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Review

Label Free Biosensor Methods in Detection of Food Pathogens and *Listeria monocytogenes*

Rajeswaran Radhakrishnan [1] and Palmiro Poltronieri [2,*]

[1] Faraday Technology Inc., Englewood, OH, USA; Rajeswaran@faradaytechnology.com

[2] ISPA-CNR, National Research Council, 73100 Lecce, Italy; palmiro.poltronieri@ispa.cnr.it

* Correspondence: palmiro.poltronieri@ispa.cnr.it; Tel.: +390832422609

Abstract: Food pathogens contaminate food products that allow their growth on the shelf and also under refrigerated conditions. Therefore, it is of utmost importance to lower the limit of detection (LOD) of the method used and to obtain the results within hours to few days. Biosensor methods exploit the available technologies to individuate and provide an approximate quantification of the bacteria present in a sample. The main bottleneck of these methods depend on the aspecific binding to the surfaces and on a change in sensitivity when bacteria are in a complex food matrix in respect to bacteria in a liquid food sample. In this review we introduce Surface Plasmon Resonance (SPR), new advancements in SPR techniques, and Electrochemical Impedance Spectroscopy (EIS), as label-free biosensing technologies for the detection of *L. monocytogenes* in foods. The application of the two methods has made possible the detection of *L. monocytogenes* with LOD of 1 log CFU/mL. Further advancement are envisaged through the combination of biosensor methods with immunoseparation of bacteria from larger volumes.

Keywords: *Listeria monocytogenes*; label-free biosensors; Surface Plasmon Resonance (SPR); Electrochemical Impedance Spectroscopy (EIS)

1. Introduction

Food pathogens, either anaerobic, microaerophilic or aerobic species, are contaminating food processing plant surfaces or originate from food sources. Therefore, a limit has been fixed for the presence of colony forming units of pathogens. *Listeria monocytogenes* can contaminate a wide range of foods, including yogurt, cheeses, meat, ham, smoked salmon, poultry, seafood and vegetable products, especially their surfaces. It's presence poses a real threat to ready-to-eat foods, since the bacteria can survive and proliferate in adverse environmental conditions during food production and storage (such as low pH, refrigerated temperatures and high salt concentration).

International regulation for ready-to-eat foods requires corrective actions in the presence of *L. monocytogenes:* the bacteria count should be lower than 0.04 CFU g^{-1} for food that supports the growth of the microorganism, and 100 CFU g^{-1} for food not supporting the survival. It is necessary to determinate absence of *L. monocytogenes* in foods destined to infants [1-4]. Microbiological criteria for *L. monocytogenes* in food safety are based on microbiology laboratory culture methods. *L. monocytogenes* detection methods, i.e. UNI EN ISO 11290-1:2005, include a pre-enrichment step of a food sampling followed by growth on selective solid medium: the protocol takes 4 days for completion. Molecular methods have taken the lead in pathogen detection, thanks to shorter times of handling, with results obtained in few hours. Real Time PCR is the main molecular method applied in food analysis: Recently highly reliable diagnostic kits, such as the AFNOR validated iQCheck, allowed to limit the pre-enrichment step to 18 hours, eliminating 1 day of culture, combined with reliable Real Time PCR analysis kits [3-5].

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Biosensors have been applied to pathogen detection in liquid and solid food samples, but only in few cases a low Limit of Detection (LOD) in the range of the LOD achieved by PCR has been obtained. Recently, performing immunomagnetic separation on pre-enrichment cultures of L. monocytogenes, combined with protein chip detection, the methods obtained a good sensitivity with values reaching the sensitivity of PCR analysis [3]. Therefore, it is the interest of food industry players to see novel methods able to provide a fast response and high sensitivity comparable to the iQcheck and AFNOR validated methods for real time diagnosis of contamination in foods [3, 4].

In this review we will introduce label free biosensor methods applied to bacteria detection, either focused on *L. monocytogenes* [6-8], or other pathogens contaminating food products.

Biosensors often rely on label-free detection methods, such as Surface Enhanced Raman Scattering (SERS), electrochemical methods, Lateral Flow Immuno-Assays, and on applications of metal nanoparticles, quantum dots, and nanomaterials for surface modification [9-23]. Among the detection methods providing signal enhancements at low bacteria contamination, the electrochemical methods, in which signal amplification is achieved through enzymes and redox cycling, have been recently extensively reviewed [17-20].

In this review we will focus on Surface Plasmon Resonance (SPR) and on SPR combined with immunoseparation from pre-enrichment broths [1-3, 8] and on Electrochemical Impedance Spectroscopy (EIS).

2.1.1. SPR methods

The system of optical approach based on surface plasmon resonance (SPR).is performed with a light source and thin metallic material (Au). SPR is an optical technique that uses the evanescent wave produced by an incident, monochromatic light beam. The light beam interacts with free electrons (plasmons) in the metal film at the α angle (SPR angle) of incident light. The angle is dependent on the metal-dielectric interface. The prism based Kretschmann configuration is based on the excitation of a surface-bound electromagnetic wave from the metal side. The binding event between the investigated antigen and the capture antibody is recorded through the detection of a shift of the reflected light toward higher values of the SPR angle, or through a change in reflectance at a fixed angle, measuring changes in refractive index close to the sensor surface. SPR, exploiting as capture ligands either species-specific antibodies, as well as nucleic acid aptamers, has been extensively applied to pathogen detection.

However, using SPR methods, the results have been often limited to clear solutions with inoculated bacteria, and the LOD has been often too high and unsatisfactory for food safety applications. In various reports, SPR immunosensor measurements detected *L. monocytogenes and Salmonella* spp. cell suspensions at a concentration of 3 to 4 log CFU/ml [7, 24-27].

In addition, low analytical sensitivity is either the result of a small refractive index, slow diffusion driven mass transfer, or the insufficient depth of the influenced layer. These are intrinsic problems in the conventional SPR methods. For the extension of SPR applications to the food safety, either new surfaces [28-30] have been tested, either new portable instruments have been developed [31].

2.1.2. SPR Imaging in multiplex: multichannel SPR biosensors

SPR methods are able to evaluate bacterial presence in multiplex. Grating-coupled surface plasmon resonance imaging (GCSPRI) has been applied to multiplexed detection of microbes, toxins and

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viruses. In GCSPRI, disposable grating systems are obtained by deposition of thick metal films. Then, the wave is excited from the transparent material side: the sample is placed on a topographically-located position on the surface [32-34]. This system does not require a prism. GCSPRI was able to measure at the same time the binding of multiple regions of interest (ROIs) through an array of specific capture molecules immobilized on the surface.

2.1.3. Enhancement of Sensitivity by Combining SPR with A Labeling or Capturing Method

Standard SPR detection has been performed at relatively high concentrations of bacteria, often leading to non-specific binding. To circumvent this limitation, For this reason, improvement of the SPR detection power has been achieved with an additional hybridization step. The sensitivity was significantly increased through the addition of antibody-nanoparticle conjugates (gold NP), as signal enhancers exerting a mass effect. The enhancement of the signal from bacteria bound to the antibodies on the gold surface [35] has been applied also to other detection methods based on capture antibodies bound to a gold surface, such as the Quarz Crystal Microbalance (QCM) [35].

As reported previously, antibody-functionalized gold nanoparticles (immuno-AuNP) have been added to bacteria captured on the surface, to enhance the SPR signal, producing an increase in the reflectance units, with a LOD at an *L. monocytogenes* concentration of 2 log CFU/mL [1].

A new, indirect method capable of enhancing SPR sensitivity is Localised Surface Plasmon Resonance (LSPR) [36-38]. The LSPR approach has been applied to label-free, real-time pathogen detection, and small, cost-effective LSPR biosensor systems have been constructed. Although LSPR has problems similar to SPR, especially the rapid decay of surface plasmon, employing small binders, such as the Fab portion of antibodies and aptamers, and by surface modification of the nanoparticles, an enhanced signal has been obtained .

Long-range SPR was also combined with magnetic nanoparticles and Au nanoparticles. In the detection of pathogens, the SPR propagated along a thin metal film, embedded in a symmetrical layer architecture with optimised refractive index, resulting in a dark-field light-scattering imaging technique: detection was achieved within 30 min [23].

2.2. EIS

Electrical biosensors rely solely on the measurement of currents and/or voltages to detect binding [39-41]. Electrical biosensors can be further subdivided according to how the electrical measurement is made, including voltammetric, amperometric/coulometric, and impedance sensors. Among these, *impedance biosensors* measure the electrical impedance of an interface in AC steady state with constant DC bias conditions. Most often this is accomplished by imposing a small sinusoidal voltage at a particular frequency and measuring the resulting current; the process can be repeated at different frequencies. The current-voltage ratio gives the impedance. This approach, known as Electrochemical Impedance Spectroscopy (EIS), has been used to study a variety of electrochemical phenomena over a wide frequency range [42]. If the impedance of the electrode-solution interface changes when the target analyte is captured by the probe, EIS can be used to detect that impedance change. Alternatively, the impedance or capacitance of the interface may be measured at a single frequency. Impedance measurement does not require special reagents and is amenable to label-free operation. Due to their low cost, low power, and ease of

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miniaturization, impedance biosensors hold great promise for applications where minimizing size and cost is crucial, such as point-of-care diagnostics and biowarfare agent detection.

2.2.1. Fabrication of Impedance Sensor

Impedance biosensors are fabricated by immobilizing a biorecognition molecule onto a conductive and biocompatible electrode and then detecting the change in the interfacial impedance upon analyte binding. Biorecognition molecules may include antibodies, receptor proteins, single-stranded DNA, aptamers, or peptides. Impedance biosensors can detect a variety of target analytes by simply varying the probe/bio recognition molecules used. This makes impedance biosensors ideal for detection of food pathogens [43], food allergens [44], and environmental monitoring of species such as endocrine disrupting chemicals (EDCs) [45].

The right choice of electrode materials is the key to the successful performance of any biosensor. It is important to choose the right material, so that its functionalisation can be achieved with relative simplicity. For electrochemical biosensors, the electrode materials are constrained by the requirements for both high electrical conductivity and biocompatibility. Biomolecules often denature with prolonged exposure to metal surfaces. To date, this has limited the electrode materials in electrochemical biosensors primarily to gold, platinum and carbon [46]. Though the immobilization of biomolecules onto carbon electrodes may provide excellent stability [47-48], they have the well-known drawback of exhibiting complex electrochemistry. That mainly depends on type of carbon, surface preparation and on chemical treatment [49]. Si also has been widely used as a biosensor material. Although, many combinations of metal surfaces and organic molecules have been studied for impedance sensing, noble metal surfaces (especially Au) have attracted the the highest interest for preparing structurally well-defined chemical interfaces for biosensing.

2.2.2. Linkage of Bio-molecules onto Au Surface

There are two different ways of attaching molecules onto a gold surface. One is the direct attachment method, where the biomolecules are chemically modified, such that they have -SH groups as their terminal. One such example is shown in figure 1. Protein molecules can directly attach to the gold surface due to the hydrophobic and electrostatic interactions between the protein molecule and the gold surface [50]. While this direct attachment method serves as the simplest immobilization scheme, it is not suitable for EIS based sensing due to several reasons.

The surface coverage might be low. This is especially true for proteins and antibodies, which differ in shape, size, and orientation [51]. Direct attachment is more suitable for molecules, such as DNA, which have a well defined structure [52]. It is challenging to block the active surface sufficiently. Since the molecules can be randomly arranged over the gold surface, it is difficult to prevent the non-specific adsorption of interfering molecules [51]. The adsorption of proteins onto the gold surface can be reversible. The proteins can be easily removed from the surface using certain solvents such as acetone and detergents such as Tween [53].

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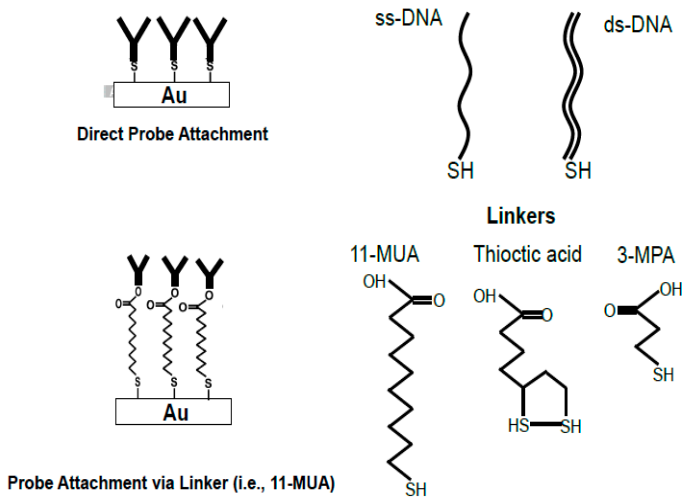

Figure 1. Direct and indirect attachment of molecules onto gold surfaces.

One way to overcome this issue is to use linker molecules. Functional alkanethiols serve as suitable linkers. Sulphur compounds (eg., thiol compounds) have a strong affinity to gold surfaces [54]. The gold-sulphur attachment occurs due to the oxidative addition of -SH bond to the gold surface. The bonding of the -SH group to the gold surface is very strong (the bond strength is approximately 10kT) [54].

Figure 1 shows some examples of functional alkanethiols (11-MUA, 3-MPA and thioctic acid). All of these linkers have -SH groups, which can bind to the gold surface, and a functional COOH group. This COOH group can be modified to form NHS esters, using the EDC/NHS protocol [53]. The covalent binding between the NHS ester group and the amine group in proteins is irreversible and hence the use of linker molecules enhances the stability of protein attachment to the gold surface. Furthermore, the alkanethiols form self-assembled monolayers on the gold surface. These SAM layers have a well-defined composition, structure, and thickness [54].

2.2.3. Attaching Bio-Molecules

To covalently attach bio-molecules (probes) such as antibodies and proteins, NHS/EDC coupling chemistry has been most commonly used to form amine-reactive sites on functionalized gold or Si electrodes. These amine-reactive sites are subsequently exposed to probes [55], resulting in protein covalently bound to the surface. Most published reports use target proteins of real-world interest but in highly purified conditions. Much effort is still required to bring about robust analysis of clinical samples using impedance biosensors to enable point-of-care applications. Key challenges include poor reproducibility, non-specific binding, and the complex and highly variable nature of clinical samples.

2.2.4. Technical challenges for Impedance Biosensors

Although impedance biosensors have been well studied in the academic literature [46-48], it is widely considered to have some technical limitations that have hindered their commercial introduction, including: Susceptibility to nonspecific adsorption in complex matrices, Stability and reproducibility for biomolecule immobilization onto a conductive electrode material and Complexity of impedance detection. These challenges are not only specific to impedance

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biosensors. For example, non-specific adsorption is a common problem for all biosensor transduction methods.

2.2.4.1. Susceptibility to Non-specific Adsorption

The most frequently cited practical concern regarding impedance biosensors is the perception that this method is particularly susceptible to interference arising from non-specific adsorption. Non-specific adsorption is without question a common limitation for a wide variety of different biosensor methodologies [56-58]. Non-specific adsorption is typically ascribed to proteins contained in a complex test matrix binding to the sensor interface though unwanted process not involving biomolecular recognition. Thus non-specific adsorption can be studied by control experiments using either complex test matrices or mixtures of different proteins or analytes.

While non-specific adsorption may cause spurious signals during impedance biosensing, several methods have been employed to mitigate this, including sample dilution [59-60], adsorption of a blocking reagent such as bovine serum albumin (BSA) [58], and use of a control electrode at which biomolecular recognition is unlikely [61]. The utility of sample dilution depends on the particular application and the desired detection limit. When a monoclonal antibody is used for biomolecular recognition, a control electrode can be used with another antibody from the same animal and sub-type whose antigen is unlikely to be found in the text matrix of interest. Recently, Suni et al. reported impedance detection of *Listeria monocytogenes* in tomato pulp with limit of detection of 4 CFU/ml [43], and demonstrated that non-specific adsorption was un-measurable. Non-specific adsorption was quantified by comparing the impedance change at the measurement electrode (mouse monoclonal IgG_1 antibody to *L. monocytogenes*) to that at a control electrode (mouse monoclonal IgG_1 antibody to GAPDH). This approach depends on the availability of adequate control electrodes whose antigen is not present in the samples of interest, and with no cross-reactivity to the analyte of interest. It should be noted that the use of multiple measurement antibodies with different binding epitopes, and multiple control antibodies, are both relatively straightforward.

2.2.4.2. Stability of Biomolecule Immobilization onto a Conductive Electrode Material

For impedance biosensing, biomolecule immobilization onto a conductive and biocompatible electrode material is most commonly accomplished through Au-thiol self-assembly chemistry [62]. However, the limited stability of Au-thiol self-assembly chemistry to date limits its application to impedance biosensors [63]. Depending on storage conditions, the shelf life is limited to days to weeks. Durable chemistry for biomolecule immobilization is also needed for sensor calibration, which often involves the use of aggressive chemicals.

A significant increase in stability can be achieved using multidentate thiols relative to monodentate thiols [64-65]. In most cases, these multidentate alkanethiols readily generate SAMs on both flat and curved gold surfaces at room temperature, and show an enhanced ability to withstand exposure to elevated temperatures in thermal desorption studies. The driving force of their stability is the chelate effect, which is the free energy of the entropically favored bidendate binding can be twice that of monodendate binding [66]. Prof. T. Randall Lee's research group at the University of Houston recently reported SAM formation on Au from the bidendate thiol 16-[3,5-bis(mercaptomethyl)phenoxy]-hexadecanoic acid (BMPHA) [44]. With the aforementioned

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results in mind, Dr. Radhakrishnan, et.al, recently characterized the carboxylic acid-terminated alkanethiol, BMPHA, in an effort toward the generation of highly stable carboxylic acid-terminated organic thin films [67]. To provide a more complete analysis of the effectiveness of this class of adsorbate, they prepared and compared this SAM against the monothiol 16-mercaptohexadecanoic acid (16-MHA) based SAM. The detection limit for *Ara h* 1 allergen using the BMPHA linker is approximately 0.71 ng/mL (0.01 nM), which is about 10x lower than that obtained using the monodendate thiol, 16-mercaptohexadecanoic acid (16 MHA).Structures of both adsorbates are shown in figure 2.

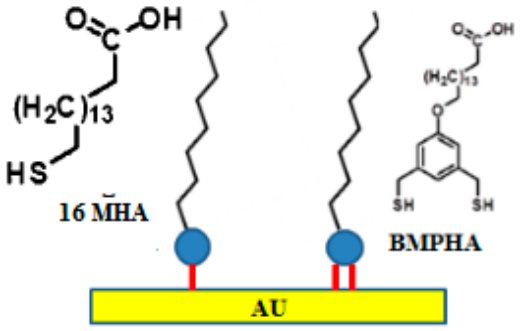

Figure 2. Structures of two carboxylic acid terminated alkanethiols.

Other substrate materials that have been reported for impedance biosensors include carbon [68-69], Si [70-71], Pt [72-73], Ti [74-75], and ITO [76-77]. Recently, degenerate (highly doped) Si was reported as an alternative electrode material for impedance biosensors[78]. Degenerate Si behaves as an electrical conductor, albeit a poor one, rather than a semiconductor, preventing formation of a space charge layer during AC interrogation of the sensor interface. Radhakrishnan and Suni illustrated results demonstrating the ability to regenerate antibody coated Si electrode during a 30-day trial period of storage using KSCN based solution [79]. This illustrated the potential for this methodology to be used for storage of antibody-coated degenerate Si electrodes, with calibration on the day they are used and reusability of same electrodes.

2.2.4.3. Complexity of Impedance Detection

Although substantial progress has been made in impedance based sensors, there are still some obstacles to be overcome for them to be used towards on-site detection. Firstly, many impedimetric tests would require sophisticated and often time consuming data processing to extract binding related information. Expensive benchtop impedance analyzers are usually needed if high test frequencies (>1 MHz) are used. Secondly, most of the reported impedance assays are conducted with highly processed samples. To be viable as an on-site diagnostic system, sample preparation must be simple enough to be performed on site. Thirdly, most of the currently tested systems still require at least 30 minutes to perform a single assay on abundant molecules or longer time for more diluted analytes. While this is an improvement over the standard enzyme-linked immunosorbent assay (ELISA) method, an incubation time of half an hour or more may still be too long under some circumstances. The assay time can be improved by incorporating the AC electrokinetic (ACEK)

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microfluidics [80] which was emerged in the 1990s, with impedance sensor. The ACEK effects have been intensively studied as a means to manipulate particles or macromolecules. It has been demonstrated by several groups [81-84] that ACEK working with microelectrodes can induce in situ concentration of particles for improved detection sensitivity and throughput. ACEK, as a particle and fluid manipulation mechanism, has minimal requirements on the device fabrication and operation to be incorporated into a detection system – only microelectrodes and their AC signal source need to be added. ACEK effects use an AC electric field to induce particle and fluid movement, so that macromolecules can be in situ concentrated onto microsensors [85]. When an inhomogeneous AC electric field is applied to an aqueous solution, both particle movement and microflows can be induced to transport particles. Direct particle movement can be caused by dielectrophoresis (DEP), and particle can also be carried by microflows such as AC electroosmosis or AC electrothermal flows to reach the microsensor. The manipulation of particles by DEP is based on the difference between the particle polarizability and that of the medium solution at a certain frequency. An AC electric field can generate microflows through one or both of two ACEK mechanisms known as AC electroosmosis (ACEO) and AC electrothermal (ACET) effect. ACEO typically dominates at low ionic strengths. The flow velocity of ACEO has been observed to decrease significantly with increasing conductivity and eventually drop to zero above 0.085 S m^{-1}[86, 87]. Many medical and biological applications involve the use of solution with high conductivity, so the ACEO flow will be negligible. The ACET effect arises from inhomogeneous heating of electrolytic fluids by passing electric current. Therefore, the ACET flow is almost frequency-independent, and scales with the electrical conductivity of fluid. With planar electrodes, the ACET effect will induce vortices above each electrode, and the microflows will convect the embedded particles towards the electrode surface [86]. Because fluidic forces have no dependence on particle size, ACET microflows will be well suited for transporting macromolecules to the electrodes. Recently, Liu, et.al successfully showed that the ACET effect has played an important role in increasing detection sensitivity [87]. Thus, Combining DEP and ACET effects, the ACEK-based impedance sensor can be very effective in enriching nanoscale particle concentration over a large range and realizing accelerated detection.

3. Conclusions

The application of biosensing methods, such as enhanced SPR and EIS biochips, has made possible the detection of *L. monocytogenes* with LOD as low as 1 log CFU/ml [88]. Further advancement are envisaged through the application of superior surface modification methods, such as the use of bidendate thiol 16-[3,5-bis(mercaptomethyl)phenoxy]-hexadecanoic acid (BMPHA), the application of proper solutions to stabilize the capture ligands, and the application of ACEK microflow mechanisms, such as AC electroosmosis (ACEO) and AC electrothermal (ACET) effect. Furthermore, an increase in the limit of detection may be achieved by combining the biosensor methods with immunoseparation of bacteria from larger volumes. It is envisaged that improved biosensing methods can respond to food safety issues in the shortest time possible and provide safety certification to the food chain even at retailer shops and refectory level.

Author Contributions: R.R. reviewed the work done using EIS, P.P. described the techniques for SPR biosensors.

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

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