methods for biosensing purposes are limited to few dozens of publications. The search for publications in this topic was particularly time-consuming since Scopus database search results for "electrochemical surface plasmon resonance

(bio)sensors" were poor and restricted to less than ten articles. The same limitation was observed for search in Web of Science (Clarivate Analytics). As complement, exhaustive search within massive results returned by Google Scholar was performed. More relevant literature was addressed in this Review highlighting the significant achievements of this technology that can be of potential interest for researchers in this field.

2. Electrochemical Surface Plasmon Resonance Biosensors

2.1. Concepts behind eSPR biosensors

To fully understand eSPR biosensors operation it is fundamental to understand the basic principles of SPR and electrochemical detection alone. SPR affinity biosensors are able to monitor in real time refractive index (RI) changes resulting from the interactions between a bioreceptor (oligonucleotide strands, antibodies, proteins, etc.) immobilized on the surface of a metal film (usually gold, up to 50 nm) and a ligand in solution [4-6]. The SPR physical process arises from the interaction of light with such metal surface. Some concepts about SPR and instrumentation design are briefly discussed in this Review. Moreover, many recent reviews provides detailed description of SPR theory and fundamentals [7-10]. These reviews can be consulted for further information.

Most commercial SPR and research instruments are still based on classical Kretschmann´s configuration which is based on the phenomenon of total internal reflection. Considering the Kretschmann configuration as example to illustrate the SPR sensing principle (shown in Fig. 1A), the plane-polarized light from a light source passes through the (high RI) glass prism and is reflected from the thin metal layer back through the prim to the detector. At a specific angle of incidence, the SPR angle, part of the radiation couples with electrons in the metallic film, which start free collective oscillations due to excitation. These charge-density fluctuations, called surface plasmon polaritons (SPPs), are extremely sensitive to RI changes close to the sensing interface allowing the monitoring of binding events (e.g. protein binding to immobilized antibodies as bioreceptors; Fig. 1A). For quantification purposes, the linear relationship between the amount bound (bio)molecules and SPR angle shift is used.

These optical sensors offer several advantages relatively to other biosensing approaches, such as (i) wide variety of surface chemistry procedures for attaching of biorecognition

element; (ii) high robustness and stability in complex biofluids (plasma, serum and whole blood); (iii) high degree of automation; (iv) possibility of parallel sensing, among others. These unique features greatly contributed to many innovative applications of plasmonic biosensors over recent years, including food quality and safety analysis [5, 11], environmental monitoring [5, 12], drug discovery [4] and the early disease diagnosis [5, 13-15].

Meanwhile, electrochemical (bio)sensing emerged during the last decade as a powerful and versatile analytical tool, being a very promising alternative to optical approaches. Thus, it is not surprising that many reviews can be found in the literature highlighting the great importance of electrochemical methods for detection of several relevant (bio)molecules, namely, disease biomarkers [16], pathogens [17], pharmaceutical residues [18], environmental contaminants [19], among others. Similar to SPR biosensors, bioreceptors selective to target analyte, such as antibodies and nucleic acids, are commonly immobilized on the surface of different transducers (gold, carbon, silver, etc.) for construction of electrochemical biosensors (see Fig. 1B). The set-up used for electrochemistry experiments usually includes a three-electrode electrochemical cell. A potential difference is applied between the working electrode (WE) and the counter electrode (CE, typically made of Pt) while the current resulting from an electrochemical reaction flowing through the circuit is measured. For precise controlling the working electrode potential, a reference electrode (RE) of defined electrode potential, such as the Ag/AgCl electrode or the saturated calomel electrode (SCE), is employed.

Electrochemical methods offers (i) good precision, robust and accurate surface modification procedures (based on polymers, SAMs, electrocatalytic nanomaterials, etc.); (ii) fast analysis and high detection sensitivity using common techniques, such as cyclic voltammetry (CV), chronoamperometry, square wave voltammetry (SWV), etc., and; (iii) enhanced performance in complex samples due to straightforward signal amplification strategies [16, 20-22]. Besides, relatively simple and low cost instrumentation is needed, enabling large-scale production of electronic devices along with possibility to miniaturize modern microelectronics for high-throughput analysis in point-of-care (PoC) [18-20, 23, 24].

Common detection schemes used in clinical and biomedical context are depicted in Fig. 1B. Briefly, the measured electroanalytical signals can arise from:

(i) enzyme labels (e.g. HRP, ALP, etc.) linked to secondary antibodies [20, 21] (or DNA probes[16, 22]) for sandwich immunoassay detection of target antigen by monitoring the

redox signals of electroactive product from enzymatic reaction. Furthermore, receptors (antibodies, oligonucleotides, aptamers, etc.) and enzymes can be conjugated to nanomaterials (CNTs, metallic NPs, quantum dots, magnetic beads, among others) acting as amplification tags to enhance detection sensitivity [25];

(ii) redox active reporters (usually MB or Fc) covalently linked to oligonucleotide probes whose flexibility is reduced after duplex formation leading to inhibition of redox tag electron transfer as hybridization proceeds [16, 22];

(iii) external biocompatible redox probe (such as $[Fe(CN)_6]^{3-/4-}$, $[Ru(NH_3)_6]^{3+}$, ferrocene, etc.) that is introduced into the bioanalytical system to access its diffusional behavior upon immunocomplex formation [16, 17, 20].

The hyphenation of SPR with other analytical techniques (Fig. 1B) has become increasingly popular over recent years [8, 26]. Particularly, the often used Kretschmann configuration allow the simple and fruitful combination of SPR with electrochemical methods, for (i) real-time, non-destructive, *in-situ* characterization of ultrathin films at nanometric scale [27], (ii) to investigate metal deposition process [28] and (iii) for biosensing purposes, as discussed in this Review.

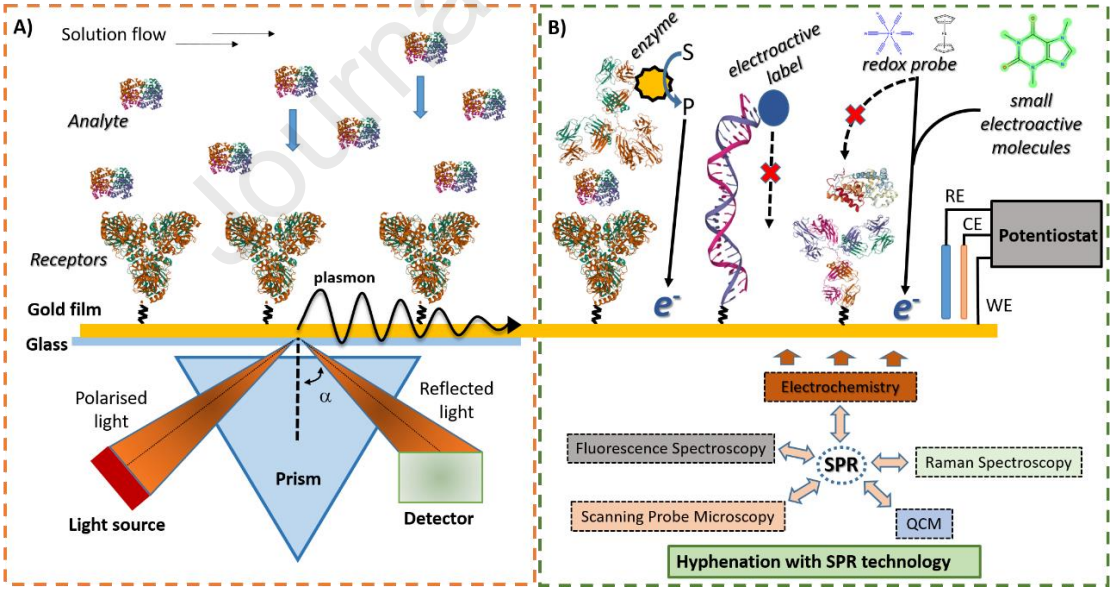

Fig. 1. A) Schematic illustration of a typical SPR experiment for real-time measuring antibody-antigen molecular interactions, including SPR instrumentation (based on Kretschmann´s configuration) and operation mode; B) Examples of SPR combination with other analytical techniques is given, with particular focus for electrochemistry. The set-up used for electrochemistry experiments is shown along with common detection schemes used for electrochemical biosensing.

2.2. Set-up configuration and eSPR principles

Although the versatility and advantages of synchronized electrical and optical readout has been discussed and reviewed for several bioanalytical systems [29, 30], the application of eSPR approach for analytical purposes is a much less explored topic and the available literature is restricted to few papers (see Table 1). This is probably due the particular specificities of the eSPR setup and/or problematic monitoring of the electrochemical process after the SPR gold chip modification using well-described procedures.

The combination of electrochemical readout with SPR response (eSPR) in the same instrument is technically very simple. Some home-made or commercially available SPR instruments (e.g. SPR Autolab ESPRIT, KEI bv) have an open architecture allowing the integration of an external potentiostat to control the potential applied to the cuvette, as schematically represented in Fig. 2A. Thus, in eSPR experiments, the thin gold layer on SPR chips has the dual function of generate the evanescent wave and being the working electrode (of the standard three-electrode cell) where electrochemical processes take place [29, 30] (see example in Fig. 2B). Indeed, the basic principle of eSPR approach relies on the quantitative dependence between the RI and the current measured [31-33] when conventional electrochemical methods are applied.

Early work specifically addressed the relationship between the electrochemical potential (and current) and the SPR response. Several authors reported changes in RI under electrode potential modulation in distinct electrochemical environments [34-38] while others observed a good correlation between time differential SPR data and the faradaic current during CV potential ramping [39-42]. The concept was first validated for electrochemical interfacial reactions (of MB and $[Fe(CN)_6]^{3-/4-}$) [39, 40] and then transposed to redox proteins monolayers [41, 42] covalently immobilized on a gold surface, allowing the differentiation of changes in protein electronic states from redox-induced conformational changes.

Efforts were made by several researchers, with great merit's for Professor N. Tao [43] and collaborators, to fully understand how SPR parameters depends on external electrochemical variables, providing the theoretical background for both non-Faradaic and Faradaic processes. In this context, Wang et al. [44] stated that for capacitive reactions at the electrode/solution interface, the modulation of the electrode potential (ΔV) can shift the resonant angle ($\Delta\theta$), via changes in the RI (Δn), average layer thickness (Δd), and surface charge density ($\Delta\sigma$) at the electrode, according to the relationship:

$$\frac{\Delta\theta(\lambda)}{\Delta V} = c1\frac{\Delta n(\lambda)}{\Delta V} + c2\frac{\Delta d}{\Delta V} + c3\frac{\Delta\sigma}{\Delta V}$$

(1)

where λ is the wavelength of the incident light and c1, c2 and c3 are constants. Later, Garland et al. [45] extended the concept for transient conditions by direct probing optical changes in the double layer structure of a gold film-aqueous electrolyte interface under potentiostatic control.

Meanwhile, Foley et al. [46] showed that SPR signal depends on the double layer charging current density:

$$\Delta\theta = \frac{1}{\alpha}c\Delta V$$

(2)

where α is a constant ($\alpha \sim 47$ C m^{-2} deg^{-1} for a bare gold surface), c is the interfacial capacitance per unit area, and ΔV is the potential change.

A basic formalism of electrochemical SPR Moreover was established by Wang et al. [31], which provided a quantitative relationship between the shift of SPR angle ($\Delta\theta$) and electrochemical current $i(t)$ signals, given by:

$$\Delta\theta(t) = B\left(\alpha_R D_R^{-\frac{1}{2}} - \alpha_O D_O^{-\frac{1}{2}}\right).\left(nF\pi^{\frac{1}{2}}\right)^{-1}.\int_0^t i(t')\,(t-t')^{-\frac{1}{2}}dt'$$

(3)

where α_o and α_R are the changes in the local RI per unit concentration for the oxidized and reduced species, respectively, D_O and D_R are the corresponding diffusion coefficients and F is the Faraday constant. This means that, mathematically, the shift in the SPR signal is proportional to the semi-integral of the faradaic current, providing a straightforward way to directly measure the electroactive species concentrations at the electrode surface in convolution voltammetry. More importantly, their work greatly contributed to pioneer use of SPR imaging of local electrochemical current (with high spatial resolution and electrochemical sensitivity), a powerful functional imaging tool that found several chemical and biological applications [46-50].

The principle reasoned by Wang et al. [31] was later applied for eSPR measurements of diffusion-controlled fully reversible, quasi-reversible and irreversible redox reactions [32]. Hexaammineruthenium and 4-nitrotoluene were used model systems to corroborate theoretical predictions for all types of redox reactions as long as RI of reduction and oxidation states of a redox molecule are distinct to generate the eSPR signals.

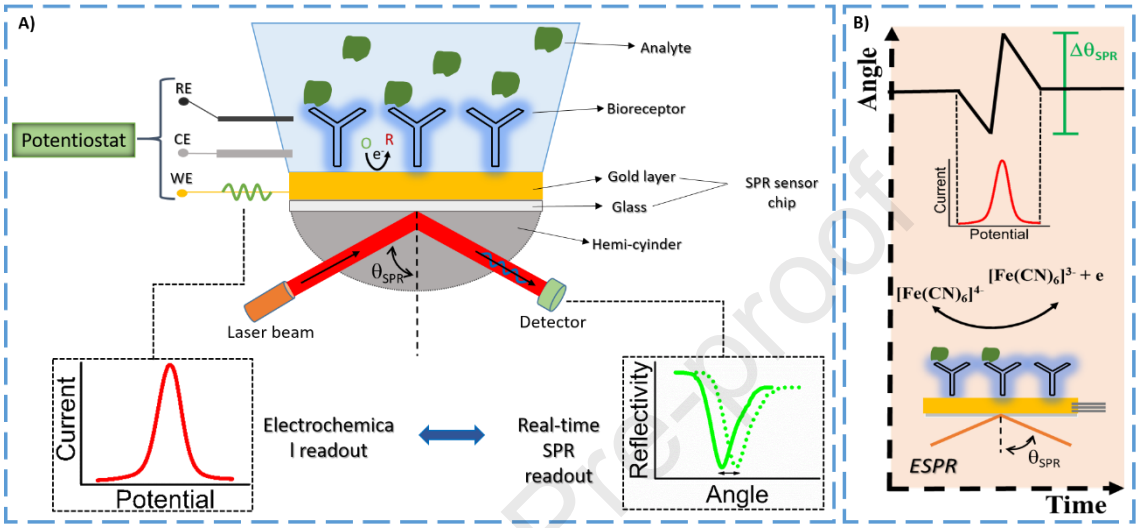

Fig. 2. A) Schematic illustration of the multi-parametric eSPR apparatus. A potentiostat is used to apply and control the voltage on the gold surface and measure the electrochemical voltammograms while the resulting SPR optical changes are recorded in real-time. The standard three-electrode electrochemical cell is composed by a reference electrode (RE), a counter electrode (CE) and a working electrode (WE), all inserted in the aqueous medium (under static or flow conditions); B) During eSPR measurements, if a redox reaction occurs (here ferrocyanide/ferricyanide redox couple), the change in RI of oxidized and reduced states of species is monitored in real-time.

2.3. Applications of eSPR biosensors

New sensing strategies combining SPR and electrochemistry have been developed over the last two decades. As stated before, it is not our intention to provide a comprehensive review of main applications of eSPR technique. In fact, we focus on this Review to eSPR systems used as analytical tool for screening biological markers in clinical practice. An overview of representative examples of eSPR biosensors in this context is summarized in Table 1 with mention to chemistries used to attach bioreceptors to biosensing surfaces,

reporting strategy employed for electrochemical readout, procedure for sensor regeneration, electrochemical technique used and detection levels achieved (LOD).

Table 1: List of more representative works reported in literature for the eSPR determination of clinically relevant (bio)molecules

Analyte	**Chip modification**	**Reporting strategy**	**Regeneration**	**EC technique**	**LOD**	**Ref.**
Biotinylated DNA targets	Thiolated PNA probe; MCH as lateral spacer (d = 2.1 nm)	Ferrocene-streptavidin conjugates	50 mM NaOH (5 min)	SWV	10 pM	[51]
25-base DNA target	Stem-loop DNA probe or linear DNA probes; MCH as lateral spacer	MB at modified DNA probes	5 mM HCl	ACV	20 nM	[52]
Pathogenic DNA sequences *(whole blood)*	Stem-loop DNA probe; MCH as lateral spacer	MB at modified DNA probe	8 M urea (5 min)	ACV	5 - 10 nM	[53]
miRNA-145 biomarker *(human serum)*	Thiolated RNA probe; ME as lateral spacer	Ferrocyanide/ferricyanide redox couple	---	E_1 = +0.5 V; E_2 = -0.3 V	0.56 fM	[54]
IgG	Covalent immobilization of anti-human IgG on PABA (d_{PABA} = 140 Å)	Label-free detection at constant applied potentials	---	E = −0.2 V; E = +0.4 V	1 μg mL^{-1}	[55]
HA protein antigen from influenza virus	Covalent immobilization of anti-H5 (H5N1) capture antibody on MPA SAM	Sandwich immunoassay using a MB-labeled secondary antibody	---	E = -0.17 V	300 pM	[56]

HRP-conjugated IgG *(undiluted serum)*	Covalent immobilization of IgG on mixed monolayer of MPO and cysteamine/PDT	TMB oxidation after "membrane cloaking" method for NSB	0.5% Triton X-100/10 mM glycine-HCl (pH 1.7) buffer	E = +0.175 V	5 fM	[57]
IgG	Covalent immobilization of anti-mouse IgG on PPA (d_{PPA} = 14 nm)	Sandwich immunoassay using a ALP-labeled secondary antibody that generates electroactive PAP	---	CV	SPR: 602.6 ng mL^{-1} EC: 589.3 ng mL^{-1}	[58]
CA 15-3 biomarker *(human serum)*	Covalent immobilization of anti-CA15-3 antibody on MSA SAM (d = 1.4 nm)	Ferrocyanide/ferricyanide redox couple	0.1 M glycine-HCl (pH 2.0) buffer	SWV	SPR: 21 U mL^{-1} EC: 0.0998 U mL^{-1}	[59]
H_2O_2	Os-gel-HRP film coated gold nanohole array	Redox change in the Os complex mediator induced by changes in oxidized state of HRP	---	---	---	[60]
DNR *(live cancer cells)*	Bare surface	Redox profile of DNR	---	CV	---	[61]

d = thickness; MCH: 6-mercaptohexanol; MB: methylene blue; ME: 2-mercaptoethanol; PABA: poly(3-aminobenzoic acid); HA: hemagglutinin; MPA: 3-mercaptopropionic acid; HRP: horseradish peroxidase; MPO: 3-mercapto-1-propanol; PDT: 1,3-propanedithiol; TMB: 3,3‘,5,5‘-tetramethylbenzidine; PPA: polypyrrole propylic acid; PAP: p-aminophenol; ALP: alkaline phosphatase; MSA: mercaptosuccinic acid; DNR: daunorubicin.

To obtain multidimensional data from the eSPR system, the chemical functionalization of the biosensing platforms is a critical step for developing combined optical and electrochemical (bio)sensors [30]. SAM approach is by far the most used for effective immobilization of bioreceptors at the SPR platforms [51-54, 56, 57, 59]. These ultrathin films (from 1.4 to 2.1 nm) can be easily attached to the chip surface through SH-Au covalent bond and allow accurate and sensitive monitoring of electrochemical processes occurring at the electric double layer. Mixed monolayers composed by thiolated oligonucleotides probes and short hydrophilic SAMs as lateral spacers (MCH, ME, etc.) [51-54] can provide stable platforms for detection of nucleic acids while short-chain alkanethiols having terminal carboxylic acid groups (such as MPA, MSA) can be used for covalent immobilization (by EDC/NHS reaction) of capture antibodies [56, 57, 59] for detection of large biomolecules. In addition, the *in situ* electrosynthesis of thin conducting polymers (such as PABA, PAP, among others) [55, 58] as support for covalent immobilization of antibodies can be a straightforward approach to build the sensing platforms taking advantage of eSPR monitoring for easy optimization of polymer thickness.

Relatively to voltammetric methods, the use of alternating current voltammetry (ACV) [52, 53] or the application of a constant electrochemical potential [54-57] to the bioanalytical systems provided a simple mean for SPR optical signals enhancement induced by the redox processes occurring at the sensor surface. Moreover, the integration of SWV [51, 59] or CV [58, 61] technique with SPR allowed to obtain electrochemical and optical responses independently. For the electrochemical readout, the recorded redox signals can be generated from (i) redox reporters (MB, ferrocene) [51-53, 56] conjugated to oligonucleotides or proteins, (ii) oxidation of common substrates (TMB) [57] or reaction sub-products (PAP) [58] catalyzed by enzymes (HPR, ALP, etc.), or (iii) from commonly used biocompatible redox probes, namely the ferrocyanide/ferricyanide redox couple [54, 59].

Common strategies for SPR sensors regeneration, such as the use of acid/basic solutions [51, 52, 59], or solutions containing surfactants [57] or high concentration of chaotropic agents [53], can also be employed for eSPR biosensors reusability.

3. Detection of Proteins

Early work on synchronized electrical and optical readout relied on the fruitful combination of CV electrochemical technique with SPR for the *in situ* study of redox proteins adsorbed to gold surfaces previously modified with alkanethiol SAMs [41, 42]. Meanwhile, the hyphenation of SPR with EIS technique was extensively used to study molecular binding interactions between proteins [62-64]. For example, simultaneous label-free SPR-EIS measurements were used for the development of a flow injection analysis (FIA) sandwich immunoassay to detect interferon-γ (IFN-γ) [63]. In this study, EIS was successfully used to monitor the binding of IFN-γ to surface immobilized MD-2 capture antibody while poor SPR response was observed due to small amount of mass bound. By opposition, IFN-γ subsequent binding to liposome, coupled to a secondary antibody, resulted in enhanced SPR resonance angle shift without increasing the impedance signal. A possible explanation to this phenomenon suggested by authors was that the binding event to liposomes occurs far from the electric double-layer (depth of tens of nanometers) but within the generated SPR evanescent field (depth of hundreds of nanometers). Also, the results highlighted the usefulness of FIA systems for eSPR immunosensing investigations. In another study, plasmonic-based EIS measurements were able to detect and follow the kinetic of direct binging of solution anti-IgG to surface immobilized IgG [64]. The authors concluded that, in contrast to conventional SPR monitoring, the plasmonic-based impedance response was less affected by bulk RI changes and NSB effects. Although complementarity of impedance and SPR is already an established technology for investigation of affinity binding processes [65, 66], the studies reported so far lacks of application of developed methodologies for biosensing of large molecules. The long measurement times required, the nonspecific impedance changes and the complicated (and sometimes problematic) data interpretation can explain the limited use of EIS technique as analytical tool [67, 68].

3.1. Immunoglobulin G detection

SPR immunoassays performed under constant electrochemical potential emerged in the literature perhaps as the simplest approach for eSPR detection of proteins [55, 56, 69, 70]. Such methodology exclusively records the SPR angles changes (and sometimes current) when an external potential is applied to the bioanalytical system. Immunosensors

operating label-free were developed for the eSPR detection of IgG [55, 69]. To build the biosensing platforms the capture antibody was immobilized on electrosynthesized PABA [55], and GO/PEDOT/PSS [69] polymer films on gold surface. The surface heterogeneity and disorder resulting from the electrochemical polymerization enhanced the binding of IgG and relatively low detection levels were achieved (LOD between 0.35 – 1 μg mL^{-1}), allowing detecting IgG at physiological levels. The same detection concept was used for detecting adrenaline making use of its specific reaction with electropolymerized PABA [71]. Furthermore, the combination of electrochemical techniques with transmission surface plasmon resonance (TSPR) [72] was used for investigation of poly(pyrrole-3-carboxylic acid) properties and detection of human IgG at concentration levels lower than conventional eSPR technique (~ 1 ng mL^{-1}).

Recently, in 2017, the covalent coupling of probe biomolecules glucose oxidase (GOD) and anti-human IgG on electropolymerized carboxylated conducting polymers was employed for the simultaneous electrochemical and optical detection of glucose and human IgG, respectively [70]. After injection of a mixture of glucose and IgG into the dual sensor, the eSPR monitoring at ~+0.7 V showed that the SPR response was sensitive to the binding of IgG whereas the recorded amperometric measurements selectively responded to glucose. However, the described approaches lack of applications of developed eSPR biosensors in close-to-real conditions (urine, serum and blood samples) for analytical validation the technology for clinical purposes. After potential application to the bioanalytical system, the NSB of matrix proteins to the sensor surface can be rather problematic and compromise the biosensor performance.

In contrast to previous methods described, a more appealing eSPR approach for detection of large biomolecules emerged in the literature between 2007 and 2008 [57, 58] and consisted in simultaneously and independently measure the SPR optical signal and the electrical current from voltammetry at the same immunosensing spot. The detection concept used enzymes, namely horseradish peroxidase (HRP) and alkaline phosphatase (ALP), conjugated to antibodies, that generates the redox signals or produced *in situ* the redox reporters (in the presence of a substrate) for the electrochemical readout.

Phillips et al. [57] developed a very innovative methodology based on the use of a temporary supported lipid membranes as a new route to suppress background signals from NSB of serum matrix proteins towards the trace detection of target proteins. The phospholipid membrane disruption after treatment with surfactant removed adsorbed matrix proteins from the gold sensing platforms before detection, leaving intact the

immunocomplexes at the short-chain SAM surface (see Fig. 3A). The overall membrane cloaking method concept was employed to develop an amperometric immunoassay for detection of HRP-conjugated IgG in undiluted serum samples. To do so, the potential at the developed eSPR flow cell was set at +0.175 V and the currents from TMB substrate oxidation recorded (Fig. 3B). From the calibration plot of the immunosensing assay, detection of low concentrations levels of IgG (5-10 fM) was achieved. Besides, the restoring of electron transfer at the electrode surface after membrane removal with Triton-X allowed surface regeneration previously to each measurement avoiding the need of a reference channel. This was not possible after (irreversible) surface blocking with BSA. Thus, the SPR monitoring of the cloaking process combined with complementary sensitive electrochemical readout showed to be very promising for routine biomolecules analysis in complex biofluids (such as undiluted blood serum), having a great potential for PoC disease diagnosis.

Pioneer work performed by Dong et al. [58] reported for the first time an eSPR sandwich immunosensor for detection of IgG where the redox label was generated *in situ* as a product of enzyme catalytic activity (see Fig. 3C). After IgG interaction with target antibody, the introduction of a secondary antibody, conjugated with ALP enzyme, amplified the SPR response and allowed the amperometric detection of enzymatically generated p-aminophenol (PAP) from p-aminophenol phosphate (PAPP). Polypyrrole propylic acid (PPA) polymer on gold surface was used as support for covalent immobilization of probe antibody. The polymer film was prepared by CV electropolymerization, a straightforward approach that allowed the easy monitoring of the polymer thickness (d = 14 nm) for optimized sensor architecture and performance. The binding of secondary antibodies induced a SPR angle variation proportional to the amount of surface antibody-antigen complexes. Moreover, the enzymatically generated PAP was detected by CV technique. The redox peaks observed in the voltammograms, depicted in Fig. 3D, corresponded to the reversible oxidation of PAP to p-quinone imine (PQI). From data collected data, a logarithm dependence of the IgG concentration and the peak current (Fig. 3E, 3F) was obtained. To fully assess the performance of the combined optical and electrochemical method, the detection sensitivity of the two sensing approaches was compared. The results obtained indicated that lower LODs can be achieved using electrochemical readout relatively to SPR detection (~602.6 ng mL^{-1}), since electrochemical signals can be continuously amplified in time by the enzyme (for example, $LOD_{1st\ CV\ cycle}$: ~676.1 ng mL^{-1}; $LOD_{2nd\ CV\ cycle}$: ~589.3 ng mL^{-1}).

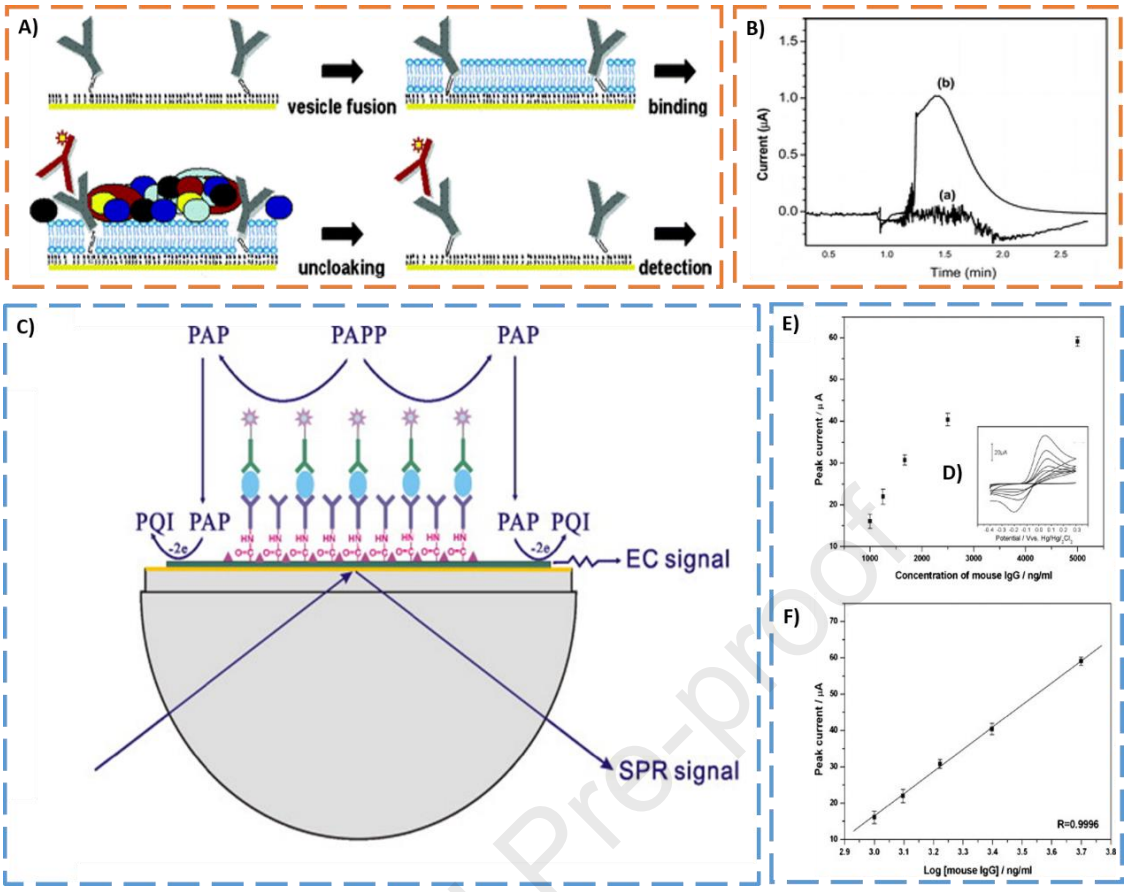

Fig. 3. A) Schematic representation of the membrane cloaking method used for target IgG protein detection directly in undiluted serum; B) Amperometric response to *in situ* TMB oxidation in the absence (control, curve a) and presence (curve b) of HPR conjugates at the detection platforms; C) Schematic representation of the *in situ* eSPR immunosensor for IgG detection based on secondary antibodies conjugated with ALP; D) CVs of enzymatically generated PAP obtained for the several concentrations of IgG tested (1 to 5 μg mL^{-1}); E-F) Electrochemical calibration curves obtained in E) linear reference frame and in F) semi-logarithmic reference frame. Adapted from [57] and [58] with permission from American Chemical Society and Elsevier, respectively.

3.2. Virus detection

Meanwhile, in 2019, Qatamin et al. [56] reported the detection of hemagglutinin (HA) protein from the H5N1 avian influenza A virus by sandwich immunoassay using a MB-labeled secondary antibody that displayed an enhanced reversible change in RI due to MB oxidation-reduction state transition after applying a modulated electric potential. Maximum SPR optical signal intensity was observed at ~ -0.17 V (formal potential of

MB). The high detection sensitivity (LOD of 300 pM) and selectivity of the eSPR immunosensor indicated that it can be a promising alternative to biosensing approaches currently being used in viral disease diagnostic (PCR, ELISA, etc.).

3.3. Cancer biomarker detection

Very recently, in 2021, Ribeiro et al. [59] developed an eSPR approach for the label-free and sensitive screening of breast cancer biomarker CA 15-3 in biological samples. The bioreceptors immobilization chemistries at the SPR gold substrates were fully developed taking into account the need to optimize both electron transfer and surface plasmon processes. Thus, ultrathin films (with an overall thickness of 1.4 ± 0.4 nm) were prepared taking advantage of short-chain carboxyl acid-terminated SAM employed (mercaptosuccinic acid, MSA) instead of commonly used 11-MUA, in order to avoid surface blocking to electron transfer process.

The overall fully automated detection scheme is schematically represented in Fig. 4A and consisted in recording the SPR response of the binding event between CA 15-3 (in solution) and surface immobilized bioreceptors (Fig. 4B, left), followed by electrochemical readout in the presence of the ferrocyanide/ferrocyanide redox couple (Fig. 4B, right).

Fast and sensitive SWV measurements were performed after the SPR monitoring in order to enhance the detection sensitivity of the eSPR approach. Thus, the eSPR approach does not needed either, secondary antibodies or labels, on amplification tags surface to enhance optical signals, which usually makes detection expensive, complex and time-consuming. The SWV voltammogram collected after the immunocomplex formation are shown in Fig. 4D and a decrease of redox peak currents with the increasing antigen concentration was observed due to increasing surface blocking to the diffusional redox probe at the electrode surface. After recording the optical and electrochemical responses independently, both calibration curves were obtained (Fig. 4C and inset of Fig. 4D). The LOD obtained by the electrochemical readout for detection of CA 15-3 was much lower (about ~210 times) than the obtained by SPR sensing, emphasizing previous conclusions reported, that electrochemical techniques can be a suitable label-free amplification strategy to enhance sensitivity of SPR biosensors for quantification of low levels of analyte. In addition, a protocol was implemented to reduce NSB to the sensor surface for

improved detection of the circulating protein biomarker in human serum samples, allowing the application of the eSPR biosensor under clinical and diagnosis context.

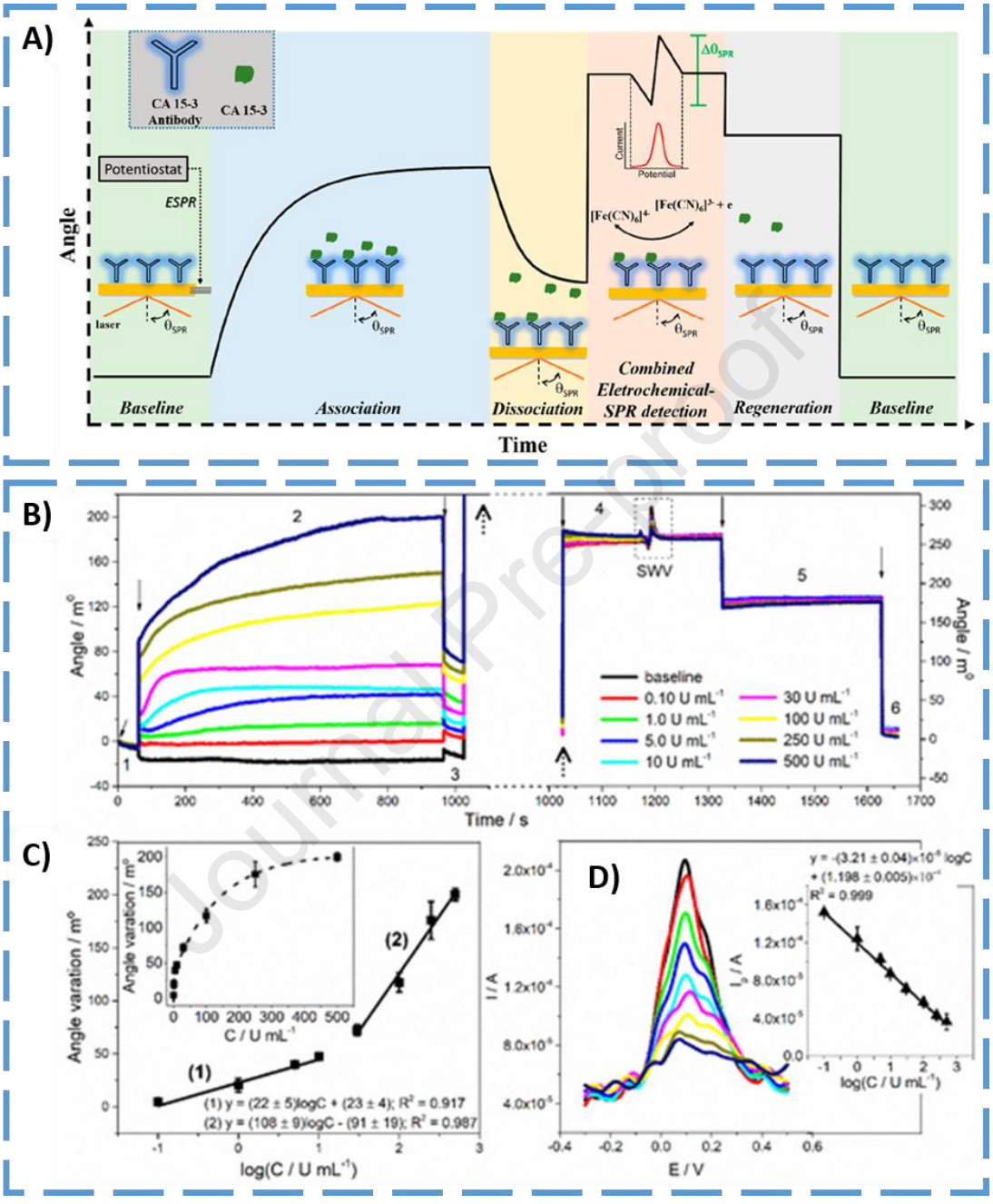

Fig. 4. A) Scheme of the fully automated eSPR detection procedure for screening cancer biomarker CA 15-3; B) Direct SPR monitoring of the binding event for the CA 15–3 concentrations tested (left) followed by electrochemical control (SWV)-SPR measurements in the presence of redox probe (right); C) Non-linear (inset) and linear calibration curves obtained by direct SPR; D) SWV voltammograms recorded after the immunocomplex formation. Inset: Calibration curve obtained by electrochemical readout. Adapted from [59] with permission from American Chemical Society.

4. Detection of Nucleic Acids

Preliminary eSPR hybridization studies were performed by Georgiadis and collaborators [73, 74], in 2001, reporting the effect of applied potential (electrostatic charging) on nucleic acid probe immobilization on gold [73] and its interaction (hybridization/electrostatic denaturation) with unlabeled DNA target oligonucleotides [74]. After that, combining SPR and EIS allowed the detection of oligonucleotides hybridization on nanoscale SiOx layers on gold (Au/SiOx) [75, 76]. The Faradaic impedance spectra, collected in the presence of the ferrocyanide/ferricyanide redox couple, was used to discriminate kinetics of DNA hybridization (and dehybridization) with non-complementary strands. Although SPR data provided a means to validate the results obtained by EIS [75, 76], oligonucleotide quantification by eSPR was not attempted.

The first eSPR DNA biosensor emerged in 2005. Liu et al. [51] developed a sensitive method by tagging target protein with ferrocene (Fc) as a means for synchronized electrochemical and optical amplified detection of DNA hybridization. The overall detection procedure is schematically represented in Fig. 5A. After the hybridization event, the specific binding of biotin (at target DNA strands) to streptavidin-ferrocene conjugates provided the amplification of both, SPR and electrochemical, signals. SPR kinetic experiments (Fig. 5B) showed that the injection of protein conjugates upon the hybridization of nonspecifically bound DNA (BT15, control) induced a small increase on SPR reflectivity while the hybridization with target fully complementary DNA (BT2) biotinylated sequences resulted in a strong increase of reflectivity. Furthermore, protein conjugation with electroactive Fc allowed simultaneous electrochemical monitoring of the binding event. SWV was selected as electrochemical technique, and the voltammograms recorded for each concentration tested (Fig. 5C) revealed that the Fc redox signal increased with the increasing amount of target DNA bound to the sensor surface. From the calibration plot (Fig. 5D), a linear concentration range, between 10 and 200 pM, and a LOD of 10 pM (2 fmol) were obtained. In addition to the high sensitivity (and reproducibility), the developed eSPR DNA biosensor showed high selectivity since discrimination at single-base mismatch level was achieved. The research group used the same detection strategy to perform combined electrochemical and surface plasmon field-enhanced fluorescence spectroscopy (SPFS) studies concerning short DNA structure transformation [77].

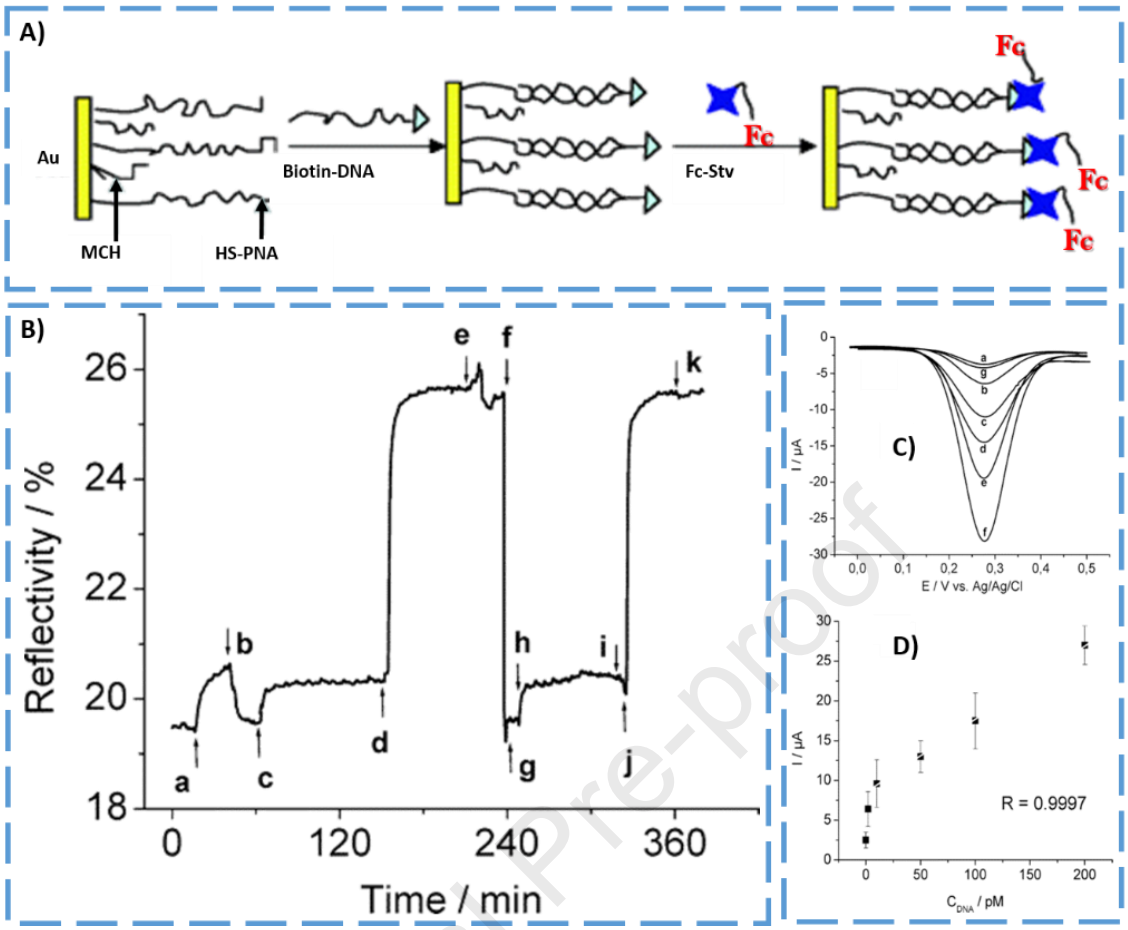

Fig. 5. A) DNA hybridization detection strategy based on the formation of an electroactive streptavidin layer for optical and electrochemical signal amplification. Thiolated peptide nucleic acid probes (HS-PNA) at the electrode surface were used for effective hybridization with target sequences and MCH was used lateral spacer that optimized film organization; B) SPR curves recorded (a) after injection of BT15 (non-complementary biotinylated target; reference; C = 1 µM), followed by injection of ferrocene-streptavidin conjugates (C = 200 nM); (b) surface wash with buffer solution; (c) injection of BT2 (target sequence; C = 500 nM); (d) after surface wash, followed by injection of electroactive conjugates; (e) same as (b); (f) after injection 50 mM NaOH for surface regeneration; (g–k) repetition of steps (b–e); C) SWV voltammograms collected at the Au/PNA surface after hybridization event with BT2 (fully complementary biotinylated target and after binding of ferrocene-streptavidin conjugates. From (a) to (f): 0 to 200 pM. Curve g represents the voltammogram obtained for BT15 (noncomplementary target, control); D) Graphical representation of the relationship I_{peak} vs. C_{BT2}. Adapted from [51] with permission from American Chemical Society.

On/off bioelectrochemical switches were considered to be very innovative platforms for electrochemical biosensing in complex media [78] and the structure-switching technology was adapted to the eSPR sensing method [52, 53, 79]. In 2014, the eSPR technique allowed optimal nucleic acid probe selection, between one stem-loop DNA probe (SLP) and two linear DNA probes (LP and 3×LP), all modified with methylene blue (MB) organic dye as terminal group (electroactive reporter) and containing a sequence complementary to target 25-base DNA. ACV was used to monitor the MB redox activity (maximal at ∼−0.27 V) after duplexes formation at the sensor surface. Best results were achieved by the longer linear DNA probe (3×LP), however, a poor LOD (of 20 nM) was achieved by the developed sensor [52].

4.1. Bacteria detection

Meanwhile, in 2015, Dallaire et al. [53] developed an eSPR biosensor for detection of pathogenic oligonucleotides directly by the use of a thiol-immobilized DNA stem-loop structure possessing MB as redox label. Upon hybridization with pathogenic DNA sequences, the stem-loop structure disrupts hindering the electron transfer process (see Fig. 6A). The employed structure-switching electrochemical process was converted into a measurable plasmonic response, taking into account the different RI of reduced (MB_{red}) and oxidized (MB_{ox}) states of MB (ΔRI_{redox}) and the distinct electron transfer rates at "ON" or "OFF" configurations ($\Delta RI_{redoxON} >> \Delta RI_{redoxOFF}$). From detection studies performed in buffer solution, the LOD of the switch-based eSPR sensor was estimated to be 5 nM and 10 nM for *Mycobacterium Tuberculosis* (Target_1) and *Escherichia coli.* (Target_2), respectively. Then, the eSPR sensor was directly applied for detection of DNA strands from *Escherichia coli.* in whole blood samples. The ACV voltammograms (Fig. 6B) and the real-time hybridization dynamics recorded by impedance method (Fig. 6C) during incubation confirmed the high selectivity of the electrochemical structure-switching plasmonic sensing in complicated matrices, highlighting that eSPR is able to resist better to NSB than conventional SPR, a crucial advance for biosensing applications in clinical context.

Taking advantage of the eSPR technology to map the redox potential amplitude with high spatial resolution, the developed eSPR sensor was also applied for the simultaneous detection of oligonucleotides from drug-resistant tuberculosis and *Escherichia coli*., in complex samples, using the experimental setup represented in Fig. 6D. The DNA probes

for each pathogen were placed on separated spots of the same working electrode but in compact with the same sample solution. The real time response for the successive injection of target DNA sequences (Fig. 6E) showed that fast and reliable multiplexed detection was successfully achieved by the switch-based eSPR approach. However, although the overall concept was successfully demonstrated by the research group, showing very promising results even in complicated matrices (whole blood), however, it is important to further improve this technology since the LOD achieved by the sensing approach (in the nM range) can compromise its application under diagnosis context, where detection sensitivity at fM level is often needed.

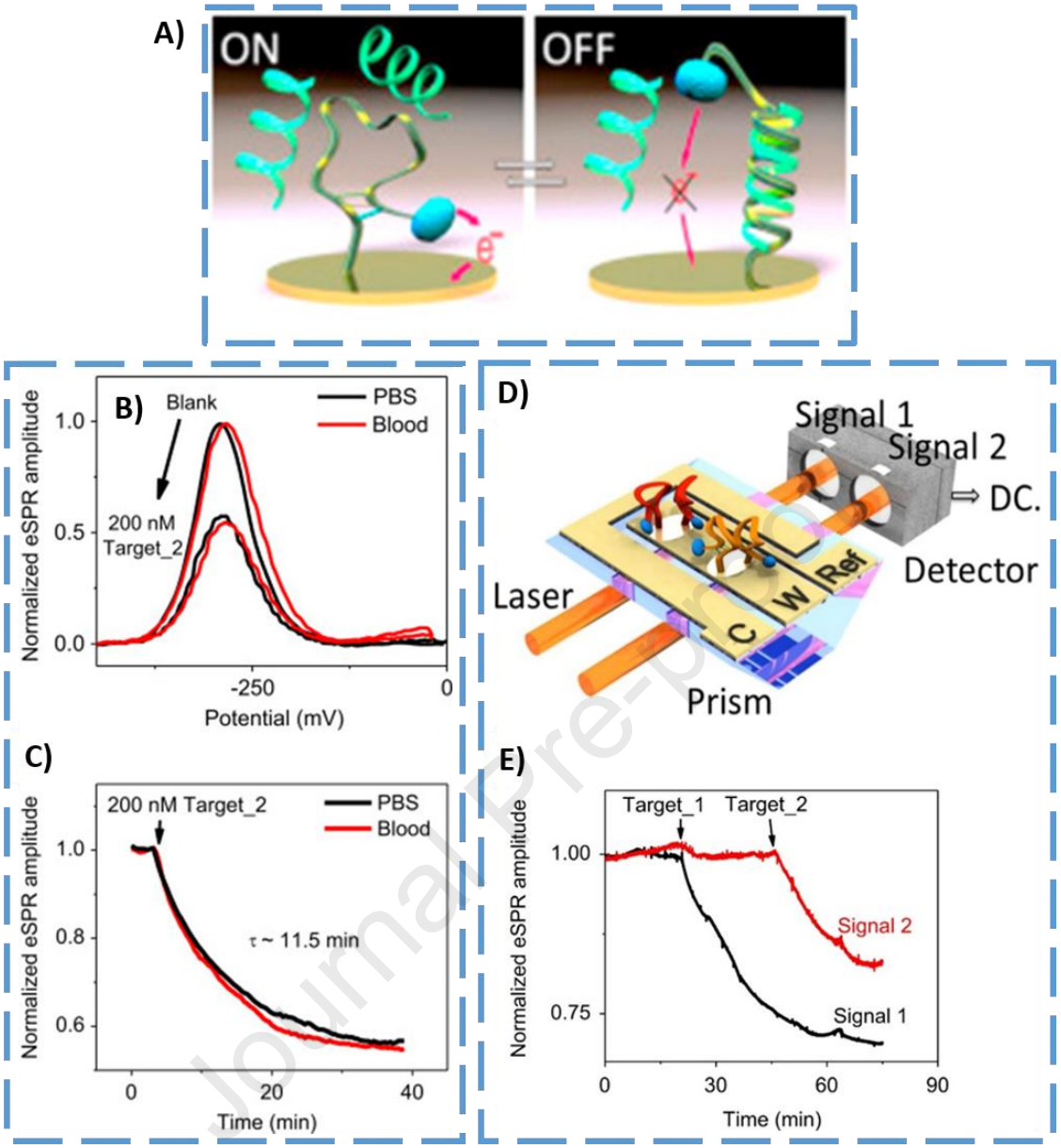

Fig. 6. A) Schematic representation of the sensing mechanism based on the MB nanoswitches. MB electron transfer is maximal for the "ON" state and minimal for the "OFF" state as the immobilized probe suffers conformational change; B) Normalized eSPR voltammograms obtained for detection of *Escherichia coli.* DNA strands in PBS and blood. Electrochemical measurements were recorded before (blank) and after surface incubation with target oligonucleotide for hybridization. Maximum eSPR signal at ~-275 mV (V_{redox} of MB); C) Real-time eSPR response to the hybridization event between immobilized probe and target oligonucleotide; D) Set-up used for eSPR multiplexed detection of drug-resistant tuberculosis (Target_1) and *Escherichia coli.* (Target_2); E) Responses obtained after addition of Target_1 and Target_2 sequences (C = 100 nM) to the sample media. Adapted from [53] with permission from Elsevier.

4.2. Cancer biomarker detection

Recently, in 2020, an eSPR approach operating label-free was developed by Ribeiro et al. [54] for the simple and sensitive detection of breast cancer biomarker miRNA-145. The preparation of the sensing surfaces was achieved by covalent attachment of thiolated RNA probes to the sensing surface using 2-mercaptoethanol (ME) as lateral spacer to reduce steric hindrance. Fully automated *in situ* preparation of sensor platforms and detection procedures for the miRNA-145 quantification were carried out by the robotic autosampler, increasing reproducibility and avoiding contamination and/or operator's errors. The overall detection scheme is represented in Fig. 7A. By controlling the electrode potential, it was possible to induce the accumulation of the redox probe molecules at the electrode surface after the hybridization event which resulted in enhanced optical response as consequence of the electrochemical process. As hybridization proceeds, the electrostatic repulsion between negatively charged redox probes and the strongly negatively charged sensor surface increasingly hindered the diffusion-controlled redox process thereby decreasing the overall angle variation. The real-time data collected from the automated detection scheme is displayed in Fig. 7B for the several concentrations of miRNA-145 biomarker tested. Furthermore, the SPR optical changes obtained under hybridization equilibrium and the following eSPR monitoring upon application of the electrochemical potentials (E_1 = +0.5 V; E_2 = -0.3 V) were represented as function of the analyte concentration, as shown in Fig. 7C and 7D, respectively. As can be seen, direct SPR only provides reliable response to concentration values above 10 nM. However, the control of applied electrochemical potentials to the redox system clearly influenced the plasmon resonances providing a simple and label-free amplification strategy thereby improving the sensitivity of the hybridization assay from nM to femtomolar concentration levels (LOD = 0.56 fM), allowing the application of the eSPR biosensor for clinical purposes. In addition, the developed eSPR biosensor showed high selectivity since it was able to discriminate sequences having single-base and two-base mismatch. Successful eSPR quantification of miRNA-145 in human serum was reported in this work.

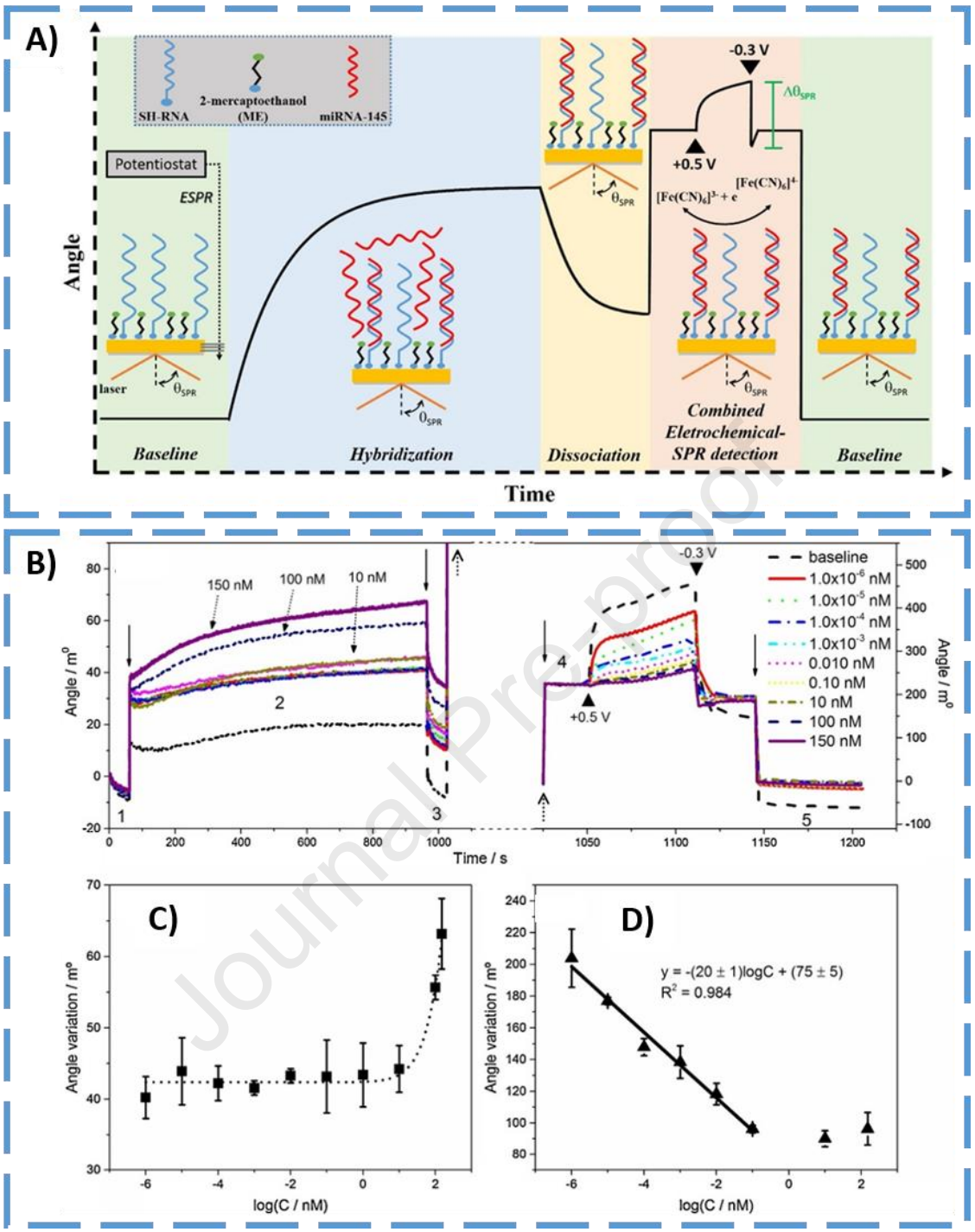

Fig. 7. A) Sensing approach used for the amplified eSPR detection of miRNA-145. After monitoring target miRNA hybridization in real-time (first detection step) signal amplification was achieved by potential-assisted deposition of a biocompatible redox probe on the sensor chip (second detection step); B) Real-time direct SPR response to the hybridization event (left) followed by combined electrochemical control (vs. Ag/AgCl) SPR measurement (right) in the presence of ferrocyanide/ferricyanide redox couple (C = 5 mM); C)-D) Calibration curves obtained for miRNA-145 by (C) direct SPR and (D) eSPR readout. Adapted from [35] with permission from Elsevier.

5. Detection of small molecules

Detection of small molecules can be rather challenging using conventional SPR approach since small differences in the RI imposes limited sensitivity. However, eSPR method can easily overcome this limitation as long as the target analyte is redox active for direct electrochemical detection using common voltammetric techniques. This simple and straightforward biosensing strategy has already found some relevant applications in clinical context [61, 80] that can expand knowledge to provide new sensing solutions.

Another popular approach for eSPR detection of small molecules rely on enzyme based biosensors integrating conductive polymers acting as wiring matrixes for the electrochemical activation of redox enzymes. Early work demonstrated changes of the optical dielectric properties of several polymers, such as poly(aniline) [81, 82] poly(aniline)/poly(acrylic acid) [83] and poly(pyrrole) [84], by reactions catalyzed by enzymes, mostly HRP and glucose oxidase (GOx), enabling to indirectly determine the substrate concentration quantitatively.

Baba et al. [85] reported the development of a eSPR glucose biosensor based on a multilayer thin film composed of N-alkylaminated poly(pyrrole)/GOx. Layer-by-layer (LbL) electrostatic assembly was used for simple and stable immobilization of GOx and signal enhancement was mediated by doping-dedoping events on the poly(pyrrole) (see Fig. 8A). Importantly, the eSPR biosensor was able to operate at potentials lower than +0.3 V, a crucial advantage to avoid oxidation of (nonenzymatic) glucose, generated H_2O_2 or coexisting species in biofluids (AA, UA, acetaminophen). Best sensitivity (of 0.3 μA/10 mM) was achieved by using the polymer film in doped state before sensing. This work however lacks of long-term stability studies and application of eSPR device for glucose analysis in blood and/or urine.

An ingenious eSPR approach for detecting H_2O_2 and glucose involved the use of a horseradish peroxidase-osmium redox polymer (Os-gel-HRP) operating without the need of electrochemical instrumentation [60, 86-88]. The combined measurement of electric current and SPR optical signals on a gold nanohole array, incorporated into a microfluidic channel (see Fig. 8B), was used for H_2O_2 measurement [60]. The array was previously modified with a Os-gel-HRP film that was responsible for the electrochemical processes occurring at the gold nanohole. The immobilized HRP consumes the H_2O_2 (catalytic reduction into water) and subsequently returns to its native form by accepting an additional electron from osmium complex. The SPR dip shift due to the Os complex redox

state change decreased with the increasing analyte concentration (Fig. 8C). The relative dip shift was linearly proportional to H_2O_2 concentration in the range from 10 to 250 μM, after which saturation occurred (Fig. 8D). The overall eSPR detection concept, successfully demonstrated for H_2O_2 quantification, can be further extended to a higher number of chemical species by immobilizing one or more oxidase enzymes (GOD, glutamate oxidase, etc.) on the gold nanoarray.

In another work, Nakamoto et al. [86] took advantage of the great features of the Os-gel-HRP redox polymer to developed a one-chip SPR biosensor (containing two circular gold thin films) for simultaneous PoC detection of two biomolecules with very different molecular sizes in human urine, the disease biomarker transferrin (MW = 75 kDa) and a calibration marker, creatinine (MW = 113 g mol^{-1}). Transferrin was detected by simple immunoreaction with surface immobilized antibody (direct SPR) while creatinine was converted by a tri-enzyme layer to H_2O_2, being detected by eSPR approach using the above mentioned Os-gel-HRP film. For successful detection of creatinine, several enzymes were employed to remove interferences (such as AA and UA) from urine samples. The biosensor was capable of detecting transferrin (from 20 ng/mL to 10 μg/mL) and creatinine (from 10 μM to 10 mM) at clinically relevant levels in real human urine with improved accuracy by calibrating the transferrin concentration with the creatinine.

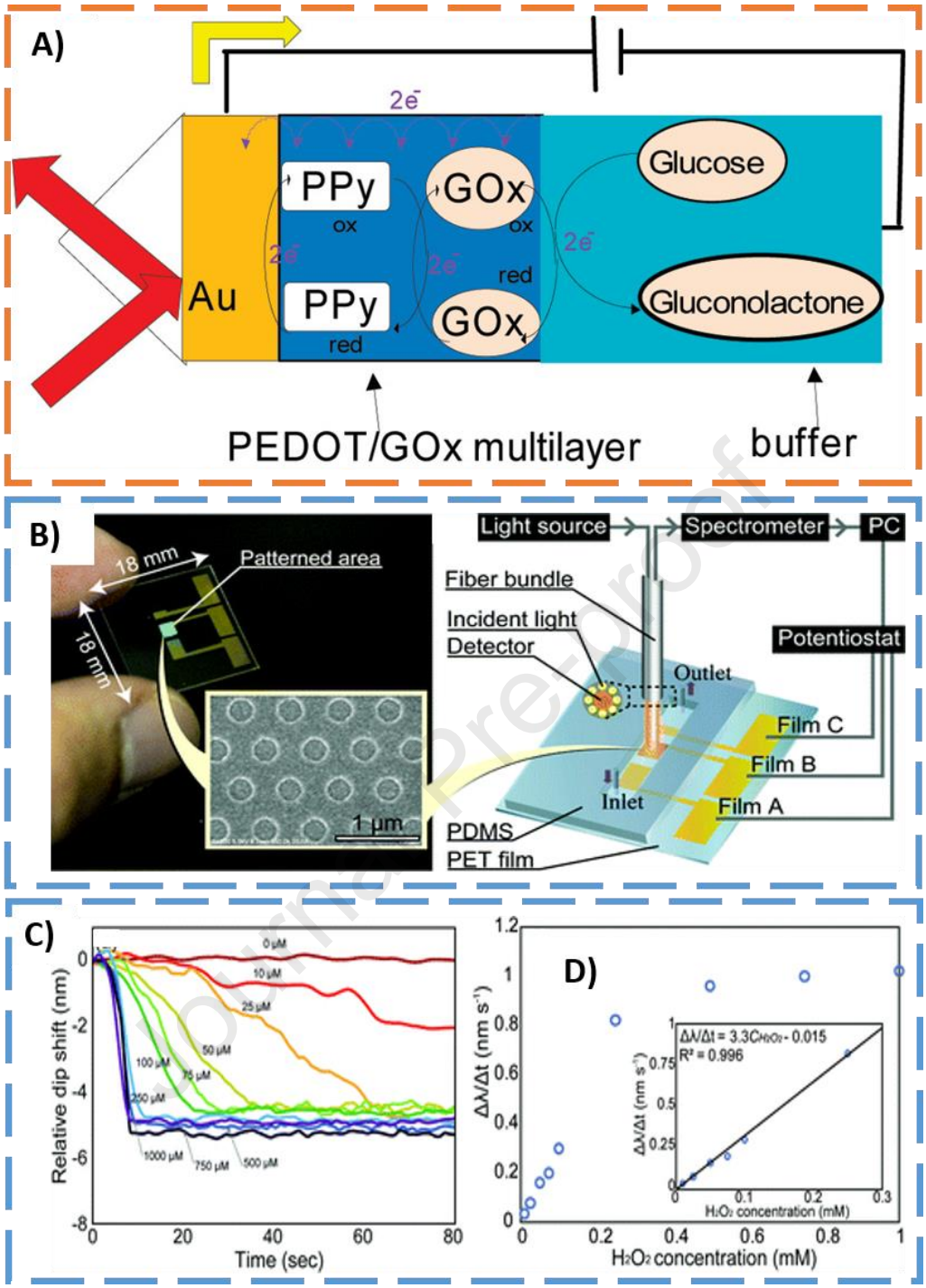

Fig. 8. A) Schematic diagram of the multilayer composite electrode cascade of events for sensing of glucose using glucose oxidase (GOx). B) Detection chip (left), including SEM image of gold nanohole array (inset) and schematic representation of the eSPR apparatus (right). Films A, B and C corresponds to CE, WE and RE, respectively; C) Dip variation after injection of increasing concentrations of H_2O_2 onto the gold nanohole array, previously modified with Os-gel-HRP; D) Calibration curve obtained for H_2O_2. Adapted from references [85] and [60] and with permission from American Chemical Society.

6. Living-cell Analysis

SPR can provide real-time, non-invasive and label-free evaluation and monitoring of living cells, a very appealing not only for research purposes in live sciences but also for several medical practices [89]. In this topic, the combination of electrochemical methods with SPR [61] and SPR imaging (SPRI) [48, 90] has the unique advantage of multi-parametric cell analysis.

A very inspiring eSPR application was developed by Wu et al. [61] for the real-time evaluation of treatment efficiency of live cancer cells directly adsorbed over SPR substrates. The measurement of electroactive daunorubicin (DNR) by CV technique was used for assessment of drug uptake into living cells and SPR method allowed to monitor signal variation induced by detachment of apoptotic cells from the chip surface, after incubation with the anticancer drug (see Fig. 9A). SPR signals induced by CV oxidation and reduction of extracellular DNR (at ~ −0.6 V) were found to be linearly related to cell survival rates (Fig. 9B). Thus, the innovative eSPR methodology was able to provide reliable and label-free information about treatment effectiveness and cytotoxicity of bioactive drugs in real-time, a unique advantage over commonly used colorimetric methods.

In another work, Zhang et al. [90] developed an electrochemical SPR imaging (eSPRI) method to obtain direct information about the changes of dielectric properties and the redox character of the cells. CV technique was used in their work. The authors observed that cell oxidation effect recorded in CV measurements correlated well with optical signal changes found by SPR detection.

Furthermore, recent literature highlights the great potential of plasmonic imaging of electrochemical impedance (P-EIM) [43, 66] for several applications, including the analysis of single cells and intracellular events.

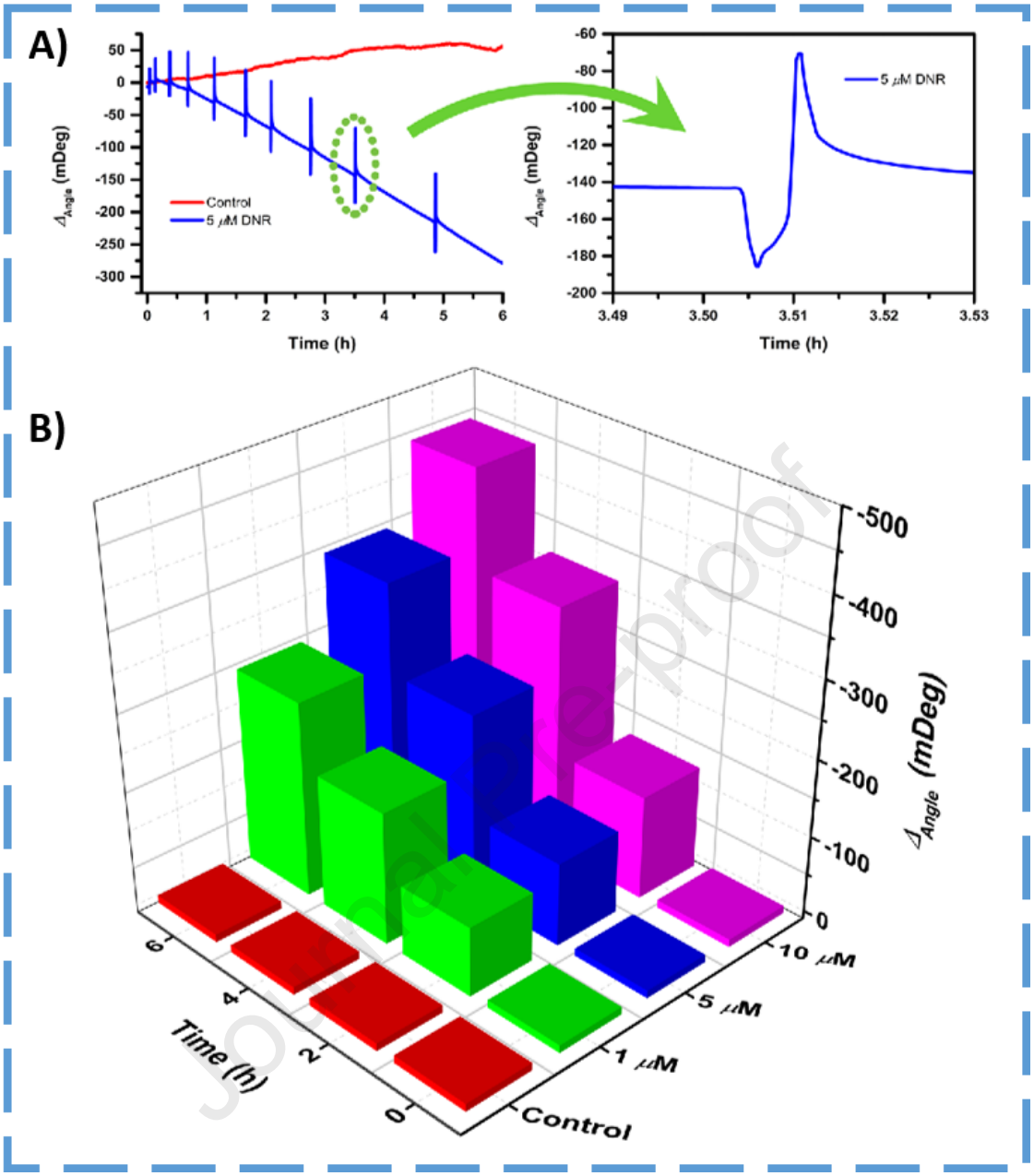

Fig. 9. A) Real-time SPR curves obtained after HepG2 cells treatment with 5 μM DNR, showing the optical signal variation when CV measurements were collected. Control experiment were conducted and show the SPR signal increase due to the cell growth and attachment to the SPR platforms; B) SPR signals collected as a function of DNR concentration and cells exposure time. Adapted from reference [61] with permission from American Chemical Society.

7. Limitations and optimization

For implementation of eSPR technology in routine clinical practice, the eSPR biosensors need to overcome the challenges of biomarkers detection in complex biological samples, such as serum. Concerns related to nonspecific binding (NSB) of serum components (mostly proteins, such as albumin) to biosensing surfaces have been clearly identified for SPR biosensors (leading to false/negative results, data misinterpretation, etc.) [13, 91] and several antifouling materials, based on PEG/OEG, peptides, polysaccharides (e.g. hyaluronic acid), polyacrylates, among others, have been developed to improve optical biosensing in blood-based media [91-93]. However, most of these strategies, some of them commercially available [91], are of limited use for eSPR method since the electron transfer process is inhibited due to the high thickness of antifouling layers, compromising electrochemical detection. Thus, for the successful implementation of eSPR detection it is fundamental that surface modification procedures used can efficiently attach bioreceptors to the plasmonic platforms, and resist to NSB of unwanted biomolecules coming from biofluids (by using antifouling compounds [93, 94], for example), while providing a measurable reversible electron transfer kinetics of the label through the film to the electrode [17, 21, 95].

Common procedures for preparation of optimized eSPR biosensing surfaces rely on gold functionalization with (i) electrosynthesized polymeric films [55, 58] and (ii) SAMs, in particular short-chain thiols [56, 59]. The *in situ* electrochemical polymerization of functional monomers (PABA, PPA, etc.) allows the easy modulation of the polymer film thickness with respect to electron transfer [27] while short-chain SAMs tend to form monolayers with considerable amount of defects being very attractive for investigation of redox reactions [96]. Besides, SAMs of short carboxyl-terminated thiols, such as MPA and cysteine, were recently used as alternative linker molecules (replacing the widely used MUA [94, 97, 98]) in SPR biosensors, and proved to be effective in reducing NSB of proteins composing biological fluids [99-102].

Although no eSPR biosensors based on polymeric films was yet applied for analysis in clinical samples, some have succeeded using SAM approach for biomarkers analysis in serum [54, 59] and whole blood media [53] (see Table 1). Other surface treatments and strategies were used to further resist to serum proteins adsorption, such as de-activation the SAM head group (-COOH) with ethanolamine [59], co-immobilization of hydrophilic SAMs [54], surface block with blank serum [59], use of NSB binding buffers [59], sample

dilution [54, 59] or applying a "membrane clocking" method prior to detection [57]. Still, more work reporting eSPR biosensors application to real clinical samples is currently needed, aiming not only to address detection in serum, but also in other biofluids. The application of eSPR technology to urine and saliva samples remains unexplored. The design of new eSPR sensors working directly in crude biofluids with minimal sample preparation should be envisioned. For validation, the eSPR biosensors application in real clinical samples from patients is needed [13].

To further improve eSPR biosensing in biofluids, new SAM-based surface chemistries and polymeric functionalizable antifouling coatings that recently emerged in the literature can be of potential interest for successful eSPR biosensing in biofluids, namely:

(i) monolayers composed of mixed SAMs of MPA:MUA [103] and/or the use of ethylene glycol-based thiols (~ 2.5 nm) [104-106]. The optimized surface design uses the longer thiol for effective immobilization of biomolecules at the gold surface through -COOH terminal groups (via EDC/NHS coupling) and the hydrophilic shorter SAM is used to limit the occurrence of NSB [103, 106];

(ii) dopamine polymer, poly(dopamine), used over recent years as antifouling coating for several biomedical applications, including biosensing [107-110].

The use of synthetic bioreceptors, such as aptamers and molecularly imprinted polymers (MIPs), may also contribute to increase the assay selectively and stability in complicated matrices [13, 16, 21]. Thiol-terminated aptamers [111] and MIPs [112, 113] already showed their potential for eSPR biosensing of drugs and herbicides and the concept can be further extended for detection of small molecules and proteins acting as biomarkers. Furthermore, redox-labeled MIP NPs (nanoMIPs) [114] were recently developed with the purpose of combining specific recognition and reporting functions that can be measured by electrochemical techniques. The ingenious detection concept, already validated for electrochemical sensing, can be, in principle, adapted for eSPR biosensing as well.

To provide precision diagnosis it is fundamental to move from single to multiplex biomarkers screening [115, 116]. Although electrochemical [117] and SPR [118] methods already started the journey towards the simultaneous multi-biomarker detection in the same biofluid sample, no eSPR multiplex approach was reported so far. To achieve this level of technology, new eSPR cells and SPR gold substrates (containing electric insulated working electrode areas) [119] need to be intentionally designed (or adapted) to allow the user to perform multiple-electrode measurements. Bi-potentiostats are currently commercially available to collect the simultaneous electrochemical measurements

independently at corresponding SPR channels. The continuous developments in SPR and electrochemical fields may contribute in near future for the development of eSPR multiplex devices in order to became a valuable tool for personalized medicine, enabling label-free, low-cost and accurate screening of multiple biomarkers in parallel.

8. Conclusions and perspectives

Although not yet widely spread, information gathered (and summarized in Table 1), revealed that eSPR method is a very versatile analytical tool since a widespread number of clinically relevant analytes were detected so far, namely nucleic acids, disease biomarkers, pathogens, virus and small chemical species, such as drugs and H_2O_2.

To develop combined electrochemical and plasmonics biosensors, the biofunctionalization strategy is a key factor for experimentalists to increase the bioassay performance. SAM approach is by far the most used for effective immobilization of bioreceptors at the SPR platforms. In addition, the *in situ* electrosynthesis of thin conducting polymers (such as PABA, PAP, among others) as support for covalent immobilization of antibodies can be a straightforward approach to build the sensing platforms taking advantage of eSPR monitoring for easy optimization of polymer thickness. Furthermore, SPR devices having robotic autosampler can offer the unique advantages of rapid, easy, fully automated and reproducible *in situ* procedures to build the artificial architecture assembled to the sensor surface.

For the electrochemical readout, the recorded redox signals can be generated from (i) redox reporters (MB, ferrocene) conjugated to oligonucleotides or proteins, (ii) oxidation of common substrates (TMB) or reaction sub-products (PAP) catalyzed by enzymes (HPR, ALP, etc.), or (iii) from commonly used biocompatible redox probes, namely the ferrocyanide/ferricyanide redox couple. Relatively to voltammetric methods, the use of ACV or the application of a constant electrochemical potential to the bioanalytical systems provided a simple mean for SPR optical signals enhancement induced by the redox processes occurring at the sensor surface. Moreover, the integration of SWV or CV technique with SPR allowed to obtain electrochemical and optical responses independently.

Parallel studies showed that the electrochemical readout was far more sensitive than optical detection, thus, acting as simple and cost-effective (and many times label-free) amplification strategy for the very sensitive detection of low physiological concentration

levels of analyte in biofluids. Although the reported studies (see Table 1) succeed to show the great potential of eSPR as analytical tool, the application of developed biosensors in complex samples (serum, whole blood) was scarce and remains challenging due to lack of sensitivity of devices and/or due to NSB of serum proteins to detection platforms. Still, some strategies were successful implemented to block NSB of serum proteins to SPR chips, such as the use of NSB hindering buffers, surface treatment with blank serum or apply a "membrane clocking" method prior to detection.

For further improvement of eSPR technology, both, SPR and electrochemistry, may contribute with new tools to enhance the sensitivity, selectivity and accuracy of biosensors, operating label-free in biological samples (plasma, serum, blood). In this context, the use of new antifouling materials, such as ethylene glycol-based thiols/poly(dopamine)-based materials, can be attempted to minimize NSB to SPR substrates. Also, the incorporation of electrocatalytic nanomaterials on the sensor surfaces should provide enhanced electrochemical response to redox reactions of reporting molecules while electrochemical techniques can be further optimized to achieve faster detection with improved sensitivity (such as pulse techniques). Moreover, the introduction of novel (bio)receptors can maximize detection performance in biofluids in a cost-competitive manner. The use of aptamers, peptide nucleic acids (PNAs) and MIPs for screening oligonucleotides and proteins, was not fully explored in this context.

Another demand for the eSPR approach is to envisage the simultaneous detection of multiple biomolecules in a single assay to improve diagnostic precision and efficiency and reduce detection costs. This can be easily achieved by taking advantage of eSPR approach to map redox processes with high spatial resolution in combination with promising microfluidics technology for improved throughput and simplified operation in manufactured eSPR cells.

Although conventional SPR systems (Biacore, BioNavis, Reichert, etc.) are being commercialized worldwide and many research laboratories routinely uses them to study biomolecular interactions or for analytical purposes, the implementation of eSPR approach has not yet reached an expressive application. In our opinion, the eSPR setup is technically very simple since it only requires the integration of an external potentiostat to control the potential applied to the cuvette. Furthermore, we consider the eSPR approach to be very innovative and brings new and important contributions to the SPR field for detection of clinically relevant (bio)molecules in order to rapidly report several health hazards with high sensitivity (to identify disease at the early stages) in a simple, cost-

competitive and reproducible manner, offering great advantages relatively to conventional immunoassays (mainly ELISA methods) routinely used for screening disease biomarkers in hospital labs. Based on the above mentioned evidences, we think that current technology deserves the attention of scientific community as a biosensing methodology with a great potential to reach a widespread commercialization level and intensive use in medical diagnosis, environmental monitoring and food quality fields. Moreover, the simplicity and robustness of electrochemical and optical approaches should allow, in a near future, the development of compact and miniaturized devices that can become a new standard in PoC testing.

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References

[1] G.A. Kwong, S. Ghosh, L. Gamboa, C. Patriotis, S. Srivastava, S.N. Bhatia, Synthetic biomarkers: a twenty-first century path to early cancer detection, Nature Reviews Cancer 21(10) (2021) 655-668.

[2] O. Hansson, Biomarkers for neurodegenerative diseases, Nature Medicine 27(6) (2021) 954-963.

[3] M. Ignatiadis, G.W. Sledge, S.S. Jeffrey, Liquid biopsy enters the clinic — implementation issues and future challenges, Nature Reviews Clinical Oncology 18(5) (2021) 297-312.

[4] S.G. Patching, Surface plasmon resonance spectroscopy for characterisation of membrane protein-ligand interactions and its potential for drug discovery, Biochimica Et Biophysica Acta-Biomembranes 1838(1) (2014) 43-55.

[5] J. Homola, Surface Plasmon Resonance Sensors for Detection of Chemical and Biological Species, Chemical Reviews 108(2) (2008) 462-493.

[6] J. Homola, Surface Plasmon Resonance Based Sensors, Springer Berlin Heidelberg2006.

[7] Y. Xu, P. Bai, X. Zhou, Y. Akimov, C.E. Png, L.-K. Ang, W. Knoll, L. Wu, Optical Refractive Index Sensors with Plasmonic and Photonic Structures: Promising and Inconvenient Truth, Advanced Optical Materials 7(9) (2019) 1801433.

[8] S.S. Hinman, K.S. McKeating, Q. Cheng, Surface Plasmon Resonance: Material and Interface Design for Universal Accessibility, Analytical Chemistry 90(1) (2018) 19-39.

[9] B.A. Prabowo, A. Purwidyantri, K.-C. Liu, Surface Plasmon Resonance Optical Sensor: A Review on Light Source Technology, Biosensors 8(3) (2018) 80.

[10] M. Li, S.K. Cushing, N. Wu, Plasmon-enhanced optical sensors: a review, Analyst 140(2) (2015) 386-406.

[11] J. Ladd, A. Taylor, S. Jiang, SPR Biosensors for Food Safety, in: J. Homola (Ed.), Surface Plasmon Resonance Based Sensors, Springer Berlin Heidelberg, Berlin, Heidelberg, 2006, pp. 207-227.

[12] J. Dostálek, J. Homola, SPR Biosensors for Environmental Monitoring, in: J. Homola (Ed.), Surface Plasmon Resonance Based Sensors, Springer Berlin Heidelberg, Berlin, Heidelberg, 2006, pp. 191-206.

[13] J.-F. Masson, Surface Plasmon Resonance Clinical Biosensors for Medical Diagnostics, ACS Sensors 2(1) (2017) 16-30.

[14] N. Bellassai, R. D'Agata, V. Jungbluth, G. Spoto, Surface Plasmon Resonance for Biomarker Detection: Advances in Non-invasive Cancer Diagnosis, Frontiers in Chemistry 7 (2019) 570.

[15] H. Vaisocherová, J. Homola, SPR Biosensors for Medical Diagnostics, in: J. Homola (Ed.), Surface Plasmon Resonance Based Sensors, Springer Berlin Heidelberg, Berlin, Heidelberg, 2006, pp. 229-247.

[16] S.N. Topkaya, M. Azimzadeh, M. Ozsoz, Electrochemical Biosensors for Cancer Biomarkers Detection: Recent Advances and Challenges, Electroanalysis 28(7) (2016) 1402-1419.

[17] P. Skládal, Advances in electrochemical immunosensors for pathogens, Current Opinion in Electrochemistry 14 (2019) 66-70.

[18] H.M. Mohamed, Screen-printed disposable electrodes: Pharmaceutical applications and recent developments, TrAC Trends in Analytical Chemistry 82 (2016) 1-11.

[19] M. Li, Y.-T. Li, D.-W. Li, Y.-T. Long, Recent developments and applications of screen-printed electrodes in environmental assays—A review, Analytica Chimica Acta 734 (2012) 31-44.

[20] F. Ricci, G. Adornetto, G. Palleschi, A review of experimental aspects of electrochemical immunosensors, Electrochimica Acta 84 (2012) 74-83.

[21] F.S. Felix, L. Angnes, Electrochemical immunosensors - A powerful tool for analytical applications, Biosensors & Bioelectronics 102 (2018) 470-478.

[22] Y. Wu, N. Arroyo-Currás, Advances in nucleic acid architectures for electrochemical sensing, Current Opinion in Electrochemistry 27 (2021) 100695.

[23] R.A.S. Couto, J.L.F.C. Lima, M.B. Quinaz, Recent developments, characteristics and potential applications of screen-printed electrodes in pharmaceutical and biological analysis, Talanta 146 (2016) 801-814.

[24] M.M. Khoo, Z. Rahim, N.T. Darwish, Y. Alias, S.M. Khor, Non-invasive control of protein-surface interactions for repeated electrochemical immunosensor use, Sensors and Actuators B: Chemical 224 (2016) 683-691.

[25] C. Fenzl, T. Hirsch, A.J. Baeumner, Nanomaterials as versatile tools for signal amplification in (bio)analytical applications, TrAC Trends in Analytical Chemistry 79 (2016) 306-316.

[26] A. Abbas, M.J. Linman, Q. Cheng, New trends in instrumental design for surface plasmon resonance-based biosensors, Biosensors and Bioelectronics 26(5) (2011) 1815-1824.

[27] A. Chieng, M. Chiang, K. Triloges, M. Chang, Y. Wang, Recent progress in the studies of electrochemical interfaces by surface plasmon resonance spectroscopy and microscopy, Current Opinion in Electrochemistry 13 (2019) 94-99.

[28] M. Kitta, K. Murai, K. Yoshii, H. Sano, Electrochemical Surface Plasmon Resonance Spectroscopy for Investigation of the Initial Process of Lithium Metal Deposition, Journal of the American Chemical Society 143(29) (2021) 11160-11170.

[29] S.S. Hinman, Q. Cheng, Bioinspired assemblies and plasmonic interfaces for electrochemical biosensing, Journal of Electroanalytical Chemistry 781 (2016) 136-146.
[30] A.B. Dahlin, B. Dielacher, P. Rajendran, K. Sugihara, T. Sannomiya, M. Zenobi-Wong, J. Vörös, Electrochemical plasmonic sensors, Analytical and Bioanalytical Chemistry 402(5) (2012) 1773-1784.
[31] S. Wang, X. Huang, X. Shan, K.J. Foley, N. Tao, Electrochemical Surface Plasmon Resonance: Basic Formalism and Experimental Validation, Analytical Chemistry 82(3) (2010) 935-941.
[32] J. Lu, J. Li, Charge Transfer Kinetics from Surface Plasmon Resonance Voltammetry, Analytical Chemistry 86(8) (2014) 3882-3886.
[33] B.W. Davis, M.J. Linman, K.S. Linley, C.D. Hare, Q. Cheng, Unobstructed electron transfer on porous polyelectrolyte nanostructures and its characterization by electrochemical surface plasmon resonance, Electrochimica Acta 55(15) (2010) 4468-4474.
[34] J.D.E. McIntyre, Electrochemical modulation spectroscopy, Surface Science 37 (1973) 658-682.
[35] R.M. Corn, M. Romagnoli, M.D. Levenson, M.R. Philpott, The potential dependence of surface plasmon-enhanced second-harmonic generation at thin film silver electrodes, Chemical Physics Letters 106(1) (1984) 30-35.
[36] D.G. Hanken, R.R. Naujok, J.M. Gray, R.M. Corn, Synthesis, Spectroscopic Characterization, and Electro-Optical Properties of Noncentrosymmetric Azobenzene/Zirconium Phosphonate Multilayer Films, Analytical Chemistry 69(2) (1997) 240-248.
[37] D.G. Hanken, R.M. Corn, Electric Fields and Interference Effects inside Noncentrosymmetric Multilayer Films at Electrode Surfaces from Electrochemically Modulated Surface Plasmon Resonance Experiments, Analytical Chemistry 69(18) (1997) 3665-3673.
[38] V. Lioubimov, A. Kolomenskii, A. Mershin, D.V. Nanopoulos, H.A. Schuessler, Effect of varying electric potential on surface-plasmon resonance sensing, Applied Optics 43(17) (2004) 3426-3432.
[39] Y. Iwasaki, T. Horiuchi, M. Morita, O. Niwa, Analysis of electrochemical processes using surface plasmon resonance, Sensors and Actuators B: Chemical 50(2) (1998) 145-148.

[40] Y. Iwasaki, T. Horiuchi, M. Morita, O. Niwa, Electrochemical reaction of Fe(CN)3−/4−6 on gold electrodes analyzed by surface plasmon resonance, Surface Science 427-428 (1999) 195-198.

[41] D.D. Schlereth, Characterization of protein monolayers by surface plasmon resonance combined with cyclic voltammetry 'in situ', Journal of Electroanalytical Chemistry 464(2) (1999) 198-207.

[42] S. Boussaad, J. Pean, N.J. Tao, High-Resolution Multiwavelength Surface Plasmon Resonance Spectroscopy for Probing Conformational and Electronic Changes in Redox Proteins, Analytical Chemistry 72(1) (2000) 222-226.

[43] E.S. Forzani, H. He, J. Hihath, S. Lindsay, R.M. Penner, S. Wang, B. Xu, Moving Electrons Purposefully through Single Molecules and Nanostructures: A Tribute to the Science of Professor Nongjian Tao (1963–2020), ACS Nano 14(10) (2020) 12291-12312.

[44] S. Wang, S. Boussaad, S. Wong, N.J. Tao, High-Sensitivity Stark Spectroscopy Obtained by Surface Plasmon Resonance Measurement, Analytical Chemistry 72(17) (2000) 4003-4008.

[45] J.E. Garland, K.A. Assiongbon, C.M. Pettit, D. Roy, Surface plasmon resonance transients at an electrochemical interface: time resolved measurements using a bicell photodiode, Analytica Chimica Acta 475(1) (2003) 47-58.

[46] K.J. Foley, X. Shan, N.J. Tao, Surface Impedance Imaging Technique, Analytical Chemistry 80(13) (2008) 5146-5151.

[47] X. Shan, U. Patel, S. Wang, R. Iglesias, N. Tao, Imaging Local Electrochemical Current via Surface Plasmon Resonance, Science 327(5971) (2010) 1363.

[48] W. Wang, K. Foley, X. Shan, S. Wang, S. Eaton, V.J. Nagaraj, P. Wiktor, U. Patel, N. Tao, Single cells and intracellular processes studied by a plasmonic-based electrochemical impedance microscopy, Nature Chemistry 3(3) (2011) 249-255.

[49] X. Shan, S. Wang, W. Wang, N. Tao, Plasmonic-Based Imaging of Local Square Wave Voltammetry, Analytical Chemistry 83(19) (2011) 7394-7399.

[50] Y. Wang, H. Wang, Y. Chen, Y. Wang, H.-Y. Chen, X. Shan, N. Tao, Fast Electrochemical and Plasmonic Detection Reveals Multitime Scale Conformational Gating of Electron Transfer in Cytochrome c, Journal of the American Chemical Society 139(21) (2017) 7244-7249.

[51] J. Liu, S. Tian, L. Tiefenauer, P.E. Nielsen, W. Knoll, Simultaneously Amplified Electrochemical and Surface Plasmon Optical Detection of DNA Hybridization Based on Ferrocene−Streptavidin Conjugates, Analytical Chemistry 77(9) (2005) 2756-2761.

[52] S.E. Salamifar, R.Y. Lai, Application of electrochemical surface plasmon resonance spectroscopy for characterization of electrochemical DNA sensors, Colloids and Surfaces B: Biointerfaces 122 (2014) 835-839.

[53] A.M. Dallaire, S. Patskovsky, A. Vallee-Belisle, M. Meunier, Electrochemical plasmonic sensing system for highly selective multiplexed detection of biomolecules based on redox nanoswitches, Biosensors & Bioelectronics 71 (2015) 75-81.

[54] J.A. Ribeiro, M.G.F. Sales, C.M. Pereira, Electrochemistry-assisted Surface Plasmon Resonance Detection of miRNA-145 at Femtomolar Level, Sensors and Actuators B: Chemical (2020) 128129.

[55] S. Sriwichai, A. Baba, S. Phanichphant, K. Shinbo, K. Kato, F. Kaneko, Electrochemically controlled surface plasmon resonance immunosensor for the detection of human immunoglobulin G on poly(3-aminobenzoic acid) ultrathin films, Sensors and Actuators B-Chemical 147(1) (2010) 322-329.

[56] A.H. Qatamin, J.H. Ghithan, M. Moreno, B.M. Nunn, K.B. Jones, F.P. Zamborini, R.S. Keynton, M.G. O'Toole, S.B. Mendes, Detection of influenza virus by electrochemical surface plasmon resonance under potential modulation, Applied Optics 58(11) (2019) 2839-2844.

[57] K.S. Phillips, J.H. Han, Q. Cheng, Development of a "Membrane Cloaking" Method for Amperometric Enzyme Immunoassay and Surface Plasmon Resonance Analysis of Proteins in Serum Samples, Analytical Chemistry 79(3) (2007) 899-907.

[58] H. Dong, X.D. Cao, C.M. Li, W.H. Hu, An in situ electrochemical surface plasmon resonance immunosensor with polypyrrole propylic acid film: Comparison between SPR and electrochemical responses from polymer formation to protein immunosensing, Biosensors & Bioelectronics 23(7) (2008) 1055-1062.

[59] J.A. Ribeiro, M.G.F. Sales, C.M. Pereira, Electrochemistry-Assisted Surface Plasmon Resonance Biosensor for Detection of CA 15–3, Analytical Chemistry 93(22) (2021) 7815-7824.

[60] K. Nakamoto, R. Kurita, O. Niwa, Electrochemical Surface Plasmon Resonance Measurement Based on Gold Nanohole Array Fabricated by Nanoimprinting Technique, Analytical Chemistry 84(7) (2012) 3187-3191.

[61] C. Wu, F.u. Rehman, J. Li, J. Ye, Y. Zhang, M. Su, H. Jiang, X. Wang, Real-Time Evaluation of Live Cancer Cells by an in Situ Surface Plasmon Resonance and Electrochemical Study, ACS Applied Materials & Interfaces 7(44) (2015) 24848-24854.

[62] T. Vandenryt, A. Pohl, B. Van Grinsven, R. Thoelen, W. De Ceuninck, P. Wagner, J. Opitz, Combining Electrochemical Impedance Spectroscopy and Surface Plasmon Resonance into one Simultaneous Read-Out System for the Detection of Surface Interactions, Sensors 13(11) (2013).

[63] M. Bart, P. van Os, B. Kamp, A. Bult, W.P. van Bennekom, Development of a confined wall-jet flow-through cell for simultaneous electrochemical and surface plasmon resonance applications, Sensors and Actuators B-Chemical 84(2-3) (2002) 129-135.

[64] J. Lu, W. Wang, S.P. Wang, X.N. Shan, J.H. Li, N.J. Tao, Plasmonic-Based Electrochemical Impedance Spectroscopy: Application to Molecular Binding, Analytical Chemistry 84(1) (2012) 327-333.

[65] C. Polonschii, S. David, S. Gáspár, M. Gheorghiu, M. Rosu-Hamzescu, E. Gheorghiu, Complementarity of EIS and SPR to Reveal Specific and Nonspecific Binding When Interrogating a Model Bioaffinity Sensor; Perspective Offered by Plasmonic Based EIS, Analytical Chemistry 86(17) (2014) 8553-8562.

[66] L. Yuan, N. Tao, W. Wang, Plasmonic Imaging of Electrochemical Impedance, Annual Review of Analytical Chemistry 10(1) (2017) 183-200.

[67] S. Wang, J. Zhang, O. Gharbi, V. Vivier, M. Gao, M.E. Orazem, Electrochemical impedance spectroscopy, Nature Reviews Methods Primers 1(1) (2021) 41.

[68] A. Bogomolova, E. Komarova, K. Reber, T. Gerasimov, O. Yavuz, S. Bhatt, M. Aldissi, Challenges of Electrochemical Impedance Spectroscopy in Protein Biosensing, Analytical Chemistry 81(10) (2009) 3944-3949.

[69] C. Pothipor, C. Lertvachirapaiboon, K. Shinbo, K. Kato, F. Kaneko, K. Ounnunkad, A. Baba, Development of graphene oxide/poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate) thin film-based electrochemical surface plasmon resonance immunosensor for detection of human immunoglobulin G, Japanese Journal of Applied Physics 57(2) (2018).

[70] S. Sriwichai, R. Janmanee, S. Phanichphant, K. Shinbo, K. Kato, F. Kaneko, T. Yamamoto, A. Baba, Development of an electrochemical-surface plasmon dual biosensor based on carboxylated conducting polymer thin films, Journal of Applied Polymer Science 135(1) (2018).

[71] A. Baba, T. Mannen, Y. Ohdaira, K. Shinbo, K. Kato, F. Kaneko, N. Fukuda, H. Ushijima, Detection of Adrenaline on Poly(3-aminobenzylamine) Ultrathin Film by

Electrochemical-Surface Plasmon Resonance Spectroscopy, Langmuir 26(23) (2010) 18476-18482.

[72] R. Janmanee, A. Baba, S. Phanichphant, S. Sriwichai, K. Shinbo, K. Kato, F. Kaneko, In situ Electrochemical-Transmission Surface Plasmon Resonance Spectroscopy for Poly(pyrrole-3-carboxylic acid) Thin-Film-Based Biosensor Applications, ACS Applied Materials & Interfaces 4(8) (2012) 4270-4275.

[73] A.W. Peterson, R.J. Heaton, R.M. Georgiadis, The effect of surface probe density on DNA hybridization, Nucleic Acids Research 29(24) (2001) 5163-5168.

[74] R.J. Heaton, A.W. Peterson, R.M. Georgiadis, Electrostatic surface plasmon resonance: Direct electric field-induced hybridization and denaturation in monolayer nucleic acid films and label-free discrimination of base mismatches, Proceedings of the National Academy of Sciences of the United States of America 98(7) (2001) 3701-3704.

[75] M. Manesse, V. Stambouli, R. Boukherroub, S. Szunerits, Electrochemical impedance spectroscopy and surface plasmon resonance studies of DNA hybridization on gold/SiOx interfaces, Analyst 133(8) (2008) 1097-1103.

[76] S. Patskovsky, V. Latendresse, A.M. Dallaire, L. Doré-Mathieu, M. Meunier, Combined surface plasmon resonance and impedance spectroscopy systems for biosensing, Analyst 139(3) (2014) 596-602.

[77] J. Liu, L. Tiefenauer, S. Tian, P.E. Nielsen, W. Knoll, PNA−DNA Hybridization Study Using Labeled Streptavidin by Voltammetry and Surface Plasmon Fluorescence Spectroscopy, Analytical Chemistry 78(2) (2006) 470-476.

[78] A. Vallée-Bélisle, F. Ricci, T. Uzawa, F. Xia, K.W. Plaxco, Bioelectrochemical Switches for the Quantitative Detection of Antibodies Directly in Whole Blood, Journal of the American Chemical Society 134(37) (2012) 15197-15200.

[79] S. Patskovsky, A.-M. Dallaire, M. Meunier, Electrochemical surface plasmon resonance sensing with absorptive redox mediator film, Sensors and Actuators B: Chemical 222 (2016) 71-77.

[80] Y. Mao, Y. Bao, W. Wang, Z. Li, F. Li, L. Niu, Layer-by-layer assembled multilayer of graphene/Prussian blue toward simultaneous electrochemical and SPR detection of H2O2, Talanta 85(4) (2011) 2106-2112.

[81] X. Kang, G. Cheng, S. Dong, A novel electrochemical SPR biosensor, Electrochemistry Communications 3(9) (2001) 489-493.

[82] J. Wang, F. Wang, H. Chen, X. Liu, S. Dong, Electrochemical surface plasmon resonance detection of enzymatic reaction in bilayer lipid membranes, Talanta 75(3) (2008) 666-670.

[83] O.A. Raitman, E. Katz, A.F. Bückmann, I. Willner, Integration of Polyaniline/Poly(acrylic acid) Films and Redox Enzymes on Electrode Supports: An in Situ Electrochemical/Surface Plasmon Resonance Study of the Bioelectrocatalyzed Oxidation of Glucose or Lactate in the Integrated Bioelectrocatalytic Systems, Journal of the American Chemical Society 124(22) (2002) 6487-6496.

[84] L. Tian, J. Qiu, Y.-C. Zhou, S.-G. Sun, Application of polypyrrole/GOx film to glucose biosensor based on electrochemical-surface plasmon resonance technique, Microchimica Acta 169(3) (2010) 269-275.

[85] A. Baba, P. Taranekar, R.R. Ponnapati, W. Knoll, R.C. Advincula, Electrochemical Surface Plasmon Resonance and Waveguide-Enhanced Glucose Biosensing with N-Alkylaminated Polypyrrole/Glucose Oxidase Multilayers, ACS Applied Materials & Interfaces 2(8) (2010) 2347-2354.

[86] K. Nakamoto, R. Kurita, O. Niwa, One-chip biosensor for simultaneous disease marker/calibration substance measurement in human urine by electrochemical surface plasmon resonance method, Biosensors and Bioelectronics 26(4) (2010) 1536-1542.

[87] S. Koide, Y. Iwasaki, T. Horiuchi, O. Niwa, E. Tamiya, K. Yokoyama, A novel biosensor using electrochemical surface plasmon resonance measurements, Chemical Communications (9) (2000) 741-742.

[88] Y. Iwasaki, T. Tobita, K. Kurihara, T. Horiuchi, K. Suzuki, O. Niwa, Imaging of electrochemical enzyme sensor on gold electrode using surface plasmon resonance, Biosensors and Bioelectronics 17(9) (2002) 783-788.

[89] X.-L. Zhou, Y. Yang, S. Wang, X.-W. Liu, Surface Plasmon Resonance Microscopy: From Single-Molecule Sensing to Single-Cell Imaging, Angewandte Chemie International Edition 59(5) (2020) 1776-1785.

[90] L.L. Zhang, X. Chen, H.T. Wei, H. Li, J.H. Sun, H.Y. Cai, J.L. Chen, D.F. Cui, An electrochemical surface plasmon resonance imaging system targeting cell analysis, Review of Scientific Instruments 84(8) (2013) 085005.

[91] M. Couture, S.S. Zhao, J.-F. Masson, Modern surface plasmon resonance for bioanalytics and biophysics, Physical Chemistry Chemical Physics 15(27) (2013) 11190-11216.

[92] Q. Chen, D. Zhang, J. Gu, H. Zhang, X. Wu, C. Cao, X. Zhang, R. Liu, The impact of antifouling layers in fabricating bioactive surfaces, Acta Biomaterialia 126 (2021) 45-62.

[93] B. Liu, X. Liu, S. Shi, R. Huang, R. Su, W. Qi, Z. He, Design and mechanisms of antifouling materials for surface plasmon resonance sensors, Acta Biomaterialia 40 (2016) 100-118.

[94] T. Špringer, M. Bocková, J. Homola, Label-Free Biosensing in Complex Media: A Referencing Approach, Analytical Chemistry 85(12) (2013) 5637-5640.

[95] J.E. Contreras-Naranjo, O. Aguilar, Suppressing Non-Specific Binding of Proteins onto Electrode Surfaces in the Development of Electrochemical Immunosensors, Biosensors 9(1) (2019).

[96] L.R. Schoukroun-Barnes, F.C. Macazo, B. Gutierrez, J. Lottermoser, J. Liu, R.J. White, Reagentless, Structure-Switching, Electrochemical Aptamer-Based Sensors, Annual Review of Analytical Chemistry 9(1) (2016) 163-181.

[97] S.C. Jena, S. Shrivastava, S. Saxena, N. Kumar, S.K. Maiti, B.P. Mishra, R.K. Singh, Surface plasmon resonance immunosensor for label-free detection of BIRC5 biomarker in spontaneously occurring canine mammary tumours, Scientific Reports 9 (2019).

[98] U. Eletxigerra, J. Martinez-Perdiguero, R. Barderas, J.M. Pingarrón, S. Campuzano, S. Merino, Surface plasmon resonance immunosensor for ErbB2 breast cancer biomarker determination in human serum and raw cancer cell lysates, Analytica Chimica Acta 905 (2016) 156-162.

[99] S. Choi, J. Chae, Reusable biosensors via in situ electrochemical surface regeneration in microfluidic applications, Biosensors & Bioelectronics 25(2) (2009) 527-531.

[100] O.R. Bolduc, J.F. Masson, Monolayers of 3-Mercaptopropyl-amino Acid to Reduce the Nonspecific Adsorption of Serum Proteins on the Surface of Biosensors, Langmuir 24(20) (2008) 12085-12091.

[101] S. Choi, J. Chae, Methods of reducing non-specific adsorption in microfluidic biosensors, Journal of Micromechanics and Microengineering 20(7) (2010).

[102] P. Lin, L. Ding, C.-W. Lin, F. Gu, Nonfouling Property of Zwitterionic Cysteine Surface, Langmuir 30(22) (2014) 6497-6507.

[103] D. Blasi, L. Sarcina, A. Tricase, A. Stefanachi, F. Leonetti, D. Alberga, G.F. Mangiatordi, K. Manoli, G. Scamarcio, R.A. Picca, L. Torsi, Enhancing the Sensitivity of Biotinylated Surfaces by Tailoring the Design of the Mixed Self-Assembled Monolayer Synthesis, ACS Omega 5(27) (2020) 16762-16771.

[104] H. Vaisocherová, E. Brynda, J. Homola, Functionalizable low-fouling coatings for label-free biosensing in complex biological media: advances and applications, Analytical and Bioanalytical Chemistry 407(14) (2015) 3927-3953.

[105] L.R. St. Hill, H.-V. Tran, P. Chinwangso, H.J. Lee, M.D. Marquez, J.W. Craft, T.R. Lee, Antifouling Studies of Unsymmetrical Oligo(ethylene glycol) Spiroalkanedithiol Self-Assembled Monolayers, Micro 1(1) (2021).

[106] S.C. Freitas, S. Maia, A.C. Figueiredo, P. Gomes, P.J.B. Pereira, M.A. Barbosa, M.C.L. Martins, Selective albumin-binding surfaces modified with a thrombin-inhibiting peptide, Acta Biomaterialia 10(3) (2014) 1227-1237.

[107] J.H. Ryu, P.B. Messersmith, H. Lee, Polydopamine Surface Chemistry: A Decade of Discovery, ACS Applied Materials & Interfaces 10(9) (2018) 7523-7540.

[108] H. Lee, M. Dellatore Shara, M. Miller William, B. Messersmith Phillip, Mussel-Inspired Surface Chemistry for Multifunctional Coatings, Science 318(5849) (2007) 426-430.

[109] M.E. Lynge, R. van der Westen, A. Postma, B. Städler, Polydopamine—a nature-inspired polymer coating for biomedical science, Nanoscale 3(12) (2011) 4916-4928.

[110] J. Byun, S. Cho, J. Moon, H. Kim, H. Kang, J. Jung, E.-K. Lim, J. Jeong, H.G. Park, W.K. Cho, T. Kang, Zwitterionic Polydopamine/Protein G Coating for Antibody Immobilization: Toward Suppression of Nonspecific Binding in Immunoassays, ACS Applied Bio Materials 3(6) (2020) 3631-3639.

[111] A. Blidar, B. Feier, M. Tertis, R. Galatus, C. Cristea, Electrochemical surface plasmon resonance (EC-SPR) aptasensor for ampicillin detection, Analytical and Bioanalytical Chemistry 411(5) (2019) 1053-1065.

[112] R. Pernites, R. Ponnapati, M.J. Felipe, R. Advincula, Electropolymerization molecularly imprinted polymer (E-MIP) SPR sensing of drug molecules: Pre-polymerization complexed terthiophene and carbazole electroactive monomers, Biosensors and Bioelectronics 26(5) (2011) 2766-2771.

[113] D. Balciunas, D. Plausinaitis, V. Ratautaite, A. Ramanaviciene, A. Ramanavicius, Towards electrochemical surface plasmon resonance sensor based on the molecularly imprinted polypyrrole for glyphosate sensing, Talanta 241 (2022) 123252.

[114] A. Garcia-Cruz, O.S. Ahmad, K. Alanazi, E. Piletska, S.A. Piletsky, Generic sensor platform based on electro-responsive molecularly imprinted polymer nanoparticles (e-NanoMIPs), Microsystems & Nanoengineering 6(1) (2020) 83.

[115] C.A.K. Borrebaeck, Precision diagnostics: moving towards protein biomarker signatures of clinical utility in cancer, Nature Reviews Cancer 17 (2017) 199.

[116] M. Adamcova, F. Šimko, Multiplex biomarker approach to cardiovascular diseases, Acta Pharmacologica Sinica 39(7) (2018) 1068-1072.

[117] P.S. Pakchin, S.A. Nakhjavani, R. Saber, H. Ghanbari, Y. Omidi, Recent advances in simultaneous electrochemical multi-analyte sensing platforms, TrAC Trends in Analytical Chemistry 92 (2017) 32-41.

[118] J.-H. Qu, A. Dillen, W. Saeys, J. Lammertyn, D. Spasic, Advancements in SPR biosensing technology: An overview of recent trends in smart layers design, multiplexing concepts, continuous monitoring and in vivo sensing, Analytica Chimica Acta 1104 (2020) 10-27.

[119] S. Wang, E.S. Forzani, N. Tao, Detection of Heavy Metal Ions in Water by High-Resolution Surface Plasmon Resonance Spectroscopy Combined with Anodic Stripping Voltammetry, Analytical Chemistry 79(12) (2007) 4427-4432.

Highlights

- Label-free detection by combining electrochemical techniques with SPR (eSPR);
- Simple and autonomous performing of the eSPR measurements;
- Application of the detection methodology in clinical diagnosis context;
- Detection of disease biomarkers at clinically relevant levels in biological samples.

Conflicts of Interest: The authors declare no conflict of interest.