Review

Rapid and label-free bacteria detection by surface plasmon resonance (SPR) biosensors

Fahriye Ceyda Dudak and İsmail Hakkı Boyacı

Department of Food Engineering, Hacettepe University, Beytepe, Ankara, Turkey

Surface Plasmon Resonance (SPR) biosensor technology has been successfully used for the detection of various analytes such as proteins, drugs, DNA, and microorganisms. SPR-based immunosensors that coupled with a specific antigen-antibody reaction, have become a promising tool for the quantification of bacteria as it offers sensitive, specific, rapid, and label-free detection. In this paper, we review the important issues in the development of SPR-based immunoassays for bacteria detection, concentrating on instrumentation, surface functionalization, liquid handling, and surface regeneration. In addition, this review touches on the recent advances in SPR biosensing for sensitivity enhancement.

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1 Introduction

The identification and quantification of microorganisms has become a key point in biodefence, food safety, diagnostics, and drug discovery researches. Detection of pathogens and indicator microorganisms in water and food samples plays a vital role in public and environmental health. For 2005, it has been reported that 1.8 million people died from diarrhoeal diseases, mostly related to the contamination of food and drinking water (http://www.who.int/mediacentre/factsheets/fs237/en). Centers for Disease Control and Prevention estimate that 325 000 hospitalizations and 5000 deaths occur each year in the United States related to foodborne diseases [1]. The accurate detection and identification of bacteria in food and water is required for public safety.

Identification of bacteria is usually realized by traditional methods such as culture-based and colony-counting methods which, in most cases, require several handling steps. Therefore, the detection by traditional methods is time consuming and inconvenient [2]. To overcome these difficulties, many novel and rapid methods have been developed such as polymerase chain reaction (PCR) methods and immunoassays. PCR, a nucleic acid amplification technique, has gained acceptance for the detection of pathogens [3, 4]. Although PCR methods shorten the analysis time compared with traditional techniques, it often requires trained personnel for the analysis [5]. Immunoassays, which rely on the specific reaction of antibody and antigen, have been commonly used in rapid detection of bacteria. Enzyme-linked immunosorbent assays (ELISA), which are commercially available, have been used for the detection of various microorganisms. Immunomagnetic separation (IMS), another analytical tool used for the detection of bacteria, is based on the capturing of target bacteria by antibody-coated magnetic beads. IMS was coupled with various detection methods such as PCR [6], methods based on fluorophore-labeled secondary antibodies [7], enzyme-labeled antibod-

Correspondence: Dr. İsmail Hakkı Boyacı, Hacettepe University, Department of Food Engineering, Beytepe, TR 06800, Ankara, Turkey
E-mail: ihb@hacettepe.edu.tr
Fax: +90-312-299-2123

Abbreviations: ATR, attenuated total reflection; **GST**, glutathione S-transferase; **IMS**, immunomagnetic separation; **NTA**, nitrilotriacetic acid; **QCM**, quartz crystal microbalance; **SAM**, self-assembled monolayer; **SIA**, subtractive inhibition assay; **SPR**, surface plasmon resonance; **SPRi**, surface plasmon resonance imaging; **SPW**, surface plasmon wave

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ies [8], and quantum dot-labeled antibodies [9, 10]. Although these immunoassay techniques are quite sensitive and specific, the procedures involve a lot of incubation steps which make the assays inconvenient.

Biosensors, that combine biological materials such as antibodies, peptides, and nucleic acids with a physicochemical transducer, have become an important tool for the rapid, sensitive, and selective detection of microorganisms. Biosensors can be classified according to the transducing system as optical, electrochemical, thermometric, piezoelectric, or magnetic [11]. Figure 1 shows the studies concerned with bacteria detection using different types of biosensors over the period of 20 years. One of the most popular methods are electrochemical biosensors which have been used for the detection of various bacteria [12] such as *Escherichia coli* O157:H7 [13] and *Staphylococcus aureus* [14, 15]. Although electrochemical biosensors offer rapid detection of microorganisms, they could not arise popularity because of their low sensitivity and label or mediator requirements [11]. Quartz crystal microbalance (QCM) biosensors, piezoelectric sensors, have been developed for the label-free detection of a wide range of bacteria [16]. The detection of *E. coli* O157:H7 in 30-50 min with a detection limit of 10^3 cfu ml^{-1} was reported using QCM immunosensors [17]. Similar immunosensors have been reported for the detection of other pathogens such as *Salmonella enteritidis* [18] and *Listeria monocytogenes* [19].

Another option for the rapid and label-free detection of bacteria are surface plasmon resonance (SPR) biosensors. SPR sensors, a type of optical sensors, measure the changes of the refractive index at the sensor surface. This review highlights the major principles of SPR biosensors for bacteria detection and future prospects of SPR biosensor technology.

2 SPR instrumentation

SPR is a charge-density oscillation that exists at the interface of two media, a metal and the other a dielectric [20]. Although silver, copper, and alluminium are suitable metals for surface plasmon, the most commonly used metal is gold due to its oxidation stability and, being inert which is a desire, characteristic while working with biological samples.

The charge density wave, called surface plasmon wave (SPW), is maximum at the interface and diminishes exponentially into both media. SPW can be excited by an optical wave, if the component of the wave vector that is parallel to the interface, matches that of SPW [21]. Attenuated total reflection (ATR) by prism couplers or optical wave guides and diffraction at the surface by diffraction gratings can be used for the excitation of SPW by an optical wave [20]. The Kretchmann configuration (Fig. 2), where the excitation of SPW is achieved by using ATR in prism couplers, is widely used in SPR sensors [22].

When a SPW exists at the metal-dielectric interface, its fields penetrate into the dielectric. A change in the refractive index of the dielectric medium results in a change in the propagation constant of the SPW. Those changes in propagation constant are determined by measuring one of the characteristics of the light interacting with SPW.

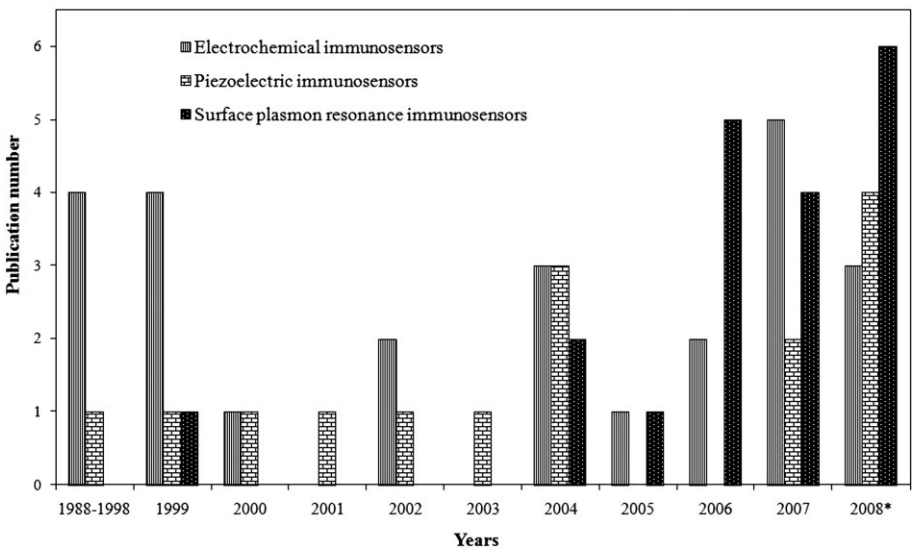

Figure 1. Number of studies published on detection of bacteria using different types of biosensors from 1988 until November 2008. (Source: ISI Web of Science; Keywords: Electrochemical, immunosensor and bacteria: ▥, Piezoelectric, immunosensor and bacteria: ▤, Surface plasmon resonance, immunosensors, and bacteria: ■).

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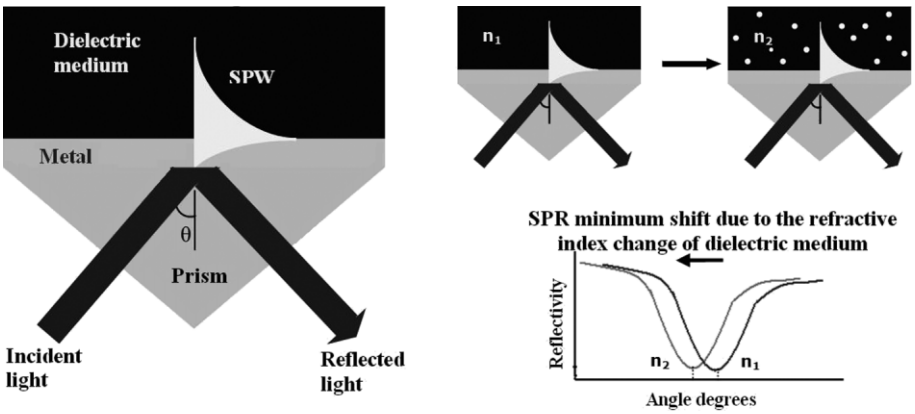

Figure 2. The Kretchmann configuration and the shift in SPR minimum angle due to change in the refractive index of the dielectric medium.

Therefore, SPR sensors mainly can be classified as the sensors with angular, wavelength, and intensity modulation [21]. At angular modulation, when the incident light at a fixed wavelength and certain angles is coupled to SPW, a minimum intensity in the reflected light occurs at an angle, called SPR minimum. A change in the refractive index of dielectric medium results in a change of the angle at which the SPR minimum occurs (Fig. 2). For wavelength modulation, change in the refractive index is determined by measuring the intensity of the reflected light at multiple wavelengths, keeping the angle of the incident light constant. Lastly, the intensity of the reflected light can be measured to determine changes in the refractive index. In this type of SPR sensors, angle and wavelength of the incident light are kept constant.

Nowadays, three groups of SPR and SPR-like technologies compete with each other for real-time label-free monitoring of biomolecular interactions. They are SPR, SPR imaging (SPRi), and non-SPR instruments [23–25]. Non-SPR instruments are not the topic of this text and are described briefly elsewhere in the literature [25]. In SPR sensors, multiple measurements are limited by the number of channels on the sensor surface. Most of the commercial SPR instruments have single or double channels. Multiple channels are critical for user to monitor angle shifts for more than one sample and references in the same time. In the SPRi instrument, the sensor surface is monitored with a CCD camera, and the reflectivity changes of the spots on the sensor surface are measured. Although SPRi enables high numbers of measurements in a single run [25], in practice, it has some drawbacks, such as the difficulties related to the preparation of homogenous spots on the sensor surface for the detection of different targets and the construction of a sensing surface in fluidic systems.

SPR optic construction has critical importance for the performance of the analytical system. However, liquid handling systems and complementary devices such as temperature controller and degasser units have also a vital role for the success of the instrument [26–29]. Although the design of the flow system varies in different commercial products, the common requirement is pulse free liquid handling. Any pulse or change in the flow of the liquid on the sensor surface influences the sensor response. Some of the commercial SPR systems such as Biacore products, Autolab SPR instruments, SPRi-Plex (Genoptics), SensiQ (Nomadics) are often associated with microfluidic channel flow injection system [25, 30]. And some others (Reichert, BI-SPR of Biosensing Instruments, Bio-Suplar) are in the market without flow control unit and flow control is supplied additionally with a syringe or peristaltic pump. Due to the technological requirement in both SPR and SPRi instrument, only small surface areas are used for monitoring of biomolecular interaction. Any air bubble on this area causes the total loss of output. For that reason, usage of degassed solutions and continuous buffer degassing improves sensitivity and enables extended sample runs [31]. Besides, it is possible to eliminate this problem by using a small degasser unit. Another supplying device in SPR instrument is a temperature controller. Temperature has to be controlled during SPR measurements as any change in the temperature affects the SPR outputs. Naimushin and coworkers visualized the effects of temperature on the refractive index and also the biomolecular interaction in their study [28]. It was reported that the binding rate of a biochemical reaction was increased approximately threefold when the temperature was increased from 5°C to 40°C. Similar results were also reported by other researchers [32–34]. Lack of temperature regulation is one of

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the main reasons for the high standard deviation in SPR measurements.

3 SPR-based immunosensors for bacteria detection

SPR immunosensors, comprising antibodies coupled to the transducer, have been used for the detection of various bacteria. For direct detection of bacteria, SPR immunosensors are fabricated by immobilizing antibodies which are specific to the target bacteria, on the gold film surface. The SPR response after the immobilization of antibodies is recorded as a reference. When a solution containing bacteria is brought in contact with the sensor surface coated with antibodies, the target bacteria bind to the antibody. After washing the surface and removing the unbound bacteria, a shift in the SPR response is monitored due to the binding of bacteria to the sensor surface (Fig. 3). The shift in the SPR response is proportional to the concentration of the target bacteria. One can employ the linear relation between the number of the bacteria attached to the surface and the response of the SPR immunosensor to prepare a calibration curve. The success of a SPR immunosensor, like all other biosensors, highly depends on the experimental design. Handling of the solutions and surface processes are key points in the efficiency of the SPR based immunosensor.

3.1 Antibody immobilization

One of the factors determining the success of an SPR biosensor is the immobilization of antibodies to the sensor surface. The sensitivity and selectivity of an SPR immunosensor for the detection of bacteria can be improved by controlling the orientation and providing a good degree of freedom for the antibodies on the sensor surface so that recognition sites are not sterically hindered [35]. A large variety of methods have been applied to prepare a sensing layer with desired and controlled characteristics [36]. The sensitivities reported for the detection of a wide range of microorganisms using different surface modifications and assay types are shown in Table 1. In the first approach, the antibodies were non specifically attached to the sensor surface by simple physical adsorption, where the –SH groups of antibodies reacted with the gold atoms. In this method, the attachment of the antibodies to the sensor surface is very fast and easy. But, this is a random process, so the orientation of the antibodies on the surface is barely in right direction. This results in the attachment of the antibodies with their active sites [37]. In addition, antibodies may be denaturated when binding directly to the metal surface. So the sensitivity of the biosensor is generally unusable.

The other more specific way for a reasonable immobilization of the antibodies is the chemical conjugation to the sensor surface. In this method, antibodies are attached covalently to the metal surface by forming a self-assembled monolayer (SAM) on the sensor surface containing free anchor groups, such as thiols, amines and acids, and activating the surface depending on the chemical groups. SAM on the surface of the sensor may help in controlling the binding orientation of antibodies and minimizing the non-specific adsorption problem [22]. Moreover, the immobilization of antibodies by covalent binding increases the stability of protein on the sensor surface. Construction of a

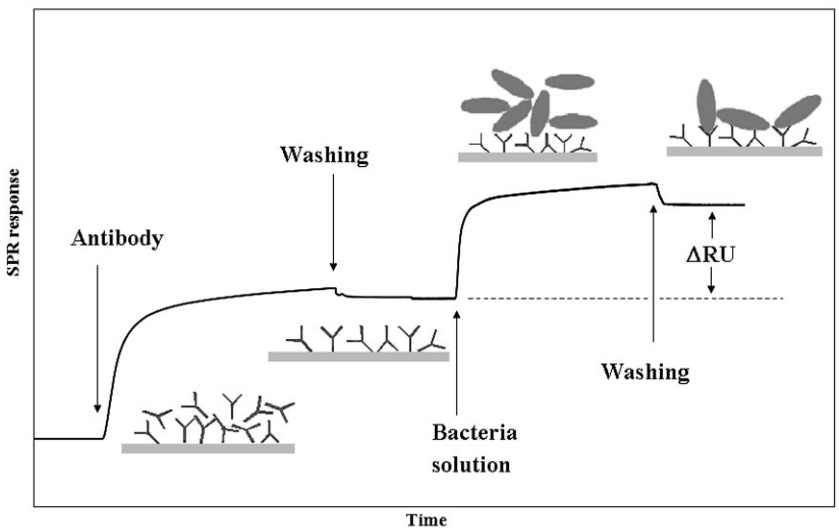

Figure 3. The shift in SPR response during the binding of antibody and bacteria to the sensor surface.

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Table 1. SPR immunosensors developed for bacteria detection

Bacteria type	SPR brand	Assay type	Immobilization method	Linker molecule	LOD (cfu/ml)	Reference
Escherichia coli O157:H7	Spreeta	Direct	Physical adsorption	Neutravidin	10^2–10^3	[44]
Escherichia coli O157:H7	Reichert SR7000	Direct Sandwich Direct	Covalent binding	– – Protein G	10^6 10^3 10^4	[49]
Vibrio cholera O1	Multiskop	Direct	Covalent binding	Protein G	10^5	[41]
Salmonella enteritis *Escherichia coli*	Biacore	Direct	Covalent binding	Protein A	25 23	[48]
Escherichia coli	Spreeta	Direct	Physical adsorption	Streptavidin	90	[43]
Salmonella typhimurium	Spreeta	Direct	Physical adsorption	Neutravidin	10^6	[67]
Escherichia coli O157:H7, *Salmonella choleraesuis* serotype typhimurium *Listeria monocytogenes*, *Campylobacter jejuni*	Custom–built SPR sensor	Direct	Covalent binding	Streptavidin	1.4×10^4 4.4×10^4 1.1×10^5 3.5×10^3	[42]
Salmonella paratyphi	Multiskop	Direct	Physical adsorption	Protein G	10^2	[68]
Escherichia coli O157:H7	Multiskop	Direct	Covalent binding	Protein G	10^4	[69]
Salmonella typhimurium	Multiskop	Direct	Covalent binding	Protein G	10^2	[46]
Yersinia enterocolitica	Multiskop	Direct	Covalent binding	Protein G	10^2	[47]
Escherichia coli	Bio–suplar	Direct	Covalent binding	Neutravidin	10^7	[70]
Staphylococcus aureus	Reichert SR7000	Sandwich	Covalent binding	–	10^5	[39]
Listeria monocytogenes	Biacore	Direct	Covalent binding	–	10^7	[38]
Listeria monocytogenes	Biacore	Subtractive inhibition assay	Covalent binding	–	10^5	[60]

recognition surface by using SAM has been used in a vast range of SPR-based immunosensors, such as the biosensors developed for the detection of *Listeria monocytogenes* [38] and the detection of *Staphylococcus aureus* [39]. The immobilization efficiency of a protein to the sensor surface may be varied by using molecules with different length and different tail groups [37]. The immobilization of antibodies can also be controlled by forming mixed SAMs on sensor surface by using molecules terminated with active head groups, which are used to anchor antibodies, and molecules terminated with hydroxyl head groups which form non-fouling spaces [40]. Mixed SAMs may increase the sensitivity of the sensor by decreasing the steric hindrences. Using mixed SAM, Jyoung *et al*. [41] developed an immunosensor for *Vibrio cholerae* O1 with a detection limit of 10^5 cfu/ml. Taylor *et al*. [42] could detect four foodborne pathogens at 10^3 to 10^5 cfu/ml using SAM with oligo(ethylene glycol) alkanethiol and biotinylated alkanethiol.

One of the most remarkable techniques for the immobilization of antibodies is to employ the high affinity molecular linkers such as protein A, protein G, Fc-specific antibody, and streptavidin. Using linker molecules may decrease the sensitivity as they put the antibody and target molecule out of the sensing surface. However, these linkers provide the ordering of the antibodies in a controlled way so that the active sites of the antibodies are exposed to the liquid side on the surface. In addition, the molecular linker acts as a spacer arm and binding efficiency may be enhanced due to abrogation of steric hinderence effects. Detection of *Escherichia coli* in water samples at 10^2 cfu/ml [43] and *E. coli* O157:H7 at 10^2 to 10^3 cfu/ml [44] has been achieved coating the gold surface with streptavidin and immobilizing the biotin-labeled antibodies on the assembly of streptavidin. The adsorption is carried out at high concentrations of streptavidin to reach a high surface coverage which minimizes the nonspecific binding. Protein A and protein G have been successfully applied in immobilizing antibodies to sensor surfaces for the detection of bacteria [41, 45–48]. Subramanian *et al*. [49] reported that the detection limit of the developed immunosensor

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for *E. coli* O157:H7 decreased from 10^6 to 10^4 cfu/ml when protein G is used as the linker molecule between SAM and the antibody.

3.2 Optimization of the process conditions

In SPR sensing are a number of different factors affecting the sensing capability of the sensor and the binding kinetics of the analyte in the solution. Following the preparation of the sensing layer, the conditions to be used during the detection of the microorganism need to be optimized. The ionic strength, protonation state of the entities and the flow rate of the analyte should be carefully optimized both to increase the detection limit as well as to improve the response time of the sensor.

Ionic strength, the salt concentration of the buffer carrying the analyte in it, is an important factor for the binding of the microorganism to the antibody. The ionic strength of the buffer affects the supramolecular interactions between the surface protein of the bacterium and the antibody on the surface. Therefore, buffer solutions should be carefully prepared and the binding efficiencies in different buffer solutions should be determined by correlating with binding affinity constants [50]. Another issue is the protonation and deprotonation of the amino acids at the core site of the antibodies which affect the binding strength of the antibodies. Electrostatic interactions between bacteria and antibodies can be tuned by changing the pH of the buffer solution [50, 51].

Beside the physicochemical conditions of the binding buffer, the flow in the SPR sensor system affects the sensitivity and the response time of the developed immunosensor [37]. The flow rate basically affects the mass transfer conditions in the biosensor [35, 36]. By changing the flow rate, one can overcome the adverse effect of the mass transfer limitations. At low flow rates, the diffusion of the microorganisms will be slow on the immobilized antibodies and this will increase the response time of the sensor. High flow rates can be good to minimize the mass transfer limitations, however, this time, the association between bacteria and antibody may be adversely affected by the fluid forces [35]. The optimization of the flow rate can be carried out by testing a range of different flow rates.

3.3 Regeneration of immunosensors

The regeneration of the sensor surface is critical in the development of the immunosensor. Performing multiple measurements with high repeatability is important for reducing the cost and decreasing the analysis time. The regeneration of the SPR sensor can be achieved by dissociating the antigen-antibody interaction with suitable solutions such as HCl [52], NaOH [49, 53], glycine-HCl [54], and SDS solution [43]. The optimal regeneration solution of an immunosensor varies with several factors such as antibody immobilization technique, nature of antibodies, and strength of antigen-antibody reaction [22]. Concentrated and strong regeneration solutions may cause leaching of antibodies from the surface. In that case, immobilization of tthe antibodies by covalent binding provides the stability of the sensing surface during regeneration. In addition, eluents cause reversible denaturation of antibodies which dissociates the antibody-antigen complex. However, special care has to be taken in order to prevent the irreversible denaturation of the antibodies which shortens the life time of the immunosensor.

4 Recent developments

SPR-based immunosensors hold a great potential for detecting microorganisms in food and water samples in a rapid way. However, the detection limits achieved with SPR immunosensors are far from competing with those of traditional methods. Many different procedures have been used to increase the sensitivity of the immunosensor such as sandwich assays [55–57] and subtractive inhibition assay (SIA) [58, 59]. In the sandwich assay, secondary antibodies bind to the analyte on the sensor surface, previously captured with antibodies. Subramanian *et al*. [49] demonstrated that the detection limit of the developed immunosensor for *E. coli* O157:H7 decreased from 10^6 to 10^3 cfu/ml by the sandwich assay procedure. The SIA have also been used successfully for the detection of bacteria by SPR sensor [60]. In this method, bacteria cells are first incubated with antibodies, and unbound antibodies are separated from the solution by filtration. The amount of unbound antibodies is determined by SPR sensor in which antibody-specific antibodies are used as ligands. Although the sandwich assay and SIA increase the assay sensitivity in SPR measurements, they suffer from high assay cost and long analysis times, because of using secondary antibodies and applying multistep injections.

The selectivity and the sensitivity of an immunosensor depend primarily on the properties of recognition surface. Novel approaches by exploiting some new tagging methods of the antibodies are under consideration for the enhancement of the sensitivity. Controlled assembly of histidine-tagged antibodies, produced by recombinant DNA technology, on Ni(II)/nitrilotriacetic acid (NTA)

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chelator decorated surface [61, 62] and glutathione *S*-transferase (GST)-tagged antibodies on glutathione-rich gold surfaces [63] are some of the examples of novel methods for antibody immobilization.

Another approach is to develop new biomolecular recognition elements with higher stability and higher affinity to the target bacteria. For this purpose, new technologies, like phage display and molecular imprinting, have been recently integrated in SPR biosensors. Phages can be used in bacteria detection as alternatives to antibodies [64]. Balasubramanian *et al.* [65] reported the detection of *Staphylococcus aureus* by SPR using lytic phage as the recognition element. Phage display technology is another tool for developing single-chain antibodies for bacteria. Phage displayed antibodies have various advantages on antibodies such as, *in-vitro* production, low cost, low molecular weight, and high stability. Phage-displayed antibodies have been used for the detection of *Listeria monocytogenes* in the SPR biosensor, indicating the potential of non-antibody recognition elements [66].

5 Conclusions

Development of a biosensor with the necessary properties for reliable and effective use in routine applications is the challenge of the researchers. A glucose biosensor is the only one commercially available on the market with the potential of covering the requirements of the end user. In the case of bacteria detection, it is not easy to satisfy the standards. For example, *E. coli* must be less than 1 cfu in 100 mL of drinking water according to the water quality standards in most of the countries. In biosensor applications, most of the time, it is not possible to take 100 mL sample into measurement. To eliminate this problem, volume reduction with filtration or immunomagnetic separation is performed. Even so, it is difficult to achieve such low detection limits by biosensors. At this point, SPR biosensors stand out with a number of advantages such as high sensitivity, rapid and label-free detection. It is believed that integration of new technologies in other research fields, such as, material science and molecular biology, with SPR will provide the development of biosensors with detection limits compatible with traditional techniques. In the near future, SPR biosensors will undoubtly take its place in commercial market.

The authors have declared no conflict of interest.

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İsmail Hakkı Boyacı is an associate professor of Food Engineering Department at the University of Hacettepe in Turkey. He received his PhD (2001) from the University of Hacettepe working on the development of carbohydrate biosensor for multiple detections. He worked as a post doctoral researcher on microbial immunosensors in the group of Prof. Dr. William R. Heineman at the Chemistry Department, Cincinnati University, OH, USA. Current research interests include bacterial biosensor based on optical and electrochemical sensoring. Recently, he focused on label–free detection of bacteria in water samples using surface plasmon resonance and surface–enhanced Raman scattering sensing devices.

Fahriye Ceyda Dudak, born in 1981, completed her undergraduate degree in Hacettepe University, Turkey. At Hacettepe University, she studied rapid immunoassays for bacteria detection under the supervision of İ. H. Boyacı. She is currently a PhD student in the Food Engineering Department of Hacettepe. Her research interests include biosensors with focus on the use of new ligands in biosensor applications.

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