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REVIEW

Surface plasmon resonance applications in clinical analysis

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Abstract In the last 20 years, surface plasmon resonance (SPR) and its advancement with imaging (SPRi) emerged as a suitable and reliable platform in clinical analysis for label-free, sensitive, and real-time monitoring of biomolecular interactions. Thus, we report in this review the state of the art of clinical target detection with SPR-based biosensors in complex matrices (e.g., serum, saliva, blood, and urine) as well as in standard solution when innovative approaches or advanced instrumentations were employed for improved detection. The principles of SPR-based biosensors are summarized first, focusing on the physical properties of the transducer, on the assays design, on the immobilization chemistry, and on new trends for implementing system analytical performances (e.g., coupling with nanoparticles (NPs). Then we critically review the detection of analytes of interest in molecular diagnostics, such as hormones (relevant also for anti-doping control) and biomarkers of interest in inflammatory, cancer, and heart failure diseases. Antibody detection is reported in relation to immune disorder diagnostics. Subsequently, nucleic acid targets are considered for revealing genetic diseases (e.g., point mutation and single nucleotides polymorphism, SNPs) as well as new emerging clinical markers (microRNA) and for pathogen detection. Finally, examples of pathogen detection by immunosensing were also analyzed. A parallel comparison with the reference methods was duly made, indicating the progress brought about by SPR technologies in clinical routine analysis.

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Abbreviations

Ab	Antibody
AChE	Acetylcholinesterase
Ag	Antigen
ALCAM	Activated leukocyte cell adhesion molecule
ALL	Aleuria aurantia lectin
ALP	Alkaline phosphatase
APS	Antiphospholipid syndrome
ATR	Attenuated total reflection
CEA	Carcinoembryonic antigen
CF	Cystic fibrosis
CFU	Colony-forming unit
CRP	C-reactive protein
CVD	Cardiovascular disease
DL	Detection limit
D-PBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
EPO	Eritropoietina
EW	Evanescent wave
GMO	Genetically modified organism
GNP	Gold nanoparticle
HBS-EP	HEPES buffer saline-HEPES containing EDTA and polysorbate 20
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HNA	Hexitol Nucleic Acid
HNSCC	Head and neck: squamous cell carcinoma
HSA	Human serum albumin
HRP	Horseradish peroxidase

IMA	Immunometric assays
LC	Liquid chromatography
LNA	Locked nucleic acid
mAb	Monoclonal antibody
MAC-ELISA	IgM antibody capture ELISA
MDS	Myelodysplastic syndromes
MIP	Molecular imprinted polymer
miRNA	Micro RNA
MNP	Metal nanoparticle
MORF	Phosphoramidates morpholino
MPA	Mercaptopropionic acid
MS	Mass spectrometry
MUA	Mercaptoundecanoic acid
MW	Molecular weight
NA	Nucleic acid
OEG	Oligo(ethylene glycol)
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PHA-L4	*Phaseolus vulgaris* lectin
PNA	Peptide nucleic acid
POC	Point of care
RA	Rheumatoid arthritis
RIU	Refractive index unit
RSD	Relative standard deviation
RT	Radiation therapy
RU	Resonance unit
SAT	Slide agglutination test
SNA-1	*Sambucus nigra* lectin
SELEX	Systematic evolution of ligands by exponential enrichment
SNP	Single nucleotide polymorphism
SPR	Surface plasmon resonance
SPRi	Surface plasmon resonance imaging
SPRCD	Surface plasmon resonance coupler and dispenser
SPW	Surface plasmon wave
TE	Tris HCl-EDTA
TM	Tris HCl-Mg

Introduction

Why SPR in clinical analysis?

Surface plasmon resonance (SPR) appeared as revolutionary technology almost 25 years ago, when the first commercial instrumentation was launched on the market by Pharmacia Biosensors AB, a Swedish company, derived from Pharmacia AB. The company developed an innovative technology by the joint effort of physicists, chemists, biologist, engineers, and computer scientists. Since then, many scientists joined the SPR to test new applications in various analytical fields, such as food safety [1] (e.g., mycotoxins [2], genetically modified organism (GMO) [3]), microbial contamination such as *Escherichia coli* [4], doping analysis [5], laboratory medicine [6, 7], proteomics [8, 9], bacteria detection [10], and also environmental monitoring [11]. Among them, assuredly, clinical analysis has also been explored as a fruitful application field.

The advantages brought about by current SPR technology include real-time monitoring of the analyte/molecular markers, label free and parallel analysis (with SPRi), minimal sample pretreatment, quantitative response, and very good sensitivity and reproducibility, (reported detection limits are in atto- or femtomolar ranges and coefficient of variations below 10 %). These features, coupled to miniaturization, make SPR suitable for point of care (POC) diagnostics [12], where fast analysis and multi-analyte detection are mandatory.

In this review, we focused on the analysis of target of interest in molecular diagnostics in complex and real matrices (e.g., serum, saliva, blood, and urine) but also in standard solution when the detection strategy is innovative and involves the improvement of analytical performances. So the panel of revised analytes includes hormones (steroids and peptides), protein clinical markers, antibodies involved in immune disorders, nucleic acids for genetic disease and as clinical markers (i.e., miRNA), bacterial cells, and viruses for pathogens detection. So far, most of the research articles come from academic exercises but we are more and more confident that the application of SPR to the clinical and medical analysis will gain momentum in the next future.

Principles of SPR biosensing

The physical principles and the state of the art of surface plasmon resonance [13–17] and surface plasmon resonance imaging-based [18–21] biosensors were reviewed in many excellent works.

At the beginning of the 20th century (1902), Wood was the first scientist who described the inhomogeneous distribution of light in a diffraction grating spectrum caused by surface plasmon wave (SPW) [22]. Sixty-six years later (1968), Kretschmann [23] and Otto [24] rigorously demonstrated the optical excitation of surface plasmons (SPs) with the method of attenuated total reflection (ATR).

SPR is defined as a charge-density oscillation at the interface between two media, with dielectric constants of opposite signs (e.g., metal and a dielectric), which generate a surface plasmon wave (SPW) with a propagation constant β, expressed by the following equation [13]:

$$\beta = \frac{\omega}{c}\sqrt{\frac{\varepsilon_M \varepsilon_D}{\varepsilon_M + \varepsilon_D}}$$

where ω is the angular frequency, c is speed of light in vacuum, and ε_D ε_M are the dielectric constants of dielectric and metal, respectively.

At the metal-dielectric interface the electromagnetic field of the SPW has as a maximum intensity that exponentially decreases (evanescent wave, EW) into both media with a variable penetration from 100 to 600 nm (for VIS and NIR wavelengths) [25].

Prism couplers, grating couplers, or metal-dielectric waveguides are the configuration used for the excitation of surface plasmons but the first one represents the most common approach for the plasmon excitation via the attenuated total reflection method (ATR) with Kretschmann geometry becoming also the most suitable for sensing and biosensing applications.

In the Kretschmann geometry, the light wave is totally reflected at the boundary between a thin metal layer (typically gold or silver with 50 nm of thickness) and a high refractive prism coupler (typically in glass). The reflected light excites the surface plasmons of the metal film generating an EW (or SPW) penetrating the metal layer (Fig. 1).

SPR based biosensor belongs to refractometric devices, since the propagation of the SPW is sensitive to changes in the refractive index of the dielectric. The binding between analytes and bioreceptors immobilized on the sensing surface causes a local change in refractive index (Fig. 1) and the

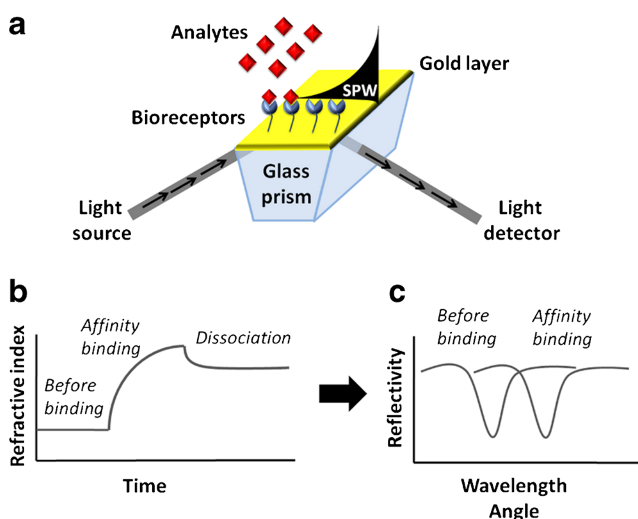

Fig. 1 In a SPR biosensor based on a prism coupler and working in Kretschmann geometry with the ATR method, light is totally reflected by a thin gold layer (50 nm) that excites the surface plasmons (SPs) and generates a SPW (**a**). The affinity binding between bioreceptors and analytes causes a change of refractive index vs. time (**b**) entailing a shift of the reflectivity curve vs wavelength of source light or angle of reflected light (**c**)

variation of the propagation constant β, generating a real time signal in label-free way, since no labels have to be added in solution for the development of the analytical signal. Classic SPR spectroscopy is based on the monitoring of coupling angle, coupling wavelength, intensity, phase [15], whereas SPR imaging (SPRi) is based on the measurement of reflectivity of monochromatic incident light at a fixed angle by means of a CCD camera. Compared with traditional SPR, SPRi offers main breakthroughs such as multiplexed detection (by designing 2-D patterned microarrays of molecular probes) and the real-time visualization of the whole biochip surface to monitor simultaneously multiple bioreceptor/analyte bindings with the parallel support of a digital image, representing the intensity of binding in a color scale [19]. On the other side, SPRi suffers from order of magnitude worse resolution than classical SPR (10^{-6} versus 10^{-7} RIU) [17].

Bioreceptors

Biosensor selectivity depends on the bioreceptors immobilized on the sensing surface. Here we will present and discuss works that appeared in the last years aiming at developing robust and reliable SPR assays in clinical diagnostics. Among bioreceptors, we can enumerate antibodies, nucleic acid probes or synthetic receptors (molecular imprinted polymers (MIPs), aptamers, or artificial DNA (XNA). Accordingly, it is customary to talk about immunosensors, nucleic acid sensor, aptasensor, and so on. An immunosensor uses the affinity interaction between an antigen and an antibody [26, 27]. Nucleic acid (NA) sensors are based on oligonucleotidic probes and NA hybridization reaction [25, 28]. In NA sensors, artificial nucleosides (XNA) oligomers have also been employed as bioreceptor for clinical applications. XNAs increase sensor stability by avoiding biodegradation or exploiting improved affinity of XNA versus the target sequence compared with conventional DNA probes. D'Agata et al. reviewed works dealing with the great potential of artificial DNA (PNA, LNA, HNA, and MORF) in nucleic acid SPR-based sensing, exhibiting higher selectivity and sensitivity in detecting complementary or mismatching nucleic acid targets [29].

Behind that, aptasensors emerged in the last years as an interesting alternative to immunosensing. Aptamers are NA molecules that can bind to predetermined targets (small molecules as well as proteins and peptides) with high affinity and specificity [30, 31]. These nucleic acid aptamers are engineered through repeated rounds of in vitro selection or equivalently with systematic evolution of ligands by exponential enrichment (SELEX) [32, 33].

Finally, MIP sensors take advantage of totally synthetic molecular recognition elements, constituted by highly cross-linked polymers engineered to bind one target compound or

a class of structurally related target compounds with high selectivity [34, 35].

Interesting applications to clinical diagnostics have recently been reported for synthetic receptor such as XNA, but still very few are using aptamers and MIPs instead.

Bioreceptors have first to be immobilized on the sensing surface and for this purpose, different approaches were reported. Furthermore, the suitable assay format has to be applied depending on the clinical application. Both aspects will be discussed in the next paragraphs.

Bioreceptors (biomolecules) immobilization

In SPR-based biosensor development, the immobilization of the biomolecule on the sensing surface is a key step essential for the success of the analysis. In particular the features to be considered for the optimal bioreceptors immobilization procedure deal with the retentions of biological activity for the analyte after immobilization, the compatibility between binding activity and range of analyte detection, amount of immobilized bioreceptor (to avoid steric hindrance), reproducibility of immobilization (to assure reproducibility of results), and finally the regenerability of the biosensing surface (receptor-analyte dissociation without compromising the biological activity of the receptor). For the sensor applicability to real matrices, the immobilization chemistry should be optimized to also prevent unspecific interaction on the surface arising from matrix components (i.e., proteins in blood, serum, etc.).

The binding of biomolecules to gold-sensing surface can be achieved through many suitable immobilization approaches, such as physical absorption, hydrophobic (via lipid layer), electrostatic interactions, covalent coupling (coupling of nucleophiles to carboxylic groups, to thiol groups or to aldehyde groups), and coupling of native and tagged molecules (avidin/biotin) [36]. Among them, the last two approaches are surely the most exploited in clinical diagnosis application for cheapness, ease of realization, stability, and robustness.

In particular, nucleotides are efficiently immobilized after modification with the thiol group, exploiting the high affinity between sulphur and gold surface or, alternatively, with biotin labeling via affinity interaction with avidin or streptavidin coated chip surface. In both strategies, the formation of a self-assembled monolayer (SAM) increases the degrees of freedom of the bioreceptor and, consequently, the bindings with analytes.

Proteins (e.g., antibodies) and other peptides are widely immobilized by covalent amine coupling between amino groups of proteins and activated (with NHS/EDC solutions) carboxylic linkers of alkanethiols (or disulfide) in SAM format or carboxymethylated dextran (3D matrix immobilization). These approaches are rapid, simple, and inexpensive but bring about random orientation of bioreceptors (attributable to multiple functional groups on the protein), which could decrease the conformational flexibility, hindering the bioreceptors/analytes interaction and thus lowering the sensor sensitivity. An alternative strategy for a controlled orientation is, instead, based on the affinity reaction between biotin-conjugated protein and (strept)avidin-coated chip. In addition, the interaction between Fc and protein G or A could be also exploited for the Abs immobilization.

Preventing nonspecific adsorption of biomolecules (e.g., protein) on the SPR sensing surface is another key-step for the development of specific biosensor, with real application to clinical diagnostics where complex matrices (such as serum, blood, and urine) are analyzed. A strategy frequently adopted is the fuctionalization of surface with non-fouling materials resistant to nonspecific interaction such as glycol-derivate oligo/polymers: oligo(ethylene glycol) (OEG), poly(ethylene glycol)(PEG), EG6-COOH, EG3-OH but also poly(L-lysine) grafted with poly(ethylene glycol) (PLL-g-PEG) [17]. The success of protection from nonspecific interaction is due to steric-entropic barrier properties coupled with a high degree of hydration of PEG molecules as reported by Blättler et al. [37].

Assay format

Two assay formats, direct and indirect, were mostly adopted on SPR biosensor for the detection of analyte in clinical diagnosis (schemes in Fig. 2). The assay selection is based on the MW analyte, on the bioreceptors/analyte affinity, and on the matrices under investigation.

In the direct assay, bioreceptors are immobilized on the sensing surface and analytes are added in solution. The affinity interaction causes a refractive index variation, providing analytical signals proportional to analyte concentration. This strategy is usually adopted for analyte with MW greater than 5000 Da, sufficient to develop a refractive index variation and resulting in detectable signal. Moreover with a multi-step direct assay (Fig. 2) DLs can be further lowered using a secondary bioreceptor (specific for analyte) in a sandwich format. Labeled secondary bioreceptors (with biomolecules or nanoparticles) are often employed to increase the assay sensitivity.

Indirect assays are preferred for low MW analytes (55×10^3 Da). Here, purified analytes (or conjugates) are immobilized on the SPR surface, while specific bioreceptors are incubated with the samples to be analyzed and injected to sensing surface; when the solution reaches the surface, only remaining free bioreceptors bind to immobilized analyte, providing a signal inversely proportional to its concentration in the sample. Besides, secondary bioreceptors (labeled or not) can be added in solution to further improve sensitivity and lowering DLs with multi-step assays.

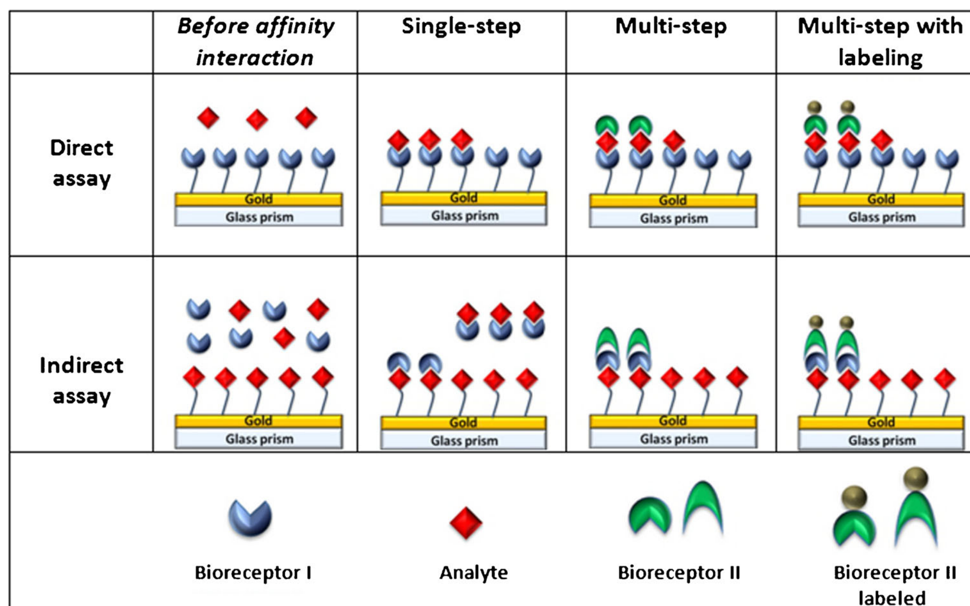

Fig. 2 Direct and indirect assay schemes in single-step and multi-step (with labeling) detection in SPR sensing

Trends for improvements of analytical performances in SPR sensing

Recently, many developments in SPR technology were performed for the improvement of sensor analytical performances in terms of sensitivity, specificity and detection in real matrixes [38]. A strategy was offered by nanoparticles coupled to plasmonic sensors in miniaturized and low cost systems [39].

In 2011, Gao et al. examined recent works in engineering hybrid SPR interfaces such as oxide-based hybrid SPR interfaces and nanocomposite films, analyzing the optical properties and the suitable applications in sensing [40]. More recently (2012), Bedford et al. have specifically reviewed the recent trend of incorporation of GNPs within the sensing surface itself or as tagging molecules in SPR biosensor. Significant sensitivity increases and DLs decreases were described for a variety of analytes [41]. As reported by Minunni and Spoto, the increase of sensitivity is explainable by both the mass enhancement of the NPs in the EW as well as by the optical coupling between surface plasmons (SPs) and electric fields of localized surface plasmons (LSPs) of NPs when they are close to the metallic surface [38]. LSPs are defined as collective electron oscillations confined in the MNP surface and promoted by interaction with the light. If the MNP dimension is much smaller than the wavelength of the excitation light, the local electromagnetic fields are strongly enhanced. A further contribution to the increase of sensitivity is also expected if NPs clustered since it was proven that clusters of spherical metal and dielectric colloids show strong electric, magnetic, and Fano-like resonances from the electromagnetic coupling between closely spaced particles [38].

Zanoli et al. reviewed the employment of functionalized gold nanoparticles for ultrasensitive DNA detection on different transduction systems (including SPR), useful to bypass PCR amplification steps [42]. Since we have previously defined the SPR-based biosensor as a label-free system, for the sake of clarity we specify that the addition of bioreceptors labeled with nanoparticles is not employed for signal development itself but for the improvement of system analytical performance (sensitivity increases and DLs decreases), really essential in clinical diagnostics where analytes are in complex matrices at very low concentrations.

Some drawbacks of NPs uses could be found in the NPs synthetic step, in tuning dimensions, and absorption wavelengths (for coupling between plasmons), since these steps are time- and reagent-consuming. Furthermore, it seems constrictive to employ NPs in reusable sensors since surface poisoning could occur, compromising serial measurements on the same chip: no data of regeneration are available in literature. Very recently, graphene-based surface plasmon resonance interfaces were also studied for suitable application in SPR sensor. The functionalized surfaces showed several advantages such as high surface-to-volume ratio, adsorption of organic or biological molecules (pi stacking), passivation against oxidation, and controlling the number of graphene layers [43]. Other strategies, different from NPs-based approach, have been also adopted in this last decade for the improvement of SPR based sensor. In 2011, Abbas et al. collected the last forefront tendencies in SPR instrumentation with advanced performances identified in: new excitation schemes and optical configurations, liquid handling by microfluidics, and microsystems for integrated SPR chips generally based on glass or poly(dimethylsiloxane) (PDMS). New material such conducting metal oxides ZnO and ZnO/Au in the adhesion layer to replace Cr or Ti were also discussed as well as forefront SPR-related optoelectronic components (light source

and detector) and finally the SPR coupling with other analytical techniques (hyphenation approach with scanning probe microscopy, electrochemistry, fluorescence and Raman spectroscopy) were also evaluated for the achievement of higher sensitivity and resolution [44].

After focusing on different features of the SPR technology (physical principle and instrumental details with the most recent trends) on bioreceptors immobilization approaches and on assay designs (indirect and direct, simple, multistep), we move on to review the applications of these strategies to real cases of interest in clinical diagnostics.

Hormones detection

Hormones and small peptides analysis is a key area in diagnostic and, more recently, also in anti-doping controls. Endocrine diseases diagnosis may be difficult, and it requires to directly assaying hormone levels in blood or eventually making indirect measurements. For example, diabetes mellitus is diagnosed via measurements of blood glucose rather than direct assays of plasma insulin.

Some interesting examples of hormones detection are provided by different authors, demonstrating the ability of SPR to provide interesting solutions also in this clinical area.

Frasconi et al. developed a SPR immunosensor for the detection of cortisol and cortisone levels in urine and saliva samples with an Eco Chemie Autolab SPR system. The measurement of free cortisol level in these matrices was an indicator for adrenal or pituitary gland disorder, and in doping analysis a marker of glucocorticoids illicit use. Urine samples (3 mL) were hydrolyzed by an incubation for 1 h at 50 °C with 1 mL of PBS (pH 7.4), 50 μL of 17α-methyltestosterone (internal standard), and 50 μL of β-glucuronidase from *Escherichia coli*. Saliva samples were analyzed without pretreatment. Specific antibodies were immobilized on polycarboxylate-hydrogel-based coatings, and the proposed method resulted to be simple, inexpensive, reproducible (RSD% <10 %), and sensitive, with detection limits less than 10 μg /L (~2.8 nM). A linear detection range from 5 to 154 μg/L for cortisol and 30–174 μg/L for cortisone was found and a very good correlation with the reference LC/MS-MS method was proven, confirming the potential utility of SPR for clinical, pharmacological and anti-doping application [45].

Estriol-16-glucuronide, a hormone for the monitoring of ovarian function, was detected by Jiang et al. with Biacore X-100 system in urine samples from nonpregnant and pregnant subjects. The authors developed an inhibition (indirect) immunoassay by the steroid immobilization on the sensor surface, through estriol-16-glucuronide-ovalbumin conjugate with an oligoethylene glycol (OEG) as linker. Au nanoparticle-Ab conjugate (Au-Ab) was used to enhance the sensitivity of the SPR assay. A detection limit of 0.016 μg/L (~34 pM) in urine diluted with TM buffer (1:5, 1:10 and 1:30) was rapidly achieved in 2 min without complicated sample pretreatment and with a good reproducibility (CV% <0.71 %). The assay was fast and inexpensive compared with traditional method based on RIA, HPLC-MS, or HPLC with UV and fluorescence detection. It required less clean-up steps, resulting an efficient way to monitor E3-16G production rates in urinary samples of a normal menstrual cycle [46].

Besides the analysis of very low MW hormones, SPR sensing has been also applied to the detection of peptide hormones such as insulin (MW 5750 Da), which regulates carbohydrate metabolism. Insulin sensing in serum is greatly important for clinical diagnostics and to follow-up patients affected by various types of diabetes but also for doping control.

Recently (2010) Frasconi et al. detected insulin with indirect immunoassay in human serum samples from healthy and diabetic patients using Eco Chemie Autolab SPR system. Hydroxyl/LC-PDP-functionalized G4-PAMAM dendrimer-encapsulating GNP was covalently bound by amino coupling to an alkanethiolates (carboxylated) SAM-modified gold surface. Then insulin was covalently immobilized on the functionalized surface exploiting the amino-coupling chemistry for the immunoassay (Fig. 3).

The innovative surface chemistry reduced nonspecific surface interactions and the effect of coupling between localized plasmon of the NPs and surface plasmon lowered the DL down to 0.5 pM (~2.9 ng/L). Ten-fold diluted sera were analyzed in the 2–43 pM linear concentration range and radioimmunoassay reference method confirmed the reliability of the analytical device; indeed the biological level for insulin in the diabetic patients is 85–500 pM, corresponding to a range 8.5–50 pM in a 10-fold diluted sample. Moreover, the sensor provided reproducible results (CVs ranged between 3.5 % and 4.9 %) and it was reusable up to 40 times [47].

Other high MW peptide hormones, such as human pituitary hormones, were recently detected with SPR. In particular, Lechuga et al. developed binding inhibition (indirect) immunoassays on a SPR from Sensia SL, where hormones were immobilized on the sensor surface by amino-coupling and working conditions (assay buffer and regeneration solution) were optimized. They detected first hGH (growth hormone) in human serum samples (mixed 1:1 in PBST), essential for development and normal growth [48]. Then they also performed, using the same strategy, the single and multi-analyte determination of two other gonadotropic hormones in urine and serum sample (both mixed 1:1 in PBST): follicle stimulating hormone (hFSH) and luteinizing hormone (hLH) involved in the development and function of the reproductive system [49]. Finally they detected hGH, hLH, hFSH, and hTSH (thyroid gland stimulation for thyroxine production) in urine and serum sample (again both mixed 1:1 in PBST)

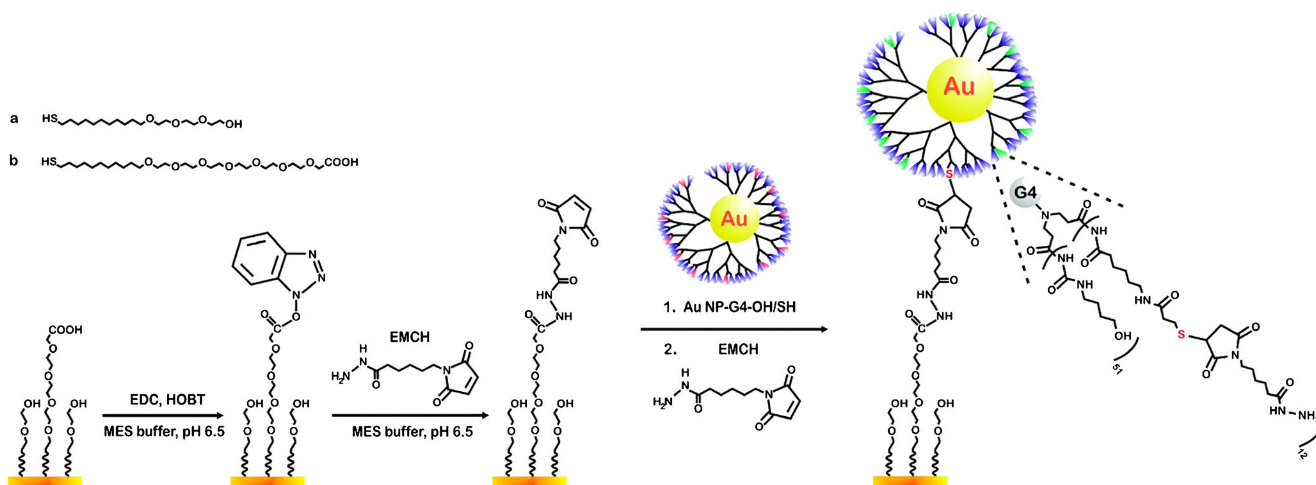

Fig. 3 Immobilization of hydroxyl/LC-PDP-functionalized G4-PAMAM dendrimer-encapsulated Au nanoparticle onto mixed SAMs of alkanethiolates on gold derived from tri(ethylene glycol)-terminated thiol (**a**) and the hexa(ethylene glycol) carboxylic acid-terminated Thiol (**b**). Reprinted with permission from Frasconi M, Tortolini C, Botre F, Mazzei F (2010) Multifunctional Au nanoparticle dendrimer-based surface plasmon resonance biosensor and its application for improved insulin detection. Anal Chem 82:7335–7342. Copyright (2010) American Chemical Society [47]

using an indirect immunoassay coupled to multi-analyte sensor. All biosensors resulted sensitive (about μg/L (~40 pM) as DL for each analyte), reusable from 50 to 100 consecutive assay cycles and specific for each analyte. The SPR based immunoassays resulted reproducible with mean intra- and inter-day CVs about 7 %, appearing as a highly reliable tool for endocrine real-time and daily monitoring in clinical laboratory compared to official methods as RIA, IMA or ELISA [50].

As reported by Segura in 2009, there are many areas of common interest and overlaps between clinical analysis and anti-doping monitoring such as methodological similarities, analysis of human biological matrices, and detection of the same analytes (e.g., hormone). The author was confident that the mutual interaction between the fields would be able to bring mutual improvements in terms of technological solutions and innovative strategy [51].

In the anti-doping code, the list of prohibited substances, under the category Peptide Hormones, Growth Factors and Related Substances, a variety of hormones and their releasing factors can be found: (1) erythropoiesis-stimulating agents [e.g., erythropoietin (EPO), darbepoetin (depo), hypoxia-inducible factor (HIF) stabilizers, methoxy polyethylene glycol-epoetin beta (CERA), peginesatide (hematide)]; (2) chorionic gonadotrophin (CG) and luteinizing hormone (LH) and their releasing factors, in males; (3) corticotrophins and their releasing factors; (4) growth hormone (GH) and its releasing factors and insulin-like growth factor-1 (IGF-1). In addition, several growth factors are also banned [52].

An excellent review on the potential use of SPR in anti-doping analysis is provided by the Segura group where it is highlighted how conventional analytical methods for detecting human biotics as doping agents (i.e., recombinant products), may hardly distinguish between endogenous and exogenous origin. For larger molecules (i.e., protein hormones) the innate structural complexity, the heterogeneous nature, and the extremely low levels in biological fluids made the analytical procedures heavily dependent from the immunological approaches [5].

Protein biomarkers

A protein biomarker is an indicator of a specific biological stage used in clinical diagnosis to monitor the stage of related diseases, to guide molecularly targeted therapy, and to evaluate a therapeutic response [53]. The increasing number of clinically relevant protein biomarkers and the advances of proteomics techniques indicate the need of reliable methods for their detection in complex matrices. At this purpose, SPR and SPRi provide a suitable platform for daily routine clinical analysis thanks to real-time and label-free detection [54].

In this section, we focused on the most recent assays based on SPR biosensor for the detection of protein biomarkers for multiple diseases first and then specific for cancer and cardiac diseases.

Protein biomarkers for multiple diseases

The C-reactive protein (CRP, 115 kDa) is an important blood serum marker for inflammatory processes (e.g., cardiovascular disease (CVD), inflammatory bowel diseases), currently detected by latex-enhanced turbidimetric immunoassay.

Jung et al. developed a label-free array system using amide-linked (AL) NHS-dextran Biacore CM5 chip for CRP detection in human sera. After AFM studies, they found this surface

more suitable for proteins immobilization than a normal epoxide-linked carboxymethyl-dextran layer. Monoclonal anti-CRP antibodies were immobilized onto the surface and rapid analysis of CRP was performed down to 20 μg/L (~0.8 nM) concentration in 120 human sera diluted (1:10 in PBS, pH 7.4); results showed a good correlation with the turbidimetry reference immunoassay [55].

Bini et al. immobilized a biotinylated 44 mer RNA aptamer for CRP on a carboxylated dextran-streptavidin-modified CM5 chip of a Biacore X. In the assay optimization they evaluated the effect of different buffers, the effect of Ca^{2+} ion on the interaction between the aptamer and CRP, and finally the specificity of the aptasensor against putative interfering biomolecules. The highest SPR signals and the lowest detection limit (5 μg/L, ~0.2 nM, suitable for clinical applications) were recorded with the following experimental condition: serum diluted 1:100 with HEPES buffer at pH 6.5 and 2 mM Ca^{2+} concentration. CV% was estimated to be about 11 % and a liner correlation was found within 0–500 μg/L range of concentration. Experiments in serum solution showed an interfering effect caused by IgG, suggesting the need of sample pretreatment [56].

The two assays described above were suitable for clinical monitoring of CRP because the protein is about 0.8 mg/L in adult serum (above the achieved DLs) [56].

Circulating Annexin A5 (anxA5, 35 kDa), a biomarker related to various diseases (acute myocardial infarction, trauma, thrombosis, inflammation, and cancer) was rapidly and inexpensively detected in human blood samples (diluted 1:10 in HBS-EP) by Trouvé et al. using a Biacore 3000. They immobilized anxA5 antibody on a carboxymethylated dextran CM5 sensor chip by amino coupling, and they found for the first time that the level of circulating anxA5 was higher in a male 5.43 (±0.02) μg/L than in a female 4.41 (±0.20) μg/L, indicating a difference by gender. A good linearity was observed between 3.3 and 3.8 μg/L with a good correlation (R^2=0.9511) and CV% below 5 %. These results proved that the label-free SPR biosensor was a very good alternative to the label-based conventional ELISA immunoassay with advantages such as very good sensitivity, high reproducibility, and fast responses (in few min) [57].

Cancer biomarkers

The long-term outcome of cancer therapy depends on early diagnosis and the response to therapy. An important aspect of cancer management includes careful monitoring of cancer biomarkers in physiological fluids [58]. Vaisocherová et al. used a home-built SPR instrument for the immunodetection in human serum of ALCAM [59] (activated leukocyte cell adhesion molecule/CD 166), a 100–105 kD transmembrane glycoprotein biomarker of pancreatic cancer, typically with a concentration of 100 μg/L in human serum [60]. Anti-ALCAM was immobilized via physical adsorption on positively charged amine-terminated alkanethiol SAM surface. Despite nonspecific binding recorded, a sufficiently low DL was achieved (10 μg/L, ~98 pM in serum diluted 1:10 in PBS) for the discrimination of ALCAM levels in cancer cases from control sera. A good reproducibility was also confirmed by the CV% less than 5 %. The direct detection (without signal amplification) showed also an excellent correlation with ELISA reference method [59].

In the same year, Ladd et al. developed an immunosensor for the simultaneous and specific detection of ALCAM and transgelin-2 (TAGLN2), a 22-kDa protein that has been a biomarker of breast and colorectal carcinoma). Specific antibodies were immobilized by amino-coupling on COOH-OEG-coated gold sensor chip of a home-built SPRi platform. DLs for ALCAM and TAGLN2 in PBS buffer were 6 μg/L (~59 pM) and 3 μg/L (~0.1 nM), respectively. No cross-reactivity was recorded but high levels of nonspecific adsorption were found with serum solution (1:10 in PBS), indicating the need of highly non-fouling surfaces in sensor applications [60].

In 2010, Piliarik et al. combined high-resolution home-built SPRi sensor and high-density protein array with low-fouling background for the parallel detection of protein cancer biomarkers ALCAM and hCG (human chorionic gonadotropin) in diluted blood plasma samples (10 % in TE buffer). Specific antibodies were immobilized by amino coupling on a low-fouling chip surface (BSA immobilized on carboxyl-terminated thiols). The biosensor showed a very low nonspecific protein adsorption (less than 5 ng/cm^2), and a linear dependence between response and analyte concentration was found (below 250 μg/L for hCG and below 500 μg/L for ALCAM). The following DLs were reached: 45 μg/L (0.44 nM) for ALCAM and 100 μg/L (4.6 nM) for hCG and a very good reproducibility was confirmed by the CVs% less than 10 % [61].

In 2011, Kazuno et al. developed a novel SPR biosensor based method for multi-sequential detection of SNA-1, AAL, and PHA-L4 lectins to estimate the glycosylation of haptoglobin (a complementary marker to CA125 in ovarian cancer) in sera of patients with prostate cancer disease. The method involved anti-haptoglobin immobilization by amino-coupling, sera dilution 1:1400 in HBS-P buffer, filtration, and finally detection of the sugar chain by lectin solution. A calibration curve for haptoglobin was obtained in 0–500 mg/mL concentration range. Multi-sequential analysis of SNA-1 and haptoglobin represented an accurate diagnosistic approach for prostate cancer. The label-free SPR biosensor was less complex and time-consuming than conventional ELISA, where labeling step with enzymes, radioactive isotopes, or fluorescein are required [58].

Gill et al. quantified p38αMAP kinase, a prognostic marker in head and neck squamous cell carcinoma (HNSCC), by an

immunosensor (Biacore 2000). They investigated the correlation between p38α and HNSCC in diluted serum (1:99 in HBS-EP) of patients undergoing radiation therapy (RT). They immobilized anti-p38α Abs by amino-coupling on a CM5 sensor chip and used Western blot and ELISA as reference methods. Patients with HNSCC showed 3-fold increase in p38α levels (0.61 mg/L ~ 16 nM) compared with control sample while values during-RT and post-RT treatments decreased to 0.35 mg/L (~9.2 nM) and 0.30 mg/L (~7.9 nM), respectively, showing a reduction attributable to a clinical tumor regression by RT. These results confirmed the suitability of p38α as a serum marker in HNSCC, and that if coupled to SPR sensing offers higher sensitivity than traditional antibody-based methods such as ELISA and Western blotting [62].

Pimková et al. developed SPR biosensor with dispersionless microfluidics (home-built) for the detection of sVEGFR-1 (soluble vascular endothelial growth factor receptor), a biomarker abnormally produced from cancer cells in myelodysplastic syndromes (MDS). VEGF-A (homodimeric glycoprotein) was covalently immobilized on the sensor surface by amino coupling, allowing the detection of sVEGFR-1 in human blood (2 % in PBS) down to a DL of 25 μg/L (0.14 nM) and with a good reproducibility between 85 % and 96 %. The approach suggested a model for future protein multi-array for MDS diagnosis based on protein–protein interactions. The assay resulted in being less sensitive than conventional detection of sVEGFR-1 based on ELISA, capable of detecting 0.02 μg/L and up to 0.5 μg/L, respectively, for healthy individuals and MDS patients [63].

An innovative approach for the same biomarker detection was presented by Liu et al. where VEGF was released in solution by living SKOV-3 ovarian cells (i.e., cancer cell) (Fig. 4). Anti-VEGF Abs were immobilized on a protein G-coated chip surface, and the analyte produced in the flow chamber (in PDMS) was directly monitored. The linearity of the response in the 0.5–4.0 mg/L VEGF concentration range

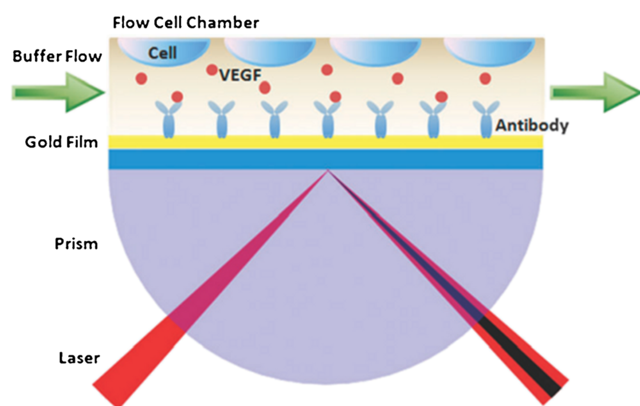

Fig. 4 Configuration of the SPR based biosensor integrating a mini cell culture module for direct measurement of biomarker from living cells. Reproduced by permission of The Royal Society of Chemistry [64]

and reproducibility (inter-assay RSDs% less than 13.6 %) were assessed in PBS buffer. Further, the cell viability was demonstrated and then the in vivo microenvironment of the VEGF signaling pathways was mimicked. From the quantification of VEGF released by cells, the carcinoma cell number was accurately predicted, showing the suitability of an innovative strategy for the real-time monitoring of biomarker expressed from living cells [64]. We underline here VEGF is also a target molecule in anti-doping analysis, present in the WADA list of prohibited substances [52].

Battaglia et al. developed a fiberoptic SPR biosensor for the simultaneous detection of three cytokines related to chronic wound healing, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-r (TNF-r), reaching a detection limit below 1 μg/L in HBS buffer and in spiked cell culture medium (CCM). Specific antibodies were immobilized on the SPR fiberoptics by aminocoupling, and no nonspecific bindings from the cell culture medium proteins were observed on the biosensor surface. The sensor emerged as a reliable device for multiple and specific biomarker detection since the IL-1 and TNF-R concentrations are ~ 1 μg/L in normally healing wounds and 50 and 15 μg/L, respectively, in chronic wounds. Moreover, the IL-6 levels are ~ 0.2 μg/L (not detectable) in a normally healing wound and ~ 3 ng/mL (detectable) in not healing wounds [65].

Cardiac biomarkers

Cardiac markers are proteins released by injured myocardial cells into the bloodstream during cardiovascular diseases (CVDs) and so are useful to better diagnose CVD conditions promptly and efficiently [66].

In particular, brain natriuretic peptide (BNP, 3.5 kDa) is a hormone secreted mainly from the cardiac ventricle into the blood, frequently monitored as marker in cardiac failure [67].

Teramura et al. developed a sandwich-type immunosensor for BNP detection in donor plasma (not pretreated) using nanobeads for signal amplification (Fig. 5). Primary anti-BNP Abs were immobilized on COOH-SAM chip surface (amino coupling) of a home-built SPR instrument, and a sandwich assay was performed with a secondary biotinylated antibody after immunoaffinity interaction with BNP. Finally, streptavidin-conjugated nanobeads (50 nm in diameter) were added for signal amplification, enhancing sensitivity, and lowering DL from 10 μg/L (2.9 nM) to 25 ng/L (7.2 pM). Linearity was observed in the 0–1000 ng/L BNP concentration range, confirming the suitability of this immunosensor for clinical necessities since the normal level of BNP in plasma is about 20 ng/L but it increases approximately to 1 μg/L during acute or chronic cardiac failure. Sensitivity was comparable to commercially available reference assays for BNP (RIA and chemiluminescent reactions (4–2000 ng/L) [68].

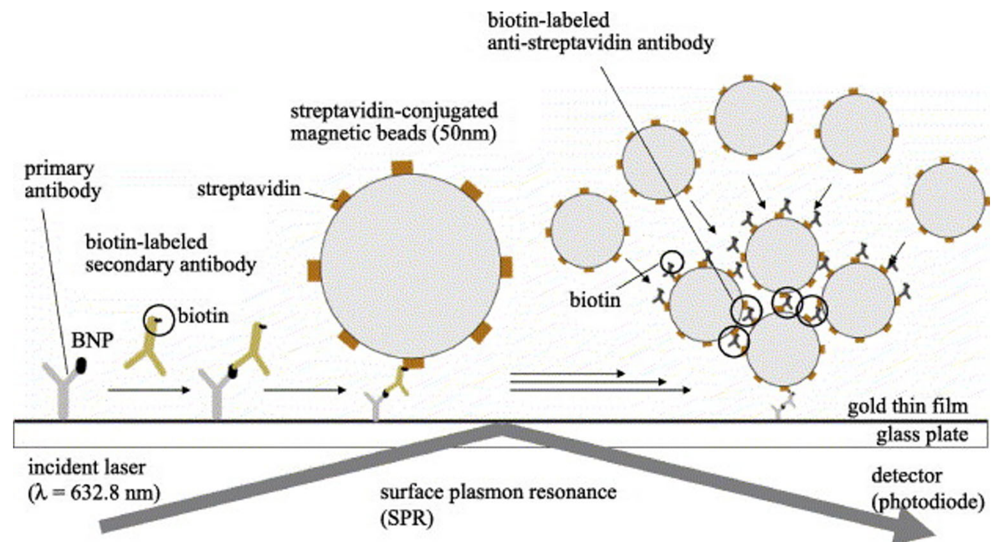

Fig. 5 Schematic illustration of SPR-based sandwich-type immunoassay for BNP and amplification of SPR signal by accumulation of streptavidin nanobeads. Reprinted with permission from Elsevier [68]

In addition, an indirect immunoassay coupled to portable SPR instrumentation (with developed microfluidic) was also reported for BNP detection in human serum (1:10 in 0.1 M PBS). Samples were incubated with acetylcholine esterase-labeled antibodies and then introduced into the microchannel of the device so that only free-BNP conjugate bound to BNP previously immobilized on the surface (by amino-coupling). Then acetylthiocholine was added as substrate and hydrolyzed by acetylcholine esterase. The thiol compound (thiocholine) produced covalently bound to a thin gold layer located downstream in the microchannel, generating a SPR angle shift monitored as analytical signal. A concentration range from 5 to 100 ng/L was examined and trace levels of analyte (15 fg) were detected in about 30 min. CV% for five measurements of 100 ng/L BNP was 33.1 % and although this value was high compared with a commercial immunosensing system, the authors considered the RSD% acceptable for clinical diagnosis since BNP concentration significantly increases in heart failure patients [69], as mentioned above.

Two other proteic myocardial infarction biomarkers, myoglobin (MG) and cardiac troponin I (cTnI), were quantified at biological levels in undiluted serum and without any sample pretreatment, using a fiberoptic SPR immunosensor (Jobin-Yvon SPEX 270 M Spectrometer). Specific antibodies were immobilized via amino coupling on a 16-mercaptohexadecanoic SAM, minimizing the nonspecific signal the serum proteins. DLs were 0.9 μg/L (50 pM) for MG and 0.7 μg/L (30 pM) for cTnI, well below threshold limits to detect myocardial infarction disease (approximately 15–30 μg/L for MG and 1–3 μg/L for cTnI). A good reproducibly (CV% <13 %) and linear correspondences between signals and concentrations up to 50 μg/L were also assessed for both analytes [70].

Liu et al. designed a SPR biosensor with high anti-fouling ability for the detection of the cardiac marker troponin T in D-PBS buffer. Anti-troponin T antibodies were immobilized by amino-coupling on the chip surface (Navi 200 SPR) coated with a homogeneous SAM of oligo(ethylene glycol) (OEG)-terminated alkanethiolate and mercaptohexadecanoic acid (MHDA). The system showed interesting high anti-fouling properties (high resistance to nonspecific adsorption of HSA) and high affinity and specificity for the detection of troponin T. A good linear correlation (R=0.991) below 50 μg/L and a DL of 100 μg/L (5.6 nM) were also confirmed [71].

Antibodies for immune disorders

Biosensing plays a crucial role in the analysis of autoantibodies in human serum for the real-time monitoring of autoimmune diseases. An increasing number of researches were available in literature. Schlichtiger et al. recently reviewed different biosensor-based approaches in this field. Authors also emphasized the main advantage of SPR for monitoring bindings in real-time and for multiplexed analyses of autoantibodies by use of microarrays (e.g., SPRi) [72].

The detection of pathogenic anti-dsDNA Abs in sera of patients affected by lupus erythematosus (SLE) was reported Buhl et al. An antigenic construct was formed as follow: a synthetic oligonucleotide 5′-aldehyde-modified (CHO) was coupled to biotinylated human transferrin using 4-hydrazinonicotinate acetone hydrazone (SANH) as covalent linker; then it was hybridized with the complementary anti-strand and ligated with a human recombinant dsDNA fragment 233 bp in length. The conjugate was finally immobilized on a gold surface coated with carboxymethyldextran functionalized with streptavidin, and diluted sera (1:100 in diluents from SLE patients and healthy donors) were analyzed with the SPR biosensor system (Biacore X). As a result, authors confirmed SLE in patients with 98.2 % specificity at a sensitivity

of 83.3 % compared with positive results in the Farr RIA assay with 88.1 % specificity at a sensitivity of 87.5 %. Moreover, a very good reproducibility was confirmed by intra-assay imprecision (1.2 %–1.8 %) and day-to-day variation (8.0 %), making the SPR biosensor really suited for anti-dsDNA Abs detection in SLE disease. In addition, the device provided information about binding kinetics and affinities of the specific autoantibodies [73].

The same group also studied interactions between dsDNA and anti-dsDNA autoantibodies from SLE sera of patients, again with Biacore X and with the same dsDNA immobilization strategy previously described. They characterized kinetics, off-rates, and functional affinities (avidities) for three anti-dsDNA mAbs, confirming the importance of kinetic properties for the explanation of behavior of mAbs in traditional methods such as the Farr RIA and ELISA [74].

Metzger et al. developed a biosensor for the diagnosis of antiphospholipid syndrome (APS) through the discrimination of disease-relevant autoantibodies (anti-β2-GPI) from cross-reactive antibodies developed in other infections. Human β2-GPI (β2-glycoprotein I) was covalently linked to a CM5 chip surface coated with n-alkanethiol SAM (Biacore X). Sera (1:100 in HBS) from APS patients or patients with SLE, syphilis, parvovirus B19, and healthy donors were analyzed and the results were compared with those from ELISA test. No significant antibody bindings (signal <35 RU) were recorded in samples from healthy individuals or patients with other infections, whereas response levels in the range of 50–500 RU were recorded from positive APS patient sera, with a correlation coefficient of 0.87 with reference ELISA test. The SPR-based assay was also reproducible since no significant loss of activity (<8 %) was recorded after 50 measurement cycles of patient sera [75].

In a later study, Müller et al. used the same instrumental setup to prove the correlation between the affinity of anti-β2-GPI in patient sera (1:100 in HBS) and the antigen β2-GPI preparations (manufacturer, origin, and purification method). The study explained the inter-assay differences of anti-β2-GPI ELISAs, confirming that only one common β2-GPI preparation would have improved the inter-assay comparability [76].

In the same year, the authors also demonstrated that the employment of β2-GPI-derived domain-specific peptides would have offered diagnostic advantages in primary autoantibody screening for APS and in discrimination of sera of APS patients from sera of healthy patients [77].

Schlichtiger et al. detected antiphospholipid antibodies (aPL, a serological indicators of the disease) with an immunosensor developed on Biacore X platform. In particular, they detected cardiolipin antibodies (aCL) in sera (1:10 in HEPES) from healthy donors and APS patients. Cardiolipin was immobilized by amino coupling (after activation with EDC/NHS) on 11-mercaptoundecanoic acid SAM-coated chip surface. The binding Ag/Ab was monitored and the assay confirmed APS with 100 % diagnostic specificity, equal to the standard ELISA method used in routine diagnostics but showing more sensitivity than ELISA (100 % versus 85.7 %). Moreover, the chip was regenerable (up to 15 measurements) with excellent intra-chip reproducibility (RSD%=3.6 %) and chip-to-chip reproducibility (RSD%=2.6 %) [78].

Rutgers et al. reported kinetic analysis of anti-GBM autoantibodies, from sera of nephritic patients, performed with Biacore 2000 instrumentation. Purified bovine collagen α1(IV)NC1 (control) and α3(IV)NC1 (Ag) were immobilized onto a carboxymethylated dextran hydrogel surface after EDC/NHS activation. Autoantibodies from patient sera bound to α3(IV)NC1 whereas no binding was recorded to α1(IV)NC1 (control). From the estimation of the dissociation and association constants, they demonstrated the high affinity Ab/Ag, the high velocity of association and the slowness of dissociation, explaining the rapid course of the disease as well as the resistance to therapy (persistent Ab glomerular aggregation could generate potentially incessant inflammation) [79].

Alaedini et al. detected autoantibodies against the monosialotetrahexosyl ganglioside GM1 (anti-GM1) related to acute and chronic motor neuropathies. GM1 (Ag) and GM2 (control) gangliosides were absorbed on a methyl dextran layer of a chip and the discrimination between patients sera and healthy controls was possible with sensitivity and specificity comparable to classic ELISA tests [80].

Antiglutamic acid decarboxylase (GAD) autoantibody detection, an indicator of type I diabetes mellitus, was developed by Lee et al. with Biacore 2000. The ratio between 3-MPA and 11-MUA (10:1) thiols was first optimized for covalent binding of biotinylated GAD via streptavidin immobilization (after NHS/EDC activation). Anti-GAD Abs were analyzed in HBS buffer within 0.5–4.0 μM concentration range and the interference with other biomolecules (BSA as control) was found to be negligible [81].

Carlsson et al. detected insulin autoantibodies (IAA) in serum samples from individuals at high risk of developing type 1 diabetes (T1D). They designed an indirect competitive immunoassay to bypass nonspecific adsorption of matrix proteins that could mask the analytical signal. Insulin was immobilized on CM5 chip of a Biacore X after activation with NHS/EDC and the sensor was calibrated with IAA (0–40 U/mL) in sera derived from pooled nondiabetic serum spiked with pooled high IAA-positive serum to obtain identical matrix composition. Excellent assay performances were reported since analysis time was 100-fold reduced from 4 days to 50 min, and the sensitivity was comparable to that offered by RIA; the cut-off for positivity was 6.45 U/mL (in healthy Swedish children) [82].

A parallelized immunoassays on SPR microarray imaging (IBIS Technologies) was used by Lokate et al. to detect anti-

citrullinated protein antibodies (ACPA) in sera of patients (1:50 in PBST) affected by rheumatoid arthritis (RA). Carboxylated Xantec HC 200 nm sensor chips were activated with NHS/EDC and then two different linear citrullinated peptides (CitA and CitB) were immobilized on the surface; ArgA and ArgB (with arginine instead of citrulline) were spotted as corresponding control peptides. Interactions between citrullinated peptides and serum autoantibodies of 50 RA patients and 29 controls were monitored with an experimental DL of 0.5 pM. Although the sensitivity was slightly lower than that of the reference ELISA test, the SPR system presented the advantage of automation and surface regeneration by repetitive incubations with 10 mM glycine•HCl for 30 s, making the test really suitable for daily routine clinical control [83].

Three years later, a similar approach was applied by van Beers et al. with IBIS-iSPR (imaging) for the analysis of the autoantibodies against peptide fractions in RA patients diluted sera (50-fold in PBST). Peptides obtained from citrullinated human fibrinogen were immobilized on a dextran hydrogel surface (after activation with NHS/EDC) and three major citrullinated epitope were identified. The multi-array enabled the simultaneous detection of 24 autoantibodies/peptides interactions with a significant reduction of analysis time, resulting in less time-consuming and more accurate than reference ELISA screening methods [84].

SPR imaging-based sensing was also used by Scarano et al. for anti-bovine IgG detection in untreated human serum and milk since high levels of these antibodies are related to Type 1 diabetes in serum of children. Bovine IgGs were immobilized in microarray-mode (SPRi Lab$^+$, Horiba) by amino coupling on MUA-coated gold chip surface. The nonspecific adsorption and the matrix effect were evaluated by a dedicate software [85] aimed to optimize a guided automated selection of best-reacting spot (Fig. 6). Anti-bovine IgG was detected in a range of concentrations from 0.1 to 1.0 mg/L in real diluted matrices (1/40 for serum and 1/100 for milk in HEPES buffer) with the standard addition method. A good linear response (R^2=0.998), a good reproducibility among experiments (CV%=3.0 %), and a DL of 2.8 mg/L (18 nM) were achieved in serum while in milk the equally good performances were evaluated as R^2=0.997, CV%=5.6 %, and DL=11 mg/L (18 nM). The assay opened new horizons for the detection of protein in complex real matrices with SPRi-based biosensing, since clinical data (e.g., in serum) displayed anti-bovine igG values up to 258 mg/L with median value of 19 mg/L [86].

Carcinoembryonic antigen (CEA) autoantibodies (specific for CEA, a biomarker associated to colorectal, gastric, and pancreatic cancer) were monitored in cancerous human serum samples by Ladd et al. with a home-built SPR instrument. CEA was immobilized on a carboxylated gold surface by aminocoupling and autoantibodies were detected by a sandwich assay adding a secondary antibody (goat anti-human IgG-HRP conjugate). Sera were diluted in PBSA buffer (1:100, 1:200, and 1:400) and tested; CEA autoantibody concentrations were around 1 mg/L, resulting ~2.5 μg/L after dilution (1:400). Autoantibodies were distinguishable in cancerous sera samples from the healthy sample, using ELISA as reference assay. This finding could be used as a diagnostic criterion for early cancer detection as well as for the diagnosis of disease progression [87].

Nucleic acid sensing in clinical diagnosis

Recently, two excellent reviews summarized the progresses made by SPR- and SPRi-based nucleic acid (NA) sensing [25, 28] and their employment in clinical analysis.

Many oligonucleotidic analytes of interest in medical diagnostics have been detected by NA SPR based sensing. Here we reviewed the NA assays in two main classes: assay for the detection of chromosome anomalies (i.e., point mutations and SNPs) and assay for the detection of genetic material as biomarkers (i.e., microRNAs and biomarkers of pathogens). As for the other analytes, we focused only on NA assays applied to real matrices with the exception of those developed in standard solution that in our opinion improved on the analytical approach or the system analytical performance.

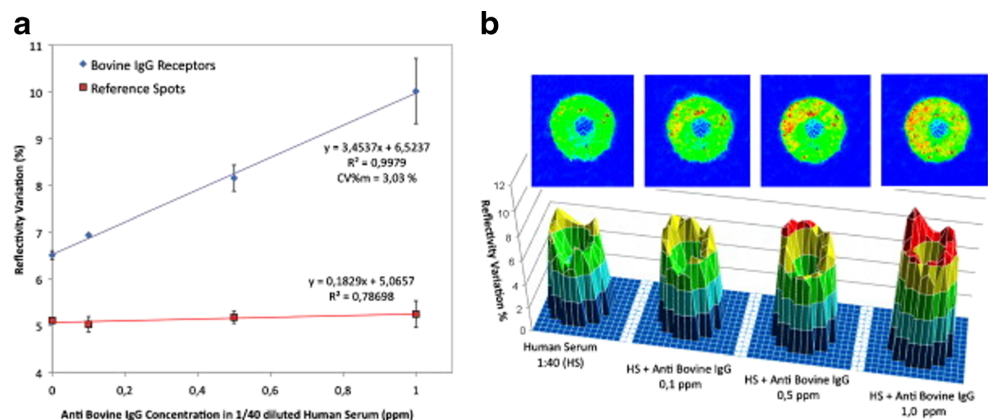

Fig. 6 (**a**) Anti-bovine IgG calibration in 1/40-diluted human serum. (**b**) Difference images of one bovine IgG spot of the array after the binding event with anti-bIgG at different concentrations and, lower, 3D elaboration of the same binding data obtained by "Data Analyzer." Reprinted with permission from Elsevier [86]

Point mutation detection

The study of chromosome anomalies by SPR in real samples deals with the detection of point mutation and single nucleotide polymorphisms (SNPs), which are point mutations that occur with frequencies >1 %. Both mutations may have remarkable involvements in clinical diagnosis if they occur in a specific coding region of chromosome-related to common diseases [88, 89].

In particular, SPR was used as a tool for the detection of point mutation related to tumor suppressor genes (e.g., TP53 [90–93] and K-Ras [94]), cancer disease [95, 96], and hereditary disease [97–102].

The tumor suppressor gene TP53 is the mostly mutated gene in presence of human cancers [103] and, for this reason, it is one of the most studied genes in cancer research also with assays-based on SPR transduction systems.

In 2005, Jiang et al. detected TP53 point mutation with a DNA SPR-based sensing using portable instrumentation (a Texas Instruments Spreeta). Synthetic thiolated oligonucleotides (26 mer) were immobilized on the gold chip surface as capturing probe for the SNP analysis of real DNA samples (extracted and amplified at a 0.15 μM concentration) from both normal wild-type (Jurkat) and mutated (Molt 4) cell lines. The biosensor distinguished between sequences differing by only one base in the SNP position (signal lowered by 34 % for the Molt 4) in only 2 min with a very low nonspecific response and with the ability to regenerate the sensor up to 50 times. These findings coupled to the good reproducibility (CV%=8 %) proved that the method has potential for routine clinical analysis [90].

In the same year, Wilson et al. reported the use of the MutS, a natural protein able to recognize selectively only mismatching sequences, for the detection of SNP in 1 μM synthetic oligonucleotides (TP53 analogue) with the same instrument. The prior injection of SSB (single strand binding) protein prevented the nonspecific interaction between Muts and thiolated ssDNA immobilized on the sensor. The sensor was regenerable for 50 times (at least) making the whole approach really inexpensive for the detection of clinically relevant mutations [91].

Sipova et al. developed a sandwich-like assay for the detection of SNP (codon 273) in a short (20 mer) TP53 synthetic analogue with a four-channel SPR biosensor developed at the Institute of Photonics and Electronics (Prague). Immobilized thiolated probes hybridized the target while streptavidin–oligonucleotide (SON) complexes investigated the polymorphic site with a secondary hybridization (enhancement). A good sensitivity down to 40 pM and a good specificity were achieved in the detection [92].

With the beginning of the nanotech era, metal noble nanoparticles assumed a central role in the development of more sensitive assays for point mutation discrimination. Yao et al. developed a method based on sandwich-like assay coupled to nanoparticles, for the detection of SNPs again in TP53 gene (excised from the pC53-SN3 plasmid and 1794 bp) with a home-built SPR. Thiolated oligonucleotides were linked to GNPs (signal enhancer) for the SNP discrimination down to 1.38 f. target concentration in 15 μL of sample. The method was adequately reproducible (RSD% <16 %) and specific thanks to the carboxylated dextran film that prevented the nonspecific adsorption of nucleotide-capped GNPs. Therefore, the SPR-based test was attractive when analyte concentrations were very low and the sample availability was very limited [93].

Another original approach, based again on NPs coupled to SPR imaging DNA sensor (SPR imager, GWC Technologies), was developed by the Corn group for the high sensitive detection of SNPs of clinical interest. In particular, picomolar detection of SNP in BRCA1 gene, associated with breast cancer, was reached in non-amplified human DNA samples by measurements of surface enzymatic ligation reactions enhanced by gold nanoparticles. The coupling of SPRi and thiolated DNA microarrays for SNP genotyping was very attractive because it could be multiplexed for the simultaneous analysis of multiple SNPs [95].

The same instrument was used by Spoto and coworkers to further enhance SNP detection with the employment of streptavin-coated GNPs linked to biotinylated oligonucleotide and PNA probes immobilized on a gold surface previously functionalized with dithiobis(*N*-succinimidylpropionate) (DTPS). Femtomolar sensitivity was reached with synthetic DNA target in PBS buffer, making the strategy suitable for detecting DNA samples without PCR amplification [104]. Indeed, 3 years later the same approach (Fig. 7) was applied for the SNP discrimination in the human β globin gene involved in several type of β thalassemia, directly in non-amplified genetic material (isolated from leucocytes in peripheral blood) and down to attomolar concentration bypassing the PCR step [97].

As mentioned in the Introduction, the labeling with NPs was demonstrated to be useful for the improvements of analytical performances such as increase of sensitivity and lowering of DLs. With this strategy, genomic DNA was directly detected without any PCR amplification step but, on the other side, synthesis of NPs was time- and reagent-consuming. Interesting examples of the combined use of artificial DNA and SPR, able to enhance sensitivity of diagnostic SNP investigations, were eventually reported in literature [29].

In 2001, Feriotto et al. reported the first application of PNAs and SPR to human hereditary mutations. In particular, these authors detected the W1282X SNP on CF gene, associated to cystic fibrosis, with Biacore 1000 SPR. PCR-amplified samples were biotinylated and immobilized on the sensor surface with streptavidin-biotin chemistry while PNA probes were hybridized on the immobilized PCR products for the

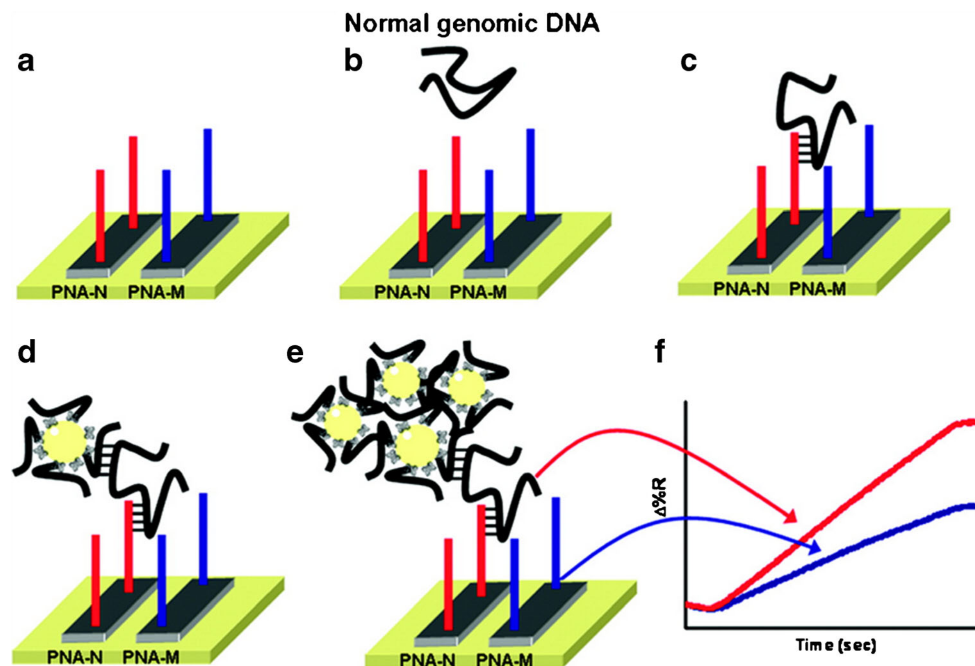

Fig. 7 Pictorial description of the nanoparticle-enhanced SPRI strategy used to detect the normal βN/βN, heterozygous β°39/βN, and homozygous β°39/β°39 genomic DNAs. In order to simplify the pictorial representation, only specifically adsorbed DNA is shown. Nonspecifically adsorbed DNA is also present on the surface and contributes to generate the SPRI-detected signal. PNA-N and PNA-M specifically recognize the normal β-globin and the mutated β°39-globin genomic sequences, respectively. Reprinted (adapted) with permission from D'Agata R, Breveglieri G, Zanoli LM, Borgatti M, Spoto G, Gambari R (2011) Direct detection of point mutations in nonamplified human genomic DNA. Anal Chem 83:8711–8717. Copyright (2011) American Chemical Society [97]

recognition of polymorphism. The reported results suggested that SPR was an easy, fast (few min), and automatable platform for detecting mutations with real-time monitoring of hybridization, unlike other methods based on gel electrophoresis and/or dot-spot analysis [98].

Three years later, Corradini et al. used Biacore 1000 to discriminate the same SNP in CF gene (5 μM) with chiral chains modified PNA (based on D-lisine), improving the recognition ability compared to simple PNA [99].

In other approaches reviewed below, the SNP discrimination was performed without the employment of enhancing factor (i.e., protein or nanoparticles) or artificial DNA, speeding the assays and lowering costs.

Recently (2013), Ermini et al. have developed a very sensitive (140 aM) and label-free DNA sensor for the specific detection of non-amplified ABCB1 gene fragment (extracted from human lymphocytes) on SPRi Lab$^+$ platform (Horiba Scientific) [105]. ABCB1 is a key gene in pharmacogenomics, with many SNPs implicated in a common set of problems such as altered drug levels and susceptibility to diseases (e.g., inflammatory bowel disease, Parkinson's disease, refractory seizures, and CD4 cell recovery during human immunodeficiency virus therapy) [106]. The sandwich assay was based on the optimization of sample pretreatment (fragmentation and denaturation) coupled to a rational design of very selective and performing probes. In particular, thiolated capturing probes, immobilized on the sensor surface, hybridized the target (ABCB1 gene fragment) while a secondary probe hybridization confirmed the selectivity of the capturing [105].

In the same year, the assay described before was optimized (secondary probes labeling and length) and successfully applied for the detection of rs1045642 SNP (in the ABCB1 gene) related to susceptibility of many diseases such as colorectal [107], breast, and renal cancer [108]. SNP discrimination was performed in genomic DNA extracted from human lymphocytes and randomly enriched at 6 mg/L (~2.8 fM) by whole genome amplification (WGA). The measures were reproducible (CV% <9 %) and the sensor was reusable for up to 20 times [96].

MicroRNAs as clinical marker

MicroRNAs (miRNAs) are important gene regulatory nucleic acids (about 22 mer) in humans, affecting transcriptional and post-transcriptional regulation of gene expression [109]. miRNA also has a key role as marker of serious disease (i.e., cancer [110, 111], heart disease [112, 113], diabetes [114], nervous system diseases [115], and liver disease [116], included from high femtomolar to low nanomolar range of concentration in human blood [25, 117]. However, miRNAs are not yet mentioned in literature coupled to SPR for clinical routine analysis; below, we reviewed some academic researches for the detection of miR-122, the most abundant miRNA in

hepatocytes with an achievable future key role in clinical diagnosis as a predictive marker for viral, alcohol, and chemical-induced liver disease [116].

A fast and simple assay was recently (2013) described by Zhang et al. for the detection of miR-122 extracted from human breast tumor cells. The detection was based on a simple and hybrid sandwich-like assay: miR-122 was hybridized from a fully complementary thiolated DNA probe immobilized on the sensor surface using a Biacore X. A secondary biotinylated DNA probe (linked to streptavidin) was hybridized to the target, enhancing sensitivity and lowering the detection limit down to 17 pM [118]. In addition, the assay was PCR-free and showed a good reproducibility (CV%=5.7 %).

Corn and coworkers described an innovative approach for the detection of miRNA, extracted from mouse liver tissue, based on thiolated LNA microarray immobilized on the gold chip surface (SPR imager, GWC Technologies). Simultaneous detection of miR-16, miR-122b, and miR-23b from mouse liver tissue, was achieved down to 10 fM, enhancing SPRi transduction with poly-(A)enzyme chemistry and T30-coated Au nanoparticles (Fig. 8). Despite the high sensitivity and specificity, the method required an elaborate multistep protocol [119].

A portable NA sensor based on SPRCD (coupler and dispenser) with a special diffraction grating was developed by Sipova et al. Nonamplified miR-122 extracted from mouse liver tissue was captured by thiolated DNA immobilized on the sensor surface. A specific antibody recognized DNA/RNA-formed hybrid, enhancing the response and allowing the detection in less than 30 min and down to 2 pM (results that were in good agreement with those obtained using qPCR) [120].

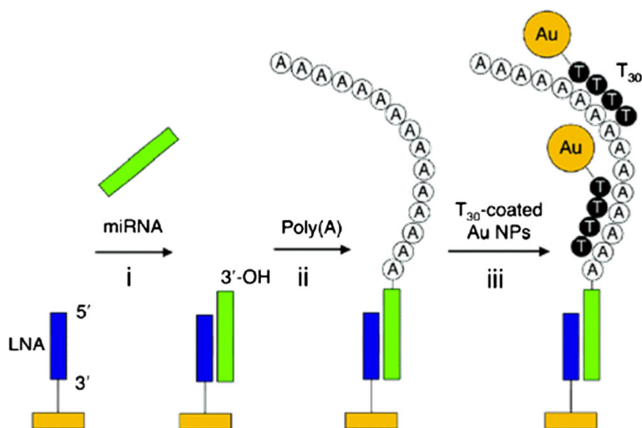

Fig. 8 Schematic showing the detection of microRNAs using a combination of surface polyadenylation chemistry and nanoparticle amplified SPRI detection: (*i*) hybridization adsorption of miRNA onto a complementary LNA array element; (*ii*) addition of poly(A) tails to the surface bound miRNAs using poly(A) polymerase; and (*iii*) hybridization adsorption of T30-coated Au nanoparticles to poly(A) tails detected by SPRI measurements. Reprinted (adapted) with permission from (Fang S, Lee HJ, Wark AW, Corn RM (2006) Attomole microarray detection of micrornas by nanoparticle-amplified SPR imaging measurements of surface polyadenylation reactions. J Am Chem Soc 128(43):14044–14046). Copyright (2006) American Chemical Society [119]

Although the two described assays were developed for the detection of miRNA from mouse liver, they represented excellent model systems for the improvement of analytical performance (e.g., DLs lowering) suitable for next applications to detection of human miRNA samples.

Nucleic acid as bacterial marker

Since current methods for the detection of pathogens are expensive, time consuming, and involve culture-based techniques [121–123], SPR platforms were also employed for the survey of bacteria (e.g., *Escherichia coli*) in environmental monitoring and in clinical diagnostics, through the detection of bacterial genetic material as biomarker [124].

Kai et al. developed a method for the rapid detection of verotoxin-producing *Escherichia coli* O157:H7 isolated from stools, amplified by PCR, and using TE as sample buffer. Biotinylated PNAs (bioreceptors) were immobilized on a streptavidin-coated sensorchip SA of Biacore 2000. A good correlation was found with positive results for PCR (10^2 CFU per 0.1 g of stool) samples from patients and healthy carriers. The sensor was reusable for 100 times after regeneration with a washing solution (50 mM NaOH) [125].

Wang et al. detected simultaneously four pathogenic microorganisms (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clostridium tetani*, and *Clostridium perfringens*). DNA was extracted from the four pathogens, amplified simultaneously by universal primers (16S RNA) and then hybridized on a SPR-based multichannel sensor with specific DNA probes immobilized by thiol chemistry. The assay was specific, fast, and with DLs down to 10 pM (for *C. tetani*). In addition, a good linear dependence (R^2>0.99) was recorded between signals and PCR products concentrations (0.1–100 nM). Although the DNA was extracted from commercial bacterial strains and not from human matrices, the study clearly showed potential of multi array for high analytical productivity in clinical diagnostics (15 min for four specific interactions) [126].

Finally, in 2012 Zhang et al. developed a label-free and sensitive method for the detection of invA gene, extracted from bacterial culture of *Salmonella* and amplified by PCR. Biotinylated ssDNA probes were immobilized on streptavidin-coated dextran chip surface of a Biacore X. The sensor was previously calibrated with synthetic target DNA and a good linearity from 5 o 1000 nM and a detection limit of 0.5 nM were assessed. Detection of *Salmonella* was possible as low as 100 CFU/mL within 4.5 h and the excellent regeneration of sensor surface (up to 300 cycles binding/regeneration) allowed sensor reuse with cost reduction [127].

Pathogens detection by immunosensing

Beyond the identification of pathogens via NA detection, other approaches based on the developing of different SPR immunosensors were reported. In particular, we reviewed the pathogens detection in real matrices, and examples in buffer solution were reported only when these approaches were found very promising.

Viruses

SPR-based detection of antibodies against viral pathogens in medical diagnostics was previously reviewed by Homola in 2008. In particular, the review focused on antibodies detection for hepatitis G and C, hepatitis B, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), Epstein-Barr virus, Varicella-Zolter virus, human respiratory syncytial virus, and adenovirus [17].

Antibodies for herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) were detected with Biacore X in diluted human serum (1:100 in HBS buffer). Biotinylated peptides, from corresponding segments of the N-terminus of HSV-1 and HSV-2 glycoprotein B (gB), were immobilized as antigen on chips coated with streptavidin. SPR biosensor discriminated specific Ab/Ag binding better than conventional reference method (ELISA), evaluating low-avidity background reactivity recorded frequently in human sera [128].

Abad et al. detected anti-adenoviral antibodies (rAd/p53) in diluted sera of patients (1:10 in HEPES buffer) after dosing with an adenoviral-based gene therapy vector. Dextran and amino-coupling chemistry were used for the immobilization of intact virus on chip surface of a Biacore 3000. The assay was useful for designing more efficient dosing schedules automated and requiring less analysis set-up time than reference ELISA method [129].

Antibodies detection against hepatitis G in patient serum was performed by Rojo et al. with synthetic peptide (antigens) immobilized on Biacore 1000 via dextran and aminocoupling chemistry, finding a good correlation with the ELISA reference test [130].

The same chemistry of immobilization was used by McGill et al. for the detection of human respiratory syncytial virus (RSV) in diluted serum (1:10 in HBS buffer) with Biacore 2000. Antibodies against virus glycoproteins (F and G) were covalently immobilized on dextran for the discrimination of the antigenic differences between the two virus genotypes (F- and G-glycoproteins) [131].

An indirect assay was described by Chung et al. for the detection of antibodies against human hepatitis B virus (hHBV) in diluted patient serum (5 % in PBS). hHBV antigens were immobilized (via aminocoupling) on the Spreeta surface platform and DLs of 9.2 nM or 0.64 nM (after amplification with avidin-biotinylated secondary antibodies) were achieved, with very similar results of the reference ELISA kit for the diagnosis of hepatitis [132].

Regnault et al. developed an assay for the detection of anti-protein S antibodies, expressed after Varicella-Zolter viral diseases in diluted plasma (20 % in HBS buffer) of infected patients. A dextran layer was employed for the immobilization of protein S via amine coupling on the surface (with Biacore X). High binding (700 RU) was observed in serum of patients whereas low signals ranging from 0.35 to 10 RU were recorded in plasma from healthy donors [133].

Vaisocherová et al. described the detection of antibodies for Epstein-Barr virus (anti-ENA) in diluted human serum (1 % in PBS buffer) with a home-built SPR sensor platform based on the wavelength division multiplexing (WDM). Synthetic peptides (bioreceptors) were immobilized on the surface via hydrophobic and electrostatic interaction and anti-EBNA antibodies were detected down to 0.2 μg/L (~1 pM). Inter-chips RSDs% of the sensor response was estimated to be within the 8 %–18 % range whereas intra-chip RSD% was found to be lower (~10 %), showing a good reproducibility in both situations. In summary, the analytical performances were comparable with that of a conventional peptide-based immunoassay (ELISA) [134].

Kumbhat et al. developed an immunosensor for the detection of dengue virus specific IgM antibodies in infected patients serum (different dilutions in PBS), using an Autolab Model SPRINGLE. Dengue antigen-BSA conjugates were immobilized as bioreceptor via aminocoupling on gold sensing surface functionalized with a mercaptoundecanoic SAM. In addition, the sensor was regenerable with pepsin solution in glycin-HCl buffer (pH 2.2), and the results were comparable with those obtained by MAC-ELISA [135].

Nilsson et al. designed an inhibition assay (indirect) for the quantification of hemagglutinin (HA), a glycoprotein on the capsid of the seasonal influenza viruses. Recombinant HA proteic Ags (A/H1N1, A/H3N2, and B) were immobilized via aminocoupling on the dextran matrix of a Biacore T100 chip while specific antibodies were mixed in serum solution of virus antigen (Fig. 9). The detection limit was 0.5 mg/L and the method resulted in more precise (intra-CV=2 %) and faster (9 h) than single-radial immune-diffusion (SRID) reference method (intra-CV=2 %; analysis time=22 h) [136].

Park et al. reported a preliminary study in PBS buffer for the detection of SARS coronaviral envelope protein (SCVme) in the diagnosis of severe acute respiratory syndrome (SARS). SCVme was anchored to gold chip surface (SPRi, K-MAC) by gold binding protein (GBP) and selective and sensitive detections of anti-SCVme antibodies were performed down to 200 μg/L (DL) in 10 min. AFM and SPR imaging analyses confirmed the anti-SCVme-specific capturing by antigen domain with an appropriate orientation [137].

Wang et al. employed high-resolution SPR microscopy (SPRM) for the label-free and imaging detection of single

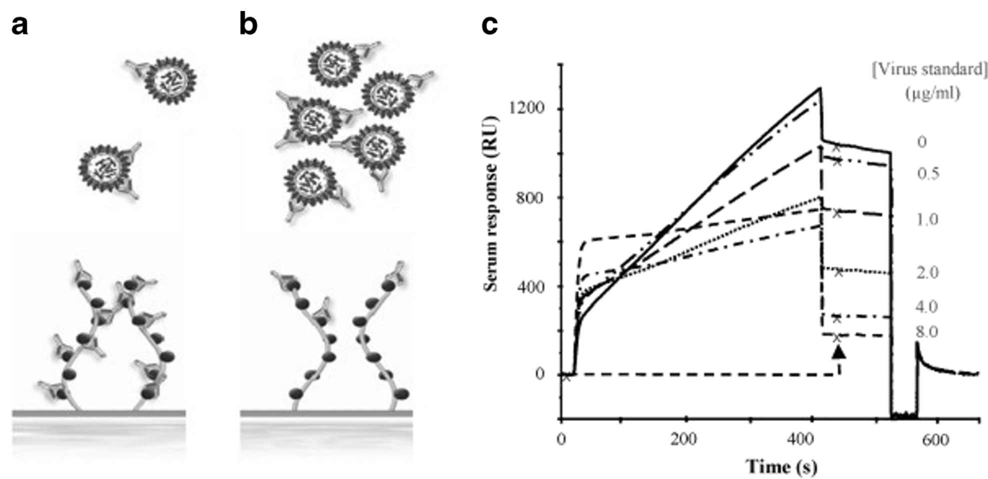

Fig. 9 Inhibition assay principle (**a**) and (**b**). HA is first immobilized on the dextran matrix (black filled circles). Virus is then mixed with a fixed concentration of serum and injected over the surface. Free antibodies (not bound to virus at equilibrium) bind to the surface HA, giving a response. Low concentration of virus in the sample (**a**) gives high antibody binding, whereas high virus concentration (**b**) results in low binding level. (**c**) Overlay plot showing sensorgrams of injected serum mixed with a concentration series of virus standard. Report points (marked as X) are taken before and after injection and response levels are measured between those (arrow). After each injection, the surface is regenerated in preparation for a new injection. Reprinted with permission from Elsevier [136]

viruses particles (H1N1 Influenza A/PR/8/34 and HCMV) in PBS buffer down to ~0.2 fg/mm^2 (mass DL). Anti-influenza A antibodies were covalently immobilized on PEG/PEG-COOH coated gold chip surface via aminocoupling. This study demonstrated the suitability of the method to assess masses and binding activity of individual viral particles [138].

Bacteria and parasites

In this section detections of pathogenic bacteria or other microorganisms were reviewed focusing on immunological assays for direct pathogen or anti-pathogen antibodies detection.

Salmonella, a frequent gastrointestinal pathogen involved in many diseases such as diarrhea, gastrointestinal inflammation, and life-threatening typhoid fever [139], has been widely reported in literature coupled to SPR-based sensing.

A SPR-based sandwich immunoassay was developed in PBS buffer by Muzumdar et al. for serotyping of *Salmonella* B, C, D. Polyclonal antibody for *Salmonella*, immobilized on the SPR chip (Plasmonic Biosensoren AG), captured the *Salmonella* bacterial cells further probed with antigen-specific antibodies for serotyping. The method was rapid and quantitative with a detection limit of about 10^5 cell/mL, and the SPR immunosensor provided more comprehensive and quantitative information than conventional slide agglutination test (SAT) [140].

More recently (2012), Gupta et al. designed an immunosensor for *Salmonella typhi* antibody detection in PBS buffer and then in patients sera. *Salmonella typhi* antigen was immobilized on a 4-mercaptobenzoic acid (4-MBA) modified gold surface chip of an electrochemical surface plasmon resonance system (Autolab ESPRIT). The discrimination between positive (Widal positive) and negative sera (Widal negative) was achieved in less than 10 min. Experimental DLs were, respectively, 1:102400 dilution for Mab and 1:204800 dilution for *Salmonella typhi* antibodies, and a good reproducibility (5 measures) was recorded (CV%=1.23 %) [141].

Boer et al. detected glycan-specific serum antibodies, biomarkers of exogenous pathogens, in human sera infected by the parasite *Schistosoma mansoni*. Sera were diluted (1:50 in PBST) while 144 glycans samples were printed on an epoxide-activated chip (Biacore Flexchip). Different serum antibody profiles between infected sera and control sera were revealed in real-time and without fluorescent labeling [142].

Conclusions

Up to now, ISI Web of Knowledge reported almost 40,000 publications related to SPR sensing and, if we analyze the distribution of the topics, the technology impacts significantly on clinical chemistry. However, studies demonstrate proofs of principle and only very recently, reports on applications to real matrices have appeared.

In this review, we considered a panel of analytes of interest for clinical diagnostics, also focusing on emerging technological innovations such as coupling between SPR and nanoparticles, microfluidics, or new chip design, for improving the analytical performance.

The detection of hormones related to endocrine disorders, protein biomarkers in relation to various conditions (inflammation states or traumas) or, more specifically, for cancer and

cardiac diseases were discussed as case studies. Antibody detection was also reported in relation to diagnosis of infections and immune system diseases. In addition, we reviewed nucleic acid sensing for the detection of genetic disorders, but also for recognizing specific sequences for pathogens (virus, bacteria) detection, or for miRNA as emerging biomarkers. Finally, we discussed immunosensing approaches for pathogens detection.

In our selection, we privileged studies conducted on real matrices (blood, serum, and urine) with a few exceptions, when the analytical strategies appeared especially original and innovative.

The development of innovative chip chemistry and antifouling strategies guarantees reduced nonspecific binding, which is essential for complex biological fluids analysis. Amine coupling allows for covalent immobilization of receptors, ensuring repeated measurement on the same chip, after a simple dissociation of the receptor–ligand complex with chaotropic reagents. Interestingly, sample pretreatment is reduced to a minimum: dilution, sometimes a short heating or filtration.

In summary, SPR-based biosensors offer analytical performances (sensitivity, DLs, and reproducibility) comparable to conventional methods employed in clinical analysis (electrophoresis, chromatography, ELISA, RIA, etc.); in addition, they ensure real-time monitoring, label-free solutions, parallel analysis (SPRi), high throughput, little sample pretreatment, fast responses, and cheapness. For all these reasons, we believe that in the near future, SPR will emerge as an efficient, powerful, and alternative tool for daily routine clinical analysis, opening also new horizons for future developments in personalized medicine and in point of care diagnostics.

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