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REVIEW

Application of selected biosensor techniques in clinical diagnostics

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ABSTRACT

Introduction: Examination of disease biomarkers mostly performed on crude materials, such as serum, meets some obstacles, resulting from sample complexity and the wide range of concentrations and sizes of the components. Techniques currently used in clinical diagnostics are usually time-consuming and expensive. The more sensitive and portable devices are needed for early diagnostics. Chemical sensors are devices that convert chemical information into parameters suitable for fast and precise processing and measurement.

Area covered: We review the use of biosensors and their possible application in early diagnostics of some diseases like cancer or viral infections. We focus on different types of biorecognition and some technical modifications, lowering the limit of detection potentially attractive to medical practitioners.

Expert opinion: Among the new diagnostic strategies, the use of biosensors is of increasing interest. In these techniques, the capture ligand interacts with the analyte of interest. Measuring interactions between partners in real time by surface plasmon resonance yields valuable information about kinetics and affinity in a short time and without labels. Importantly, the tendency in such techniques is to make biosensor devices smaller and the test results apparent with the naked eye, so they can be used in point-of-care medicine.

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1. Introduction

Surface plasmon resonance (SPR) is an optical analytical technique that tracks the interaction between biomolecules. Developed in the second half of the 20th century, this technique is now experiencing a rapid boom. Of the two specifically interacting partners, the target analyte is captured even from the complex biological material by the immobilized partner (ligand), and further the physicochemical part of the device records the signal. This phenomenon is referred to as biosensing. Such techniques may be thus applied to analyze the molecular mechanisms of interactions. SPR provides invaluable services in the field of pharmaceutical research, allowing the screening of potential candidates for new drugs, as well as precisely tracking the kinetics of binding a potential drug to its biological target [1–3]. Currently, there are also attempts to apply plasmon resonance in the area of medical diagnostics. Biosensing with SPR can be an alternative to the enzyme-linked immunosorbent assay (ELISA). Its advantage is much higher sensitivity, associated with a low limit of detection (LOD), which could reach even the femtomolar level [4,5]. This is desired especially in the early diagnostics, when immediate implementation of treatment is the most effective and associates with much better prognosis. Such attempt may be critical for treatment planning, for example, in neurodegenerative disorders [5–7], oncology [8–10], or highly contagious diseases [9,11]. Biosensors consist of a biologically active ligand part (also called a bioreceptor) that binds specifically to the analyte, capturing it from the other compounds. The transducer element converts the biochemical intermolecular recognition event into an analytically useful signal. Usually, the detection consists in measuring electrical, thermal, or optical signals [12]. In biomedical applications, the popular analyses include SPR, imaging surface plasmon resonance (iSPR), localized surface plasmon resonance (LSPR), or surface-enhanced Raman spectroscopy (SERS) [4,5,13,14].

For diagnostic purpose, it is important to distinguish the analyte in the presence of other compounds because of the complexity of the biological material, whether in serum or saliva [4]. For this reason, regarding body fluids, the surface of the sensor needs some additional functionalization. Also, the ligand construct should be designed in an appropriate way to avoid false-positive results. When large molecules (like proteins) are analyzed, things become more complicated because the proximity of the ligand should not be limited [4]. On the other hand, regarding smaller particles, the ligand should be very specific to pick them up from the complex surroundings because bigger molecules can mask their presence in the sample [6].

The growing area of SPR application creates a demand to make the wider group of readers familiar with the possibilities of biosensing, including practitioners directly involved in diagnostics, who – hopefully – will soon be able to use such methods. Our goal was to introduce the reader to the basics of the common version of plasmon resonance (with the most popular experimental Kretchmann system) and some new modifications of the method that can potentially be useful in

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Article highlights

- Biosensors can be an effective tool in point-of-care medicine.
- Chemical modifications of the sensor surface decrease significantly the limit of detection.
- SPR based biosensors are promising in early diagnostics of some diseases (e.g. viral infections, cancer, neurogenerative disorders)
- The most challenging issue of today is integration of biosensor techniques with microfluidic measurement cells under smartphone control

the field of medical diagnostics, enabling the development of miniaturized devices with a simple reading system that can be used in point-of-care medicine. We focus on the variety of biosensing systems that can be used for diagnostic purposes. We also show examples of diagnostic applications of methods based on the plasmon resonance technique.

2. Principles of SPR

SPR allows identification of the interactions of the molecules that specifically recognize each other and also to evaluate the binding kinetics. One of the interaction partners, called a ligand in bioresonance techniques, is immobilized on an appropriately prepared sensor surface. When a component of the test sample, recognizable by the ligand (called an analyte), appears in the dielectric liquid flowing over the sensor (in the measuring cell), both molecules start to form a complex, which results in a change in the parameters of plasmon resonance.

The resonance phenomenon takes place at the interface between the metal and the liquid phase flowing over it – the dielectric. Metal electrons form the so-called electron gas, only weakly bound to the atomic nuclei, treated as nodes of the crystal lattice. Providing a quantum of energy with a precisely defined value to the metal allows the surface electrons (plasmons) to enter the resonance: they create an evanescent plasmon wave while absorbing the energy delivered. The device exploiting the SPR phenomenon consists of an ultra-thin layer of a noble metal (sensor). Most often it is gold, but silver, aluminum, or platinum are also possible. The sensor surface is covered by a dielectric liquid in the measuring cell. The other side of the metal sensor adheres to the prism, through which a beam of polarized light reaches the sensor. The use of a prism makes it possible to exploit the phenomenon of total internal light reflection (Figure 1a). When the value of the energy delivered to the gold surface corresponds to the excitation energy of the plasmons, they absorb the energy, entering resonance. This results in decay of the reflected light beam intensity, observed as a dark spot instead of reflected light (Figure 1b). The phenomenon of plasmon resonance is extremely sensitive to even the smallest changes at the metal–dielectric interface. Such changes, namely, the interaction of the analyte with the ligand bound to the sensor surface, result in a change in the reflection angle of the decayed light (Figure 1c). Changes in the reflected light intensity are recorded by the detector in real time, thus showing the formation of a complex on the sensor surface (Figure 2). The obtained interaction image, called the sensogram, allows the observation of binding and achievement of the steady state (equilibrium) by the molecules that recognize and bind each other. At this state, in a time unit, the same number of molecules builds the complex and dissociate (Figure 2). This step completes the rinsing of the measuring cell with the sample analyte. Next, the cell is rinsed with a buffer that allows dissociation of the complex. Removal of the dissociated analyte from the reaction medium with the flowing buffer shifts the equilibrium of the reaction, allowing complete dissociation of the complex (Figure 2). The parameters of phases a and c make it possible to calculate the rate of complex

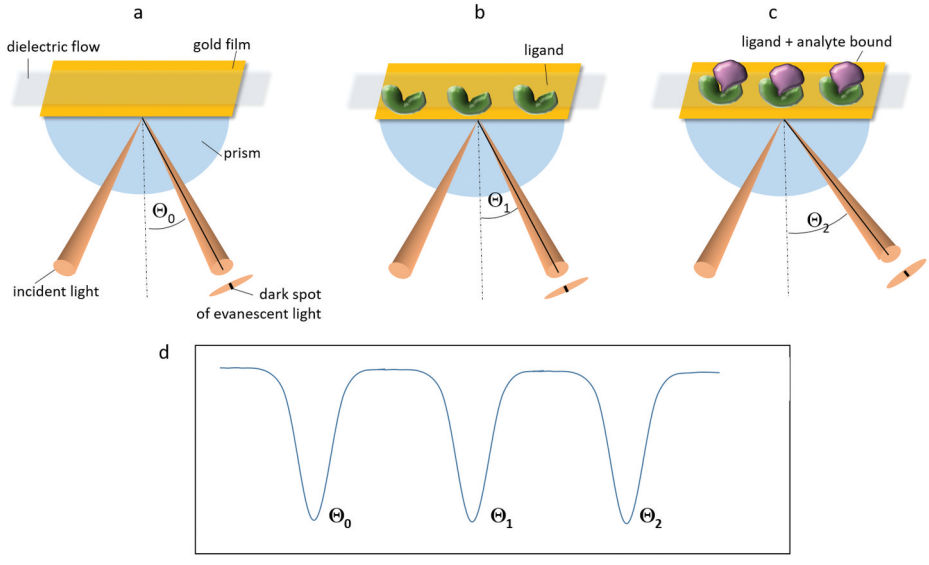

Figure 1. The scheme of the SPR sensor: a) total internal light reflection at the noble metal surface; b) angle of reflected light changed after ligand immobilization on the sensor surface; c) angle of reflected light changed after binding of the analyte to the ligand; d) the angles of light reflectance.

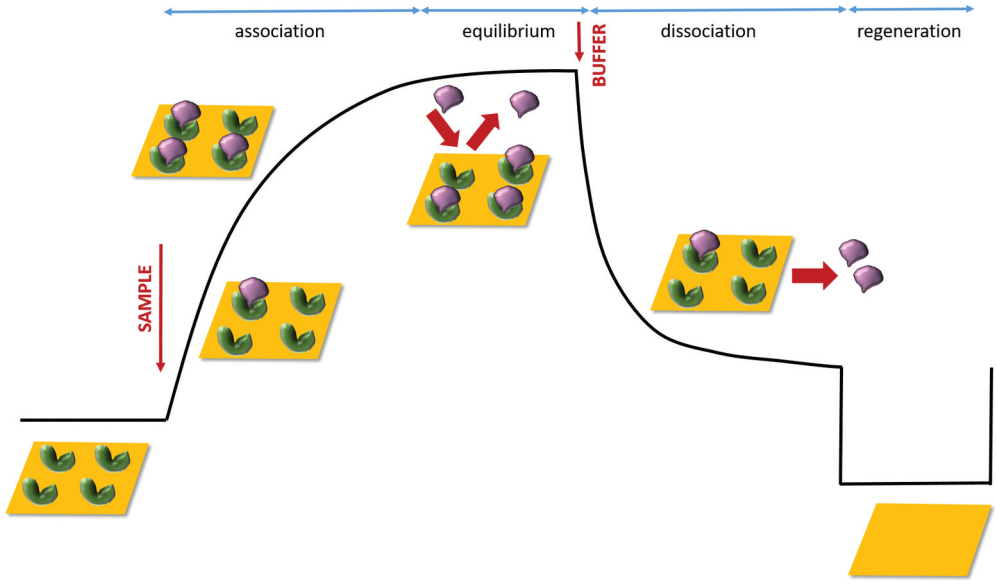

Figure 2. Scheme of typical surface plasmon resonance sensogram.

formation (association, k_a), the rate of complex dissociation (k_d), and the equilibrium constant K_D (equilibrium dissociation constant), describing the mutual affinity of the ligand and analyte, according to the equation:

$$A + B \underset{k_a}{\overset{k_d}{\rightleftarrows}} AB$$

$K_D=k_d/k_a$

The next phase of the experiment involves regeneration of the sensor, which may include the shedding of the ligand from the sensor surface (Figure 2).

The above description presents only the basic assumptions of analytical methods based on the Kretchmann SPR. Readers interested in the detailed biophysical basis of the process can find them in recent excellent reviews, focused on the technical side of the issue [7,15,16].

3. Some other techiques exploiting the SPR phenomenon

Although the SPR is a valuable method to measure ligands interaction in real time and without labels, still the traditional SPR needs sophisticated equipment and highly qualified stuff, which can be challenging in routine diagnostics. The limit of the SPR is also covering the sensor surface with expensive noble metals, such as gold or platinum. To overcome this drawback, novel biosensors are currently being designed, using alternative nanomaterials based on graphene among others [17–20]. On the other hand, SPR can have undoubtful advantages over conventional analytical techniques. An example is its ability to recognize small molecules among the rest of the complex body fluids, e.g. serum. Biosensors can work in a microfluidic scale; therefore, they only need a minimal sample size. The utmost demandable features of diagnostic tool is to obtain portable compact tools, which allow the tests to be conducted on a so-called lab-on-a-chip readable by a smartphone or the naked eye. Therefore, there are various new methods exploiting the SPR phenomenon, i.e. LSPR, field-effect transistor (FET), surface-enhanced Raman spectroscopy (SERS), and application of quantum dots with further modified surfaces of nanocomposites. In all variations of biosensing methods mentioned above, there is a significant value of nanocomposites application. Implementation of structures, such as nanoparticles (NPs), nanorods, or nanoribbons, enhances the signal of light–matter interaction and therefore increases the selectivity of such assay [21,22].

3.1. Localized surface plasmon resonance (LSPR)

In this technique, the surface of the sensor, instead of thin layer, is composed of metal NPs. As a result of their interaction with incident light, localized oscillation of electrons of corresponding frequency occurs, intead of evanescent wave. When the size of NP is smaller than the wavelength of incident light, the plasmons would swing around the nanostructure and are captivated near it which leads to 'localize' the cloud of electrons around the NP [23]. One of the factors influencing LSPR is the size and shape of NPs, so it makes possible to improve sensitivity of LSPR device by modifying these features. Changes in a distance up to 100 nm around the NP influence the LSPR signal, which is the consequence of locally enhanced plasmonic near field. When there is an interaction between the ligand and analyte on the NP surface, the maximum absorption of the plasmons is shifted toward longer waves. In this case, the maximum absorption occurs in visible light, which is desirable in designing diagnostics tests. LSPR is suitable for lab-on-a-chip application because, compared to the traditional SPR, it has a very small bulk effect and can have very small sizes. On the other hand, it also has lower sensitivity

because of the several times smaller distance of the electromagnetic field of plasmons from the sensor surface [24].

3.2. Field-effect transistor (FET)

Electrochemical biosensors are also an interesting group for analytical researchers because of their simplicity, lower cost, and potential ease of miniaturization and mass production [25–27]. In general, the principles of FET-based biosensors are based on the change in carriers of electrons between input and output electrodes on the semiconductor with a gate channel between them. Every interaction between ligand and analyte leads to the change in the flow of electrons, which is measurable. In FET-based methods, graphene or silica has broad application. The unquestionable advantage of FET biosensors is the high sensitive detection on a microfluidic chip. Such biosensors were already successfully implemented in SARS-CoV2 detection [27] or for counting the circulating tumor cells [10].

3.3. Surface-enhanced Raman spectroscopy (SERS)

The close neighborhood of nanometal surface, e.g. nanogold, leads to significant enhancement of Raman light scattering. This phenomenon, called SERS, finds out many possible applications in diagnostic methods. SERS-based biosensors have attracted a great deal of attention from researchers for their highly sensitive and quantitative analyte determination using SERS-encoded NPs (called SERS tags) as an alternative to colloidal gold for signal reporting. There are three basic parts of SERS tags: the Au/Ag NP, as a Raman-enhanced substrate, adsorbed Raman reporter dyes to generate characteristic SERS signals, and specific target-binding antibodies. Wang et al. [28] used Fe_3O_4@Ag NPs as magnetic SERS nanotags to create a SERS-based biosensor for the simultaneous detection of two respiratory viruses, influenza A (H1N1) and human adenovirus. The lowest LOD measured by the SERS-based biosensor was 50 pfu/ml and 10 pfu/ml for H1N1 and human adenovirus, respectively. The sensitivity of the biosensor was about 2000 times greater than in commonly used colloidal gold strip method. The proposed biosensor can be used directly for the analysis of biological samples without any pre-treatment [29].

3.4. Nanomaterials enhance the signal in SPR-based techniques

Despite different variants of the methods, also the new nanomaterials are of particular interest in the design of biosensors. Alternative nanocomposites often have better properties than the traditional ones. In general, they have better conductivity, mechanical strength, chemical stability, and larger hydrophilicity, and their surface is easy modifiable. Absorption of ligands also reaches a greater extent. Great potential has been reported for graphene, especially graphene oxide [18,19,30], transition metal carbides/carbonitrides (e.g. titanium carbide), also called MXenes [31], and molybdenum disulfide (MoS_2) [18]. Graphene-based sensors are faster than traditional ones because of high electron transport mobility [18]. They can also form nanostructures, e.g. ribbons or particles [20]. In biosensors with a FET, graphene shows greater 2D electrical conductivity. A gold electrode biosensor was developed, covered with silicon NPs coated with graphene oxide [32]. The individual biofunctional requirements have been properly specified and implemented so that the assay can be conducted in physiological conditions. Such preparation of NPs is necessary to allow the attachment of the antibodies to the biosensor. Given the availability of current diagnostic approaches, graphene FET-based biosensor platforms have many promising benefits, such as the ability to be very sensitive and immediately detect a small volume of the target analyte [27]. Graphene with exposed hexagonal carbon atoms on its surface, electronically conductive, with high charge mobility and specific surface area, has proven to be ultra-sensitive in sensing systems due to its ability to detect nearby changes in their surface and provide an ultra-fast sensor platform. Therefore, FET biosensors based on graphene are very important in carrying out immunological diagnostics with high sensitivity contrast. The MXenes were used in detecting carcinoembryonic antigen (CEA), where a titanium carbide based biosensor bound to protein A was immobilized with specific monoclonal antibodies a-CEA and detected the CEA concentration with LOD = 0.07 fM in the serum [33]. The reproducibility was also very promising. Molybdenum disulfide-based biosensors have properties similar to graphene, but with higher optical absorption efficiency [34]. There is a strong attachment between metal and MoS_2 which leads to more effective charge transfer between layers, resulting in the improvement of the SPR sensitivity. Covering the metal with a MoS_2 layer protects against oxidation of the metal surface [35]. Additional modification of both graphene and MoS_2-based layers with the addition of monochloroacetic acid (GO-COOH, MoS_2-COOH) results in a stronger SPR signal [18,36]. Chiu et al. [36] described the detection of lung cancer biomarkers on a carboxyl functionalized molybdenum disulfide biosensor with LOD = 0.05 pg/mL.

3.5. Toward the portable devices

Thinking about expanding the diagnostic potential of SPR techniques, the challenge is to simplify of the detection system, as well as to minimize the device size. Meeting these expectations should not only lead to lower the costs of both the equipment and the analysis itself. First of all, it will enable the transfer of diagnostic procedure from highly specialized laboratories directly to the sites of patient care, like primary care physician office. Numerous studies are now approaching this direction. Approaching down-scaling of the analytical devices size and simplifying data acquisition, it seems that the potential of LSPR in the construction of field devices is greater than that of the traditional SPR, despite the generally higher sensitivity of the latter [15]. LSPR offers a significant simplification of optical elements: plasmon excitation occurs directly through the incident light, so the device does not require a polarizer or a prism or a diffraction grating to control the angle of incidence of light on the sensor. Low-cost LEDs [15,37] may be the source of the excitation light. A good solution seems to be the use of colorimetric detection, based

on an easy to measure color change. It can be the result of aggregation, growth, or etching of NPs. A simple example is gold NPs that change their color from red to blue as a result of aggregation [37,38]. A smartphone camera [37] is enough to read such an optical signal. However, it is worth remembering that the fabrication of NPs with defined sizes and shapes will be more expensive than the usual metallic nanofilm [37]. The aspect that may pose a bigger challenge is the implementation of the microfluidic technique in minimized measuring chambers. Since, as already mentioned, the biological sample is unprocessed body fluid, its complex composition may lead to aggregation of molecules in small diameter channels and, consequently, to their clogging. These microfluidic elements must therefore be easily replaceable, and biological material may require pre-filtration [15].

4. Assay design

4.1. Preparation of sensor surface

For plasmon resonance analysis, the surface of the sensor must be prepared in an appropriate way. The concentration of the immobilized ligand should allow binding of a sufficient amount of analyte, with minimal nonspecific bindings or sterical hindrance. On the other hand, the density of immobilized elements should not be too high and the distance from the sensor surface should be well established. Also, the proper direction of immobilized element is crucial. In the perfect situation, the ligand of interest should be bound to the sensor surface in well-defined density and direction, avoiding nonspecific bindings. The unmodified gold layer is not suitable for biomolecular interactions because the ligand attachment to its surface is random and nonspecific binding is frequent. Therefore, the gold layers are usually modified before ligation. The purpose of such modification is similar to the blocking step in ELISA. The so-called antifouling layer can be prepared in a few different ways. Mixtures of different polymers with further modifications aimed to capture the ligand and provide a non-fouling background are used [39–41]. The gold standard is oligo or polyethylene glycol (OEG/PEG) and its derivatives [39,42]. Other antifouling materials are polysaccharides, such as dextran [39]. Recently described self-assembled monolayers (SAMs) included alkanethiols or disulfides [43], alkylated N-heterocyclic carbenes [44], zwitterionic thiols [39,45], dithiols [46], cellulose [47], 4′-nitro-1,1′-biphenyl-4-thiol, 11-(mercaptoundecyl)triethylene glycol (C11EG3OH) [48], or phenylboronic acid [49]. Another method of functionalization of the gold surface is covering it with hydrogels prepared from a mixture of carboxymethylated dextran chains, silanes, zwitterions, polypeptides, or other polymers that are usually more stable and decrease nonspecific binding [39,41]. Such prepared anti-fouling surfaces create a hydration layer on the SAM surface which prevents nonspecific binding and limits the steric hindrance, especially when small molecules are targets. Nowinski et al. synthesized a SAM composed of natural peptides with a ligand recognition fragment at the end [50].

The other strategy to omit nonspecific binding to the sensor surface is immobilization of ligands on a well-defined artificial structure, e.g. triangular gold NPs synthesized previously with nanosphere lithography on muscovite mica substrate [51] or DNA tetrahedron probes bound on the sensor surface [52]. In such sensor surface modifications, the distances between nanoislets are well-defined and under control.

Proteins contain numerous functional groups, so they can bind to the surface of the sensor in a random-like way. To avoid this, the immobilization procedure can be performed directly due to covalent binding or indirectly due to specific interactions such as biotin-avidin [53], antibody-antigen [54], or histidine-chelated metal ion [55]. The immobilized molecule should be stable and can be regenerated many times without losing its activity.

4.2. Biorecognition

In the biomedical area, the biggest challenge is optimization between high specificity and good sensitivity with the lowest possible LOD [4]. Traditional methods that are usually used in identification of biologically active molecules can be divided into direct and indirect procedures. Though very useful, they

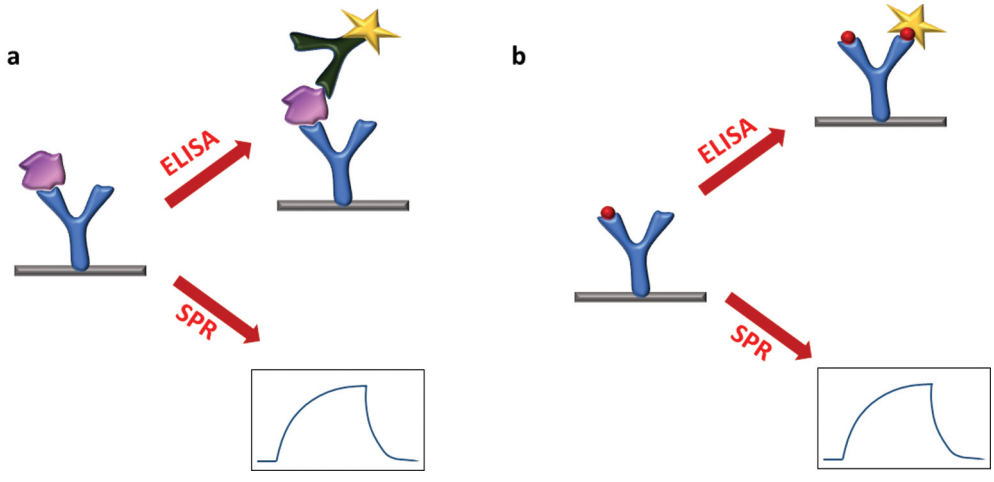

Figure 3. Comparison of the ELISA and SPR assay: additional detection step mandatory in ELISA techniques is redundant in SPR: a) Sandwich ELISA, b) competitive assay for hapten detection.

are also quite expensive and time-consuming. They usually need labels, such as enzymes or fluorescent tags. The most common indirect methods of biomolecule detection are ELISA. The first step involves immobilization of the ligand on the solid phase by passive adsorption; usually, it is a polystyrene plate. Then, the immobilized ligand is incubated with its counterpart (sample analyte) and later with secondary labeled antibody (Figure 3a). The signal is obtained by the color reaction of the label enzyme or by fluorescent signal detection. The attachment between counterparts in such a sandwich is possible through their direct specific interaction when it is an antigen and antibody or through introduced modifications, such as biotinylated lectin and avidin tagged enzyme in glycobiology research. Although, in many cases, immunochemical techniques are used as easily or even more easily than those using biosensors, the situation is different when it comes to the determination of low-molecular weight substances. As haptens, such molecules do not have the two antibody-binding sites (capture and detection), which makes impossible to use the conventional sandwich assay, that must be replaced with less convenient competitive tests (Figure 3b). The signal is measured after an equilibrium is reached. Incubation steps are separated with extensive washing, which also prolongs the assays. Compared to ELISA, SPR provides more details about the quality of the created complex, especially about the speed of its formation and dissociation [56]. Even though both methods use similar kinds of interactions between counterparts, SPR measurement of the interaction between the ligand and the analyte is possible in real time and omits the washing and some incubations steps (Figure 3) [57]. The challenge is to design a ligand that can bind its counterpart with sufficient specificity. The perfect situation is when the sensor works repetitively in a number of tests and is not too expensive [58]. Although ELISA can be modified to decrease the LOD [28,59], it still does not provide information about the dynamics of the process of interaction between molecules but only describes the equilibrium state of interaction. In general, SPR takes less time and has a lower LOD [30]. Even when a lower concentration of the analyte could be measured in ELISA, the range of linearity was wider in SPR [60].

The great advantage of the plasmon resonance methods is that the same technical strategy can be applied for various analytical purposes. In the case of classical methods, not only the test design have to be completely different depending on the type of analyte but also often completely different methods have to be used (immunochemical versus PRC techniques). For plasmonic methods, the sensor surface may need to be modified appropriately, but the assay strategy is the same for a wide variety of analytes, as shown in Figure 4. In general, the SPR allows the detection of any two mutually recognizable molecules (Figure 4a). Although the most commonly used ligand is the analyte-specific capture antibody (Figure 4b), the same technique also allows the detection of specific fragments of genetic material (Figure 4c), as an alternative to PCR or Southern/Northern blotting. The use of antigens, often exposed to polymer tails to enhance their availability (Figure 4e), is an excellent method of detecting specific antibodies and therefore identifying many infections. Advanced SPR methods enable detection of intact viral particles or even bacterial cells (Figure 4f).

4.3. Aptamers – the new tool in biosensing

Among the wide range of different types of immobilized molecules, still the most commonly used are antibodies [54,61–63]. They are commercially available and most often present well-validated affinity and specificity to their counterparts. The recently developed alternative to antibodies are aptamers, in SPR use called aptasensors. They are fragments of nucleic acids, which can be immobilized on the sensor surface [59,64–66] [Figure 4g]. Aptamers are synthesized or transcribed from double-stranded DNA (dsDNA) oligonucleotides into single-strand DNA (ssDNA) or RNA (ssRNA) fragments, respectively. They are isolated from the random bulk by exponential enrichment (SELEX). The general strategy of SELEX is selection of the best target bound sequences from the non-bound ones and then their amplification by PCR or RT-PCR. Their affinity to the ligand is then further tested in

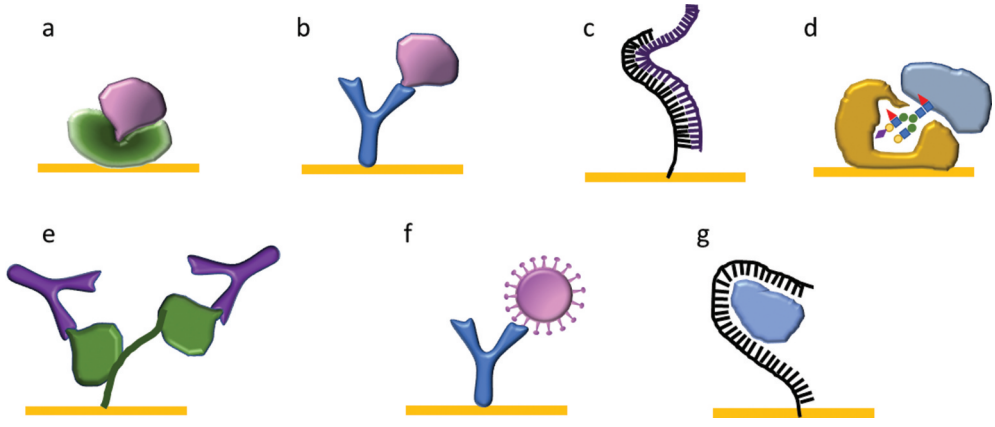

Figure 4. Biosensing types useful in SPR: a) general ligand-receptor binding; b) antibody-antigen; c) complementary oligonucleotide probe to detect gene sequence; d) lectin-glycoprotein; e) antigen protein to detect specific antibodies; f) antibodies to detect intact virus via its surface protein; g) oligonucleotide aptamer used for protein capture.

different temperature and pH conditions until they achieve the finest quality. Aptamers are folded into specific structures, so that they are able to mimic the antibodies [65,67]. Merits of aptamers are high resistance to harsh physical conditions, such as temperature or pH, and no need of an animal host in the production process. This is especially important when the availability of antibodies is limited through the toxicity of the antigen [68,69]. Aptamers, similarly to antibodies, can be modified with functional groups, for example, biotin [70] or gold NPs [66]. They can also act as structure switching systems, i.e. quenching aptamers, split aptamer-based models, or enzyme-assisted recycling procedures [71]. Last but not least, aptasensors, often in contrast to antibody biosensors, can be reused after an appropriate regeneration process, which in turn significantly reduces the costs of production and use [72,73]. Aptasensors, which detect breast cancer cells by DNA aptamers specific to HER2-positive membrane receptors, have been described: biotinylated aptamers were immobilized on neutravidin-gold NPs and detected 550 cells/mL [74]. Zhou and coworkers designed a hairpin-like structure-based aptasensor, highly sensitive in recognition of the breast cancer exosomes (LOD 3.94×10^5 particles/mL) [75]. Aptamers are also helpful in rapid diagnostics of viruses [76] or bacterial infections [77]. They can also be used reveal to protein markers, such as human gonadotropin (hCG), in serum samples [78], mucin 1 [79], or amyloid-beta oligomers in Alzheimer's disease [80]. A very promising aptasensor proved to be useful for early diagnosis of autism. The aptamer immobilized on gold nanorods (AuNR) was able to detect β-casomorphin (BCM-7) at the 1 fM level [81].

4.4. Glycobiology research and application

Recent years have brought evidence of the involvement of protein–sugar interactions in the regulation of the immune response, and thus, the association of changes in the glycosylation pattern with the pathogenesis of diseases [82–84]. This opens up a new field of diagnostic applications, and SPR is also a valuable technique in glycobiology research. In this field, however, some drawbacks must be considered. First, lectins that are most often applied as ligands present quite broad carbohydrate specificity, and thus can cross-react with glycans of similar structures, though with different affinities. The other issue is that lectin–carbohydrate interaction frequently demands cluster presentation of both counterparts. For these reasons, preparation of a functional sensor, as well as data interpretation, needs special care. In research described so far, multivalent binding between immobilized lectins and glycans was tested on synthetic glycopolymers [85,86] or lectins which were modified to increase their specificity [87]. Also screenings were performed to identify glycosylated proteins on the sensor with immobilized sugar on its surface [88].

5. Examples of biomedical assays exploiting SPR

Biomedical assays are mostly performed on body fluids, such as blood [62,78], saliva [89], semen [90,91], cerebrospinal fluid [92,93], or urine [94]. Therefore, the unique properties of each of them should be considered and the whole process should be validated individually for each body fluid, demanding special biosensor constructs according to the sample's unique composition. When SPR is used as a routine diagnostic tool, the most popular human body fluid is still blood serum or plasma [61,62]. Because of the high abundance of proteins – 60–80 mg/mL – blood serum is also the richest source of information about the patient's condition. This, however, also brings a serious challenge, as such abundant proteins can foul the surface of the sensor in a nonspecific way, making accessibility of the ligand of interest more difficult [13]. Another issue is the wide range of the sizes of target molecules which can interact with each other in a very complex manner. Obviously, dilution of the sample aimed to diminish the background of nonspecific interactions is a good strategy, but also lowers the concentration of the compounds of interest, sometimes below the detection limit [13].

5.1. Cancer biomarkers

In developed countries, cancer is still one of the main causes of death. Early diagnostics has a great impact on higher chance for recovery. A good target for tumor progression diagnostics is liquid biopsy, which analyzes the circulating cancer markers in the body fluids, not like traditional biopsy in the tumor tissue, mainly thanks to its lesser invasiveness. This is important for patients who face many invasive procedures over the years [95]. It also allows for detecting and quantifying a wide panel of tumor markers, such as proteins, circulating tumor cells, exosomes, or nucleic acids of the tumor cells, which can provide better prognostic data for the stage and grade of the tumor [8,95–97]. For monitoring tumor, proteomic markers lowering the LOD and easier detection of target molecule in complex clinical sample are advantegous. CEA, the protein marker useful in prediction of cancer development in some tumors, was established with the LOD = 0.36 ng/mL using SPR biosensors [7,98,99]. What is even more important, current cancer diagnostics is often directed into non-protein biomarkers, not easily accessible with conventional methods. SPR-based assays for miRNA detection in different kinds of cancers have been described [100–102]. These short non-coding sequences of ribonucleic acid change their concentration during some diseases and correlate with tumor progression and metastasis [103]. Cai et al. [101] designed a low-cost colorimetric test based on SPR absorptivity of gold, where an RNA fragment was attached to the gold NP and was complementary to the target miRNA from the cancer sample. As a result, the sandwich RNA-AuNP-miRNA was formed as an assay format. This complex changed the SPR signal and allowed quantitative detection of miRNA at the nanomolar level. Yeung and coworkers [100] made a step forward and designed a multiplex automated test for cancer biomarkers existing in urine. The capped gold nanoslits were immobilized with four different RNA probes through thiol bonding, aiming to capture and detect complementary miRNA sequences. As a result, miRNA biomarkers in urine were detected at the femtomolar level [100]. Another feature that seems to be important for the detailed cancer diagnostics is the assessment of DNA methylation: the epigenetic alteration frequently accompanying the disease development and

useful to predict its progress. Also in this case, the interesting reports, indicating efficient application of SPR, are available [104–107]. These examples, as well as detailed analysis of tumor-derived exosomes, show how SPR techniques may be useful for the analysis of the targets not easily assessed with conventional (like ELISA) methods.

5.2. Cardiovascular diseases

Not every diagnostic procedure requires a very low LOD. Nevertheless, by learning more and more about the molecular mechanisms of various diseases, scientists are able to develop new biomarkers that often face the fact that their concentration in biological material is very low. In such circumstances, highly sensitive biosensor methods prove to be useful. In some health conditions, such as in acute myocardial infarction, urgent estimation of troponin level lowers the risk of death, but the LOD level is femtomolar [108]. SPR was also applied in the identification and measurement of molecules present in blood in extremely low levels, e.g. brain natriuretic peptide (BNP) proposed after SPR evaluations as a prognostic marker in heart failure [12,109,110] or stroke [111]. Xu et al. performed N-terminal pro-brain natriuretic peptide (NT-proBNP) detection on gold NPs modified with a Zn-doped CdS NP cosensitized Bi_2MoO_6 nanosheet photoelectrochemical (PEC) platform, and achieved the LOD = 0.037 pg/mL [109].

5.3. Diagnostic strategies in viral infectious

In view of the SARS-cov2 virus pandemic, which has become a huge, global challenge for the health care (but also the economy) of the whole world, effective diagnosis of the infection has become an urgent issue.

Currently, three types of diagnostic tests are used in monitoring infections. The gold standard in terms of effectiveness is the RT-PCR test, which detects the presence of viral RNA in a biological assay by multiplying it with polymerase chain reaction [112–114]. Such a test presents excellent sensitivity. Also, specificity is considered satisfactory, though PCR may lead to multiplication of some non-complementary oligonucleotide sequences, and thus to some false-positive results. The key disadvantage is related to the equipment requirements and demand of highly qualified personnel, as well as the time-consuming and complicated procedure of sample transport to accredited laboratories and isolation of viral genetic material. These drawbacks are overcome by the field-applicable antigen cassette tests, which are designed to detect the presence of viral antigens through the reaction with specific labeled (usually with the colloidal gold) antibodies, which allows visual detection with the naked eye. Convenience of use and low cost are, however, offset by a much lower sensitivity. Usually, the infection cannot be detected before the onset of physical symptoms, and therefore individuals who may be vectors that propagate the infection remain undiagnosed. The third test, detecting the presence of antibodies, due to the time of the immune response and its inter-individual variability, is not suitable for the diagnosis of infections, although it may be useful for monitoring immunity [112,115].

The use of plasmon resonance methods has a chance to overcome the limitations of the aforementioned classical genetic and immunochemical methods. Appropriate selection of ligands enables detection of the genetic material of the virus with high sensitivity and without the need for its multiplication, but also an appropriately sensitive detection of viral antigens (Figure 4). Special requirements may be rendered by application of the previously described modifications of the basic method, especially localized SPR [114,116,117]. Replacing the sensor film (gold) with NPs of this metal, e.g. in the form of nanoislets, makes it possible to create a simple visual detection method. This, in turn, enables the miniaturization of the measuring device and a specific, sensitive measurement at the point-of-care in the form of a lab-on-a-chip design [114,116–119].

It is worth emphasizing that the use of SPR-based methods enables the detection of each of the aforementioned analyte targets: viral genetic material, antigens, and antibodies [112]. The first two, if a test is designed to detect them in the patient's saliva, may be applied as separate measurement channels of the same device. The detection of antibodies generally requires the use of a blood sample, but such tests will be extremely helpful in monitoring the effectiveness of the vaccine that the world is urgently waiting for [112,115,120].

Attempts at the application of SPR-based methods in the diagnosis of viral infections cover recent years, when the global threat of new viral epidemics increased. In 2013, Yu et al. [121] developed a silver nanoisland sensor for the sensitive trapping of adenoviruses. In this experiment, however, the binding was nonspecific so the method was applicable only to the evaluation of virions cultivated in vitro; the lack of specific capture prevented its diagnostic use. This barrier was overcome at about the same time by the team of Yakes et al. [122], who designed a sensor with a typical sandwich system used in ELISA immunoassay techniques. The test was used to detect feline calicivirus, the equivalent of extremely infectious human noroviruses. Intact viral particles isolated from foodstuffs were captured by specific antibodies immobilized on the sensor surface and generated a first SPR signal, further amplified by the attachment of a second (detection) antibody, which in this case did not need to be labeled. Soon there were reports on the development of sensors with hepatitis B antigens, and antibodies that specifically recognize them were imprinted on the gold surface in the form of microarrays, which allowed multiplex detection of several HB antigens and antibodies using a single chip (device) in a 20μL serum sample, with 85% agreement with ELISA data [123]. Ashiba et al. [124] used SPR-assisted fluoroimmunoassay for the sensitive detection of above-mentioned noroviruses causing severe gastroenteritis. Quantum dot fluorescence was used to enhance the sensitivity of detection. The sensor was designed to excite Q-dot fluorescent dyes by an enhanced electric field induced by SPR on Al film. Gold, commonly used as a sensor, is not compatible with the excitation (390 nm) and emission (705 nm) ranges of the Q-dots used, so it was replaced with aluminum. Shi et al. [125] used SPR to distinguish nine

respiratory viruses, which cross-reacted in PCR reactions. The viral material was precisely bound to complementary oligonucleotide probes in this study. H5N1 avian influenza virus aptasensors were used by Bai et al. [126]. A commercially available miniature chip from Texas Instruments with a streptavidin-coated gold sensor was functionalized with a biotinylated hemagglutinin-specific aptamer and thus ready to bind viral surface protein. The authors achieved binding of the analyte with a KD constant of 4.65 nM. A pair of specific aptamers was used for H5Nx viruses by Nguyen et al, [127]. Also, last year, the sensitive detection of Dengue virus protein on the graphen-based sensor was reported [9].

Last year, researchers' attention was focused on SARS-Cov2. In the articles, from 2020, regarding the diagnostic challenges of the current pandemics [112,114,116,119,128], the authors emphasize the potential benefits associated with the development and application of biosensor-based methods with simplified signal detection methods. The phenomenon of electron transfer and its change into an electric current was exploited in two in-house built devices described by Mahari et al [129]. In the first one, FTO (glass coated with fluorine doped tin oxide) with sensing areas of AuNPs, further functionalized with anti-SARS-Cov2 antibodies, was used. The SPR generated 520-nm signal of AuNPs was 9-nm red shifted after Abs binding. The variation in electron transfer after viral antigen binding was measured as the change of the current. In the second device, the FTO electrode was replaced with a graphene layer, exploiting its conductivity. LOD for both devices was about 100 fM in the saliva samples. The most detailed description so far of a biosensing platform for SARS-Cov2 detection was provided by Seo et al [27]. In this device, antibodies to SARS-Cov2 spike protein were conjugated on a graphene sheet, with 1-pyrenebutyric acid N-succinimide ester used as a linker to provide an appropriate distance from the surface and antibody orientation. A FET enabled the detection of 0.1 pg/mL spike protein in the clinical transport medium, without any sample pretreatment. Finally, the very experienced team of Das, Guo et al. reported the elegant model of sandwich plasmonic biosensor that utilizes AuNR for the detection of SARS-Cov2 spike protein [128].

The above-presented examples do not exhaust the topic of attempts to use the methods based on plasmon resonance in diagnostics. However, they allow to state that their potential is huge and is constantly growing. Unfortunatelly, no such tests have been commercialized so far. It is possible that the global investment in the development of diagnostics is incomparably lower compared to the funds allocated to the development of vaccines and effective drugs, although effective monitoring of the spread of infections seems to be an equally important goal.

6. Conclusions

Analytical methods based on plasmon resonance have experienced tremendous development in the recent years. Although their most frequently emphasized advantage, which is the ability to perform kinetic measurements in real time, is not directly applicable in clinical diagnostics, SPR seems to be gaining more and more new applications also in this area.

The frequently emphasized benefit of using SPR is the ability to develop low LOD assays. In many cases of newly discovered and predicted for diagnostic use of biomarkers, we are faced with their very low concentration in body fluids. Thus, adequately sensitive analytical techniques are essential for their detection and quantification.

Many biomarkers can be accurately determined with conventional methods, such as ELISA or PCR. The downside of these methods, however, is their time-consuming. SPR and related techniques provide a short time-to-result, which enables faster implementation of the treatment decisions, especially if the diagnostic procedure could be used in point-of-care.

The latter aspect, the development of portable and easy-to-use devices, is the greatest challenge and at the same time, the greatest development potential of the discussed methods. The possibility of quick diagnosis in many cases may be of critical importance for the patient. It is worth emphasizing also that in many countries with low incomes, access of the population to specialized diagnostic centers and to medical care, in general, is very limited, which is often provided only by mobile points. Reducing the cost of analytical devices and the ability to adapt them to work in the field can invaluably improve in the quality of medical care in countries with limited resources.

7. Expert opinion

Compared to traditional diagnostic methods, biosensors offer hope for faster diagnosis without the need to carry out long and tedious preparative procedures. They also have the advantage of allowing real-time observation of ligand–analyte interactions, which is essential for determining the dynamics of ligand interactions. This is essential when looking for new drugs or modifications to already known therapeutic molecules. Nanomaterials such as graphene used in the construction of biosensors for diagnostic purposes increase their sensitivity and selectivity, which is caused by their good conductivity and mechanical properties. It is also extremely important because, in diagnostics, most often crude material containing a lot of other ingredients is used, i.e. serum or saliva. The use of new nanocomposites can significantly reduce nonspecific bindings to the sensor surface, as well as reduce the cost and increase the time of life of such tools.

8. Five-years view

In the coming years, it is possible to create a selective rapid test against viruses based on biosensing. The current SARS-CoV2 pandemic may force this direction of portable tests development because early diagnosis of COVID-19 without going to the laboratory will significantly reduce the transmission of the pathogen and limit the pandemia. Currently, a number of patients use telemedicine and thus the usage of the mobile diagnostic tool with the possibility of immediate sending of the results to the physician would facilitate the rapid isolation of sick patients from the rest of the society. It

would also allow controlling of eventual subsequent epidemics at early stage of spreading.

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