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Review

Surface plasmon resonance sensing of nucleic acids: A review

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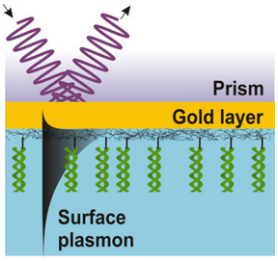
HIGHLIGHTS

- Advances of nucleic acid (NA) surface plasmon resonance (SPR) sensors are presented.
- Bioanalytical applications of NA SPR biosensors are reviewed.
- Applications for study of molecular interactions involving NAs are also discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Biosensors based on surface plasmon resonance (SPR) have become a central tool for the investigation and quantification of biomolecules and their interactions. Nucleic acids (NAs) play a vital role in numerous biological processes and therefore have been one of the major groups of biomolecules targeted by the SPR biosensors. This paper discusses the advances of NA SPR biosensor technology and reviews its applications both in the research of molecular interactions involving NAs (NA–NA, NA–protein, NA–small molecule), as well as for the field of bioanalytics in the areas of food safety, medical diagnosis and environmental monitoring.

Contents

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1. Introduction

Nucleic acids (NAs) are biomolecules that play vital role in numerous processes in cells—from the storage, transmission and expression of genetic information to regulatory roles [1,2]. Human genome sequencing has helped elucidate the links between abnormalities in an individual's DNA and genetic disorders. In addition, new families of nucleic acids (e.g. siRNA, miRNA) have been discovered and some of them were identified as prospective biomarkers of various diseases and health conditions [3,4].

An optical nucleic acid biosensor consists of specific nucleic acid (probe) and an optical transducer which serves to translate the binding of a target biomolecule to the probe into an output signal. Optical transduction methods either employ labels (e.g. fluorescent labels) or measure the binding directly. The latter are referred to as label-free optical biosensors and are typically based on absorption or Raman spectroscopy, interferometry, or spectroscopy of guided modes pertaining to dielectric and metallic waveguides [5,6]. Surface plasmon resonance (SPR) biosensors exploit a special mode of the metal-dielectric waveguide – a surface plasmon – to measure changes in the refractive index caused by the biomolecular interaction occurring at the surface of the SPR sensor. SPR biosensors represent the most advanced and mature label-free optical biosensor technology. The ability to measure biomolecular interactions directly, in real time and without the use of labels makes SPR biosensors a powerful tool for the investigation of kinetic properties of biomolecular interactions [7–12]. Various kinds of nucleic acids have been targeted by SPR biosensors [13,14]. These range from short oligonucleotides such as miRNA [14,15], through polymerase chain reaction (PCR) products [16,17], to large molecules such as cDNA [18] and 16S ribosomal RNA, which is genetic marker of specific bacteria [19].

This paper presents a review of the state of the art of NA SPR biosensing and its applications in the research of molecular interactions and bioanalytics.

2. Fundamentals of SPR nucleic acid biosensing

2.1. Concept of SPR biosensors

2.1.1. Principles of SPR sensors

Surface plasmons are special electromagnetic modes which may exist at the surface of a metal. Surface plasmon resonance (bio)sensors employ surface plasmons propagating on a planar metal-dielectric interface, which are sometimes referred to as propagating surface plasmons (PSPs) [20]. The electromagnetic field of the PSP reaches its maximum at the metal-dielectric interface and decays exponentially into both media. The penetration depth of the PSP at the boundary of gold and a dielectric (in SPR sensors, this is typically an aqueous medium) is typically between 100 and 600 nm (for the wavelengths in the visible and near infrared). In SPR sensors, PSPs are excited using prism couplers, grating couplers or metal-dielectric waveguides [20]. Of these, the most common approach to the excitation of PSPs is through the use of a prism

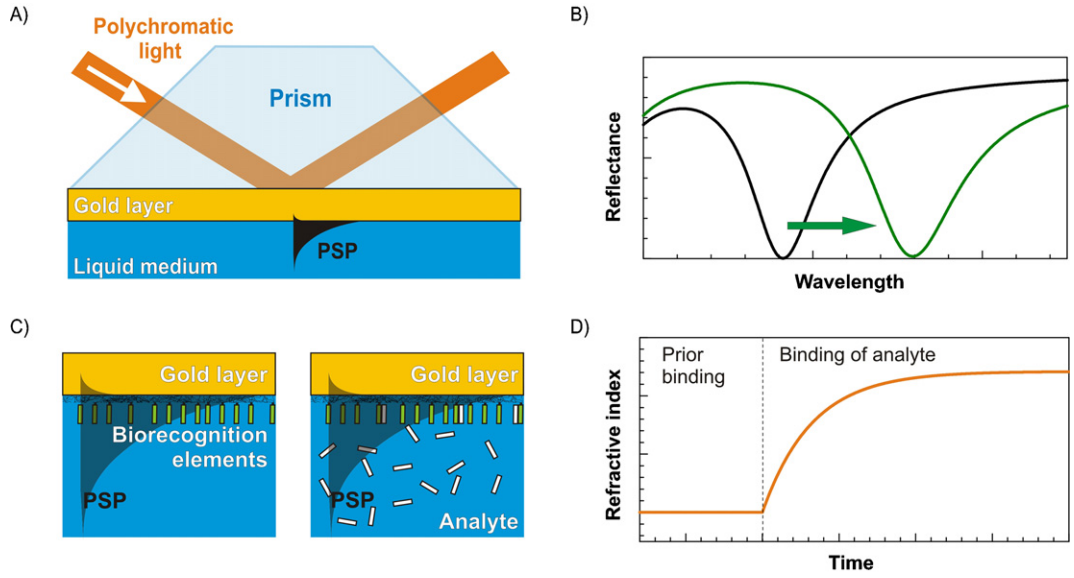

Fig. 1. Concept of a surface plasmon resonance (SPR) biosensor. (A) Excitation of PSPs by a polychromatic light in the Kretschmann geometry of the ATR method. (B) Spectrum of reflected light with a characteristic SPR dip for two different values of the refractive index at the gold layer. (C) SPR sensor surface with immobilized probes (left) and binding of the target molecules to the probes (right). (D) Temporal evolution of the refractive index caused by the molecular interaction.

coupler via the attenuated total reflection method (ATR). In the Kretschmann geometry of the ATR method, a light wave passes through a high refractive index prism and excites a PSP on the outer boundary of the metal film located on the base of the prism (Fig. 1A). The excitation of the PSP gives rise to a drop in the intensity of the reflected light. A change in the refractive index of the dielectric medium in the evanescent wave of the PSP produces a change in the propagation constant of the PSP. This change in propagation constant alters the coupling condition between the light wave and the PSP, which can be observed as a change in the characteristics of the optical wave (coupling angle of incidence, coupling wavelength, intensity and phase, Fig. 1B).

In the last two decades, a large variety of SPR sensor platforms have become available [21–24] including both laboratory systems for parallelized monitoring of molecular interactions [25–29] and compact SPR sensors for bioanalytical applications in the field [8,30–36]. A typical resolution of an SPR sensor (i.e. the smallest change in the refractive index which can be measured by the sensor) is of the order of 10^{-7} RIU for table-top laboratory systems and of the order of 10^{-6} RIU for portable SPR sensors [10,20,37].

2.1.2. SPR affinity biosensing

In SPR biosensors, an interacting molecule (probe) is immobilized on to the surface of the SPR sensor (Fig. 1C). When a solution containing the biological counterpart molecule (target) is brought into contact with the SPR sensor, the target molecules in solution bind to the immobilized probes via affinity interaction, resulting in an increase in the refractive index at the SPR sensor surface (Fig. 1D). The change in refractive index Δn_d occurring within a layer of thickness h can be expressed as

$$\Delta n_d = \left(\frac{\mathrm{d}n}{\mathrm{d}c}\right)_{\mathrm{vol}} \frac{\Delta\Gamma}{h}, \tag{1}$$

where $(\mathrm{d}n/\mathrm{d}c)_{\mathrm{vol}}$ is the increment of refractive index n with the volumetric concentration of analyte c, and $\Delta\Gamma$ is the surface concentration of the bound target [38]. The refractive index increment $(\mathrm{d}n/\mathrm{d}c)_{\mathrm{vol}}$ of proteins and nucleic acids is close to 0.18 $\mathrm{cm^3\,g^{-1}}$ [39]. The change in the refractive index causes a change in the coupling of incident light and a PSP. By tracking the characteristics of light coupled to a PSP in real time, kinetic details of the interaction between the target molecules in the solution and probes immobilized on the sensor surface can be determined. In addition, the refractive index change caused by the binding of target molecules may be used to identify the target molecules and quantify their concentrations.

The limit of detection (LOD) of SPR biosensors is typically defined as a concentration of analyte producing the sensor response equal to three standard deviations of the response to the blank sample. In the state of the art SPR biosensors, the analyte concentration corresponding to the LOD generates the surface concentration of the bound target of about 0.3 $\mathrm{pg\,mm^{-2}}$ [40]. The relationship between the surface concentration of the bound targets and the volumetric concentration of targets is given by a set of diffusion and kinetic equations [40]. These equations suggest that a state of the art SPR biosensor detecting a short 15-mer target NA (MW = 5 kDa) via a complementary probe ($k_a = 10^4\ \mathrm{M^{-1}\,s^{-1}}$, $k_d = 10^{-5}\ \mathrm{s^{-1}}$ [41]) is able to detect the target with the LOD of 400 pM [40].

2.1.3. Investigating NA interactions

The most widely used model of NA hybridization on solid surface assumes that the interaction between a single-stranded nucleic acid (probe p) immobilized on the surface of an SPR sensor and another single-stranded nucleic acid (target t) in the solution, can be described as:

$$p + t \underset{k_d}{\overset{k_a}{\longleftrightarrow}} pt, \tag{2}$$

where k_a and k_d are the association and dissociation rate constants, respectively. The surface concentration β of p–t complexes can be expressed as:

$$\frac{\mathrm{d}\beta}{\mathrm{d}t} = k_a c_t(\beta_{\max} - \beta) - k_d\beta \tag{3}$$

where c_t is the concentration of the target in the proximity of the sensor surface, $\beta_{\max}$ is the surface concentration of the probes, and t is time. Eq. (3) describes the generally accepted model of 1:1 interactions on the surface of an SPR biosensor. It describes the majority of the NA interactions with other types of molecules, such as intercalators, minor groove binders or proteins. Eq. (3) can be expanded with additional terms to account for more complex molecular interactions of nucleic acids, such as intramolecular interactions (the formation of secondary structures), the formation of triple helixes

(triplexes), quadruplexes and "kissing complexes" between two hairpins, or enzymatic interactions [42].

The process of p–t complex formation is referred to as hybridization and its rate is influenced by factors such as the sequence and length of the target, buffer composition, temperature, etc. The formation of p–t complexes is characterized by the hybridization efficiency x_{eq}, which is defined as the ratio of the hybridized probes in equilibrium and the total number of probes on the surface ($x_{eq} = \beta/\beta_{max}$). The equilibrium association constant K_{pt} and the hybridization efficiency are related through the Langmuir isotherm given by the formula [43–45]

$$\frac{x_{eq}}{1 - x_{eq}} = c_t K_{pt}. \quad (4)$$

2.1.4. Effects interfering NA sensing with SPR biosensors

As the SPR biosensors basically measure refractive index changes caused by the binding of target molecules to biorecognition elements immobilized on the sensor surface, the sensor response is a function of the concentration and size of the target molecules. When the target molecules are contained in a complex solution, the specific response due to the capture of target molecules can be concealed by the sensor response due to the adsorption of non-target molecules (which may be present at much higher concentrations than the target). Other effects that may interfere with SPR measurements include differences between the refractive indices of the sample and running buffer, sample composition variations, temperature fluctuations, etc. Various approaches have been developed to suppress the effect of these interferences. The most common approach is based on the use of a multichannel SPR sensor and referencing.

In this referencing approach, one of the sensing channels of a multichannel SPR sensor is functionalized with biorecognition elements specific for the target analyte, while the other sensing channel (reference) is functionalized with biorecognition elements that are not expected to capture any molecules from the sample, or left without biorecognition elements. If the sample is injected to both the sensing channels, the first channel records both the specific sensor response (due to the capture of target analyte) and the non-specific sensor response (due to the interfering effects), while the reference channel records the non-specific sensor response only. Finally, by subtracting the response of the reference channel from that of the sensing channel, the specific sensor response can be obtained. This approach is known to work well (error in the reference-compensated sensor response below 5%) if the interferences are caused by the background (bulk) refractive index changes. However, it is usually not sufficient to cancel the sensor response due to the non-specific adsorption from components in the complex solution. This is caused by the fact that, in general, it is not possible to produce detection and reference coatings that have identical non-specific binding properties. Therefore, multichannel SPR sensors are usually combined with functionalization methods that give rise to molecular coating with minimal non-specific adsorption in complex solutions. These include the well-established technology of self-assembled mono-molecular (SAM) films [46], or hydrogels such as modified dextran [47]. In the past decade various polymer coatings (e.g. zwitterionic polymers) grafted either on to a SAM [48] or directly on to gold [49,50] have been optimized to provide high resistance to the non-specific adsorption [48,51]. Although these coatings have been successfully used for the detection of proteins in complex real-world samples (e.g. in detection in blood plasma with non-specific adsorption below 7 ng cm^{-2} [52]) and clearly hold great promise, they have not been fully exploited in SPR sensors for the detection of NAs or their interactions, especially in complex milieu and real-world samples. In addition, no perfectly non-fouling functional coatings have been demonstrated yet and all the functional surfaces exhibit measurable levels of non-specific adsorption as a finite background signal in all real-world measurements.

2.2. Nucleic acid probes and their immobilization

2.2.1. Probes

In NA SPR biosensors, the probe is typically a oligodeoxyribonucleotide (ODN) or oligoribonucleotide with specific sequence of nucleotides (9–50 bases), which is able to hybridize with the specific target. The design of the probe is a crucial step of NA biosensor development, because the sequence specificity and yield of the hybridization are essentially dependent on the biorecognition properties of the probe. Decades of experience in designing linear probes has led to many commercially available codes that help tailor the probe-target stability for a given application and furthermore, avoid self-complementary regions on the probe [53].

The probe is tethered to the surface of the SPR sensor via a functional group attached to either of the oligonucleotide ends. The distance between the sensor surface and biorecognition area on the probe is regulated by a conjugation of spacers, such as mercaptohexyl (C_6), mercapto-undecyl (C_{11}), triethyleneglycol (TEG), or their combination [54]. Studies on thiolated probes have demonstrated an improvement in the limit of detection when using longer alkyl chains [54]. Alternatively, a blank sequence of nucleotides may also improve accessibility of the recognition area [41,55]. The thymidines should be then preferred over adenosines [55] due to their low affinity to gold surfaces [56].

Recently various alternatives to conventional DNA or RNA probes have been proposed in order to improve the target recognition and sensitivity of SPR biosensors for the detection of nucleic acids. These include synthetic DNA analogues, such as peptide nucleic acids (PNA) [57], PNA derivatives [58] and locked nucleic acids (LNAs) [59,60]. PNAs provide an attractive platform as they retain specific sequence-dependent hybridization properties and follow the Watson-Crick base-pairing rules [61] while usually maintaining higher selectivity against single-base mismatch with respect to DNA probes [62]. Moreover PNA probes offer an enhanced affinity to NA targets, originating from the decreased electrostatic repulsion between the probe and the target associated with replacing the negatively charge ribose-phosphate backbone of DNA with a pseudopeptide analogue. They may present an alternative for detection of targets with abundant secondary structures, such as rRNA [63]. Currently, the limiting factor for the use of PNAs as a biosensor probe is the high cost of custom synthesis of PNA oligomers.

2.2.2. Immobilization of probes

The immobilization of probes to the surface of the sensor is another crucial step in the development of an NA biosensor [64,65]. This process is expected to address two issues: (i) the robust and reproducible grafting of probes to well-oriented and accessible positions and (ii) the creation of a low-fouling background. As illustrated below, the most widely used approaches are the adsorption via a thiol linker, the covalent attachment on a functionalized surface of the sensor, and the use of avidin- (or streptavidin-) biotin interactions. They give rise to an ensemble of properly oriented probes attached by only one end-point. They also allow tuning the grafting density, which in turn influences the hybridization yield.

Chemisorption of probes modified with a thiol linker takes advantage of the strong affinity of thiol atoms towards metal surfaces [66]. The weakly chemisorbed probes can be removed by a small-molecule blocking agent [67], such as 6-mercapto-1-hexanol [68,69], longer alkanethiols [70] or thiolated oligoethileneglycols [71]. Those blocking agents also improve the orientation of the probes [69], reduce their density and improve the resistance of the

coating to the non-specific adsorption of nucleic acids and proteins [70,72]. The grafting density of thiolated probes is high compared to those obtained using other immobilization methods. It can reach up to 4×10^{13} molecules cm^{-2} [54,70] and it can be tuned by changing the length of the probe [67], pH [73], or and the concentration of ions in the immobilization buffer [68,74].

The surface of an SPR sensor may also be modified with a functional layer that carries various functional end-groups, such as maleimide [75], amine [76] or carboxyl [77] groups for the subsequent covalent immobilization of the probes. The functional layer can either allow for the immobilization of probes on the surface of the layer (e.g. self-assembled monolayer (SAM) of alkanethiols) or in a three-dimensional matrix (e.g. carboxymethylated dextran). Due to a greater number of reactive sites, the 3D matrix allows for the immobilization of a larger number of probes; however, the efficiency and selectivity of hybridization may be decreased due to lower accessibility of the probes and slower diffusion of the target through the matrix [78].

Immobilization via avidin (streptavidin) – a tetrameric biotin-binding protein – provides an efficient and convenient method for the immobilization of biotinylated probes. Avidin (streptavidin) is usually tethered to a functional layer containing biotin [79], or carboxylic end-groups [80] using maleimide covalent chemistry. The advantage of the streptavidin-biotin approach over direct coupling of thiolated probes is that the probes are more accessible for the target. The resulting probe layers typically exhibit lower surface densities ($\sim 5 \times 10^{12}$ molecules cm^{-2}) than thiolated probes, resulting in a higher hybridization efficiency [79]. However, the surface is less stable when subjected to multiple detection-regeneration cycles due to the gradual degradation of the anchoring protein [81].

Various alternative approaches to the immobilization of probes have been proposed to further increase accessibility of immobilized probes. For example, Mark et al. used SAMs for the immobilization of poly(amidoamine) dendrimers and the subsequent attachment of DNA probes modified with $-NH_2$ end-groups [82]. The main advantage of this approach is that dendrimers can be used as three-dimensional linkers of oligonucleotides, and thus enabling a higher binding capacity [82,83]. This ability may be very useful in microarrays when long hybridization times are used. However, SPR biosensors, which aim for short detection times and real-time readout, may only benefit slightly from improved probe accessibility. Another method was proposed by Chen et al. who immobilized NH_2-terminated probes to poly(L-glutamic acid) attached to the SAM surface [84]. The surface concentration of probes was $\sim 4 \times 10^{12}$ molecules cm^{-2}, slightly lower than surface concentration achieved by streptavidin–biotin coupling. These new methods may provide an alternative to established immobilization methods; however, further research is needed to fully assess their merit.

2.2.3. Fabrication of NA arrays

The most common method for fabrication of NA microarrays is based on microspotting of the nucleic acids onto a pre-functionalized or bare gold surface. The immobilization methods exploit adsorption via either a thiol linker or the streptavidin-biotin interaction [85,86]. The major issues are the loss of the activity of the spotted biomolecules after drying and rather irreproducible results due to variations in printing conditions [87]. Other methods for biomolecular micropatterning include pyrol/pyrol-ODN copolymerization triggered by electrostatic potential on individually addressed microelectrodes [88], deposition of anyline [89], and microcontact printing of COOH-terminated PEG-disulfide with p-carborane (COOH–CB–PEG) followed with the covalent attachment of NH_2-terminated DNA probes [90].

Various applications in protein–nucleic acid interaction require dsDNA to be immobilized on the surface. Microspotting of dsDNA on streptavidin layer formed on the surface of an SPR chip has been reported [86]. Despite the high affinity of streptavidin–biotin interaction, the efficiency of the dsDNA deposition was quite low (only 0.2–0.7 dsDNA/streptavidin, $<10^{12}$ dsDNA cm^{-2}). Other methods rely on the ability of the UV light to remove adsorbed SAM of alkanethiols. The arrays of bare gold produced by exposure to UV light can be then functionalized with disulfide-modified dsDNAs. To prevent the potential non-specific interactions of dsDNA with bare gold, amine-terminated alkanethiols might be attached to the photopatterned areas instead. Subsequently, thiol-terminated DNAs are covalently attached to those alkanethiols using a bifunctional crosslinker [76,91].

3. SPR sensors for the investigation of nucleic acids and their interactions

One of the major areas of applications for SPR biosensors is the investigation of biomolecules as well as their interactions, including the interactions of NAs with NAs, DNA/RNA-binding proteins, enzymes, and small molecules.

3.1. NA–NA interactions

The hybridization of nucleic acids on solid supports is a complex process with characteristics that may differ from those obtained for NA hybridization in a solution. The affinity constants K_{pt} of NA hybridization obtained by means of SPR biosensors have been found to differ from those obtained by equilibrium methods by several orders of magnitude [92,93]. This discrepancy originates from the nature of the model described by Eqs. (2)–(4) in Section 2.1.3, which was originally derived for interactions involving species that are infinitely diluted. In interactions occurring on solid supports, this model requires that the intermolecular distance between the immobilized biomolecules is large enough to prevent mutual physical interactions [93–96]. As the negative charges distributed along the sugar-phosphate backbones of both probes and targets form an electrostatic field, the requirement of no interaction between the immobilized probes is fulfilled only for probe densities below 10^{12} molecules cm^{-2}. Because the surface concentrations of probes on SPR chips may exceed 10^{13} molecules cm^{-2} [96,27,97], NA hybridization may be influenced by the excess of the probes and therefore depend on several factors, including surface probe density (*PD*), ionic strength of the hybridization buffer (*I*), and to a lesser extent also on the location of the complementary sequence on the probe as well as the probe length [43,98]. The cations in the buffer compensate for the negative charge of the phosphate groups and hence decrease the electrostatic repulsion between the nucleic acids packed on the SPR sensor surface. If *I* is too low for a given PD, hybridization does not occur, and conversely, at low PD and high *I* (above 0.33 M concentration of NaCl) "pseudo-Langmuir" behavior can be observed [99,100]. Between these two scenarios, "suppressed hybridization" takes place and the hybridization efficiency x_{eq} decreases with increasing PD. In the "pseudo-Langmuir" regime, the x_{eq} is independent of PD, as expected from the Langmuir model. Therefore, the "pseudo-Langmuir" regime is recommended for kinetics measurements, as hybridization proceeds in the manner most similar to that in solution. The low *PD* and high *I* conditions are also suitable for the detection of NA, as under these conditions hybridization proceeds at a highest rate. However, even under these conditions experimentally calculated hybridization rates and affinity constants are several orders of magnitude lower than the values obtained in a solution [92,93], suggesting hybridization processes on solid supports are much more complex than those predicted by the 1:1 interaction model. Several extensions of the Langmuir model have been proposed (for review, see [101–103]) to account for dispersion of probes [103], targets [104] or competitive

hybridization of ssDNA and dsDNA. Other models have described a dependence of accessibility of nucleation sites on *I* for probes of different lengths [105] and their influence on the rates of surface hybridization in the limit of widely spaced non-interacting probes [106].

Although the affinity constants obtained with SPR biosensors cannot be directly compared to those obtained with solution-based methods, SPR biosensors provide a convenient platform for the relative comparison towards the stability of complexes of natural NAs [44]. SPR biosensors have been also used to measure changes in hybridization kinetics of NAs due to mismatches of various types and positions [107,108]. In addition, complexes of chemically modified NAs with their natural counterparts [89,109–111] have been investigated to identify potential targets for the antisense drugs or novel types of probes for NA biosensing [112,113].

In order to aid the design of probes for NA biosensing, the effects of secondary structures on DNA hybridization kinetics have been investigated. It has been demonstrated that the addition of three intramolecular base pairs significantly slows down the DNA hybridization [93]. This study also concluded that the traditional 2-state model is not sufficient to describe individual strands containing four or more intramolecular base pairs.

The influence of cations and RNA modifications on the stability of kissing complexes has also been investigated using the SPR method [114,115]. Di Primo et al. investigated the loop–loop interaction between RNA I and RNA II, which regulates bacterial replication, and demonstrated that subsequent binding of Rop protein with the kissing complex follows the 1:1 model [116].

The formation of higher-order structures of nucleic acids, such as triplexes [117,118] and quadruplexes [60,119] has been also widely studied with SPR biosensors. An interesting approach was proposed by Zhao et al., who studied the influence of fold-inducing cations on the rate of G-quadruplex formation [119]. The unfolded oligonucleotide was immobilized on a sensor chip, after which a solution containing fold-inducing cation and complementary oligonucleotide was pumped across the chip. The rate of folding was deduced in theory from the rate of hybridization of the complementary ODN, which traps any unfolded immobilized G-rich oligonucleotide.

3.2. NA–protein interactions

The interactions between NAs and NA-binding proteins studied using SPR sensors are typically those related to DNA processing in cells, such as DNA replication, repair [120,121] and transcription [122]. The characteristics of those interactions on solid supports have been found to correspond well with the parameters obtained with solution-based methods.

3.2.1. RNA binding proteins

The RNA-binding proteins studied using SPR biosensors include proteins playing a role in the regulation of translation [123], mRNA and tmRNA binding proteins [122,124–126], and proteins binding ribosomal RNA (rRNA) [127,128]. In particular, the translation initiation factors binding to the 5′-cap of mRNA have been frequently studied [126,129–131]. SPR biosensors have been also applied to the investigation of affinity of aptamers selected by in vitro methods from a large pool of random sequences pertaining to the analyte of choice (e.g. proteins, drugs). This research has been described in several reviews [132,133], and is therefore not discussed here in detail.

3.2.2. Transcription factors

Binding of transcription factors (TF) has been mainly investigated by SPR imaging, which allows for multiplexed measurements concerning the binding of TF to a variety of genes [134,135]. Most studies of TF binding with SPR imaging were performed using either short dsDNA arrays [91,136,137] or whole gene promoters [135]. Even though, in the latter case, steric effects on the crowded surface affect measured kinetic parameters, such measurements allow for fast screening of whole genes for binding sites of a given TF.

One of the important features of SPR biosensor technology is its applicability to the investigation of complex multiprotein–DNA interactions. This feature was exploited in the work of Neo et al., who studied synergic binding of two transcription factors (ERα and SP1) to a DNA oligomer composed of various segments [138]. The binding studies were performed in nuclear extracts containing physiological levels of both ERα and SP1 and confirmed the formation of ternary complex of ERα/SP1 with the composite DNA.

3.2.3. HIV

SPR biosensors have been used to study protein–NA interactions involved in HIV infection [139]. The most frequently studied interactions include nucleocapside protein [140] (which has also been widely studied in other virus species [140–143]), reverse transcriptase (HIV-RT) [144,145] and HIV integrase [146,147].

Gorshkova et al. explored the mechanisms of RT binding to RNA–DNA hybrids, and determined that there is a significant difference in the binding kinetics of RT when the hybrids are immobilized on the chip surface via the RNA vs. the DNA strand (i.e. in opposite orientations) [144]. In the work of Wu et al, it was shown that the action mechanism of inhibitors could be elucidated by combining the structure and information from the kinetics of RT binding to DNA substrates in the presence of different inhibitors [145].

The stoichiometry of HIV-1 nucleocapsid protein (NC) interaction with $(TG)_4$ oligonucleotide was studied by Fisher et al. [140]. In this study the measured binding kinetics suggested that both $(TG)_4$ and (NC) are bivalent and can therefore form high-order complexes.

3.2.4. Enzymes

Current SPR biosensors make it possible to distinguish subtle changes caused by enzymatic action, which involve prolongation or cleavage of the NAs immobilized to the surface of the sensor. However, the kinetics of the enzymatic action is often difficult to separate from coincident association or dissociation of the enzyme. Most of the works therefore rely on binding assays, which study the affinity of inactivated protein to substrates of different structure or sequence [148–152].

Both the cleavage and prolongation of the NAs immobilized on the sensor surface can be monitored indirectly. For example, if the enzymatic action produces single-stranded nucleic acids, a complementary strand [153] or a tag [154] can be introduced for semi-quantitative measurement of the enzymatic activity. Vaisocherova et al. reported another indirect method to study of the integration of viral dsDNA into the host genome by HIV integrase [146]. The host genome was represented by duplexes immobilized to the sensor surface. The amount of integrated dsDNAs was read after several washing steps, which removed both the enzyme and unbound viral dsDNA.

Jorgensen et al. studied the activity of human poly(ADP-ribose) Polymerase-1 (PARP-1) on oligodeoxynucleotide substrates that mimic various double stranded DNA structures [148]. The PARP-1 was found to prefer the duplex structure over hairpin and loop structures, which was reflected in a higher amount of incorporated ADP per enzyme. Greive et al. investigated the step-by-step the DNA transcription by *Escherichia coli* RNA polymerase [155] including the release of the sigma factor during the initiation–elongation transition, the synthesis of the RNA transcript, and the release of core RNAP and nascent RNA at intrinsic terminators [155].

Only a few groups have successfully demonstrated monitoring the enzymatic action in real time. Corn's group showed that it is possible to obtain the catalytic and kinetic constants from the

kinetics of enzymatic cleavage measured by SPR sensor [42,156]. They used a simple model that coupled biomolecular adsorption and surface enzyme kinetics and was characterized by three rate constants (k_a, k_d, and k_{cat}) as well as a diffusion parameter. They applied this model to data concerning the cleavage of RNA strands in the RNA/DNA hybrid duplex by Ribonuclease H (RNase H). The enzymatic activity of RNase H was also studied by Šípová et al. [157], who proposed the detection of the products of the cleavage reaction using another sensing channel located downstream [157]. This approach offers additional information about the cleavage reaction as it allows detecting the cleavage products while also measuring their concentration.

3.3. NA–small molecule interactions

Numerous drugs are based on agents of low molecular weight that act as minor groove binders or intercalators. SPR biosensors present an attractive tool for study of these interactions; however, due to the low molecular weight of the binding molecule, direct monitoring of the interactions is challenging. In order to overcome this issue, immobilization methods based on a dextran layer have been used that increase the binding surface capacity, thus providing an increased sensor response. An increase in the number of binding sites can also be achieved by repeating the sequence of interest several times in each probe; however, this may cause the observed affinity to differ for different binding sites due to the varying levels of accessibility for different sequences along a single probe. SPR biosensors have been used to investigate the interactions between NAs and various compounds related to a wide variety of diseases, such as cancer, inflammation, thrombosis, osteoporosis, and viral infections [158–161]. SPR biosensors have also been used to measure differences in small molecule affinity towards various NA sequences [162,163]. There is also increasing interest in studying the interaction of G-quadruplex DNA with small molecules, such as porphyrines [164] or new classes of G-quadruplex binding ligands [165–167], as these small molecules may act as telomerase inhibitors and hence offer prospective routes towards cancer therapeutics.

3.4. Discussion

SPR biosensors enable the real-time observation of biomolecular interactions without labeling the interacting molecules, which makes them an attractive tool for the investigation of NAs and their interactions. SPR studies have demonstrated that NA hybridization, especially on solid supports, is a rather complex process whose aspects are not yet fully understood. Development of a rigorous physical model of solid support NA hybridization is expected not only to aid in the understanding of NA hybridization, but more importantly help understand processes occurring within cells, as those interactions also occur in the crowded environment of the cell nucleus or cytosol rather than in a diluted solution. Experimental data obtained using SPR biosensors may contribute to the development and validation of such a model.

As evidenced by a vast number of publications, SPR biosensors have become an invaluable tool for the investigation of NA–protein interactions [9,168]. In addition to the simple 1:1 NA–protein interactions which have been studied by SPR biosensors most frequently, SPR biosensors have expanded to include more complex multiprotein–DNA and enzymatic interactions. One of the challenges for further research in the area of NA–protein interactions is in the investigation of weak NA–protein interactions, which is limited by the sensitivity of SPR sensors. Although various interactions between NAs and small molecules have been successfully investigated, the utility of SPR biosensors in this area is limited by the sensitivity of current SPR sensors that do produce a weak output signal for molecules having low molecular weights. It is expected that future advances in SPR biosensor technology will lead to improvements in the sensitivity of SPR biosensors and will usher the technology to many more NA–protein and NA–small molecule applications.

A general limitation of current SPR biosensor-based interaction studies lies in the fact that, in order to avoid non-specific adsorption effects, the studies are carried out in model solutions (e.g. buffers) rather than in the native environment of the interacting molecules. Although the model studies yield main characteristics of the biomolecules as well as their interactions, they do not take into account a potentially complex interplay between other biomolecules present in biological media. This limitation remains a substantial challenge for label-free biosensor technologies, such as SPR biosensors, and tackling this challenge will require substantial advances in the development of non-fouling coatings and surface functionalization methods.

4. SPR sensors for the quantification of nucleic acids

Although there is a growing interest in applying SPR biosensors for bioanalytical tasks, the number of publications documenting applications of SPR biosensors for the detection of NAs in complex real-world samples is still rather limited. As the analytical performance of NA SPR sensors depends on a large number of factors (sensor platform, probes, functionalization method, sample composition, detection methodology, etc.), it is difficult to draw conclusions about the bioanalytical capabilities of NA SPR biosensors just on the basis of published results for real-world samples. In order to offer a reader deeper insight into the bioanalytical applications of NA SPR sensors, this chapter is organized into two sections. The former section reviews applications of SPR sensors for detection of nucleic acids in model pure media (buffers) and strategies for improving two key aspects of SPR sensor-based detection of NAs: the ability to detect low levels of NAs and the ability to distinguish the target NAs from other with a similar sequence. The latter section reviews applications of SPR sensors for detection of nucleic acids in real-world samples.

4.1. Detection of nucleic acids in buffers and detection enhancement strategies

4.1.1. Direct detection of NAs

The detection of target NA via its hybridization with a probe immobilized on the SPR sensor surface is usually referred to as direct detection. Table 1 presents the typical LODs of NAs achieved in buffer. As follows from Table 1, the demonstrated LODs are in nanomolar or high subnanomolar range depending on the target size, hybridization conditions and the SPR platform used. Despite the large set of parameters, which contribute to the achieved LODs, there are several conclusions that can be drawn from the collected data. LODs in nanomolar range have been achieved for ODNs in buffers that contained denaturants (e.g. formamide or urea) or low concentration of salts (NaCl < 300 mM). The use of high-ionic strength buffers (with NaCl content > 300 mM, or $MgCl_2$ ~15 mM) made it possible to achieve lower LODs, which is consistent with higher hybridization efficiency observed in those buffers. As suggested in Ref. [44], better LODs can be obtained for larger NAs, but the improvement in LOD is not directly proportional to the size of the molecule. For example, for 16S rRNA the demonstrated LOD was better by a factor of 5 than that for the reference ODN, in spite of the fact that the mass of 16S rRNA was larger by about 80-times than that of the ODN. The major cause was probably steric hindrance of the molecules bound to the surface and secondary structure of the 16S rRNA. Table 1 also suggests that the two most

Table 1
Reported limits of detection (LOD) for detection of various NA targets in buffer. AT: alkanethiols; PB: Phosphate buffer; SC: Sodium citrate.

Probes	Target	SPR platform	Immobilization method	Buffer	Hybridization time (min)	LOD	Ref.
DNA (18-mer)	ODN, 16S rRNA	SPR imaging	Chemical attachment of S-DNA to NH_2-AT	20 mM PB, 300 mM NaCl, 1 mM EDTA, 100 mM urea, pH 7.7	30	10 nM, 2 nM	[44]
DNA (21-mer)	ODN	Spreeta	Adsorption of S-DNA to gold	450 mM NaCl, 45 mM SC, pH 7.0	30	0.02 nM	[107]
DNA (23-mer)	ODN	Laboratory SPR	Streptavidin on AT	10 mM PB, 15 mM $MgCl_2$, pH 7.4	10	0.1 nM	[80]
DNA (23-mer)	ODN	SPR imaging	Adsorption of S-DNA on Au	10 mM PB, 15 mM $MgCl_2$, pH 7.4	10	0.1 nM	[26]
DNA (25-mer)	ODN	SENSIA SL	Adsorption of S-DNA on Au	75 mM SC, pH 7.4, 750 mM NaCl; 5% formamide	10	10 nM	[55]
DNA (25-mer)	ODN	Biacore X	Adsorption of S-DNA on Au	20 mM PB, EDTA 0.1 mM, Tween 0.005%, 150 mM NaCl, pH 7.4	10	2.5 nM	[173]
DNA (22-mer)	miRNA	laboratory SPR	Adsorption of S-DNA on Au	10 mM Tris-HCl, 1 M NaCl, pH 7.4	10	0.1 nM	[187]
PNA (22-mer)	ODN	AutoLab ESPRIT	Streptavidin. on biotin-AT	10 mM PB, 2.7 mM KCl, and 137 mM NaCl, pH 7.4	15	5 nM	[89]
PNA (17-mer)	16S rRNA	AutoLab ESPRIT	Streptavidin on amino-dextrane	20 mM PB, 300 mM NaCl, pH 7.4	30	0.5 nM	[177]

commonly used immobilization approaches – streptavidin–biotin interaction and adsorption of thiolated probes – make it possible to achieve similar LODs [169].

4.1.2. Strategies for improved limit of detection

One of the most common strategies for detection of very low concentrations of nucleic acids uses polymerase chain reaction to generate a large number of copies of a particular NA target. PCR method is typically used for the detection of specific genes, which are limited in numbers and therefore require a highly sensitive detection method. When working under optimal conditions, PCR requires only few copies of a target sequence as template. PCR may be performed in both qualitative and quantitative mode. At a lower number of amplification cycles parameters of PCR can be optimized so that the produced number of copies is proportional to the original concentration of the template. When a high number of cycles (25–40) is used, the PCR reaction often reaches a plateau and provides rather qualitative than quantitative results [170]. While PCR increases the concentration of target molecules, it may also amplify NAs with sequences similar to that of the target, which challenges the ability of NA SPR sensors to distinguish two NAs with a very similar sequence. PCR samples usually exhibit moderate non-specific adsorption of both PCR reagents as well as the negative controls (PCR-amplified DNA non-complementary to the probe) to the surface of the SPR sensor [171]. It should be noted that PCR samples typically contain target NAs in duplex with antisense ODNs, which need to be disrupted prior to the detection. High temperature treatment prior to the analysis was found to be insufficient, as the complementary strands tend to re-anneal before reaching the sensor surface [172]. It was demonstrated that the addition of formamide and urea to the PCR products can increase the sensor response to target hybridization by a factor of 2–3 [16]. A rather laborious but efficient alternative is the separation of biotinylated and non-biotinylated DNA strands using magnetic beads coated with streptavidin [172]. Another very efficient approach is based on the addition of short ODNs to the heated mixture of the PCR products. These oligonucleotides partially hybridize with the complementary strands but do not overlap with the target sequence, which remains free to bind to the immobilized probe [173].

The main strategies for increasing the sensor response to target NAs without generating additional copies of the target by PCR are amplification assays performed typically directly in the SPR sensor. They often take the form of the so-called sandwich assays using secondary proteins or enzymes that bind to the captured target or the target-probe complex to enhance the refractive index change produced by the binding of target (Fig. 2). Although this strategy introduces additional steps and prolongs the time required to complete analysis, it considerably increases sensitivity of the SPR method (Table 2). In addition, it improves the ability of SPR biosensors to quantify NAs in complex samples. Unlike the injection of complex samples, which usually causes large refractive index changes and non-specific adsorption of biomolecules from the sample to the sensor surface, the injection of an enhancement agent is performed in a pure solution (e.g. buffer) after the sensor surface has been washed with buffer. Therefore, these strategies are less prone to interferences (e.g. non-specific adsorption) and are better suited for the analysis of complex samples than the direct detection.

One of the most common enhancement strategies employs gold nanoparticles (GNPs) [11,174]. GNPs exhibit a large mass compared to biomolecular tags and produce a higher refractive index change than the commonly used dielectric materials (e.g. hydrogels). In addition, using the protocols developed for the functionalization of SPR sensors, GNPs may be readily functionalized with various molecules, such as linear DNA probes [175], aptamers [176], and functional layers [177]. In one of the earliest works, GNPs were modified with DNA probes complementary to a part of the target

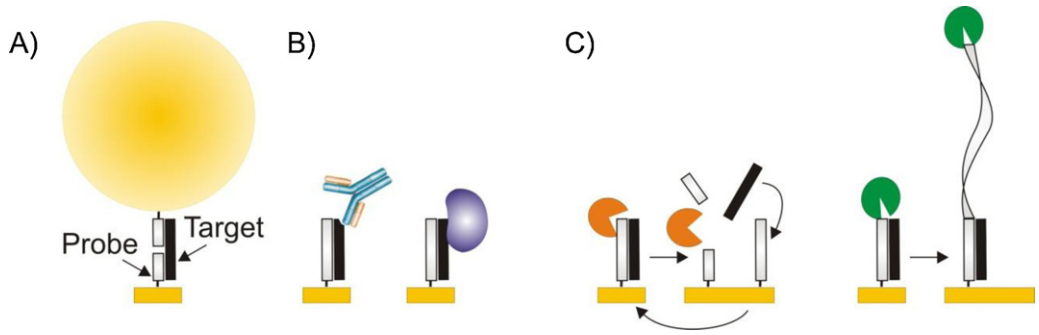

Fig. 2. Main strategies for increasing sensitivity of NA SPR sensors based on (A) nanoparticles, (B) proteins binding nucleic acids and their complexes and (C) enzymes. Figures are not to scale.

[178]. The sensor response to GNPs was found to be higher by a factor of 1000 compared to that of the target NA. However, at the same time, there was a rather high non-specific adsorption of the GNP equal to 40% and 10% of the sensor response for the untreated surface and the surface blocked with PEG, respectively. The problem of non-specific adsorption can be mitigated by employing dextran coating on the SPR chip surface [18], or also by functionalization of the GNPs with functional layers (e.g. SAMs) that prevent aggregation of GNPs as well as non-specific adsorption to the sensor surface. An enhancement factor of more than 1000 was demonstrated by combining neutral PNA probes with positively charged GNPs coated with amino-dextran that was electrostatically adsorbed to the target nucleic acids bound to the surface [177]. This method, however, requires a rather delicate design of PNA probes as both the specifically bound and non-specifically adsorbed NAs are amplified in this approach. Okumara used hydrogel nanospheres to amplify the detection of 60-mer DNA [179]. The ethyleneglycol diglycidyl ether was used in order to reduce the non-specific adsorption of the spheres on the sensor surface; however, the enhancement factor was not evaluated, as the hybridization signal of 60-mer was too low.

Another group of sensitivity-enhancing methods is based on enzymes, which can give rise to enhancement factors matching those provided by GNPs. Su et al. developed a detection strategy in which DNA hybridization on PNA probes was followed with DNA-templated polyaniline deposition and aniline polymerization by H_2O_2 in the presence of horse-radish peroxidase. This approach resulted in a 50-fold improvement of LOD [89]. Lee and coworkers used exonuclease III to cleave the DNA probe in the probe-target complex. Being released and intact, the target DNA 16-mer was reabsorbed to another probe inducing another cleavage by exonuclease III, resulting in an improvement of LODs by factors ranging from 10^2 to 10^3 [180]. A similar approach was used by Corn's group in an assay based on ribonuclease H (RNase H), which specifically hydrolyzes the RNA strand in the RNA*DNA duplex [181,182]. The array of RNA probes was exposed to a solution containing both RNase H and the DNA target. The RNA strand was then hydrolyzed, releasing the DNA target and causing reoccurring hydrolysis. Using this approach, the DNA targets were detected at levels down to 10 fM, increasing the sensitivity of the SPR sensor by 6 orders of magnitude.

The limits of detection were improved even further by combing enzymatic enhancement with enhancement methods using GNPs. In the work by Fang and coworkers, target captured by the DNA probes was elongated from the 3′-end using polyA polymerase, after which GNPs coated with polyT strands were introduced to generate an additional sensor response enhancement (Fig. 3B). This approach allowed for the detection of target NA down to attomolar concentrations [183]. Recently, Sendroiu et al. used RNA polymerase to produce multiple copies of ssRNA from each probe-target duplex which were then captured downstream, where the response of the sensor was amplified with GNPs coated with complementary cDNA (Fig. 3C) [184]. The enhancement of the sensor response achieved with this two-step strategy was as high as 10^6.

Although there are many enhancement strategies utilizing proteins with affinity to NAs or their complexes, ability of these approaches to improve the LOD is not as impressive as that of GNPs. Wilson et al. employed *Escherichia coli* ssDNA-binding protein that was introduced after the detection of the target and bound to non-hybridized DNA probes. This approach demonstrated improvement in the sensitivity of detection by a factor of 3 [185]. MicroRNA (miRNA) profiling was reported by Nasheri et al. [186], who used protein p19 with a specific sequence-independent but size-selective affinity to the RNA*RNA duplex in a sandwich detection format, enhancing the signal by a factor of 10. Another approach was proposed by Šípová et al., who amplified the sensor

Table 2
Strategies for improving the sensitivity of NA SPR sensors and the demonstrated limits of detection (LOD).

Enhancement principle	Probes	Target	LOD	
Sandwich assays:				
Streptavidin-ODN	DNA	ODN	40 pM	[189]
Streptavidin-ferrocene	DNA	ODN	10 pM	[188]
Hydrogel NPs	DNA	ODN (60 bp)	62.5 nM	[179]
GNPs	DNA	ODN (80 bp)	2.1 fM	[18]
	PNA	16S rRNA	60 pg mL^{-1} (~0.1 fM)	[177]
P19	RNA	miRNA	4 nM	[186]
Antibody	DNA	miRNA	2 pM	[187]
Enzymatic assays:				
Horse-radish peroxidase	PNA	ODN	0.1 pM	[89]
Exonuclease III	DNA	ODN	10 pM	[180]
Ribonuclease H	RNA	ODN	10 fM	[181]
PolyA polymerase, GNPs	LNA	miRNA	10 fM	[183]
RNA polymerase, GNPs	DNA	ODN	1 fM	[184]

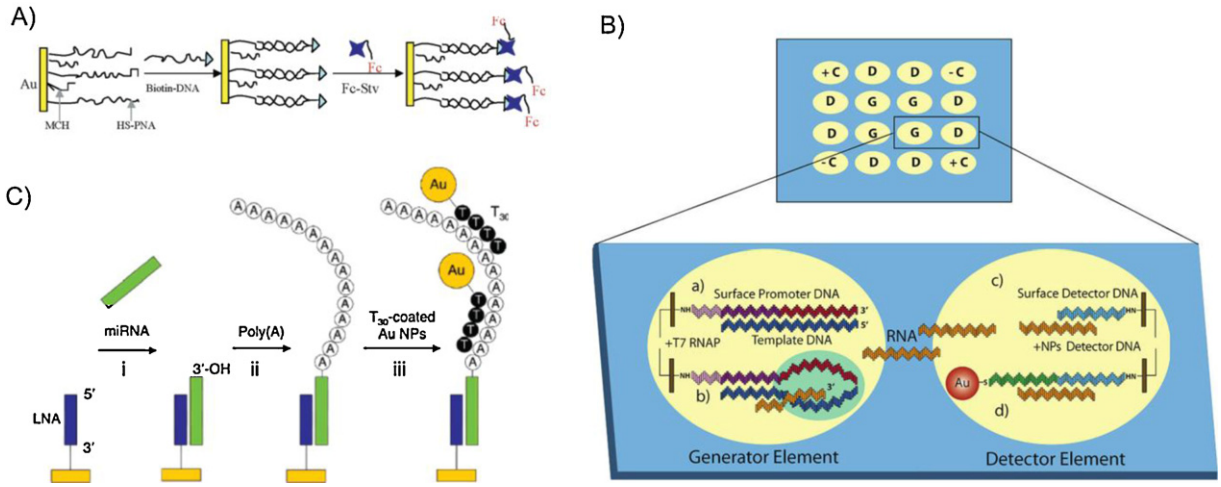

Fig. 3. Strategies for increasing sensitivity of NA SPR sensors based sandwich and enzymatic assays: (A) Detection of microRNAs using a combination of surface polyadenylation chemistry and nanoparticle amplified detection with SPR. Adapted with permission from [183]. Copyright 2006 American Chemical Society. (B) Amplified detection of PNA/DNA hybridization by the formation of an electroactive streptavidin layer. Adapted with permission from [188]. Copyright 2005 American Chemical Society. (C) Two-step enhancement method with RNA polymerase and GNPs. Adapted with permission from [184]. Copyright 2011 American Chemical Society.

response to miRNA by means of a special antibody that specifically recognizes the hybrid DNA*RNA duplex [187]. This approach was shown to improve the limit of detection by an order of magnitude. Biomolecular assemblies such as streptavidin–ferrocene complexes (Fig. 3A) [188], or streptavidin molecules interconnected with oligonucleotides [189] have also been used to enhance the sensitivity of NA SPR sensors. These methods typically improve LOD by an order of magnitude; however, they require rather laborious preparation.

4.1.3. Strategies for improved sequence specificity

The specificity of NA SPR sensors is typically related to the ability of the sensor to discriminate the target from nucleic acids with a similar sequence. The sequence specificity of the NA detection is usually defined as $[1-(SR_{MM}/SR_M)]\times 100(\%)$, where SR_{MM} is the sensor response to NA with a single nucleotide mismatch and SR_M is the sensor response to the matched NA of the same concentration. The typical values for direct detection of short ODNs are summarized in Table 3. The ability to discriminate a single nucleotide mismatch is influenced by various factors. In general, the conditions which destabilize NA complexes are those which provide higher sequence specificity. These conditions include the use of short probes [55], a low concentration of salt in hybridization buffer [74], higher hybridization temperatures [190,191], or the addition of a denaturant (e.g. formamide) to the hybridization solution [109,192]. As follows from data collected in Table 3, a similar level of specificity was achieved using ~20nt probes immobilized via thiol linker and shorter probes (~15nt) immobilized via streptavidin–biotin interaction, suggesting that steric hindrance may increase the specificity of detection. In addition, sequence specificity higher by tens of per cent can be achieved for longer targets, such as products of PCR amplification, than for short ODNs [55].

While adjusting the hybridization conditions is relatively straightforward, there are several drawbacks related to this approach—(i) the single-nucleotide specificity, in which the SR_{MM} approaches the level of non-specific binding, is achievable for rather short targets (at maximum 15 nucleotides long) [193]; (ii) the optimal conditions vary for targets of different sequence and length, limiting the applicability of this approach for multiplexed detection; (iii) the complex of the probe with the target is destabilized as well, which may significantly affect the LOD.

The methods for single mismatch detection have therefore focused on more complex detection strategies, often in combination with a "tag". The "tag" not only identifies the target-probe complex among partially matched complexes, but also enhances the sensor response to the hybridization. One approach to address this issue is based on specific proteins or mismatch-binding ligands capable of recognizing a single-base mismatch [194]. One of the rare naturally occurring single mismatch binding proteins, MutS from *Escherichia coli*, has been frequently utilized for mismatch discrimination [195–197]. However, this approach is not very reliable. Due to the affinity of MutS to ssDNA, the surface needs to be blocked prior to the detection with single stranded-binding protein (SSB) [195]. Moreover, the amount and rate of the MutS protein binding is markedly influenced by the location of the point mismatch within the duplex [196] as well as the density of the oligonucleotides on the surface [197].

Li et al. developed a detection approach based on the enzyme Taq DNA ligase (Fig. 4A) to detect a mismatch positioned at the end of the immobilized probe [198]. The target, the ligation probe and Taq DNA ligase were simultaneously introduced to the sensor. If

Table 3
Sequence specificity of direct detection of synthetic oligonucleotides in buffer. PB: phosphate buffer, PBS: phosphate buffer saline, SC: sodium citrate.

Probe length	Immobilization method	Conditions	Sequence specificity (%)	Reference
11-mer	Streptavidin-biotin	25 and 40 °C, 10 mM PBS + 150 mM NaCl,	20 (25 °C); 80 (40 °C)	[62]
13-mer	Streptavidin-biotin	10 mM PB + 150 mM NaCl; 25 °C	50	[213]
15-mer	Streptavidin-biotin	10 mM HEPES, 150 mM NaCl; 25 °C	20	[92]
20-mer	Thiol linker	PB + 0.05–1 M NaCl; 25 °C	20 (1 M NaCl)–50 (50 mM NaCl)	[74]
21-mer	Thiol linker	450 mM NaCl, 45 mM SC; 25 °C	30	[107]
25-mer	Thiol linker	30 mM PB; 15 mM SC + 150 mM NaCl; 25 °C	0 (PB); 13–35 (SC)	[55]
25-mer	Thiol linker	10 mM Tris, 1 M NaCl; 25 °C	30	[43]

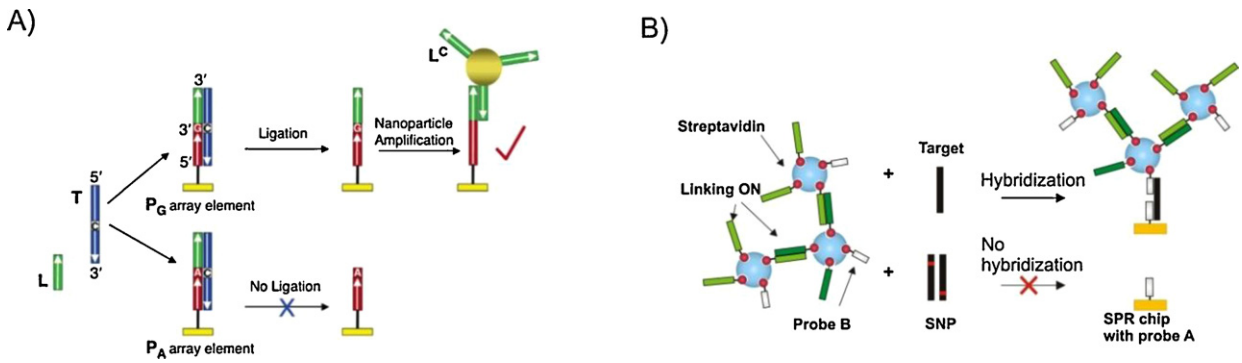

Fig. 4. SNP genotyping methods: (A) A combination of surface ligation chemistry and enhancement with gold nanoparticles. Adapted with permission from [198]. Copyright 2006 American Chemical Society. (B) An enhancement with streptavidin-oligonucleotide complexes [189].

no mismatch was present at the end of the lower probe, a covalent bond between the two probes was formed. The target was then washed away and the surface was incubated with a solution of GNPs coated with oligonucleotides complementary to the ligation probe. Using this method, the point mutation could be reliably identified down to 1 pM concentration.

The optimization of distance between two probes in sandwich arrangement was shown to offer an avenue for the improvement in sensitivity to a single-nucleotide mismatch in longer targets. It was demonstrated that, by means of an enhancement strategy based on streptavidin-oligonucleotide complexes (Fig. 4b), sensor response to ODN (20-mer) with a single nucleotide mutation can be decreased down to level of baseline noise when a single-nucleotide gap is left between the probes [189]. The base stacking interactions were used for the detection of mismatches at the end of the DNA duplexes (15-mers) immobilized on the sensor surface [199]. Those mismatches resulted in lower adsorption of the GNPs modified with different DNA duplexes onto the SPR chip due to lower stacking interactions between the duplexes on the surface and those on the GNPs. This method may help to reveal the end-point mismatches, which are in general harder to be distinguished; however, it is unclear if any benefits would be present concerning internal mismatches. Wang et al. employed a sandwich assay and GNPs for the recognition of a single base mismatch [200]. After the target hybridized with both the probes on the surface as well as the second probe on the DNA-capped GNP, an electrostatic field was applied to repulse the negatively charged GNP from the sensor surface dissociating the less stable complexes containing single nucleotide mismatch.

4.2. Detection of nucleic acids in real-world samples

4.2.1. Medical diagnostics

NA SPR biosensors have been used to detect various molecules related to medical diagnostics, including microRNAs, biomarkers of pathogenic bacteria, and mutations in human genomes related to various disorders.

Mutations in the human genome detected by SPR biosensors include point mutations in tumor-suppressor genes, such as TP53 [171,185,189,201], K-ras [62], and mutations corresponding to certain types of cancer [55,198] or hereditary diseases [193]. Detection of mutation of TP53 gene with a Spreeta SPR biosensor was described by Jiang et al. The DNA extracted from wild-type and mutated cell lines were amplified with PCR and then analyzed using a SPR chip coated with thiolated DNA probes (27-mers) and 6-mercapto-1-hexanol. Importantly, a low non-specific response was observed for both PCR blank samples (PCR samples without primer) and negative control (PCR-amplified non-complementary sequence). This work demonstrated the capability of the SPR biosensors to perform an analysis of the PCR products within 2 min and also highlighted the high stability of the functionalized SPR chip, which could be regenerated and reused 50 times. The sensor response to the target containing a point mutation was 34% lower than the response generated by the wild-type sequence [171].

Carrascosa et al. detected R1443X and BRCA1 mutations related to ovarian and breast cancer using a commercial SPR system (SENSIA SL). In their work, parameters such as spacers, probe length, and concentration of formamide were optimized to decrease the sensor response of the ODN with single nucleotide mismatch while using a direct detection format. The sensor response produced by the synthetic ODN mimicking a PCR product (a 60-mer) containing point mutations decreased by 70–92% compared to a full match at concentrations higher than 50 nM [55].

The detection of a gene mutation causing cystic fibrosis was reported by Feriotto et al. [193]. In this work, the products of PCR amplification were biotinylated and immobilized on the chip using streptavidin-biotin chemistry. The hybridization of short PNA probes (9-mers) with the immobilized PCR products was monitored using Biacore SPR biosensor. The probe complementary to the gene with a single point mutation produced a sensor response which was higher by a factor of ~70 than that produced by the probe corresponding to a non-mutated gene.

Recently microRNAs (miRNAs) have been identified as a prospective diagnostic markers of various serious diseases, including heart disease [202] and various cancers [203–205]. Clinically relevant levels of miRNAs in human blood are typically in high femtomolar to low nanomolar range, which requires the use of detection enhancement strategies. Fang et al. detected miRNA using LNA probes and enhanced the SPR signal with polyadenylation of the adsorbed RNA target along with GNPs. This approach requires a laborious multistep protocol, but made it possible to achieve an LOD in buffer as well as in total RNA extracted from mice liver tissue as low as 10 fM [183]. Moreover, the array format allowed for the detection of 3 miRNAs on one chip. MiR-122 was detected by Šípová et al. using a portable SPR sensor, DNA probes, and a special antibody recognizing the RNA*DNA duplex to amplify the miR-122 signal [187]. This approach was demonstrated to be able to detect miR-122 in buffer down to 2 pM. The biosensor was applied for analysis of the total RNA extracted from mice liver tissues and found to provide results that were in good agreement with those obtained using qPCR. In the works by Fang and Sipova, the probes were immobilized using adsorption of thiolated probes on gold surface, therefore, a moderate non-specific adsorption of RNA extracted from tissues was observed.

SPR biosensors have been also used to detect clinically relevant bacteria. For example, Kai et al. detected *E. coli* O157:H7 in stools [206]. They used a Biacore SPR sensor and PNA probes immobilized to the surface of the sensor via streptavidin–biotin interactions. The

PCR-amplified sample of DNA was analyzed and it was demonstrated that as few as 10^2 bacteria can be detected in 0.1 g of stool, which allowed for identification of both infected patients and healthy carriers of *E. coli* O157:H7.

4.2.2. Food safety

Applications of NA SPR biosensors in food safety include, in particular, the detection of foodborne pathogens [16] and genetically modified organisms (GMOs) [172,193,207].

A remarkable study was performed by Zezza et al., who demonstrated the detection of fungal pathogens in cereals [16]. A 0.57 kbp DNA fragment of *Fusarium culmorum* was first amplified using PCR, after which the products were detected with an SPR biosensor using a chip functionalized with a 25-mer DNA probe. The authors describe several issues concerning the PCR amplification of plant material. In particular, the molecules present in the matrix of the sample (from wheat) may interfere with the PCR amplification of the target gene, thus affecting both the efficiency and reproducibility of the amplification. An amount of 0.06 pg of *F. culmorum* DNA in 30 ng of durum wheat DNA was detected. That is over one order of magnitude lower than the minimum amount of starting fungal DNA (1 pg) necessary to produce a visible band using gel electrophoresis. Moreover, the detection was demonstrated to be specific for *F. culmorum* in the presence of other species of *Fusarium* or other fungal genera.

Another promising application area for SPR sensors is the detection of genetically modified organisms [172,207]. As the numbers of GM plants continues to increase, it is of great importance to develop methods which can identify specific transgenes in a multiplexed manner [208]. At present, the detection of GMOs is carried out using multiplex PCR [209] followed by gel electrophoresis or fluorescence microarrays. The ability of SPR biosensors to detect PCR amplicons was explored, by Marriotti et al., who targeted 35S promoter and NOS terminator sequences characteristic for GMOs in DNA extracted from transgenic soya flour [172]. The double-stranded PCR amplicons were separated using magnetic beads and the target DNA strands were detected with SPR biosensors down to 1 nM concentration [172]. Feriotto et al. reported the detection of Bt-176 maize genomic sequences in multiplexed PCR products using the Biacore 1000 SPR biosensor. The biotinylated PCR products were immobilized on the SPR chip and the annealed complementary sequence was removed with a regeneration solution, after which the hybridization of short DNA probes was observed. It was demonstrated, that the SPR method provides quantitative results, which were comparable to those obtained using quantitative real time PCR and were concluded to be more reliable than those obtained with Southern blotting [210]. The potential of SPR sensors for GMOs analysis can be further expanded by employing SPR sensor arrays, which offer hundreds of sensing channels and would be therefore a strong candidate for analysis of multiplexed PCR products.

4.2.3. Environmental monitoring

NA SPR sensors have been also used in environmental monitoring applications, albeit to a lesser degree. Asai and coworkers described a SPR biosensor for the detection of *Heterosigma carterae*, which belongs to a bloom-forming genus of algae [211]. The target molecule was the small subunit ribosomal DNA (rDNA), which was first amplified with asymmetric PCR and the products were subsequently analyzed with the Biacore SPR system. The lowest amount of rDNA products that was detected in this work was 6 ng. An original approach to the detection of pesticides was reported by Lim et al. [212]. In their work, *S. cerevisiae* was first treated with atrazine, which increased the expression of mRNA coding the P450 enzymes. The cells were then disrupted by boiling and the hybridization of mRNA with DNA probes (18-mers) was detected in the total RNA extracted from the cells. The smallest concentration of mRNA detected was 1 ng L^{-1} [212].

4.3. Discussion

Over the last two decades, a large number of research papers concerning NA SPR sensing have been published. These studies clearly witness strong interest of SPR sensor researchers in advancing SPR biosensor technology to bioanalytical applications and the potential label-free biosensors such as SPR hold for bioanalytics. However, as evidenced by a rather limited number of works reporting successful use of SPR biosensors in real-world bioanalytical tasks in the complex biological milieu, they also indicate that the technology has not yet reached the level which would allow its routine use as a bioanalytical tool. The fact that different types of SPR sensor platforms, probes, functionalization methods and detection methodologies have been combined to detect NAs makes the direct comparison of achieved performance figures rather difficult even for model experiments in which NA targets are detected in pure samples (e.g. buffers). This holds even more for results reported for complex biological samples, as the achieved performance is a result of a complex interplay of numerous factors—poor performance of optical platform may be offset by high performance of biomolecular coating and vice versa. This aspect should be taken in to account when drawing conclusions from the reviewed literature.

The most commonly used optical platform used in NA SPR sensors are those which offer several sensing channels (<10). SPR sensor platforms for highly parallelized measurements with a large number of sensing channels (>100) have been reported; however, their exploitation in NA sensing has been rather limited. Performance of the optical platforms lies within a rather broad range, over more than an order of magnitude when expressed in terms of the refractive index resolution. While the short DNA oligonucleotides have been used as probes in NA SPR sensors most frequently, synthetic DNA analogues with enhanced affinity to NA targets, such as peptide nucleic acids, have been introduced as attractive alternatives to DNA probes. The immobilization of probes has been most often performed by means of chemisorption of thiolated probes or immobilization via the streptavidin–biotin interaction. Both methods create stable functionalized surfaces with high density of probes, which are sufficiently accessible for binding of the target.

In the direct detection mode and model experiments performed in pure samples (buffers), the LODs offered by NA SPR sensors are in nanomolar or picomolar range (Table 1). These concentrations are higher than the typical concentrations of NA targets in many real-world applications. The most straightforward strategy for improving the LODs is based on the increasing the number of NA molecules (typically by a factor of 10^6–10^9) by means of polymerase chain reaction. However, this approach adds additional experimental steps and increases the time required for detection. Alternatively, the detection performance of NA SPR sensors has been extended by using advanced assay formats. These include faster one-step protocols requiring an additional reagent (e.g. antibody) but providing lower sensitivity, slower but more sensitive methods using multiple reagents (e.g. enzymes) or one requiring complex preparation (e.g. functionalized gold nanoparticles). These strategies have lowered LODs by several orders of magnitude, down to femtomolar or even attomolar levels, and have been shown to work well even in complex media [177,183,187]. This enables detection of various targets, such as 16S rRNA and specific miRNAs [187] and mRNAs without PCR amplification [187], thus decreasing the analysis time to several tens of minutes.

The main challenge for analysis of complex real-world samples with the SPR method is biological fouling—the non-specific adsorption of non-target molecules present in the sample (often in large

excess compared to the concentration of analyte). The fouling properties of the active surface of an SPR sensor are determined mainly by the characteristics of the functional layer. As can be deduced from a number of publications, the PCR samples [171] and cell lysates give rise to a rather moderate fouling and therefore do not pose a problem for the most commonly used functionalization methods. However, the non-specific adsorption of biomolecules from more complex samples, such as blood plasma, may considerably lower performance of NA SPR sensors, both in terms of limit of detection and false positive results. Low-fouling molecular coatings based on zwitterionic polymers demonstrated recently for SPR biosensor-based detection of proteins [52] may present a solution to this problem, but they still wait to be exploited in NA SPR biosensors.

Discrimination of the target NA from other (potentially similar) NAs present in a sample presents another challenge for NA SPR sensors. In direct detection mode, it is often impossible to differentiate between target hybridization and hybridization of NAs with long segments of nucleotides similar to the target, or NAs containing point mismatches. As follows from the reviewed literature, the sequence specificity can be improved through probe design and choice of the hybridization conditions, such as hybridization buffer, temperature, etc. The ultimate goal of achieving a single mismatch detection was so far demonstrated only by means of complex detection strategies employing sandwich assay format and/or enzymes.

5. Conclusions

Over the last decade, surface plasmon resonance biosensors have become an invaluable tool for the investigation of molecular interactions. The interactions of nucleic acids, such as NA–NA, NA–protein, NA–small molecule interactions, have been studied with SPR biosensors to deepen the understanding of complex biological processes in cells and enable the development of new drugs and therapies. The major asset of the SPR biosensors in this perspective is their ability to provide real-time measurement of the binding events without labeling of the interacting species. SPR biosensors have been routinely used for the quantification of kinetic parameters of molecular interactions; however, SPR-based studies have also demonstrated that NA hybridization on solid supports is a complex and not fully understood process.

A great deal of effort has been dedicated to the development of NA SPR sensors for bioanalytical applications. Various strategies for improving the sensitivity and sequence specificity of NA SPR sensors have been developed enabling SPR biosensors to detect NAs down to the attomolar level and allowing for the discrimination of single nucleotide difference in the target. Although the number of research papers describing these developments (carried out mostly in buffers) is quite high, the number of studies documenting applications of NA SPR sensors in complex milieu and real-world samples or providing comparison of detection performance in buffers and real-world samples is rather limited. In addition, experimental data, such as sensor's limit of detection for NAs in complex samples are a result of a complex interplay of numerous factors (sensor platform, probes, functionalization method, detection methodology, sample complexity, etc.) which makes the quantitative comparison of results achieved by different groups even more difficult. However, published data suggest that one of the major factors limiting the performance of NA SPR sensors in complex samples is non-specific adsorption of non-target molecules which forms a finite background signal. In summary, it is clear that further research and development will be needed for NA SPR sensors to reach the level which would allow their routine use as a bioanalytical tool in the complex biological milieu.

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