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Impedimetric Aptamer-Based Biosensors: Principles and Techniques

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Contents

Abstract Aptamers are a specific class of ligands with high affinities comparable to antibodies, which are selected and synthesized in vitro. In combination with impedance spectroscopy as sensitive measurement method, we gain a class of biosensors with high potential for handheld devices and point-of-care tests. In this review, we report on recent advances in aptamer-based impedimetric biosensors. Besides giving a short summary of electrochemical measurement techniques, the most exciting innovative developments of detection strategies in the last decades are reviewed. Finally, important criteria for the comparison of aptamer-based biosensors are discussed.

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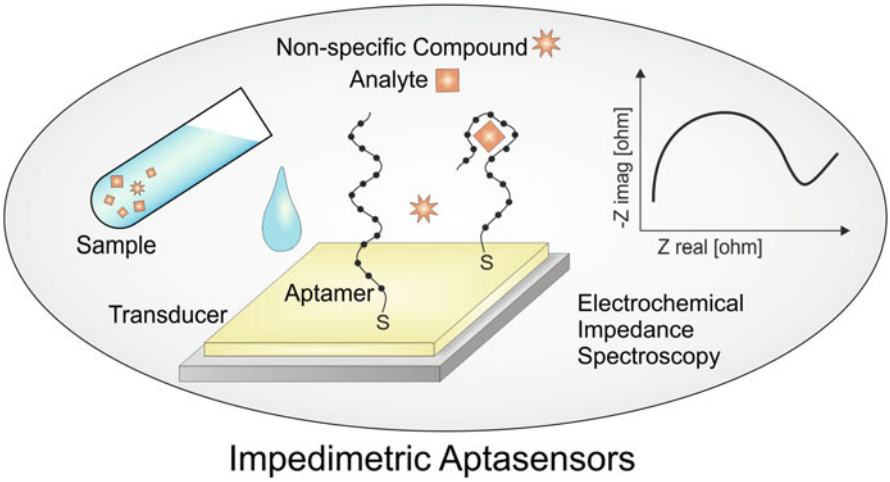
Graphical Abstract



Keywords Aptamer, Aptasensor, Biosensor, Capacitive, DNA origami, Electrode, Faradaic, Impedance, Label-free, Nanocomposites, Non-faradaic, Nanotechnology, Rapid detection, Redox mediator

1 Introduction

Impedimetric aptamer-based biosensors, also called impedimetric aptasensors, belong to the electrochemical biosensors, thus are biosensors with an electrode as transducer at which the response of an applied signal is detected. Depending on the applied signal and its response, electrochemical biosensors are categorized into different measurement methods. For example, amperometry detects current as a response to an applied constant voltage, whereas voltammetry detects current as response to a variable voltage. The electrochemical impedance spectroscopy (EIS) measures the current responses to sinusoidal voltages at different frequencies.

The advantages of electrochemical compared to other transducers are the ease of miniaturization and automation, high sensitivity, insensibility to turbidity or quenching effects, simple handling, and almost complete avoidance of complicated sample preparation. Furthermore, electrochemical biosensors captivate with their low costs, low power consumption, and fast response time.

Impedance has the advantage over other electrochemical methods of being possibly label-free and nondestructive due to the usage of low amplitudes, which enables recovery of the sample and at-line sensors in combination with lab-on-a-chip systems.

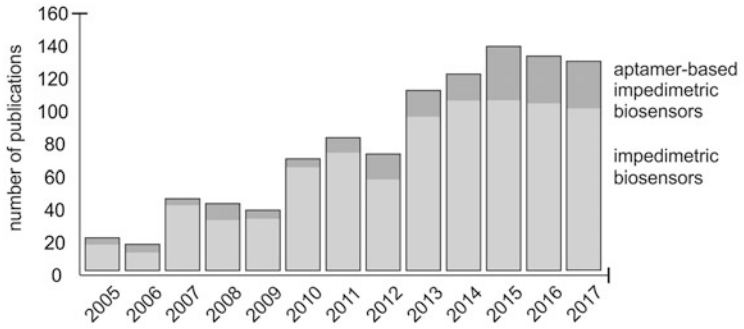


Fig. 1 Trend in number of publications on impedimetric biosensors in general (gray) and aptamer-based impedimetric biosensors (dark gray) in the last decade (searched with PubMed (all databases), Web of Science (TIB Hanover), ScienceDirect, and Springer)

The combination of aptamers with its high selectivity and specificity and EIS also merges their advantages. Thus, impedimetric aptasensors have high potential in biotechnology, e.g., in screenings, bioprocess monitoring, and quality inspection.

The first impedimetric aptasensor was described by Rodriguez et al. [1] in 2005 for the detection of lysozyme. Only 2 months later, Xu et al. [2] reported an impedance sensor using aptamers to detect the human immunoglobulin E. Since then, the number of publications on impedimetric biosensors increased continuously every year (see Fig. 1). To date, there are impedimetric aptasensors for ~85 different targets, including potassium ions, small molecules (e.g., adenosine), proteins, and whole cells.

There are several excellent reviews about electrochemical aptasensors, as, for example, by de-los-Santos-Álvarez et al. [3] who summarize the state of the art in label-free detection strategies for aptamer-ligand interaction. The book *Aptamers in Bioanalysis* by Mascini [4] contains a good selection of aptamer applications in biosensors, whereas the reviews by Hianik and Wang and Sassolas et al. [5, 6] concentrate on electrochemical detection strategies for aptasensors. The more recent review by Ferapontova and Gothelf [7] concentrates on the advances made in electrochemical aptasensors especially those based on conformational detection strategies and nanotechnology. The book chapter by Li and Ellington [8] describes electrochemical techniques for aptasensors divided into label-free and label-based detection strategies. The short review by Chiorcea-Paquim and Oliveira-Brett [9] specializes on G-quadruplex-based electrochemical biosensors. The most recent review by Muñoz et al. [10] is about impedimetric biosensors and contains a short paragraph about aptasensors.

This book chapter reviews the advances in impedimetric measurement techniques of aptasensors since 2005. Section 2 describes the basics of impedimetric measurement techniques with emphasis on different signal shapes. Since there are several reviews about detection strategies developed for impedimetric biosensors, only new developments in detection strategies in the last years will be discussed in Sect. 3 divided into non-faradaic and faradaic measurement cells. And the last section

of this chapter discusses parameters that should be determined for a developed electrochemical aptasensor to enable the comparison of different aptasensors with each other. Another chapter enclosed in this book describes the possible applications of impedimetric aptasensors in health care, food inspection, and environmental protection.

2 Impedimetric Measurement Techniques

The term impedance Z was established by Oliver Heaviside in 1886 and is defined as the ratio of the applied voltage u of a specific frequency f and its current response i [11]:

$$Z(2\pi f) = u/i \tag{1}$$

Traditionally, each frequency is applied sequentially (see Fig. 2). Therefore the duration of an impedance measurement depends on the selected frequency range and mainly on the smallest frequency, as the application of one period of a 1 Hz signal takes 1 s, whereas the application of one period of 1 mHz takes ~17 min. For a better signal-to-noise ratio, the mean value of 3–10 repetitions is measured; thus the duration increases rapidly. Therefore, normally a frequency range of 1 Hz–100 kHz is applied that takes depending on the number of repetitions about 1–3 min.

To decrease measurement time, the multisine signal is used [12], which is the addition of different frequencies to one period of the measurement frequency that is equal or smaller than the smallest frequency (see Fig. 3).

Hence, the applied multisine signal already contains various repetitions of the higher frequencies. Using Fourier transformation, the current response is transformed from time domain to frequency domain that allows the extraction of each frequency separately. Pauwels et al. [13] developed a multisine-based impedimetric aptasensor

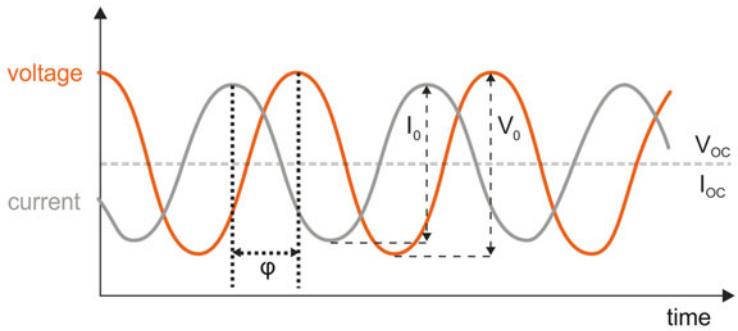


Fig. 2 Applied voltage and current response of a single sine with frequency f, V_0, and I_0 are the amplitudes, and φ is the phase shift

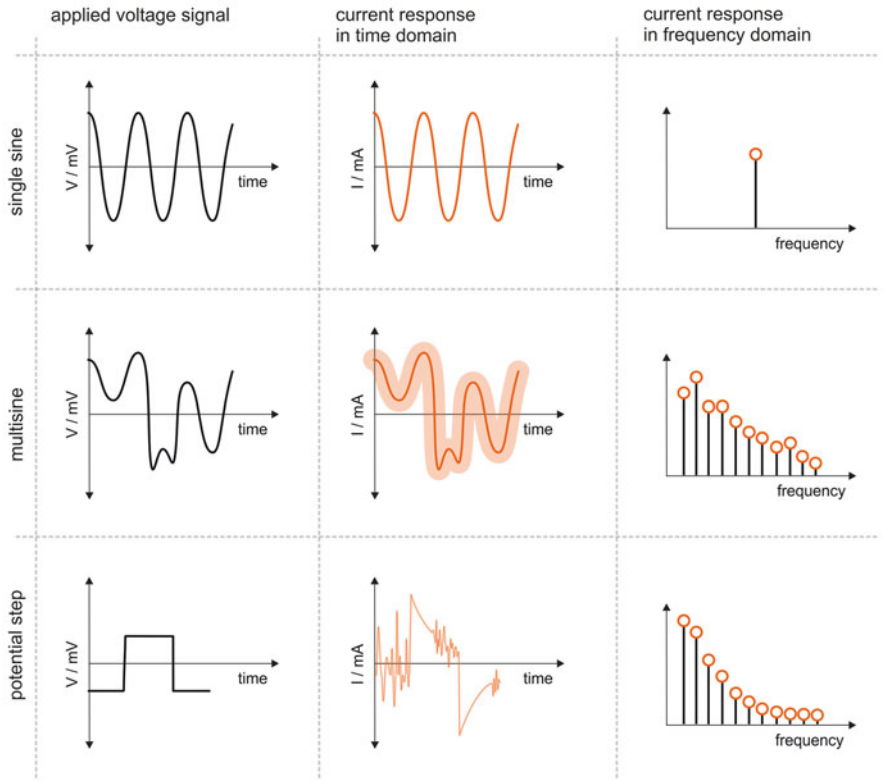


Fig. 3 Signals and responses for single-sine, multisine, and potential step techniques

by adding odd harmonic frequencies in the range 1 Hz–20 kHz and applying five periods of the created excitation signal. They modified a glassy carbon electrode with multiwalled carbon nanotubes (MWCNT) and an aptamer against 2-hydroxy-2′,3′,4′,5′,5′-pentachloro-biphenyl (OH-PCB), an environmentally harmful pollutant. For comparison, single-sine and multisine signals were applied, and the results showed no significant difference between the two techniques with errors $\leq$6%. They were able to detect OH-PCB down to 1 nM. Another advantage of using multisine instead of single sine is the gain of information about noise, linearity, and stationarity of the measurement. In that way, they found nonlinearity and non-stationarity for frequencies below 100 Hz and concluded that the selected model does not represent the data properly. In 2014, they showed the application of this aptasensor in human blood serum samples with a LoD of 10 nM [14].

A progression of the multisine signal is the addition of an infinite amount of frequencies that results in a potential step signal (see Fig. 3). In 2000, Yoo and Park [15] used the potential step technique to study an impedimetric system; thus multisine and potential step are known techniques. Despite the technological challenges, the potential step method inherits a bundle of limitations such as

the inability to detect mass transfer effects (e.g., Warburg impedance) due to the shortness of the applied signal (normally ~2 ms) [16]. Prolongation of the signal time is complicated because the current decreases exponentially and rapidly is not distinguishable from noise. However, prolongation might be applicable for systems with a very low rate constant that slows down the decrease of current and enables approximate determination of the charge-transfer resistance. However, no impedimetric aptasensor using the potential step technique has been developed yet, and further studies on this technique are necessary.

The impedimetric aptasensors, reviewed in this article, are all based on sequentially applied single-sine waves due to the absence of articles about impedimetric aptasensors using multisine or potential step techniques.

3 New Developments in Detection Strategies

In impedimetric biosensors the flow of current is measured, and depending on how this flow of current is influenced by the aptamer-target binding, impedimetric biosensors are subdivided in faradaic (FIS) and non-faradaic impedance spectroscopy (nFIS). The commonly used faradaic impedance systems are based on a charge transfer from electrode to bulk or vice versa, whereas in non-faradaic systems, no charge transfer occurs.

In this paragraph, we will discuss innovative developments in detection strategies for impedimetric aptasensors in the last decade subdivided into FIS and nFIS. To date, there are only a few publications on nFIS-based biosensors (Sect. 3.2), whereas for FIS-based biosensors (Sect. 3.1), there are several reviews on detection strategies as mentioned in the introduction, to which the reader is appointed for more details.

3.1 Faradaic Impedimetric Aptasensors

Due to the fact that in impedimetric measurements low voltages are used, no charge transfer occurs in simple buffer solutions. To enable charge transfer in faradaic measurements, a redox mediator is added to the buffer or immobilized on the surface (see Fig. 4). As redox mediators are in general easily available in big amounts and low-priced, faradaic systems with a redox mediator in solution are seen as label-free. But the addition of a redox mediator in a separated step disables online measurements. Beyond this, it is difficult to distinguish between labeled and label-free impedimetric systems. As the main advantage of label-free techniques is to spare time and costs for the labeling, the use of attached redox mediators or other electroactive molecules shall be termed labeled. But also in case of other amplification strategies, the question for time and cost consumption shall be asked. The more complex the mechanism for signal amplification is, the less useful are label-free measurements. A good overview to different detection strategies based

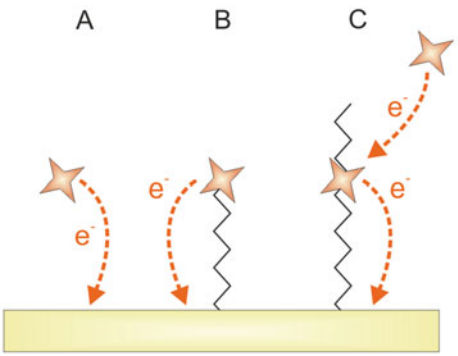


Fig. 4 The role of redox mediators (stars) in faradaic impedance spectroscopy: They promote the charge transfer between electrode (golden bar) and solution as soluble mediator (**a**), as immobilized mediator (**b**), or as a combination of both (**c**)

on labeled as well as label-free techniques is given by Mascini et al. [4] and Evtugyn et al. [61].

In opposite to salts and ions present in buffers, the special structure of redox-active molecules enables a fast electron transfer at low AC voltage amplitudes, whereas the level of the DC voltage depends on the redox molecule. In presence of a redox mediator in solution, upon binding of the target to the immobilized aptamer, mostly an increase of impedance is observed due to the hindered charge transfer by the bound target. But in some publications, a decrease of impedance was observed, for example, for thrombin detection, in which 4 of 26 publications showed a decrease of impedance [17–20]. Another example is the detection of lysozyme, in which five out of seven publications report a decrease of impedance. Rodriguez et al. stated that the positive charge of the target increased the attraction of the negatively charged redox mediator and thus decreased the impedance [1]. As the isoelectric points of lysozyme and thrombin are ~11 and ~7, respectively, lysozyme is at physiological pH more positively charged than thrombin. This point supports the statement, but no confirming experiment was performed yet.

The most commonly used redox mediator is hexacyanoferrate (Fig. 5), which is a complex of an iron (Fe) bound in the center of six cyanides (CN) arranged in an octahedral geometry. Because iron exists usually in two oxidation states,

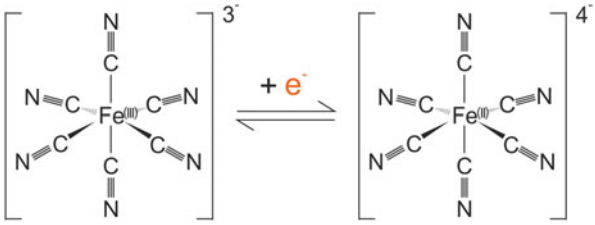


Fig. 5 The redox couple ferricyanide (left) and ferrocyanide (right) commonly used in faradaic impedance measurements

Fe^{2+} and Fe^{3+}, hexacyanoferrate has two ions, ferricyanide ($[Fe(CN)_6]^{3-}$) and ferrocyanide ($[Fe(CN)_6]^{4-}$), which are easily converted into each other. The ions are formed by the dissolution of the corresponding salts, e.g., potassium ferricyanide $K_3[Fe(CN)_6]$ and potassium ferrocyanide $K_4[Fe(CN)_6]$. This redox couple $[Fe(CN)_6]^{3-/4-}$ is widely used due to its fast electron transfer to electrodes (Pt, Au) described by the standard heterogeneous electron transfer rate constant k_0 (0.06 cm/s) and its high diffusion rate D of 6×10^6 cm^2/s [21].

As mentioned above, due to the existence of many review articles about detection strategies for electrochemical biosensors, we want to report only recent innovative strategies. One of these strategies is the so-called nanoladders that are used to enhance the signal without labeling. Peng et al. [22] developed an impedimetric aptasensor for the detection of nuclear factor kappa B (NF-κB) using this principle of enhancement. The electrode was modified with a capture DNA complement to the NF-κB aptamer. In the presence of NF-κB, the aptamer is released from the surface, and the impedance decreases due to the enhanced charge transfer via the redox mediator $[Fe(CN)_6]^{3-/4-}$ in solution. Then another oligonucleotide (O1) is added that partly binds to the free capture DNA on the surface. With two other oligonucleotides, a ladder is formed and extended through hybridization chain reaction (see Fig. 6). Due to the negative charge introduced by the oligonucleotides forming the nanoladder, the impedance increased significantly. By the addition of a peroxidase-like enzyme that intercalates into the DNA nanoladder and oxidizes

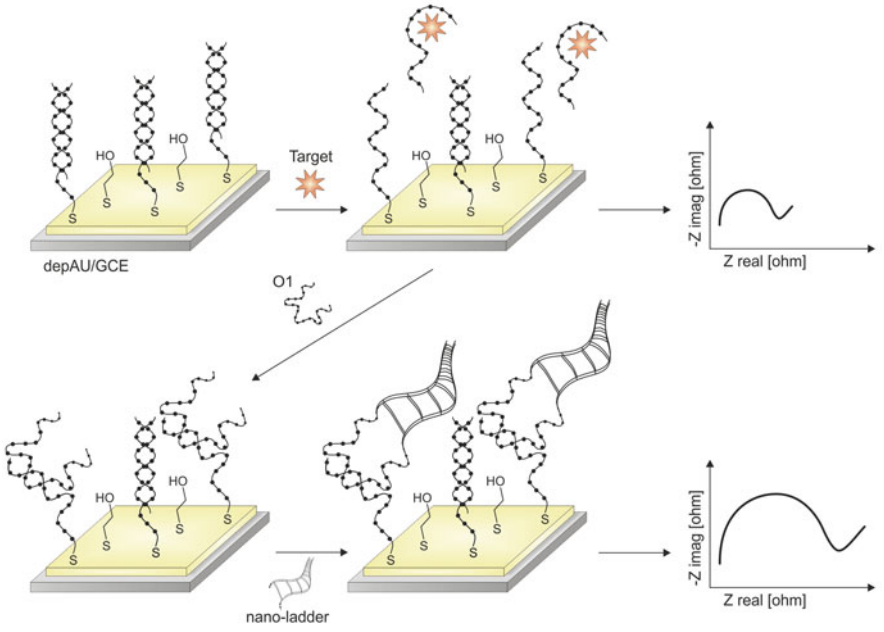


Fig. 6 Principle of signal enhancement with a DNA nanoladder. [Adapted from [22], with permission from Elsevier]

4-chloro-1-naphthol to an insoluble precipitate, the impedance is further increased. Hereby, Peng et al. reached a detection limit of 7 pM for NF-κB.

Compared to the standard polymerase chain reaction (PCR), the hybridization of the DNA fragments to a nanoladder produces multiplex nanowires which increase the negative charge on the surface significantly and thus the impedance. As oligonucleotides can be synthesized cost-effective in big amounts, this amplification method is a valuable alternative to other labels.

The artificial synthesis of oligonucleotides not only enabled the use of aptamers but also revolutionized the field of nanomedicine by engineering of three-dimensional DNA structures, also called DNA origami, for its use as synthetic vaccine, as vehicle in drug delivery or marker in cancer therapy [23]. Therefore, short oligonucleotides with specific sequences which overlap each other are used to form different structures like squares or triangles. Sheng et al. developed an impedimetric aptasensor for the detection of interferon-γ by using a triangular pyramidal oligonucleotide nanostructure [24]. The basement of the pyramidal structure is formed by four different oligonucleotides that are immobilized via thiol groups on the gold electrode surface, while the interferon-γ aptamer is the fifth oligonucleotide fragment which closes the pyramidal structure on top. The closed pyramid hinders the electron transfer to the redox mediator in solution. In the presence of the analyte, the aptamer is released from the surface and electron transfer is enabled, thus impedance decreased (see Fig. 7). By the simple addition of free aptamer, the sensor is regenerated. With this technique Sheng et al. reached an impressive wide linear range from 1 nM to 2 μM and a detection limit of 520 pM. Additionally, they were able to detect interferon-γ in diluted serum (50%).

The same principle was used by Sheng et al. for the detection of cocaine with a detection limit of 210 pM and a linear range from 1 nM to 2 μM [25], which shows that this technique can be applied for different aptamers and especially is suitable for the sensitive detection of small molecules. DNA origami offers great prospects for surface engineering in the future as there is no limit to the design of DNA nanostructures.

DNA origami can be also used to design target-induced hydrogel formation as described by Yang et al. [26]. Therefore a DNA strand was modified with acrydite to obtain a polymeric DNA structure, of which the arms are complementary to the aptamer for heparanase, a potential target in cancer treatment. In presence of the target, the aptamer is released, and the arms can hybridize with each other to form a hydrogel leading to a significant increase in impedance due to the hindrance of charge transfer between electrode and redox mediator in solution. With this method, they were able to detect heparanase down to 3 fg/mL and to detect the target in diluted serum samples with recovery rates of 90–110%. Another advantage of the use of DNA origami is that the biocompatibility of the developed biosensors is increased.

To reach lower detection limits, one strategy is to increase the effective surface, for example, by the deposition of gold nanoparticles. Gold nanoparticles exhibit special optical and physical properties, such as surface plasmons, enhanced Raman signals, fast electron transfer, magnetism, and catalytic activity [27]. If the

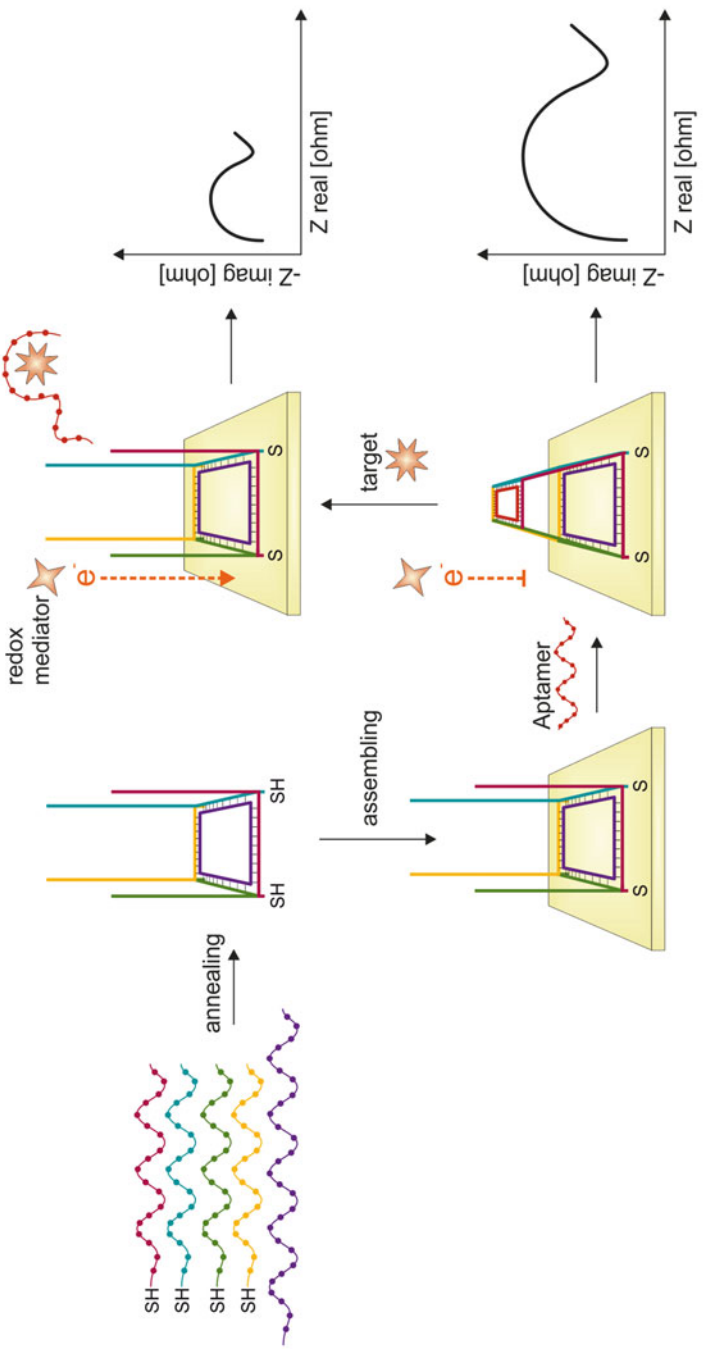


Fig. 7 Example for the use of DNA origami for aptasensor development and signal enhancement. [Adapted with permission of The Royal Society of Chemistry, from [24]; permission conveyed through Copyright Clearance Center, Inc.]

nanoparticles come in close proximity, a strong electric field is developed between them, which were used to enhance Raman signals. As for the surface plasmons, the strength of the electric field depends on the shape and size of the nanoparticles that are mostly synthesized by the condensation method, which is based on the reduction of metal salts. By the addition of different seeds and surface modifiers, a high variety of shapes can be achieved, such as rods, triangles, stars, cubes, and cages. It has been shown that nanostars give higher intensities in Raman signal than spheres, sphere aggregates, or triangles [28]. Talemi et al. [29] developed an impedimetric aptasensor for dopamine detection based on gold nanostars deposited on a pencil graphite electrode with the expectation that the charge transfer will be also enhanced, leading to a significant amplification of the impedance signal. Indeed, they were able to decrease impedance from 12 kΩ to 5 kΩ by the deposition of gold nanostars and achieved a low detection limit of 0.3 ng/L, which was one magnitude lower compared to the same sensor with gold nanoparticles (209 ng/L).

To reduce the non-specific binding but retain high surface-to-volume ratio and high electrical conductivity, the fabrication of more complex nanocomposites gained interests. Nanocomposites are made from two or more different materials with the aim to combine their properties to improve the characteristics of the transducer surface, that is, in impedimetric biosensors the electrode.

By using a nanocomposite made of reduced graphene oxide, iron particles, polyaniline, and gold nanoparticles, Hashemi et al. [30] were able to detect cocaine down to 29 pM and could also detect 25 nM cocaine in urine and serum samples.

Another example for nanocomposites is described by Aghajari et al. [31] who modified a glassy carbon electrode with a mixture of chitosan, carbon nanotubes, and palladium. Streptomycin aptamers were immobilized on the created nanocomposite via glutaraldehyde. Streptomycin is an antibiotic which is used in human therapy as well as in agriculture and as veterinary drug (e.g., in beekeeping). As it has serious side effects, the detection of trace amounts in patients or food products is required. Aghajari et al. were able to detect down to 18 pM of streptomycin by the use of nanocomposites.

In this example, the natural polymer chitosan improved the stability of the biorecognition element, but recently silk was discovered as the material for biosensors due to its mechanical and optical properties, elasticity, and biocompatibility. In the impedimetric aptasensor developed by Benvidi et al. [32], silk fibroin was combined with TiO_2 to increase conductivity and decrease solubility. This mixture was dropped on a glassy carbon electrode, and gold nanoparticles were electrodeposited to enable immobilization of thiol-modified aptamers. This aptasensor increased the sensitivity for the impedimetric detection of prostate-specific antigen (PSA) to a detection limit of 33 aM. Compared to the impedimetric aptasensor developed by [33], who used a nanocomposite of multiwalled carbon nanotubes and gold nanoparticles on reduced graphene oxide to sensitively detect PSA down to 33 fM, the nanocomposites with silk led to an increase of sensitivity of three magnitudes.

Readers, who are more interested in nanocomposites, are encouraged to read the review of Muñoz et al. [10] about the usage of nanocomposites for impedimetric biosensors.

Another interesting method for the detection of PSA was developed by Jolly et al. [34] who used the so-called hybrid molecular imprinting system. Molecularly imprinted polymers (MIPs) are synthetic receptors made by polymerization around a template. After removing the template, the created cavities recognize the target molecule. The weaknesses of MIPs are low specificity and reproducibility. By the utilization of immobilized aptamer-target complexes as template for MIPs, it is expected that these weaknesses are overcome due to the higher flexibility of aptamers compared to the usually used functional monomers. Indeed, Jolly et al. were able to increase sensitivity to 1 pg/mL (~29 fM) for PSA compared to 91 pg/mL (2.7 pM) by a MIP without aptamers [35].

In conclusion, only a small fraction of new developments in biosensing strategies was presented here. The recent advances in oligonucleotide synthesis procedures opened up a new field of surface modification. Working with oligonucleotides is, like playing with toy bricks, only limited by your imagination. Thus, using DNA origami for application-specific surface modifications is promising. Also nanocomposites are advantageous, as the combination of different materials can be used to form the characteristics of the surface according to the desired application. But it should be kept in mind that for a successful transfer of scientific developments into products, a thorough understanding of the whole mechanism is needed. Therefore, surface modifications are favored that are completely characterized and understood.

3.2 Non-Faradaic Impedimetric Aptasensors

Non-faradaic systems do not need any redox mediator and thus are favored in real-time measurements. They detect the accumulation of charge on the electrode surface. The measurement principle is based on the change of the thickness and/or dielectric properties of the material between the electrodes upon binding of the analyte, measured as a decrease in capacitance. To avoid any leaking current, proper insulation of the electrode surface is needed. And as the capacitance measured in non-faradaic systems is the sum of all capacitances, the passivation layer should be as thin and its dielectric constant as high as possible to reduce its impact on overall capacitance [36]. For details on the principles and strategies of non-faradaic biosensors, the reader is addressed to Daniels et al. [37] who published a substantial review on label-free non-faradaic biosensors including all aspects of this technology. Herein, we will review non-faradaic impedimetric aptamer-based biosensors since 2005.

In non-faradaic biosensors, mainly interdigitated electrodes (IDEs, Fig. 8) are used due to their advantages for capacitive sensing such as high contact area and collection efficiency. Interdigitated electrodes are often formed like two

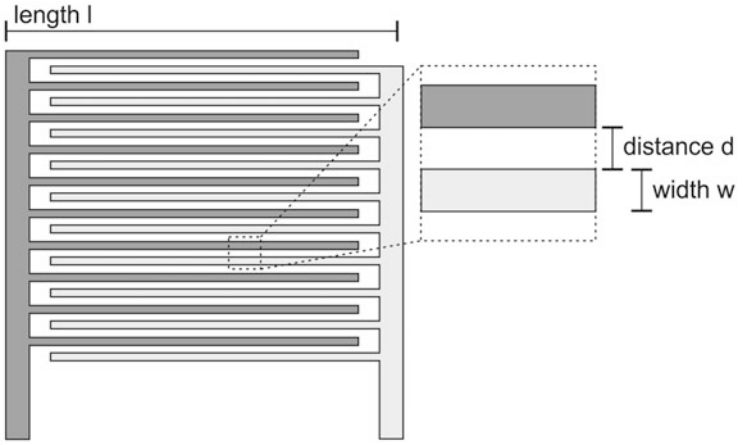


Fig. 8 Example for the structure of an interdigitated electrode (IDE)

combs with many fingers intercalated into but without touching each other. This way the surface-to-distance ratio and thus the capacity of the biosensor are increased. Grover et al. characterized different IDE designs in respect to their efficiency for capacitive sensing and found that the length l of the fingers has a high impact on sensitivity, while the number of fingers does not influence significantly. Furthermore, he found that the width w of the fingers should be small compared to the distance *d* between the fingers [38].

An example for the use of IDEs in impedimetric aptasensors is shown by Park et al. [39] for the detection of nicotinamide phosphoribosyltransferase (Nampt), an enzyme in the nicotinamide adenine dinucleotide (NAD) synthesis pathway. Nampt plasma levels are correlating to a number of diseases such as type 2 diabetes, hyperlipidemia, polycystic ovary syndrome, chronic kidney disease, obstructive pulmonary disease, and cardiovascular diseases. Additionally, it has been shown that overexpression of Nampt correlates with various cancers; thus, it is an important target for blood screenings. Park et al. described an impedimetric aptasensor for Nampt by immobilizing amine-functionalized aptamers on mercaptopropionic acid-modified gold IDE arrays. The activated electrodes were incubated with filtered human serum spiked with different concentrations of Nampt, washed, and dried, and then the capacitance measurement was performed on the dried electrodes at 700–1,000 MHz. They were able to detect down to 18 pM with a dynamic range of 18 pM to 1 nM, sufficient for the clinically relevant concentrations of Nampt, in healthy patients (0.3 nM) and patients with type 2 diabetes mellitus (0.5 nM). Similar molecules as vaspin and retinol binding protein 4 as well as bovine serum albumin (BSA) showed no significant signal.

Currently, there are various commercially available ELISA kits for Nampt detection with different detection ranges from 28 fM (EA100091 from OriGene Technologies, Inc. in Rockville, MD, USA) up to 18 nM (EIAM-VIS-1 from RayBiotech, Inc. in Norcross, GA, USA). The ELISA require 3–6 h and a sample

volume of 20–125 µL, show a reproducibility of 85–90%. As labels are needed, the prices for an ELISA kit are quite high. The impedimetric aptasensor developed by Park et al. cannot compete with the detection limit of the ELISA, but the time for detection is reduced to 1 h, the price is reduced significantly due to the absence of labels, and also the required sample volume is lower (5 µL). Further development is needed on the accuracy, sensitivity, and reproducibility, and several serum samples from different patients shall be measured to validate reproducibility. Due to the array structure, 45 IDEs on 1.4 cm^2, this biosensor could offer a worthy alternative to ELISA.

In 2015, the same group used the IDE array for the detection of human epidermal growth factor receptor 2 (HER2), a transmembrane tyrosine kinase receptor [40]. Its overexpression is a marker for breast and other cancers. This time, Qureshi et al. optimized the amount of immobilized aptamer (2 µM) and used a lower frequency range of 50–350 MHz, while they chose 242 MHz for the binding curve, reaching a detection limit of 0.2 ng/mL (2 pM) in diluted serum.

Arya et al. [41] also used IDEs to detect HER2 in undiluted serum samples. The capacitive measurement was performed with two electrodes in the frequency range 100 kHz–100 MHz with 200 mV amplitude. They found a minimum in the phase angle (about −82°) at 2 Hz, concluding that the capacitive fraction in the imaginary part of the impedance is greatest at this frequency. Compared to the previous presented system, Arya et al. detected significant higher capacitance changes (0.12 µF–6.5 pF) with a sensitivity of 35 pF per decade and reached a detection limit of 0.1 ng/mL (1 pM). Maybe this is contributed by the measurement in solution and at lower frequencies. They showed a good repeatability on different electrodes of 95%, and required sample volume is 50 µL. Although Arya et al. showed in their publication a lower detection limit than [40] and many other optical and electrochemical biosensors, already in 2013, Chun et al. [42] developed a simple faradaic impedance sensor for HER2 detection based on gold nanoparticles and reached a detection limit of 10 fg/mL (0.1 fM) in PBS. Besides that, again there are several commercially available ELISA kits with detection ranges from 8 pg/mL up to 40 ng/mL, but more prevalent are immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH).

As the last example, Liao et al. developed a non-faradaic impedance aptasensor for monitoring of platelet-derived growth factor BB (PDGF-BB) [43]. They modified a silicon electrode with silane and covalently attached the aptamer on the surface. Optimization was performed by application of an AC potential of 10 mV amplitude in the frequency range of 300 kHz–0.1 Hz at the stepwise increased DC potential of −0.4 V to 0.4 V. The DC potential is the baseline on which the sinusoidal voltage is laid. Online measurements were performed at the optimized conditions of −0.1 V and 5 kHz. A 2 µM PDGF-BB solution caused a decrease of total capacitance of about 35 nF. They were able to detect down to 40 nM PDGF-BB, low non-specific binding, and high specificity.

The same research group published 1 year later a faradaic impedance biosensor using the same aptamer on gold electrodes modified with polypyrrole [44]. In this way, they were able to detect PDGF-BB down to 0.4 nM, thus an increase of

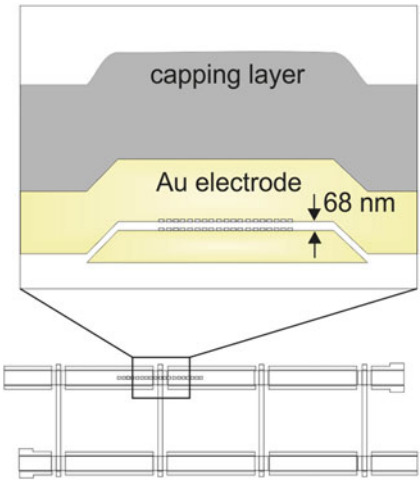


Fig. 9 Example for a nano-gap electrode—developed to increase the capacitive signal and to lower the polarization effect in non-faradaic impedance biosensors. [Adapted from [48], with permission of AIP Publishing]

two magnitudes in sensitivity. Zhang et al. developed a faradaic aptasensor for PDGF-BB and reached a LoD of 32 fM [45]. The lowest detection limit was reached with 150 aM by He et al. [46] also using a faradaic impedance biosensor.

Already in 1986, Bard et al. [47] showed for recycling IDEs using redox mediators that reducing the distance between the electrodes from 8 μm to 0.2 μm increased the collection efficiency from 60% to 90%. Therefore, high expectations were put on nano-gap devices (see Fig. 9) in reaching ultralow sensitivities. In 2005, Löhndorf et al. [48] firstly used a nano-gap electrode in combination with immobilized aptamers for the capacitive detection of thrombin. They reached a gap size of 68 nm, required a low sample volume of 3 μL, and as proof-of-principle showed a significant difference between elastase and thrombin injection. Measurements were performed at 1.2 GHz with amplitude of 20–30 mV. The same group used the developed nano-gap electrode for comparison of antibody and aptamer and showed that both ligands are highly suitable as recognition elements with similar kinetic parameters for the detection of thrombin [49]. In 2007, the group compared the performance of their nano-gap electrode with an IDE structure of 1.1 μm distance and found that for aptamer-based detection of Rev peptides, the signal was significantly increased from 0.3% to 1.3% for the nano-gap electrode [50]. The measurement was performed at 980 MHz at ~10–20 mV.

Normally in non-faradaic impedance measurements, high frequencies are used to reduce the noise due to electrode polarization and solution conductance. But biomolecular interactions are slow, and thus their detection is more sensitive at low frequencies. Decreasing the gap between the electrodes also decreases the influences of the bulk solution. Mannoor et al. compared the performance of nano-gap electrodes (20 nm) with macroelectrodes (100 μm) by impedance measurements in the low frequency range of 10 Hz to 100 kHz with amplitude

of 20 mV [51]. They showed that for measurements of distilled water at the macroelectrode, the relative permittivity approached 10^6 at 10 Hz due to polarization effects and only reached the value of 80 at high frequencies >1 kHz, whereas at the nano-gap electrode a relative permittivity of ~80 was reached in the frequency range of 10–1,000 Hz and at higher frequencies decreased down to a value of ~3. Thus, the influence of electrode polarization was diminished. Upon aptamer immobilization the relative permittivity was reduced, and upon addition of thrombin (0.2 μL), the value decreased another ~20%, while in controls with lysozyme or random oligonucleotide, no changes were observed.

In summary, the limited amount of research conducted on non-faradaic aptasensors is mainly based on big targets (molecular weight, >20 kDa) for clinical application, but also smaller targets (Rev peptides, 2.4 kDa) were detectable [50]. Until now, reached sensitivities are not as good as in faradaic impedance sensors, but the main drawback of low selectivity was resolved by the use of aptamers, and additionally it was shown that direct detection in diluted serum is possible [39–41, 45]. Although further development and research is needed, non-faradaic aptasensors offer a promising tool for point-of-care diagnostics, screenings, and online monitoring as a truly label-free technique.

4 Criteria for the Comparison of Impedimetric Aptamer-Based Biosensors

Due to the almost unlimited variety of impedimetric aptasensors based on the countless possibilities to choose electrode design, surface modification, immobilization method, detection strategy, amplification strategy, and furthermore assay design (i.e., microfluidic or batch, real time or endpoint), it is difficult to compare the performance of different biosensors. Thus, guidelines for their characterization are needed as Thévenot et al. [52] already recommended for electrochemical biosensors based on enzymes. Based on their publication, recommendations for the characterization of impedimetric aptasensors are given in this paragraph. Further suggestions are included and open for discussion.

A biosensor is defined by the integration of a biological recognition element with a transducer that converts the binding of the analyte into an electrical signal. By immobilizing the biological recognition element on the transducer, a close spatial coupling is achieved. The direct coupling enables integrated devices which distinguish biosensors from bioassays. Therefore, we recommend that a biosensor should be classified by the measurement method of the transducer and its biological recognition element in the form "receptor-based methodic biosensor" (e.g., aptamer-based impedimetric biosensor) with the addition of how the direct coupling was achieved. These key points should be stated in the abstract of a publication as well as the information if the detection strategy is direct or indirect.

As this chapter is reviewing impedimetric aptasensors, the following recommendations refer to this class of biosensors, although most criteria are common to all classes. Aptasensors belong to the affinity sensors, and the biological signal is generated by the interaction of the aptamer Apt and its target T with n binding sites for Apt:

$$n\mathrm{Apt} + T \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{Apt}_n T \tag{2}$$

After some time, the interaction will reach an equilibrium as the ratio of the dissociation rate constant k_d and the association rate constant k_a will be constant. In comparison to enzyme-based biosensors, aptasensors are not suitable for continuous monitoring, but the aptamer-analyte complex can be regenerated [53], and thus aptasensors are not necessarily single-use biosensors.

In impedimetric aptasensors the biological signal is a change in the electrical behavior of the measurement cell that is detected by electrodes, and the direct coupling is achieved by immobilization of the aptamer on the electrodes. As proven by many publications, this class of biosensors was successfully applied to biological matrices like urine, blood serum, and food extracts [54–58]. The success depends strongly on the inertness of the biosensor surface, referring to both directions. In other words, the effect of the biosensor surface on the sample and the influence of the sample on the biosensor surface should be neglectable. Aptamers are highly biocompatible and have no degrading effect on the sample, but the sample might contain enzymes leading to the degradation of the aptamer or the electrode modification. Thus, characterization of the biosensor response in different situations is very important for its optimization and transfer into industry.

The fast-growing field of biosensors still lacks standard procedures for the characterization of a biosensor. The IUPAC (International Union of Pure and Applied Chemistry) established some standard protocols, but these need revision and adaptation to the newly developed recognition elements and transducer. Guidelines for the evaluation of analytical methods can be found but are meant for the usage of a method not for its development and are application-specific, like the "Guidelines for performance criteria and validation procedures of analytical methods used in controls of food contact materials" (EUR 24105 EN) by the Joint Research Centre of the European Commission [59] or the "Guideline on bioanalytical method validation" by the European Medicines Agency [60] that is for pharmacokinetic and toxicokinetic parameter determination. Adapted from these guidelines and Thévenot et al., we recommend the following criteria for the characterization of a biosensor in development with the aim for its optimization and comparison:

- Immobilization density and capture capacity
- Response time
- Calibration curve including:
  - Apparent K_D
  - Linear and working range

  - Sensitivity
  - Detection and quantitative limits
- Selectivity/specificity
- Reliability
- Regeneration
- Repeatability/reproducibility
- Stability

In the following, every criterion will be explained in more detail.

For the development of a biosensor, the immobilization of the recognition element has to be validated. During this validation, it would be useful to determine the number of immobilized recognition elements and if possible the capture capacity which is the ratio of active to immobilized recognition elements. The number of immobilized recognition elements should be normalized to the unit of area, e.g., molecules per cm^2, whereas the capture capacity should be expressed in percentage. With these values, it is possible to evaluate the efficiency of the immobilization procedure and to determine which concentration range should be used for the calibration curve.

The time passed until the signal reached 90% of the maximal response R_{max} is called the steady-state response time. This parameter depends mainly on the diffusion of the analyte, the affinity of the aptamer, and if the sample is stirred. The calibration curve should be obtained from steady-state responses close to equilibrium, because these are unaffected by diffusion rates and analyte reassociation. The measurement time should be kept constant for every of the minimum of six freshly prepared analyte concentrations used, and each concentration should be repeated at least three times. The sample matrix should be adjusted to the final application of the developed biosensor. And most important is that the authors clearly specify the measurement procedure including the washing steps, the composition of the samples used, and how the reproducibility was determined. The calibration curve should be displayed as scatter plot of the signal versus logarithm of the analyte concentration including the standard deviation of the repeated measurements for every concentration. For better comparison of electrochemical biosensors, we suggest that the signal measured is normalized to the signal of a blank sample and to the electroactive electrode surface area.

At equilibrium, the dissociation constant K_D is the ratio of dissociation rate constant k_d and association rate constant k_a according to the law of mass action. K_D describes the ratio of unbound molecules to bound molecules as demonstrated for the affinity reaction in Eq. (2):

$$K_D = \frac{k_d}{k_a} = \frac{[\mathrm{Apt}]^n [T]^m}{[\mathrm{Apt}_n T_m]} \tag{3}$$

The smaller the K_D, the higher is the affinity of the aptamer to its target. The apparent K_D is often used to compare different biosensors, but this constant

is strongly dependent on the measurement method and measurement conditions like temperature, pH, ionic strength, flow rate, etc. Therefore, we recommend that it should only be used to compare different molecules measured with the same method under the same conditions or to compare the same molecule measured with different methods or different conditions. Besides, the conditions of the measurement should be considered carefully and stated clearly, because limitations to the binding of aptamer and target will lead to inaccurate affinity rate constants. For example, if the diffusion rate is slower than the association rate, then the limiting mass transfer will result in a decrease of the rate constants. K_D also depends strongly on the number of immobilized and/or active recognition elements; thus this should be stated.

From the obtained calibration curve, many parameters can be determined. K_D corresponds to the concentration that is equivalent to a response that is 50% of the maximum response $R_{\max}$. It can be determined by fitting the curve with a binding site model. As differences appear due to the application of different models, we recommend for all sigmoidal calibration curves to use the four-parameter logistic function as a standard model:

$$R = \frac{R_{\max} - R_{\mathrm{bl}}}{1 + \left(\frac{x}{K_D}\right)^S} + R_{\mathrm{bl}} \tag{4}$$

S is the slope in the middle of the linear range and represents the sensitivity of the biosensor response. In general, a high sensitivity and a wide linear range are desired, but as seen from Eq. (4), a wider linear range results in a lower sensitivity; thus compromises are required during optimization.

By measuring the response of a blank sample R_{bl} at least six times in repetition according to the measurement procedure, the mean $\overline{x}_{\mathrm{bl}}$ and standard deviation σ_{bl} of the measurement method are determined. According to IUPAC, the limit of detection (LoD) is defined as the concentration corresponding to a signal of $\overline{x}_{\mathrm{bl}} + 3\sigma_{\mathrm{bl}}$ which refers to a signal-to-noise ratio of 3, and the limit of quantification (LoQ) is determined from $\overline{x}_{\mathrm{bl}} + 5\sigma_{\mathrm{bl}}$. The working concentration range is determined by the lower and upper limits of quantification.

For aptasensors, the selectivity or specificity should be already examined during or after its selection. Thus, it is more important to test for unspecific binding of the biosensor surface, which is performed using random oligonucleotides of the same length as the aptamer. Besides, to test for interferences, a sample with an analyte concentration close to K_D should be spiked with the interfering substance, and the percentage change of the signal compared to a sample with analyte alone should be reported. To test the reliability of the biosensor response, the concentration of the interfering substance should be changed while keeping the analyte concentration constant. The fluctuation of the signal describes the reliability of the biosensor and can be expressed in percentage.

If the biosensor is regenerable, it is important to state the recovery time needed to return to the baseline and the regeneration repeatability defining the number of cycles performed until the signal response changed about 10%.

Reproducibility is another important parameter for evaluating the quality of a biosensor, but the expression is often misused as testing the reproducibility would require to measure the same concentrations on a different instrument and by a different operator under reproducible conditions which is easiest performed in another laboratory. What is most often measured is the repeatability, which is defined as the variation of the signal measured of an analyte concentration in the linear range on different time points.

In most publications about biosensor development, the calibration curve is obtained from analyte dilution series in buffer solutions, although it would be preferable to use spiked artificial or natural sample matrices. However, the performance of the biosensor on a real sample has to be evaluated. Therefore, measurements of a natural sample should be performed with the biosensor and a reference method. If reference methods are not available, spiked natural sample matrices may be used. The recovery is expressed in percentage.

As the last criteria, stability should be mentioned which refers to the influence of every external change on the performance of the biosensor. Mostly, the storage stability (also called lifetime) is the most important external influence. To examine a biosensor's lifetime, a batch of biosensors should be produced and used after different storage times, while the change of the response signal under identical conditions is stated in percentage.

In conclusion, a thorough characterization of a biosensor is extensive, and a publication including all criteria is seldom. What has already become established among publications is the indication of linear range and detection limit of a biosensor, presumably caused by the pursuit for single-molecule detection. But most of the published detection limits fulfill the requirements for analytical diagnostics, but lack real sample validation. Thus, especially for impedimetric aptasensor, it would be desirable to present the calibration curve from measurements in spiked natural or artificial sample matrices and perform validation with natural samples. Besides, normalization of the response to biosensor-specific characteristics like electrode area, number of recognition elements, and blank signals would be helpful for proper comparison of different impedimetric aptasensors.

5 Summary and Outlook

Most publications on impedimetric aptasensors are based on the sequential measurement of frequencies in a small range with measurement times of 1–3 min. As most biological systems are changing over time, more applications using multisine and potential step techniques are needed to avoid errors of non-stationarity and enable kinetic measurements.

To increase sensitivity and reduce non-specific binding, a special design of the electrode surface is needed. DNA origami and nanocomposites are promising tools for application-specific surface designs. However, the aim should be to keep it as simple as possible to enable thorough characterization of the surface and

mathematical modeling of the sensor response. There is a lack of models for impedimetric aptasensors, which is probably one reason for the low transfer to commercial products.

In general, impedimetric biosensors are favored due to the possibility of label-free measurements. But only non-faradaic measurement techniques are truly label-free, and recent applications of non-faradaic impedimetric aptasensors show significant lower sensitivities than faradaic impedimetric aptasensors. Thus, more research in non-faradaic impedance sensors is needed.

Despite the doubt that aptamers are not suitable for the application in real samples, their excellent performance in human serum and blood samples down to the pM range has been shown by several researchers. However, the majority of publications lack validation with real samples. As the work with real samples might often not be possible, we recommend that calibration curves are taken in spiked artificial matrices instead of buffer solution. Furthermore, we recommend that instead of absolute signal values, relative values are plotted and normalized to the electrode area or the number of aptamers to enable comparison of sensor performance.

The full potential of impedimetric aptasensors has not yet been exploited. However, the success of impedimetric aptasensors depends equally on the advances in aptamer selection and synthesis, in impedimetric measurement techniques, and in surface modifications and assay design. Besides other transducer types, the potential of electrochemical biosensors is outstanding for the application in miniaturized point-of-care devices.

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