DNA Sensors Employing Nanomaterials for Diagnostic Applications

Manel del Valle and Alessandra Bonanni

Abstract This chapter describes DNA sensors (genosensors) that employ electrochemical impedance signal as transduction principle. With this principle, hybridization of a target gene with the complementary probe is the starting point to detect clinical diagnostic-related genes or gene variants. Electrochemical impedance spectroscopy permits, then, a labeless detection, by simple use of a redox probe. As current topic, it will focus on the use of nanocomponents to improve sensor performance, mainly carbon nanotubes integrated in the sensor platform, or nanoparticles, for signal amplification. The different formats and variants available for detecting genes in diagnostic applications will be reviewed.

Keywords Carbon nanotube, DNA biosensor, Electrochemical impedance spectroscopy, Genosensor, Gold nanoparticles, Quantum dots

Contents

M. del Valle (✉)
Sensors and Biosensors Group, Chemistry Department, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
e-mail: manel.delvalle@uab.es

A. Bonanni
Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, SPMS-CBC-04-41, 21 Nanyang Link, Singapore 637371, Singapore
e-mail: a.bonanni@ntu.edu.sg

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Abbreviations

AC	Alternating current
AuNP	Gold nanoparticle
C	Capacitance
CNT	Carbon nanotube
CPE	Constant phase element
CPE	Carbon paste electrode
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDAC	N-(3-Dimethylaminopropil)-N-ethylcarbodiimide hydrochloride
EIS	Electrochemical impedance spectroscopy
GCE	Glassy carbon electrode
H1N1	Influenza A – H1N1 gene
HIV	Human immunodeficiency virus
hpDNA	Hairpin DNA
IgG	Immunoglobulin G
LOD	Limit of detection
MWCNT	Multi-walled carbon nanotube
NHS	N-Hydroxysuccinimide
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PNA	Peptide nucleic acid
QCM	Quartz crystal microbalance
QD	Quantum dot
R	Resistance
R_{et}	Electron transfer resistance
RNA	Ribonucleic acid
SPR	Surface plasmon resonance
ssDNA	Single-stranded DNA
strept-AuNPs	Streptavidin-coated gold nanoparticles
SWCNT	Single-walled carbon nanotube
TEM	Transmission electron microscopy
Z	Impedance
Z_i	Imaginary component of impedance
Z_r	Real component of impedance
αHL	α-Hemolysin nanopore
ϕ	Phase angle
ω	Radial frequency

1 Introduction

In this chapter we describe current variants of DNA sensors (genosensors) [1–3] that employ electrochemical impedance signal for detecting the hybridization event of a target DNA. In this way, the clinical diagnostic-related sought gene or gene variant can be detected in a very simple way, with an electrically addressable device, and, potentially, without the use of any label. The chapter will describe existing variants for the measure and different formats for the assay. To improve the performance of these devices, current nanobiotechnology utilizes nanocomponents, either employed at the transducer level or integrated in the procedure itself, to improve detection or to amplify its signal. Carbon nanotubes (CNTs) and nanowires, or even gold nanoparticles, can be used to produce or to modify the transducing electrodes, fostering their electrical characteristics or helping in the immobilization of the recognition element. Metal nanoparticles or even quantum dots may be used in some of the existing formats, if a better signal-to-noise ratio is required. The chapter ends with a summary of existing applications related to clinical diagnostic and discussion of late trends.

The determination of nucleic acid sequences from humans, animals, bacteria and viruses is the departure point to solve different problems: investigation about food and water contamination caused by microorganisms, detection of genetic disorders, tissue matching, forensic applications, etc. [2–4]. With a gene assay, either by a laboratory method or by a genosensor, one can ascertain the presence of a certain gene in a sample, which in turn may provide highly interesting information such as: (1) a specific gene is found, e.g. this individual is carrier of a genetically inherited disease; (2) a certain living species is present, e.g. food contamination, with cases as *Salmonella* in egg make out or *Listeria* in meat; another interesting examples can be cited as in fight against food fraud, biothreat protection, as the detection of Anthrax, or environmental protection, e.g. finding the source of an Avian Influenza outbreak; and (3) the identity of an individual is unravelled, like in crime suspect identification, in establishing paternity or degree of kinship, or in animal breeding.

All these interesting applications, which were very difficult to achieve in the past, or very laborious and time-delayed, for example if they needed microbiological culture, now can be approached with genosensor schemes, with goals of allowing a simpler and wider use.

The standard gene assay in this moment is the hybridization assay with the use of a fluorescent labelled string [4]. A single-strand DNA probe is placed over a surface and is used to hybridize with the sought DNA, or DNA target. The use of a labelled DNA sequence is used to show if the hybridization took place, a functionality that can be attained in different ways, for example in competition with the analyte gene. Other labelling strategy commonly used in designing genosensors, apart from the use of fluorescent markers [5, 6], is the use of redox active enzymes [7, 8], magnetic particles [9] or nanoparticles of different nature [10, 11]. An indirect labelling scheme consists of the use of redox couple which intercalates into DNA double helix, such as metal complexes [12, 13] or organic dyes [14, 15], or the use of redox indicators in solution which improves impedance performance [2].

When looking for a label-free approach, that is, when no modification on the DNA string used for capturing or detecting the sought gene is performed, several alternatives are also available. A first option is using the electrochemical properties of DNA by measuring the signal due to the direct oxidation of DNA bases [16, 17]. The other alternatives imply the use of transducing techniques which are sensitive to surface changes and able to detect the hybridization event. Some of these techniques are the quartz crystal microbalance (QCM) [18–20], surface plasmon resonance (SPR) [21, 22] or electrochemical impedance spectroscopy (EIS) [2, 3, 23]. For the latter, several examples of application to the labeless detection of specific DNA sequences in different fields have been demonstrated [3]. However, an amplification step is often necessary to achieve a defined response with very low analyte concentrations. In this case, approaches used to enhance the signal related to the use of nanocomponents will be also treated in this chapter. Besides, current research that focus on impedimetric genosensors using nanocomponents, considering the experimental principle, design of the device or use for its operation, and including nanotubes, nanoparticles, quantum dots or nanopores will be reviewed.

1.1 EIS Background

Impedance spectroscopy is a powerful method for characterizing the complex electrical resistance of a system, being capable to detect surface phenomena and also changes of bulk electrical properties [24]. Then, it is becoming an invaluable method in electrochemical research, where a constant growth of applications has been noticed during the last decade.

EIS has been intensively used, for example, for the elucidation of corrosion mechanisms [25], for studying electrode kinetics [26], the electrochemical double layer or batteries [27] or in solid-state electrochemistry, for characterizing charge transport across membranes [28]. In the field of sensors it may be used for characterization and optimization purposes. When used with biosensors, it is particularly well suited to the detection of binding events on the transducer surface. In fact, EIS is irreplaceable for characterizing surface modifications, such as those that occur during the immobilization of biomolecules on the transducer. We will present a short introduction to the basic principles of electrochemical impedance spectroscopy to help better understanding the signals generated in the biosensing event.

After applying an AC potential (E_t) to a system, its impedance Z is generally determined by relating the observed current crossing it (I_t), see Fig. 1. Experimentally, this is determined by applying an AC voltage perturbation with small amplitude (5–10 mV) and detecting the generated current intensity response, and the process is repeated for a number of frequencies.

$$E_t = E_0 \cdot \sin(\omega \cdot t) \tag{1}$$

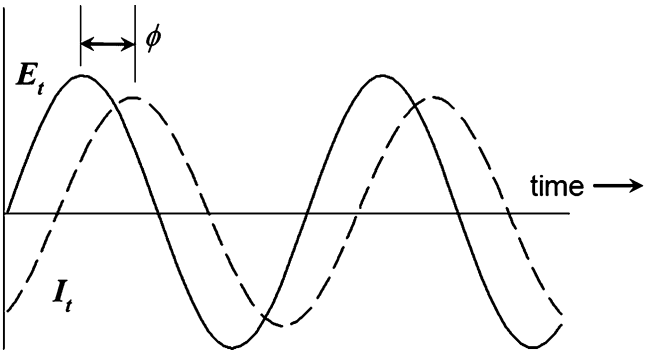

Fig. 1 Representation of the AC excitation signal, and the sinusoidal current response shown by a generic electrical circuit

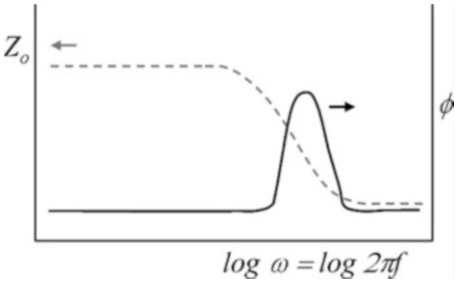

Fig. 2 Bode plot of the frequency characteristics of a given electrical circuit

$$I_{\mathrm{t}} = I_0 \cdot \sin(\omega \cdot t + \phi) \tag{2}$$

From this definition, the impedance Z, also known as AC resistance, is the quotient of the voltage and current (Ohm's law for AC current):

$$Z = \frac{E_{\mathrm{t}}}{I_{\mathrm{t}}} = \frac{E_0 \cdot \sin(\omega \cdot t)}{I_0 \cdot \sin(\omega \cdot t + \phi)} = Z_0 \cdot \frac{\sin(\omega \cdot t)}{\sin(\omega \cdot t + \phi)} \tag{3}$$

And from this equation, it is evident that final impedance may be derived in terms of a magnitude Z_{o} and a phase angle ϕ. When these two magnitudes are plotted versus the scanned frequency, a characteristic representation is obtained, known as Bode plot (Fig. 2).

More informative for the sensor practitioner than the Bode plot is the Nyquist plot. This is constructed first by applying the Euler's equivalence between trigonometry and complex numbers; in this, the impedance is now written as:

$$Z = Z_{\mathrm{r}} + jZ_{\mathrm{i}} \tag{4}$$

being $j = \sqrt{-1}$.

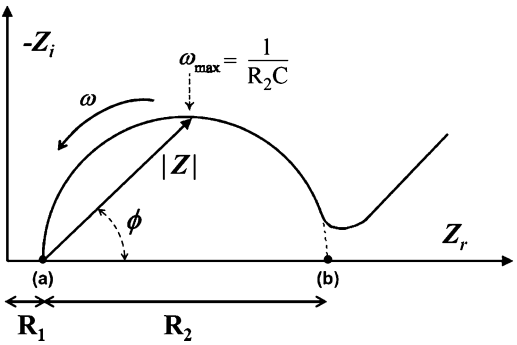

Fig. 3 Nyquist plot obtained for a typical reversible electrochemical reaction

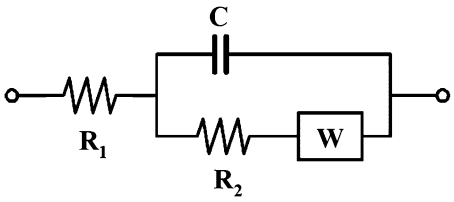

Fig. 4 Randles' equivalent circuit of a standard electrochemical reaction

And the Nyquist plot is derived when plotting for each scanned frequency, the imaginary part of the impedance ($-Z_i$) versus the real component (Z_r). Both Bode and Nyquist plots are responsible for the terminology "spectroscopy", given their use of frequency as the independent variable, thus recalling the situation with an electromagnetic spectrum. Figure 3 illustrates the typical Nyquist plot observed for a standard reversible electrochemical reaction taking place at a usual electrode. One of the valuable properties of the EIS technique is that from the shape and magnitude observed in the Nyquist plot (or alternatively in the Bode plot) one can derive which kind of electrical circuit is responsible for the profiles seen, and even calculate the electrical parameters involved. In fact, the pattern in Fig. 3 is very familiar to any electrochemist or any impedance practitioner, and it might be obtained with an electrical circuit like the one in Fig. 4. This electrical circuit, capable of providing an EIS spectrum which is equivalent to that previously seen (and so-called equivalent circuit), is well known and receives the name of Randles' equivalent circuit.

The interesting thing is that the individual elements present in it have physical meaning, illustrating the power of the EIS technique. In it, R_1 is the resistance of the solution, R_2 is the electron transfer resistance, that is, the kinetic impediment for the electrochemical reaction, C is the capacitance of the double layer, and finally, the 45° diagonal at the lower frequencies, called Warburg term, is related to the diffusion of species towards the electrode. The possibility of assigning individual elements to a circuit and finding values of their parameters is what gives to this technique the power of discriminating individual phenomena and also measuring its intrinsic characteristics. One final comment is that in many current situations, the

capacitor term C is replaced by a special term, called constant phase element (CPE), originated in the lack of ideality of the electrode systems under test.

$$Z_{\mathrm{CPE}} = (j \cdot \omega)^{-\alpha}/C \tag{5}$$

where ω is the radial frequency, C the capacitance and α an empirical coefficient, related to the ideality of the system. For a CPE situation, the exponent $\alpha < 1$, since $\alpha = 1$ corresponds to the ideal capacitor. Generally the double layer between the solution and the electrode surface in an electrochemical cell is better fitted by a CPE than a capacitor.

Now turning into the genosensing application, the goal is not the electrochemical characterization of a system, but deriving the sensor signal related to hybridization of a DNA fragment. This means relating the change of one of the impedance elements, a resistance or a capacitance, depending on the specific format and design of the sensor, to the presence of the DNA gene sought and/or its concentration. Measurements can entail scanning the whole spectra range, or, probably, can be made simpler; once the system is characterized, it may be sufficient to determine the impedance at one selected frequency or within a certain frequency range.

For the typical genosensing application, a DNA probe, complementary to the one being sought, is immobilized on a working electrode, and the interaction with the target DNA (analyte) is monitored. Here the impedance of the working electrode (biosensor modified with the biological component) must be controlling the overall changes, for which auxiliary electrodes of sufficiently large area and a high concentration of saline background are preferred. Measurements with these surface-modified sensing electrodes are normally accomplished with the help of a redox-active compound, which is used as a probe. The observed phenomenon is then the electrochemical reaction of the probe, which is affected by changes of the biologically modified surface. Hybridization is therefore translated into a change of the electron transfer resistance R_{et}, the analytical signal for this impedimetric biosensor. When the redox-active compound is not used, the alternative is to monitor changes on the capacitive impedance component (since R_{et} will become extremely large). Thus, a binding event at the electrode can be detected by following the change in R_{et} in the first case, or the change in the capacitance in the second case. The first situation, in which the electrochemical reaction of the redox probe is involved, is also referred to as Faradaic impedance, while the second situation, not involving directly a redox reaction is referred to as non-Faradaic. After representation of the impedance over a sufficiently ample frequency range, and/or altering the surface area of the devices, individual events can be separated, and the corresponding region where the impedance is dominated by the impedance element under investigation can be identified.

1.2 EIS Sensing Applications

Nowadays, EIS is a reference technique for characterization and study of any electrochemical process at the electrode–electrolyte interface [29]. Although the information that it can provide is also attainable from series of experiments

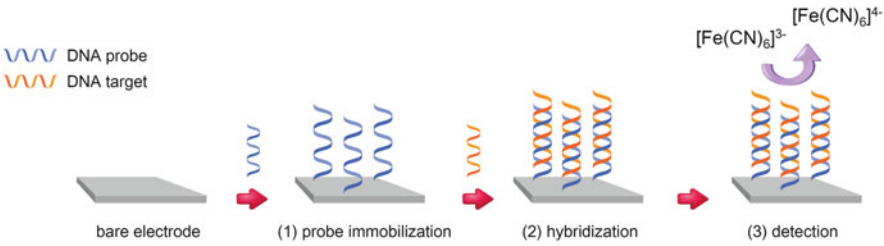

Fig. 5 Steps followed in an EIS genosensing experiment

employing the cyclic voltammetry technique at different potential scanning speeds, the powerful deductions that can be derived with the use of equivalent circuits makes EIS specially interesting to describe any electrochemical process.

Impedance spectroscopy is then mandatory for studies related to corrosion [30] semi-conducting electrodes [31], coatings [32], batteries and fuel cells [33], electrochemical kinetics and mechanism [34], biomedical and biological systems [35] and solid-state systems [36].

Due to its ability of directly probing the interfacial properties of a modified electrode, the technique is rapidly developing as a tool for studying biorecognition events at the electrode surface [23, 29, 37, 38]. In particular, EIS is becoming an attractive electrochemical tool for numerous applications either in immune-sensing [39, 40] or in genosensing field [2, 3, 41], especially in the last decade.

Generally speaking, the analysis of DNA using biosensors consists normally in a capture format, and can be described by these essential steps: (1) DNA probe immobilization onto the electrode surface, (2) Hybridization with a complementary target sequence and (3) Detection. These are schematized in Fig. 5. Normal sizes of the oligomers employed are ca. 25-mer for the probe, 20–50-mer for the target, and ca. 25 for additional fragments. The lengths specified are those typically used for PCR primers or for genetic assays, as the associated permutations assure a sufficiently high specificity. In some cases, additional steps are required in the protocol, such as sample preparation (i.e. PCR amplification), the use of other specific stages for signal amplification, or the use of systems for data treatment (i.e. Artificial Neural Network). Each genosensing step is then open to its monitoring by EIS, allowing for verification of its completeness. Figure 6 shows a typical evolution of the R_{et} observed for the ferrocyanide/ferricyanide redox probe during the steps of a genosensing experiment. In this case the redox species is considered a marker, not a label, since it is merely an accessory used for obtaining the signal and is only indirectly related to the sensing event. Each step in the experiment, associated with changes in the surface of the electrode, is responsible for altering the kinetics of this electrochemical reaction. This is due mainly to two chief effects: (1) the steric hindrance offered by the DNA probe in first instance, and the hybrid with the target, once formed; and (2) the electrical repulsion between the anionic backbone of the DNA double string and the anionic redox probe. This is the reason for the choice of the redox probe, among other options with neutral or cationic markers.

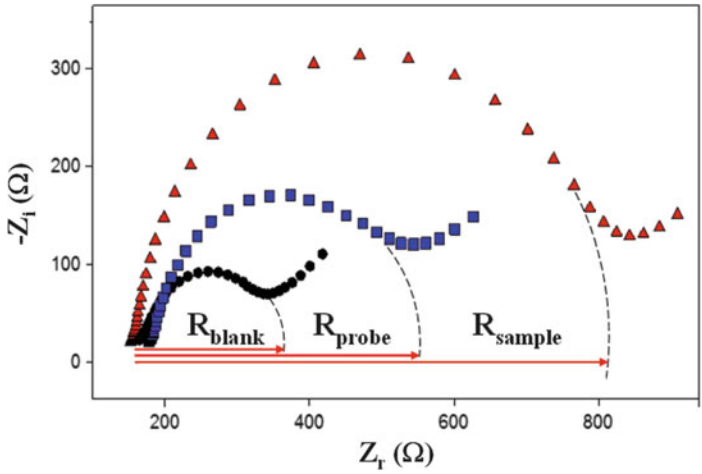

Fig. 6 Signals recorded in a typical EIS genosensing experiment

In some protocols, in order to enhance the difference in the signal obtained between the probe immobilization and the hybridization with a complementary sequence, instead of using DNA, a peptide nucleic acid (PNA) probe may be employed [42]. PNA is an artificially synthesized polymer which hybridizes equivalently to DNA, but in which the backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds, instead of deoxyribose sugar backbone. With this change PNA results uncharged due to absence of hydrolysable phosphate groups, and all R_{et} variation observed in the biosensing process may be mainly attributable to hybrid formation [43].

In fact, the detailed observation of the evolution of the EIS signal during the genosensing process gives solution to one important problem with these sensors. The problem is that different electrodes may show slightly different impedance values, if their reproducibility of construction is not good enough. Moreover, this problem becomes worse in many occasions, as different measurements are performed with different electrode units or with the same unit after renewal of the sensing surface. This issue, which is in fact originated in the high sensitivity of the technique, poses difficulties in the representation and/or comparison of results between replicated experiments, the decision of positive or negative test, or also the quantitative estimation of target DNA.

The found solution to this problematic resides in normalizing the readings to the blank measurement, which is the one that may vary with differently produced electrodes. Hence, a solution is to express the parameter of interest (i.e. charge transfer resistance or capacitance) relative to the value given by the bare electrode [44]. Results are represented then as the relative R_{et} variation between net values

obtained after DNA immobilization and hybridization. This relative variation is represented as a ratio of delta increments versus the bare electrode, as sketched on Eq. (6):

$$\Delta_{\text{ratio}} = \frac{\Delta_s}{\Delta_p} \tag{6}$$

being $\Delta_s = R_{et}(\text{sample}) - R_{et}$ (blank) and $\Delta_p = R_{et}$ (probe) $- R_{et}$ (blank). This elaboration was required for the comparison of data coming either from different electrode units or from the same unit after regeneration of surface. Briefly, when hybridization occurred Δ_s/Δ_p value should be >1 for the hybridization experiments and close to 1 for negative controls with non-complementary targets (that means $\Delta_s = \Delta_p$, i.e. no variation of R_{et} value because no hybridization occurred).

Apart of the generic sensing scheme, which in fact is the simplest concept, two additional variants should be commented. The first is the use of labelled targets, of interest when there is the need to increase the detection ability (i.e. decrease the detection limits); the second variant entails the design of more complex formats, i.e. a sandwich format with three or more DNA fragments.

For the use of labelled targets, e.g. the use of biotin to which many other functional groups may be linked is one possibility to amplify, or visualize the hybridization event by complementary techniques, i.e. fluorescence, amperometry or electron microscopy. For example, Ma and Madou [45] developed an enzymatic amplification scheme, employing a biotinylated oligonucleotide bound to a streptavidin-modified enzyme, in order to increase the sensitivity of the DNA sensor. Their approach took profit of the enzymatic precipitation of an insoluble compound on the sensing interface after hybridization, which caused an important impedance change. In a related protocol, Patolsky and Willner [46] also exploited the biocatalysed precipitation of an insoluble product on the transducer, to provide a mean to confirm and amplify the detection of a single-base mutation. The sensitivity of the method enabled the quantitative analysis of the mutant of Tay–Sachs genetic disorder without the need of PCR amplification. The same authors employed tagged, negatively charged, liposomes to amplify DNA sensing performance for hybridization and base mismatches detection [47]. One objection that may be stated here is that detection of a biotin-labelled (or any other label) DNA is unpractical, as the DNA analyte in a sample will not be biotinylated. But one should not forget that direct detection is not the unique possibility here, and in fact, an indirect, competitive assay may be used. In it, a fixed amount of biotinylated probe may be employed together with the sample, and any presence of the sought gene in the latter will produce a decrease in the finally observed signal.

An amplification that may be accomplished from a different strategy is to take profit of the ability of the double-strand DNA, or of some of its specific base-pairing points to interact with different species. For example, Bonanni et al. improved the sensitivity obtained for the detection of SNP correlated with kidney disease by performing the detection in presence of Ca^{2+} [48]. In this case, the specific binding

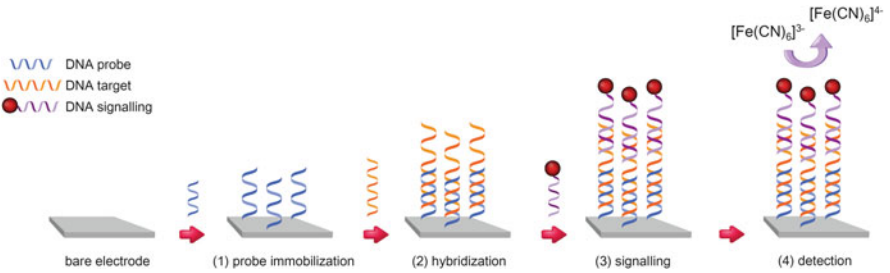

Fig. 7 Steps followed in a sandwich format EIS genosensing experiment, in this case using a labelled signalling probe

of the metal ions in the presence of A–C nucleotide mismatch induced a further impedance change, thus improving the discrimination between the mutated and healthy gene, as the signal amplification was achieved only for the former.

The second strategy that deserves comment is the use of multi-stage sandwich protocols, which simultaneously look for avoiding the use of labelled targets, to employ competitive schemes and to increase sensitivity. In essence, they employ a capture probe that hybridizes first with the sought gene in a sample, but in this case being larger than in previous examples, and using only a first half of its sequence for its capture. The second half is then free for fixing a third DNA string, named in this case signalling DNA, which may be directly detected by EIS in a labeless approach, or incorporate further labels to improve the detection. This general sandwich scheme is schematized in Fig. 7.

2 Use of Nanomaterials for Genosensing

The use of nanostructured materials for sensors and biosensor design and operation [49] is nowadays a very active field of research, where a wide variety of nanoscale or nanostructured materials of different sizes, shapes and compositions are now available [50]. The huge interest in nanomaterials is driven by their many desirable properties. In particular, the ability to tailor the size and structure and hence the properties of nanomaterials offers excellent prospects for designing novel sensing systems [51, 52] and for enhancing the performance of bioanalytical assays [53–55]. The similarity of dimensions between the involved molecules and the nanocomponents employed in these nanobiosensors is in part responsible for the increased efficiency and improved signal-to-noise ratios observed [56]. The use of these nanomaterials suggests their operation as effective mediators to facilitate the electron transfer between the active sites of probe DNA and surface of the electrodes. Moreover, the decrease in dimensions involved may show important advantages for integration of addressable arrays on a massive scale, which sets them apart from other sensors technologies available today.

The most widely utilized nanomaterials in impedance sensors are gold (Au) nanoparticles and CNTs [3]. Au nanoparticles have been employed in impedance sensors to form electrodes from nanoparticle ensembles and to amplify impedance signals by forming nanoparticle–biomolecule conjugates in the solution phase [57, 58]. CNTs have been employed for impedance sensors within composite electrodes and as nanoelectrode arrays [59, 60]. The advantages of nanomaterials in impedance sensors include increased sensor surface area, electrical conductivity and connectivity, chemical accessibility and electrocatalytic effect.

2.1 Use of AuNPs

One of the major trails of advance in nowadays nanotechnology is the use of nanoparticles. The unique chemical and physical properties of nanoparticles make them extremely suitable for designing new and improved sensing devices, especially electrochemical sensors and biosensors. Many kinds of nanoparticles, such as metal, oxide and semiconductor nanoparticles have been used for constructing electrochemical sensors and biosensors [55]. Owing to their small size (normally in the range of 1–100 nm), nanoparticles exhibit unique chemical, physical and electronic properties that are different from those of bulk materials and can be used to improve performance of sensing devices. Some important functions provided by nanoparticles include the immobilization of biomolecules, the catalysis of electrochemical reactions, the enhancement of electron transfer between electrode surfaces and proteins, the labelling of biomolecules and even their actuation as reagents. Of the different choices, one of the very relevant roles is the labelling of biomolecules, as they can retain their bioactivity and interact with their counterparts, and nanoparticles may be used for supplying the measurable signal.

Metal nanoparticle labels can be used in both immunosensors and DNA sensors. The most frequently used nanoparticles are those made of gold (AuNPs), given the extraordinary properties they present. Main use of AuNPs in genosensing is related to hybridization tagging, with added advantages of sensitivity enlargement [55]. Different from the amperometric detection [61], multiple tagging with nanoparticles of different nature is not useful for multiplexed detection of different genes in the same sample, given their nature may not be discriminated by EIS.

In a typical example of application of this type of nanobiosensor, Moreno-Hagelsieb et al. used a gold nanoparticle labelled oligonucleotide DNA target in order to amplify the capacitance signal between interdigitated aluminium electrodes imprinted over an oxidized silicon wafer [62]. As already commented, one does not expect to find gold-tagged nanoparticles in a generic sample, but their use can allow, for example, a competitive assay. In addition, a silver enhancement treatment, also useful for the electron microscopy detection, was performed offering a further signal amplification strategy. In a similar work Bonanni et al. used streptavidin-coated gold nanoparticles (strept-AuNPs) to amplify the impedimetric signal generated in a biosensor for the detection of DNA hybridization [58]. In this approach, a biotinylated target sequence was employed for the first capture by

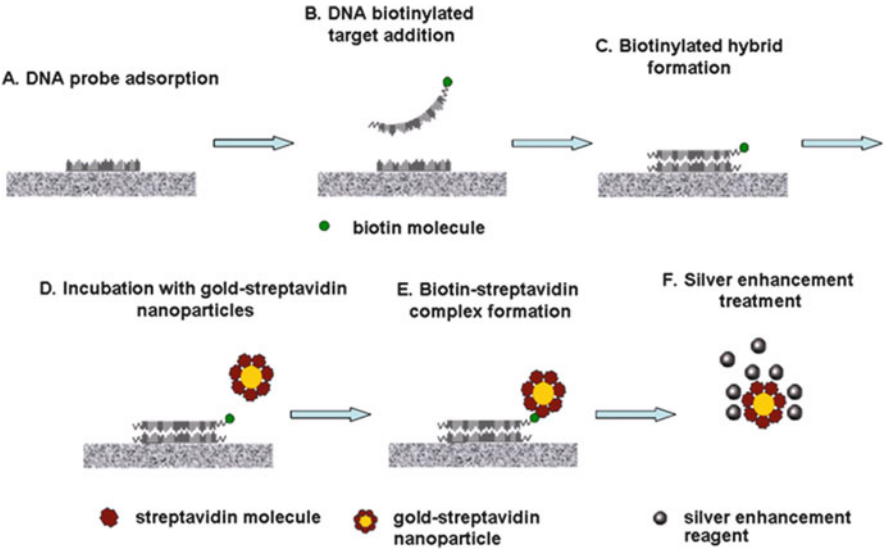

Fig. 8 Steps followed in a direct hybridization assay with impedimetric genosensor and amplification using strept-AuNPs and silver enhancement

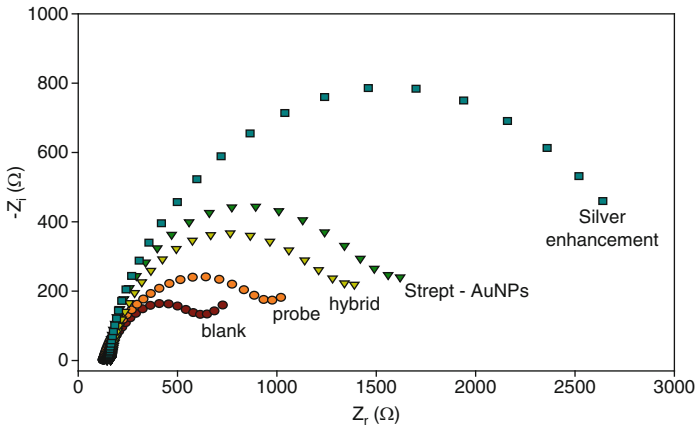

Fig. 9 Evolution of impedimetric signal after the different stages employed in the amplified genosening scheme employing strept-AuNPs

hybridization, followed by the conjugation with strept-AuNPs. The obtained impedimetric signal resulted 90% amplified in the presence of strept-AuNPs. Figure 8 schematizes the steps involved in the use of AuNPs for amplifying the impedimetric signal; the electrode used here was a simple epoxy graphite composite electrode [63, 64], and the immobilization used a simple adsorption.

Figure 9 is an illustration of the gain in impedimetric signal if the described protocol using amplification with gold nanoparticles is followed. After immobilization of the DNA probe, with little increase in R_{et}, the hybridization with the target

DNA, the primary positive signal, represents an increment similar to the fixation of the probe. The conjugation with the strept-AuNPs, still bringing a large amount of steric hindrance to the detection, is not translated in a large change in signal. It is only the amplification of the latter, with the catalytic reduction of silver onto the gold nanoparticles, which brings the largest gain in $R_{et.}$ Although not shown in here, it is obvious that even the use of non-biotinylated target or non-complementary biotinylated target produced very little signal, even lower in comparison once amplified.

In addition to labelling, a very interesting alternative of using AuNPs is to use them to construct three-dimensional networks with the nanoparticles dispersed throughout the sensing interface, in a nanostructured or molecular imprint approach, and that can be used to enhance impedance detection for biosensors. This may be accomplished through repeated use of a bifunctional gold coupling reagent, such as cysteamine or 4-aminothiophenol, where the amino group can bind to a biomolecule and the thiol group can bind to Au nanoparticles, for layer-by-layer formation of the Au nanoparticle network. Impedance detection of human immunoglobulin (IgG) using such a three-dimensional Au-nanoparticle network was recently reported using 6 nm diameter Au nanoparticles and cysteamine as the bifunctional reagent [65]. Some of the added advantages are the increased surface area for sensing, the improved electrical connectivity through the AuNPs network, the chemical accessibility to the analyte through these networks, and also the electrocatalysis.

2.2 Use of QDs

Quantum dots are nanometric scale semiconductor crystals (mainly sulphides, selenides or tellurides of heavy metals Cd, In, Zn or Tl) with unique properties originated in the quantum confinement effect that are advantageous for the development of novel bioassays, chemical sensors and biosensors [66]. Although mainly applied as fluorescent tags for biomolecules, where they bring out their exceptional properties, they have been also exploited in electrochemical sensors, normally with amperometric transduction, in which the heavy metal content after their dissolution can be detected by Anodic Stripping Voltammetry [67].

In our scope of interest, Xu et al. described a novel, sensitive DNA hybridization detection protocol, based on DNA-quantum dots nanoconjugates coupled with EIS detection. For this purpose, suitable DNA probes were covalently immobilized onto a self-assembled mercaptoacetic acid monolayer modified gold electrode; then, after hybridization with the target ssDNA-CdS nanoconjugate, they observed a remarkably increase in R_{et} value only when complementary DNA sequence was used in comparison with a three-base mismatched or non-completely matched sequences. The results showed that CdS nanoparticle labels on target DNA improved the sensitivity by two orders of magnitude when compared with nonlabelled DNA sequences [68].

For the case of the impedimetric technique, a very interesting work was reported by Travas-Sedjic's laboratory [57]. In this work, hybridization with a complementary DNA sequence is assayed employing a CdS nanoparticle label, showing a significant improvement in sensor sensitivity. In this variant, DNA probe was immobilized through entrapment during electropolymerization of conducting polymer (polypyrrole). Authors stated a limit of detection of the DNA probe of 1 nM. One important feature of their sensor is that it could be regenerated by removing hybridized DNA with NaOH, suggesting the possibility of sensor reuse.

In a similar work, Kjallman et al. employed a CdTe nanoparticle for the modification of a hairpin DNA probe. The stem–loop structured probes and the blocking poly(ethylene glycol) (PEG) molecules were self-assembled on the gold electrode through S–Au bonding, to form a mixed monolayer employed as the sensing platform. EIS was next used for characterization of the interfacial electrochemical characteristics of the modified gold electrode before and after hybridization with the target DNA [69]; this permitted to detect the target DNA with detection limit of 4.7 fm and even discrimination of non-complementary oligomers. Depending on the probe DNA to PEG ratio, the genosensor showed completely opposite response trends with regard to the change in charge transfer resistance and in the impedance at the electrode interface.

2.3 Use of CNTs

CNTs can be considered one of the most commonly used building blocks of nanotechnology [70]. CNTs are allotropes of carbon from the fullerene structural family, and can be conceived as sp^2 carbon atoms arranged in graphene sheets that have been rolled up into hollow tubes. Thanks to their extraordinary properties, like tensile strength, thermal and electrical conductivity or anisotropic conductivity behaviour, they are attracting much interest among all applied sciences and technologies. Analytical chemistry is one of the fields taking benefit of several advantages that CNTs bring for applications like chromatography, sensors, biosensors, and nanoprobes. There can be distinguished two main types of CNTs. The multi-walled CNTs (MWCNTs) behave as conductors and show electrical conductivities greater than metals. These interesting properties suggest that their incorporation into any electrical transduction scheme may be beneficial. Also, there is a second type of CNTs, the single-walled CNTs (SWCNTs), that depending on the tube diameter and chirality may behave electronically as either metals or semiconductors, complicating their use in sensing schemes. CNT synthesis methods create a mixture that includes amorphous carbon, graphite particles and CNTs, so synthesis is typically followed by a difficult and critical separation process. For electrochemical applications, CNTs are typically activated in strong acids, which opens the CNT ends and forms oxygenated species, making the ends hydrophilic and increasing the aqueous solubility of CNTs [52]. The electrochemical behaviour of CNTs varies considerably with the methods used for preparation

and purification, including oxidation treatment. For analytical applications, and in part due to difficulties in their handling, CNTs are most often used to modify other electrode materials, or as part of a composite electrode.

As a first typical application, Xu et al. [71] incorporated multi-walled carbon nanotubes (MWCNTs) into composite electrodes used for impedance detection of DNA hybridization with a redox marker. In these studies, MWCNTs were co-polymerized with polypyrrole atop a glassy carbon electrode and then ssDNA was covalently immobilized. The complementary oligonucleotide was detected with the impedance technique by the accompanying change in R_{et}.

In the work of Caliskan et al. graphite electrodes were surface-modified with carboxylic acid functionalized SWCNTs; next, amino terminated DNA probes were covalently linked with the carbodiimide (EDAC)-N-hydroxysuccinimide (NHS) reaction to form an amide bond with the terminal acid groups. Finally, DNA target hybridization was monitored employing EIS and/or voltammetry [72]. The sequence chosen as study case was a specific gene for hepatitis B virus.

In the similar work in our laboratory, we employed a screen-printed, carboxyl functionalized MWCNT electrode, in which the detected gene was the sequence identifying the genetically modified organism Bt maize, given the high demand for analysis of transgenic food products [73]. For this purpose, the capture probe for the transgenic insect resistant Bt maize was covalently immobilized using the above carbodiimide chemistry; hybridization with DNA sample was followed, and impedance measurement performed in a solution containing the redox marker ferrocyanide/ferricyanide. A signal amplification protocol could also be performed, using a biotinylated complementary target to capture streptavidin-modified gold nanoparticles, thus increasing the final impedimetric signal (LOD improved from 72 to 22 fmol, maintaining a good reproducibility (RSD $<$ 12.8% in all examined cases).

An equivalent procedure was followed for an impedimetric genosensor devised for screening the Influenza A virus outbreak on spring 2009, which created a great social alarm [74]. Although the pathogenic H1N1 virus is a RNA virus, the diagnostic tools are normally prepared for its reverse transcripted DNA, given the higher availability of custom DNA synthesis. Figure 10 shows the preparation and detection scheme, in this case a sandwich capture format. First, the aminated DNA probe was immobilized using the carbodiimide chemistry (EDAC/NHS) to the carboxylated SWCNT-modified electrode. Then, hybridization with a previously formed duplex between the virus DNA and a biotinylated DNA probe was followed, to which further amplification employing strept-AuNPs was possible.

Figure 11 illustrates evolution of impedimetric signal along the process, in a very similar sequence as in Sect. 2.1: small increases for probe immobilization, noticeable increase for hybridization with the duplex, and possibility of amplification employing Strept-AuNPs. With these, a different strategy than before was used, which was a catalytic gold reduction onto the AuNPs instead of the classical silver reduction, in this case just to show a second amplification alternative.

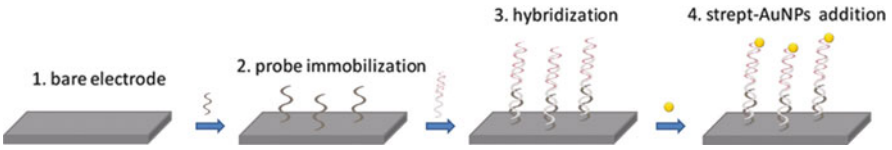

Fig. 10 Steps followed in a sandwich format EIS genosensing experiment, in this case using a labelled signalling probe

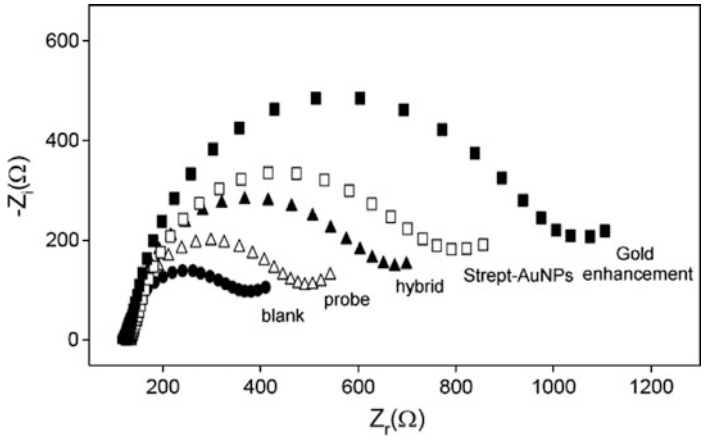

Fig. 11 Evolution of impedimetric signal after the different stages employed in the amplified genosening scheme employing strept-AuNPs

In a similar work [75], a genosensor for the impedimetric detection of the triple base deletion in a cystic fibrosis (CF)-related DNA synthetic sequence was shown. Screen-printed carbon electrodes containing carboxyl functionalized MWCNTs were used for the immobilization of an amino-modified oligonucleotide probe, complementary to the cystic fibrosis mutant gene. The complementary target (the mutant sequence) was then added and its hybridization allowed, later monitored by EIS. Results were contrasted against a non-complementary DNA sequence and a three-mismatch sequence corresponding to the wild DNA gene, present in healthy people. A further step employing a signalling biotinylated probe was performed for signal amplification using strept-AuNPs. With the developed protocol, a very sensitive detection of the triple base deletion in a label-free CF-related DNA sequence was possible, achieving an LOD around 100 pM.

A timely material very recently used to design biosensors and very much related to CNTs is graphene. Graphene is a two-dimensional lattice of carbon atoms arranged following an honeycomb pattern, and has become a star material sparked with the 2010 Physics Nobel prize, awarded to Novoselov and Geim (Manchester University) [76]. Graphene is an exceptional material in many regards, from huge charge mobility to strength and flexibility, offering a spectrum of applications ranging from flexible electronics to supercapacitors, composite materials and also biosensors, with amperometric or impedimetric transduction.

For example, the work of Muti et al. [77] used graphene oxide integrated on a graphite electrode for the enhanced monitoring of nucleic acids and for the sensitive and selective detection of the label-free DNA hybridization related to hepatitis B virus (HBV) sequences. The electrochemical behaviour of a graphene oxide-modified graphite electrode was firstly investigated using EIS and differential pulse voltammetry (DPV). The sequence selective DNA hybridization was determined voltammetrically in the case of hybridization between amino linked probe and its complementary (target), being capable of differentiating the noncomplementary target or a target/mismatch mixture (1:1).

In the work of Wang et al. [78] a reduced graphene oxide-modified glassy carbon electrode is used to detect the methicillin-resistant *Staphylococcus aureus* DNA, in this case using EIS detection. DNA probe is successfully anchored on the graphene-modified surface by simply adsorption. Hybridization with target DNA increased largely the measured impedance, with a detected amount of 100 fM.

A recent work from Bonanni and Pumera [79] investigated the suitability of different graphene surfaces for hairpin impedimetric genosensing. Electrodes modified by graphene nanoribbons were used. The hairpin DNA (hpDNA) probes were immobilized on the graphene-modified electrode surface by physical adsorption. The π-stacking interactions between the ring of nucleobases and the hexagonal cells of graphene made the platform a stable substrate for genosensing. Sensing mechanism was based on the partial release of the hpDNA probes from the graphene surface which occurs as a consequence of hybridization with complementary target, and translated in a significant decrease in R_{et}. Different DNA sequences correlated with Alzheimer's disease were used in this work, for example the mutated Apolipoprotein E gene. When hybridization was less effective, as in the case of the mutant target, a lower amount of the hpDNA probes are expected to be released, thus resulting in a less significant R_{et} decrease.

2.4 Use of Nanopores

As the last nanotechnology element to comment, the use of nanopores or nanochannels for detecting flux of ions biomolecules has to be mentioned [80]. Molecular-scale pore structures, called nanopores, can be assembled by protein ion channels through genetic engineering or be artificially fabricated on solid substrates using current nanofabrication technologies. When target molecules interact with the functionalized lumen of a nanopore, they characteristically block the ion pathway. The resulting conductance changes allow for identification of single molecules and quantification of target species. Detection can be accomplished through many different transduction mechanisms, mainly electrochemical.

A model example is the glass nanopore-terminated probe for single-molecule DNA detection designed by Takmakov et al. [81]. An array of nanopores was first prepared by anodization of aluminum, generating pores of ca. 10 nm. The inner pores were modified with biotin molecules via covalent attachment using

aminosilane/succinimide chemistry. A first model detection was done employing the biotin-streptavidin pair, detected via impedance spectroscopy of the redox probe with a gold electrode formed at the bottom of the pore network. The same principle of pore blockage was also used to detect DNA hybridization onto the DNA probes immobilized inside the pores of the device.

Nanopores are key elements in the emerging technique of 4th generation DNA sequencers [82]. In these, a voltage is used to drive molecules through nanopores separating two solutions. When nucleotide bases, ssDNA or dsDNA, are threaded through these nanopores, a specific current (ionic current or other signal) can be monitored, which can be specific for the mononucleotide interacting with the nanopore. This is in essence the technology behind the sequencers being developed by companies like Oxford Nanopore Technologies in the UK.

Compared with conventional sequencing technologies, the nanopore single-molecule approach is simpler and more cost-efficient. It does not need fluorescent labelling or amplification of the sample DNA, obviating the use of restriction enzymes or redundancy. A huge potentiality can be foreseen, as it represents a direct sequencing, just like reading a teletype that further can be parallelized. The most usable technology at this moment [83] (nothing commercially available up to now) is the use of exonuclease enzyme to fragment the ssDNA and α-Hemolysin (αHL), a protein natively used in bacteria wall pores as the nanopore. αHL defines a 2 nm wide channel, with inner peptide fragments able to interact with passing species; when immobilized in a nanopore of the proper dimension (e.g. a nano-fabricated silicon structure) and forcing the unidirectional movement of bases through potential biasing, this protein interacts and permits identification of the four A,C,T,G bases, in principle through measurement of characteristic pico-currents [84]. Reasonable mononucleotide base throughputs with acceptable signal-to-noise ratio are ca. 25 s^{-1}, a translocation velocity not easy to accelerate because of worsening of sensitivity [85]. Other nanopore protein structures, such as the porin A from *Mycobacterium smegmatis* have also been demonstrated to produce DNA translocation for the sequencing purpose [86].

More stable nanopore systems can be potentially devised on graphene [87, 88]. With the same aim, completely nanofabricated systems have been proposed by IBM researchers, in this case with FET structures built along the nanopore and using capacitance detection [89]. Also, a coupled nanopore-hybridization strategy has been described, in which a library of ca. 10-mer probes align with ssDNA fragments and pass electrophoretically driven, a technology that has been named hybridization-assisted nanopore sequencing [90].

3 Application of Impedimetric Genosensors for Medical Diagnostics

Table 1 displays a summary of employed nanomaterials and applications of above-mentioned impedimetric genosensors, as summary of the use of these types of genosensors for diagnostic and other important applications. Among the topics

Table 1 Selection of examples of impedimetric genosensors employing nanomaterials and their applications from recent literatures

Working electrode	Nanocomponent used	Application	LOD	Reference
Al/Al_2O_3	AuNPs	Cytochrome P450 2p2 gene	2 pM	[91]
Graphite epoxy composite	AuNPs	Arbitrary sequence	120 nM	[58]
Graphite	SWCNTs	Hepatitis B virus	50 nM	[72]
MWCNTs	AuNPs	Transgenic maize	2 nM	[73]
Gold	AuNPs	Arbitrary sequence	5 nM	[92]
Glassy carbon	AuNPs/polyaniline nanotubes	PAT gene (transgenic crops)	300 fM	[93]
Glassy carbon	AuNPs	PAT gene	24 pM	[94]
Gold	CdS nanoparticles	Arbitrary sequence	1 nM	[57]
Al/Al_2O_3	AuNPs	HIV gene	200 pM	[62]
Glassy carbon	MWCNTs	Arbitrary sequence	50 pM	[71]
Glassy carbon	MWCNTs	Arbitrary sequence	5 pM	[95]
Carbon paste	SWCNTs	PAT and NOS genes	300 fM	[96]
MWCNTs	AuNPs	Influenza A virus – H1N1 gene	500 nM	[74]
Carbon paste	Polyaniline nanofibers, AuNPs, CNTs	Genetically modified beans	500 fM	[97]
Glassy carbon	Nano-MnO_2/chitosan	HIV gene	1 pM	[98]
Glassy carbon	CeO_2 nanoparticles, SWCNTs	(PEPCase) gene	200 fM	[99]
Carbon paste	AuNPs/TiO_2	Cauliflower mosaic virus gene	200 fM	[100]
MWCNTs	AuNPs	Cystic fibrosis gene related sequence	100 pM	[75]
Gold	CdTe nanoparticles	Arbitrary sequence	5 fM	[69]
Gold	CdS nanoparticles	Arbitrary sequence	5 pM	[68]
Graphite	Graphene	Hepatitis B virus	160 nM	[77]
Graphite	Graphene	Alzheimer's disease-related Apo-E gene	3 pM	[79]
Graphite	Graphene	Methicillin-resistant *Staphylococcus aureus*	100 fM	[78]

covered, several applications are devoted to the detection of transgenic plants and genetically modified organisms. One of the genes detected in varied applications is the PAT gene [93, 94], specific for transgenic crops. Some other works are centered on the simultaneous determination of PAT and NOS genes [96]. The determination of genetically engineered maize, transgenic Bt corn, was shown to be possible [73]. The equivalent detection of genetically modified beans employing a specifically devised DNA sensor is also present in the literature [97].

Other important applications, regarding the medical field, include the identification of certain gene or nucleotide polymorphism correlated with specific diseases. One of the first works described from the laboratory of Itamar Willner in Jerusalem was the sandwich determination of a gene related to the Tay–Sachs mutation that

would be utilizable as a biosensor device to diagnose this genetically carried disease [101]. Similar works capable of detecting a gene cause of a inherited disease were those to detect cystic fibrosis [75]. Very recent efforts have also attempted to correlate certain gene with Alzheimer's disease and propose its detection [79]. The detection of its genetic material can be also the base for the confirmation of certain virus infections, and in this sense, impedimetric genosensors for detection of human immunodeficiency virus (HIV) to evaluate people suffering from AIDS [62, 98]. Nanobiosensors capable of detecting the hepatitis B virus [72, 77] have also been elaborated. After the pandemic Influenza A declaration in 2009, genetic assays were quickly prepared to diagnose and control the expansion of the disease; with these information, a nanobiosensor to detect its H1N1 virus genetic material was also developed [74].

The identification of microbiological species is also the other clear trend when classifying the nanobiosensors reported in the literature. As already commented in the diagnosis of illnesses of viral origin, HIV [62, 98], hepatitis B [77], Avian Influenza [102] or H1N1 Influenza [74] viruses are some of the available DNA biosensors. Also diseases to other organisms different to humans can be incorporated in this list, for example the cauliflower mosaic virus [100].

But there are not only viruses that can be detected by examining their genetic material; the presence of bacteria, or its specific variant may be evaluated also by examining their genetic material. For example, the work of Wang et al. that discriminated the strain of *S. aureus* resistant to antibiotic methicillin [78]. Also interesting is the work in the literature describing the identification of *Salmonella* spp employing capacitive detection [101], after its immunocapture with monoclonal antibodies grafted to AuNPs, these entrapped in electropolymerized ethylenediamine.

4 Outlook and Perspectives

The impedimetric genosensing topic is nowadays an active research area, where many formats and designs are reported in order to improve performance of existing biosensors. Research is still to be done in order to obtain devices with better reproducibility and stability, although any objection here can be balanced with the low detection limits achieved. Moreover, researchers should still increase efforts to get better electrode assemblies for their use in real samples, overcoming all problems associated with the complexity of matrices in various natural or commercial samples. Progress on these analytical features will accelerate their routine use, and even enable the massive production of devices using some of the principles stated in this chapter. Electrochemical impedance sensors are particularly promising for portable, on-site or point-of-care applications, in combination with simplified discrete-frequency instruments. However, there are certain impediments for solving these future applications and for the successful commercialization of useful devices, as minimizing effects of non-specific adsorption or automating all operation steps. And precisely these areas are the ones that can take more benefits from the incorporation of nanocomponents into genosensors.

A first challenge is the fabrication of useful electrochemically addressed genosensor arrays. The electrochemical impedance technique is fully compatible with multiplexed detections in electrically addressable DNA chips, which is one of the clear demands in genosensing for the next years [103]. Array sizes on the order of 10 have been described, but to be clinically useful, arrays of ca. 50 sequences are necessary. For example, a genetic disease like cystic fibrosis involves detection of around 25 different mutations plus the positive and negative controls. Microfabricated platforms can be of great help here, although issues like the mechanical reliability of the electrical contact, or reproducibility of construction and operation are still to be improved.

A second problem is related to sensitivity. DNA analysis is nowadays closely connected with PCR amplification, which is the step providing the major gain in it. Thus, two separate stages are needed, PCR, and afterwards, biosensing. Platforms are needed to integrate the two, allowing for really fast, intervention-less gene analysis. Microfluidic systems, of the lab-on-a-chip type, can be the solution here [104]. With such a platform, the goal of detecting a few viable pathogen microorganisms in a clinical sample in less than one hour might be a reality [4, 105].

The conversion of all the information which is generated with the unravelling and understanding of the functionalities yielded by the human genome is showing new achievements every day. Many of the properties found can be translated into genosensing applications to help in clinical practice and diagnostic, with small, cheap and decentralized analytical devices. But the challenge is even greater with the proteome. We are just in the beginning of its deciphering, for which high-throughput screening methods are in constant demand. Perhaps the principles used by electrochemical genosensors may be of help in the immense workload still to be done, to catalogue the human proteome in its biologically active form and to relate it to disease and cell state. Aptamer sensors, as already described, may be one starting point here. Most of the formats and strategies that have been described in this chapter are also extensible to specific detection of proteins, when the aptamer–protein interaction is exploited [106]. And impedance transduction is one of the simplest, more directly achievable transduction schemes available for their operation.

5 Conclusions

This chapter has presented current technology typically employed with genosensors which employ the EIS as the detection technique. Its operational principles and the essential protocols employed for impedimetric genosensing have been introduced. Although impedance is commonly used to investigate a variety of electrochemical systems, including fundamental redox studies, corrosion, electrodeposition, batteries and fuel cells, only recently it has been applied in the field of biosensors. Given its ability to monitor R_{et} and the double layer C, it is possible to derive applications for different types of sensing schemes, with numerous recognition

agents, by direct signal acquisition, or with the use of simple and cheap redox markers. One chief advantage of impedimetric genosensing is that it can provide potentially label-free assays, as hybridization with the DNA probe immobilized on a surface can be directly monitored. In general, impedimetric genosensors are extremely simple in operation, and capable of achieving low detection limits even when used without any amplification. If combined with additional signal amplification strategies, their absolute detection limits may be comparable to other genosensing strategies. The contribution of nanostructured materials in the development of genosensors is an active research area of activity, and the use of nanoparticles, nanotubes, graphene or other nanostructured materials has been pointed out as some of the significant research with impedimetric nanosensors.

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