Electrochemical Impedance Spectroscopy Investigations Focused on Food Allergens

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Abstract: In this article, we survey recent literature related to the development of impedimetric sensors and methods for allergen proteins in food. Electrochemical impedance spectroscopy is an electrochemical method that allows label-free monitoring of analyte-ligand interactions and was largely used for characterising various steps in the development of (bio)sensors. Both faradaic impedance spectroscopy – using a redox probe added to the analytical system to be investigated – and non-faradaic impedance spectroscopy were used for the detection of food allergens. Several approaches leading to high sensitivity and application to the analysis of real samples are particularly emphasized. The challenges in using EIS for the determination of allergens are discussed.

Keywords: Electrochemical impedance spectroscopy; Allergen proteins; Food; Biosensors.

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Introduction

Recent years marked a heightened alertness of general population and regulators towards food allergens, defined as substances that cause adverse reactions in the human body, involving

IgE-type antibodies. Allergen-related issues account for approximately 50 % of all recalls in the USA and more than 60 % in the UK. Most food allergens are proteins and the large majority of reported severe reactions in sensitive individuals [1] are due to foods from the "Big Eight" group (tree nuts, nuts, fish, shellfish, wheat, soy, eggs, milk).

On the other hand, new technologies and the emergence of functional foods set new challenges for allergen safety. For example, proteins such as lysozyme and albumin from eggs or casein from milk are sometimes used as fining agents in the wine industry. Functional flavoured water marketed in New Zealand in 2009 was removed from the market the next year after two children with allergies to cow's milk, suffered anaphylaxis. The water contained 3 times more β-lactoglobulin than cow's milk and the protein was rendered clear through a new process, thus misleading the consumers, although the bottle was properly labelled [2]. The economic impact of allergens is huge. According to a study published in 2010, the market of gluten-free food is booming, from an estimated $580m in 2004, being expected to reach $2.6bn in 2012 [3].

Currently there are no threshold levels for allergens in foods and this contributed to unnecessary recalls and over-use of "might contain" labels. This is expected to change in the future, when legal thresholds for peanuts, milk and eggs are expected to be announced as result of some European programmes like (*e.g.* Europrevall, a European FP6 project set to investigating the prevalence of food allergy in Europe, www.europrevall.org) and public initiatives (*e.g.* MoniQA, www.moniqa.eu). Europe has the strictest labelling rules (14 allergens to be labelled: buckwheat, molluscs, lupin, mustard, celery, cereals, soya, sesame, crustaceans, fish, milk, egg, tree nuts and peanuts), at the opposite pole from Japan (only 5 allergens required specific labelling in 2010) [4].

Current testing procedures for allergens are based on ELISA, DNA (using PCR), immunosensors and mass spectrometry techniques. Immunoanalytical methods (ELISA, RIE) are preferred in food industry laboratories and food control organizations to detect allergens in food due to their high sensitivity and specificity, existence of international standard methods and availability of commercial kits (*e.g.* peanut in chocolate, cookies, ice cream and cereal, hazelnut in cereal [5,6]. In the same time, methods involving a separation step (such as LC-MS) are mainly used to identify the allergen, although a method was recently described allowing the simultaneous screening by HPLC-MS of a panel of 7 allergens [7]. PCR-based methods are highly specific but take more time, require trained personnel, more laborious sample preparation, and are prone to significant matrix effects. Biosensors are increasingly used for protein detection [8,9]. Applications in food control include detection of gluten in

bakery products [10], detection of tropomysin from shrimp [11], lysozyme from egg-white, [12], casein in milk [13], detection of Ara h1 peanut protein in chocolate candy [14], detection of adulterant plant proteins, and other relevant proteins such as IgG, folate-binding protein, lactoferrin, insulin-like groth factor, beta-casein in milk but also monitoring the denaturation of bovine IgG by heat and high pressure [15] or the kinetics of milk casein interactions or interactions of lactoferrin with salivary agglutinin [16-20]. Sensitive detection of allergen proteins is complicated in processed foods by the impact of formulation and processing or storage conditions on the properties of proteins in respective matrices [21-22]. A recent review [9] summarized the use of biosensors for the detection of allergen proteins based on optical, electrochemical and mass-sensitive transducers [9].

Electrochemical biosensors for allergens rely on various detection modes such as voltammetry [23,24], amperometry [25], potentiometry [26], and electrochemical impedance spectroscopy [11,27]. Compared to other electrochemical methods, EIS was widely used to study bulk and interfacial electrical properties [28] and presents several unique advantages, *e.g.* is nondestructive and label-free, allowing to monitor biorecognition events with high sensitivity. EIS provides a very efficient, label-free electroanalytical approach for characterizing the fabrication process of a biosensor, for detecting biorecognition events between an analyte and an immobilized ligand [28], but also for quantitative detection of analytes of different sizes, from small molecules up to cells [29-32].

Electrochemical impedance spectroscopy relies on measuring the current intensity when an AC potential signal of small amplitude (typically 5–10 mV) is applied in an electrochemical cell. The electrical resistance (*i.e.* the ratio between the voltage and the electrical current) in the AC circuit defines the impedance (Z). Both non-faradaic and faradaic impedance spectroscopy measurements can be used with biosensors. In faradaic impedance spectroscopy, a redox couple – typically ferricyanide/ferrocyanide – is used to probe the electrical properties at the interface sensor-solution, whereas in the non-faradaic mode the transient electrical current is measured due to charging of the double electrical layer at the electrode-electrolyte interface [28]. Impedance spectra acquired at various frequencies contain information regarding the phenomena at the electrode-solution interface (Fig. 1a). To extract the analytical information pertaining to electrical properties at the interface, the analytical system is compared with an electrical equivalent circuit. A simple equivalent circuit for a faradaic process – the Randles equivalent circuit (Fig. 1b) – accounts for the resistance of the bulk solution (Rs), the diffusion of charged species from the bulk electrolyte to the electrode surface (Warburg impedance element), the double electrical layer at the interface electrode-

solution – which is compared to a capacitor (C for an ideal capacitor or constant phase element CPE for a non-ideal capacitor), and the resistance to electron transfer at the electrode-solution interface (*i.e.* the charge-transfer resistance, Rct). From all these parameters extracted by fitting the impedance spectra with appropriate software, the charge-transfer resistance and the capacitance are used to describe the electrical properties at the interface, with R_{CT} being in many cases the most sensitive to interfacial changes.
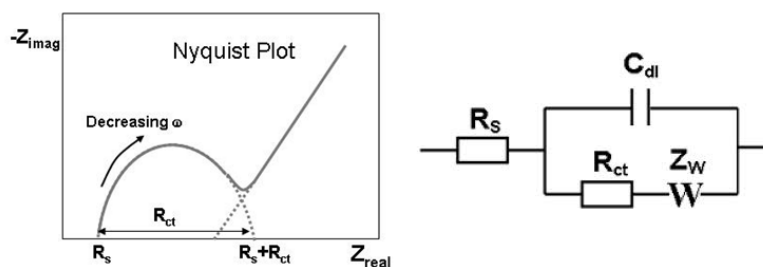

Fig. 1: *Typical electrochemical impedance spectrum for a faradaic process in a simple system in Nyquist representation (left) and equivalent Randles electrical circuit used for fitting the impedance spectrum (right).*

Excellent reviews have been published on the use of EIS in biosensors [28,33,34]. Changes in the electrical properties at the interface, determined by EIS can be associated with specific binding events due to the recognition between an analyte and a ligand. Antibodies and more recently, aptamers have been used as biorecognition elements in biosensors with EIS detection. Aptamers are small DNA sequences with high specificity and sensitivity for the target molecule and are increasingly replacing antibodies in test kits and sensors [35-38]. Aptamers are selected by in vitro methods, through chemical synthesis (Systematic Evolution of Ligands by Exponential Enrichment – SELEX), they are very stable compared to antibodies and can be labelled in many ways, without affecting their affinity and stability [39]. Aptamer-based electrochemical biosensors have been recently reviewed [40-42].

Various strategies have been used for the development of biosensors for the detection of allergens based on EIS. Two main approaches exist, where either the allergen or its specific ligand (antibody or aptamer) was immobilised onto the electrode surface. Many times, the measuring protocol includes an amplification step to increase the sensitivity of the assay, for example by using a secondary, labelled antibody or by using nanomaterials.

Most electrochemical imunosensors for allergens mimic the format of current standard ELISA procedures, which make use of enzymatic labels for obtaining sensitive readouts [25].

The enzyme used as a label (alkaline phosphatase, horseradish peroxidase) catalyses the transformation of a substrate (typically *p*-aminophenyl phosphate, or tetramethylbenzidine, TMB) that can be followed electrochemically. One advantage of such strategy is that the same enzyme labels and substrates are used in current ELISA methods, making it easier to compare the newly developed biosensors with standard testing procedures. In many biosensors for allergens, EIS measurements have been used solely for characterizing the sensor fabrication steps, while quantitative detection was ultimately done by voltammetry (especially by DPV) [43,44].

A few representative examples of EIS biosensors for allergens are presented below focussing on proteins representing four allergen groups: tropomysin (from shrimp), Ara h1 from peanuts, gliadin (gluten from wheat, barley and rye) and lysozyme (from egg white).

Tropomyosin

Pen a1 (tropomyosin) is a 36 kDa muscle protein and the major allergen in shrimp [11]. It is traditionally determined by the conventional skin test, RAST or more recently by antibody-based assays (*e.g.* ELISA, [45]). A biosensor based on an original approach has been recently described (Scheme 1), where detection by EIS is coupled to sensitivity and specificity using cells as sensing element. Rat Basophil Leukemia (RBL) mast cells were encapsulated in collagen and immobilised on sensor surface.
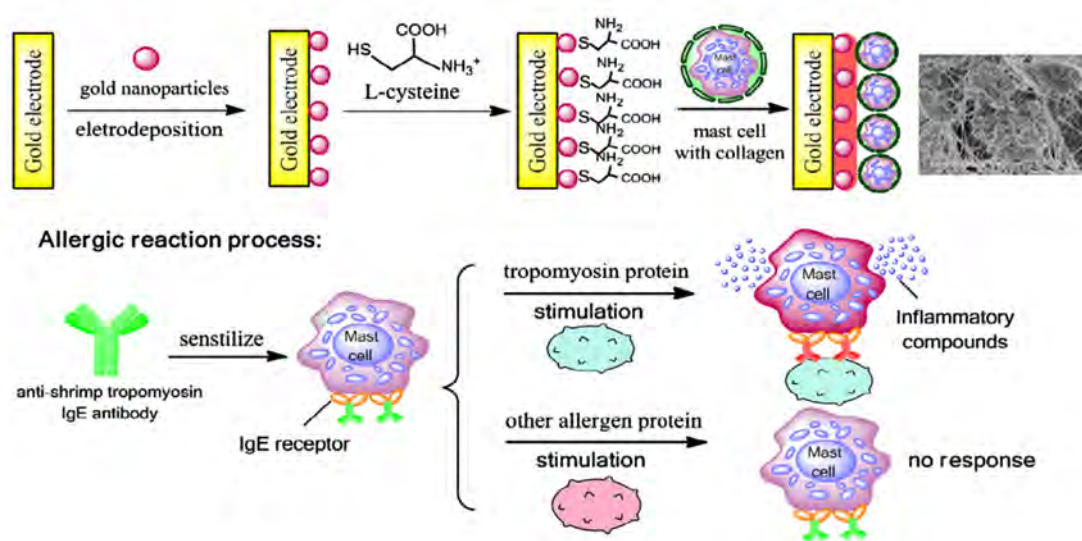
Scheme 1: *Schematic illustration of the preparation of modified electrode (above) and principle of allergen reaction process (below). Inset: SEM image of cell–collagen 3D structure.* Reprinted with permission from [11]. Copyright (2013) Elsevier.

Prior to immobilisation, cells were sensitized with anti-shrimp tropomyosin IgE, taking advantage of the abundance of high affinity IgE receptor (FcεRI) on the surface of these cells. Exposure to the allergen launched a sequence of intracellular events that could be followed using EIS. R_{CT} increased linearly with tropomyosin concentration in the range 0.5-4 µg mL^{-1} the decreased gradually from 4 to 5 µg mL^{-1}.
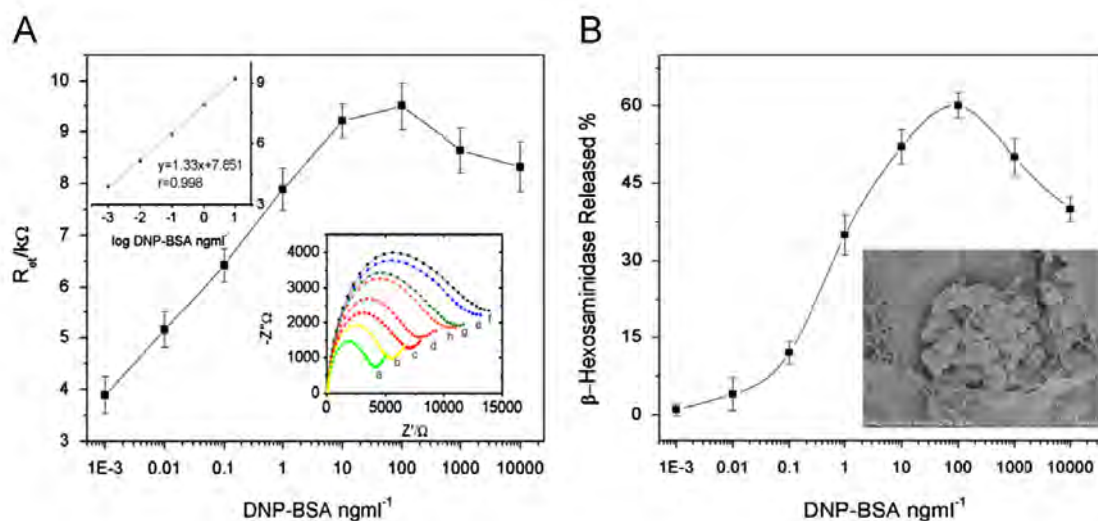

Fig. 2: *Effects of IgE-mediated hypersensitivity on RBL-2H3 cells: (A) electrochemical impedance spectroscopy for RBL-2H3 cells treated with different doses of DNP-BSA, (a→h) arrow indicates:* control = 0 ng mL^{-1} (R_{et} = 3.5 kΩ was not shown), 1×10^{-3} ng mL^{-1}, 1×10^{-2} ng mL^{-1}, 1×10^{-1} ng mL^{-1}, 1 ng mL^{-1}, 10 ng mL^{-1}, 100 ng mL^{-1}, 1×10^{3} ng mL^{-1}, 1×10^{4} ng mL^{-1}. (B) β-Hexosaminidase assay for confirmation of the cell activated by DNP-BSA. Data represent mean ± SE of three different experiments under similar condition. Reprinted with permission from [11]. Copyright (2013) Elsevier.

The authors suggested that this behaviour is related to apoptosis and cell detachment induced by high doses of tropomyosin and supported their explanation on the similarity of profiles observed by EIS and with β-hexosaminidase assay. Release of β-hexosaminidase is known to be one of the intracellular events triggered by IgE, together with cellular granulation, release of histamine and serononin [46]. The biosensor responded rapidly, within minutes of allergen addition and the detection limit was 0.15 µg mL^{-1}.

Peanut Protein Ara h1

Peanuts contain 13 allergen proteins discovered so far: from Ara h1 to Ara h11, agglutinin and oleosin [47]. Of these, Ara h1 and Ara h2 are the most important allergenic proteins [48,49]. Small amounts of protein (as low as 0.1 mg) can cause an allergic response in susceptiple individuals, thus presenting a significant risk to public health [50]. A 3 year study is currently ongoing in the UK, aiming to determine the amount of peanuts required for an allergic reaction in people affected by peanut allergies, in order to improve food labelling. The results of this study are expected in 2016 (www.tracestudy.com).

Ara h1 represents 12-16 % of total peanut proteins [47] and was therefore the focus of peanut allergen detection with biosensors. In one approach, the allergen was detected by means of electrochemical impedance measurements with a biosensor made by immobilizing a monoclonal antibody ondegenerate (heavily doped) Si wafer coated with a SAM of 10-undecenoic acid [27]. It was found that, as the concentration of Ara h1 increased, the charge-transfer resistance (R_{CT}) increased and the differential capacity C_d decreased. The lowest concentration of protein detected was 0.08 nM [27].

Another study presents the detection of Ara h1 peanut protein based on the immobilization of the Ara h1 antibody within the Au-coated pores of commercial nanoporous polycarbonate membranes. Peanut protein Ara h1 was detected through changes in the pores' conductivity as the pores are partially obscured by antigen binding [51]. This biosensor showed significant advantages (speed and simplicity) in the detection of peanut proteins over existing techniques (ELISA or lateral flow immunoassays).
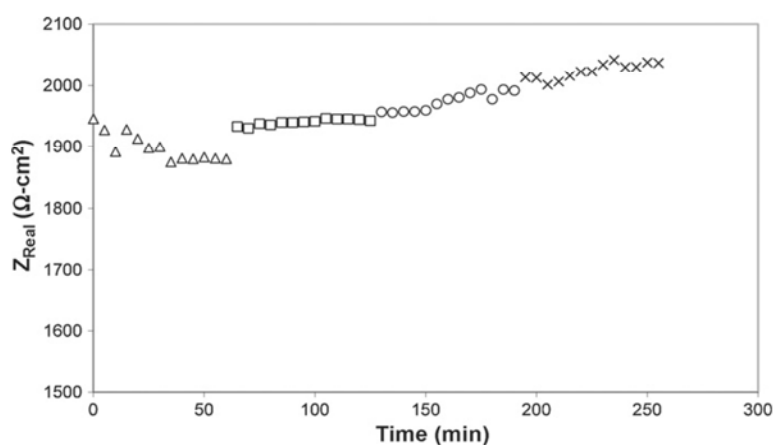

Fig. 3: *Z_{Re} at 1 kHz for membranes with 50 nm pore diameter.* 0 μg mL^{-1} (Δ), 0.04 μg mL^{-1} (□), 0.08 μg mL^{-1} (○) and 0.12 μg mL^{-1} (X) peanut protein Ara h1 in 50 mM PBS buffer at pH 7.3. Reprinted with permission from [51]. Copyright (2013) Elsevier.

An original approach for the detection of Ara h1 relies on a DNA sensor with impedimetric detection, on a principle similar to that of „molecular beacons". A stem-loop probe (a DNA sequence) was immobilised on the surface of a gold electrode. Upon hybridisation with the target DNA residues from Ara 1, the conformation of the probe changed from „closed" to „open". These conformational changes were easily tracked by EIS, the detection limit of target DNA being 0.35 fM [47].

Gliadin (Gluten)

Gluten is a protein composite consisting of gliadin (approximately 36 kDa, alcohol-soluble) and glutenin (higher molecular weight, only soluble in dilute acids or alkalis). Ingestion of gluten and similar proline-rich proteins from wheat, rye, barley, oats, spelt, kamut or their hybrids causes an exacerbated response of the immune system in people affected by celiac disease. This ultimately results in chronic inflammation and damage to the upper part of the small intestine. A gluten-free diet is mandatory for people suffering from celiac disease, therefore the systematic control and accurate labelling of food products is critical. As per January 1, 2012, a maximum content of 20 ppm gluten is allowed in products labelled as „gluten-free" – products considered safe and formulated specifically for people with gluten intolerance, part of so-called 'Foodstuffs for Particular Nutritional Uses' (PARNUTS), described in EU Regulation (EC) No. 41/2009.

In recent years, electrochemical biosensors have emerged as potential alternatives to current ELISA methods, either for the diagnosis of celiac disease or for the detection of gliadin in foods. Impedimetric [44], amperometric, [52,53] and voltammetric techniques [54-56], have all been explored. While in most cases the final goal was to detect the antibodies considered as markers of celiac disease rather than gliadin, one could use the same sensor architectures to detect the allergen in foods. For example, competitive assays could be envisaged where gliadin is immobilised on the sensor and the antibody is added in solution.

Sensor architectures range from very complex ones [44] to others that were simpler, but carefully controlled as to achieve best analytical performances. In some illustrative examples from one research group, the authors achieved the controlled covalent immobilisation of the bioreceptor [54,57], while insuring in the same time low non-specific adsorption and allowing a good diffusion of the electroactive substrates to the electrode surface [53]. This was done my modifying a gold electrode with a self-assembled monolayer (SAM) of bipodal thiol containing ethylene glycol groups and carboxylic end groups. Taking

advantage of the carboxylic end groups, gliadin was covalently immobilised by amine coupling. The ethylene glycol groups present in the thiol imparted resistance to non-specific adsorption of proteins to the formed SAM. This robust sensor architecture was used in several sensors developed by the same group for the detection of gliadin in foods [10] or for detecting marker antibodies from human serum, for diagnostic of celiac disease [57].

For example gliadin was immobilised on the sensor in order to detect anti-gliadin antibodies (Fig. 4). The interaction antigen-antibody was sensitively detected by using a secondary antibody labeled with horseradish peroxidase (HRP) and by adding 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate. After the oxidation of TMB (in the presence of hydrogen peroxide) catalysed by HRP, the oxidized TMB was reduced back on the electrode, generating a cathodic current that can be correlated with anti-gliadin concentration in the sample [57].
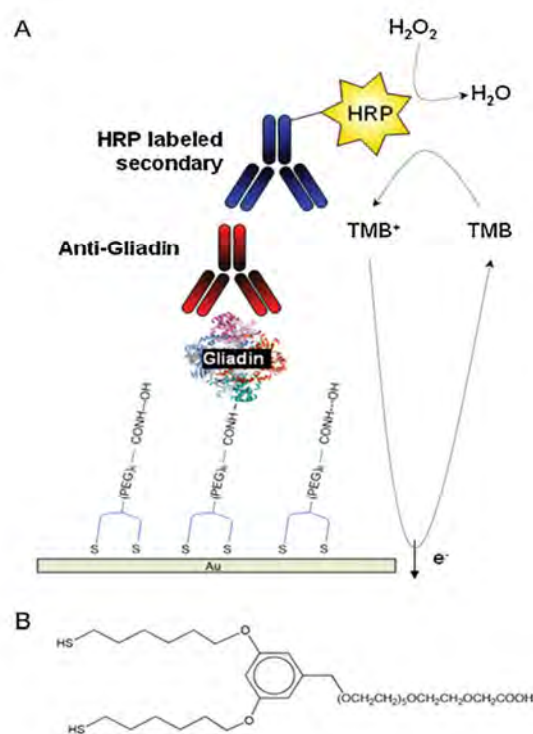

Fig. 4: *Schematic of the electrochemical immunosensor assay architecture (A). Structure of 22-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoicacid dithiol PEG-6 carboxylate (DT2) (B). Reprinted with permission from [54]. Copyright (2012) Elsevier.*

In another immunosensor described by the same group but aimed at detecting gliadin in foods [10], the authors compared two surface chemistries based on bipodal thiols and immobilised a gliadin antibody on the carboxyl-ended SAMs by amine coupling (Scheme 2).

EIS was used to follow all the steps in the construction of the biosensor: from ascertaining the time necessary for the formation of stable SAMs, to evaluation of the stability of antibody-modified SAMs kept in PBS buffer at room temperature and to the evaluation of non-specific interactions.
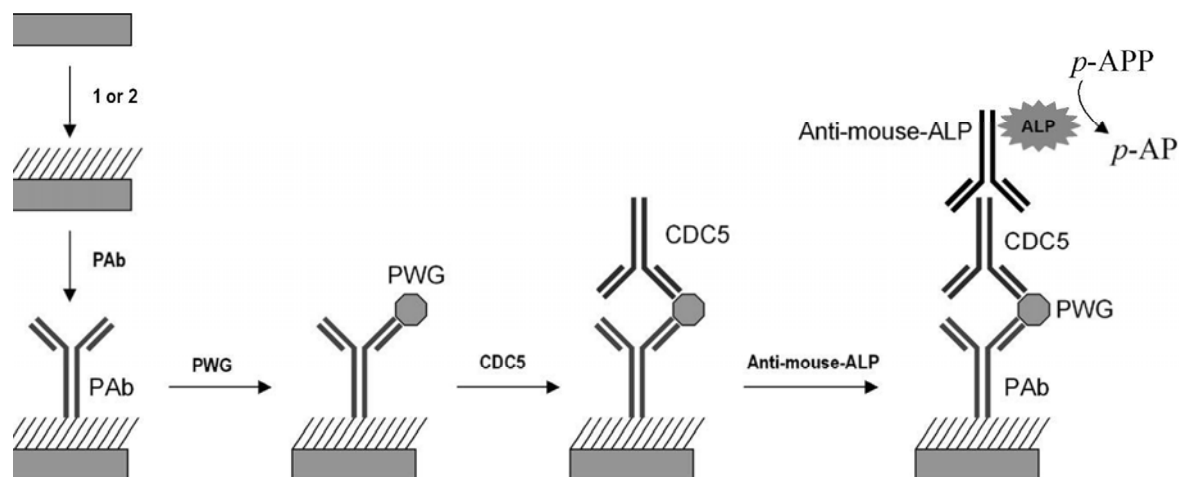
Scheme 2: *Stepwise construction of the gliadin immunosensor.* Anti-mouse-ALP: goat antimouse IgG (Fc specific) alkaline phosphatase conjugate; *p*-APP: *p*-aminophenyl phosphate; *p*-AP: *p*-aminophenol. Reprinted with permission from [10]. Copyright (2008) American Chemical Society.

The charge-transfer resistance increased with the binding of each consecutive layer in the sandwich structure (Fig. 5) from immobilisation of the polyclonal antibody on the SAM-coated gold electrode, blocking the unreacted carboxylic groups and the non-specific adsorption with ethanolamine and PBS-Tween, forming the immunocomplex by binding gliadin from the sample, binding then a monoclonal antigliadin antibody and finally up to binding a third, enzyme-labeled anti IgG antibody.

However, there was no linear relationship between R_{CT} and gliadin concentrations in the 10-60 ng mL^{-1} range, while linear calibration curves were obtained in the same conditions if the detection was done by differential pulse voltammetry (DPV). The authors linked the inability to quantitatively detect gliadin by this approach using EIS to the high sensitivity of this electrochemical method. EIS captures small changes at the interface sensor-solution which are due not only to analyte binding but also to non-specific interactions or to changes in the layer of physically adsorbed bovine serum albumin (BSA) used to block NSA.

In contrast, the conditions chosen for DPV allow only the oxidation of the substrate of the enzymatic reaction without other possible contributions. This biosensor was in fact applied for the detection of gliadin in real samples and the results were compared to those obtained by ELISA [10].
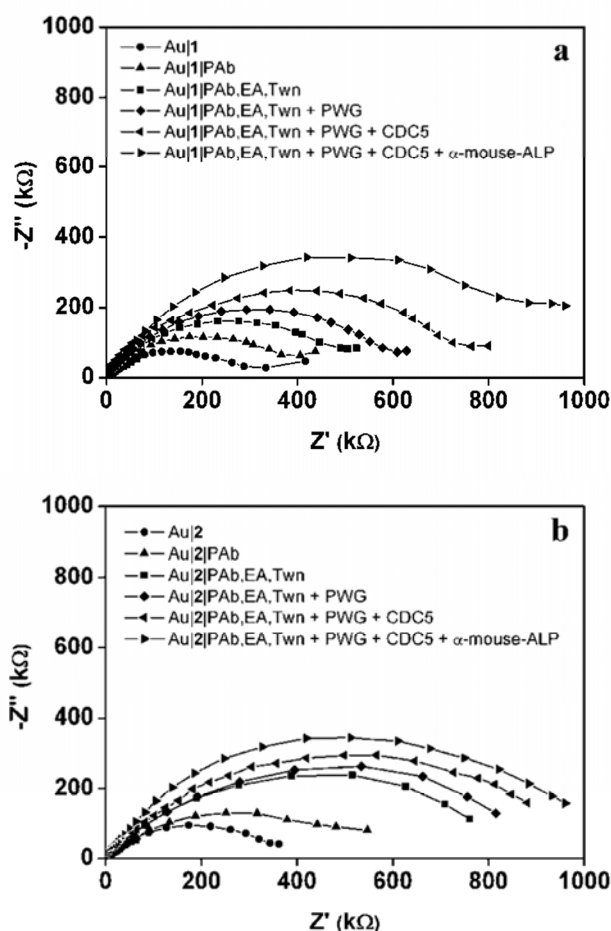

Fig. 5: *Complex impedance plots (in 1 mM $K_3Fe(CN)_6$ solution in PBS pH 7.4) recorded at a SAM of 1 (a) and 2 (b) for the sequential immobilization of biocomponents in the sandwich assay.* 1, 2: SAMs formed by thiol 1 and thiol 2, respectively; PAb: rabbit polyclonal antigliadin antibody; EA: ethanolamine; Twn: PBS-Tween; PWG: Prolamin Working Group gliadin standard; CDC5: monoclonal antigliadin CDC5 antibody; mouse-ALP: goat antimouse IgG alkaline phosphatase conjugate. Reprinted with permission from [10]. Copyright (2008) American Chemical Society.

An impedimetric sensor was developed for the detection of anti-transglutaminase antibodies (of IgG and IgA type) in human serum using screen printed gold electrodes modified with a poly(sodium-4-styrenesulfonic acid) layer. Gliadin was covalently immobilised on the sensor and, to increase the sensitivity of the detection, an extra incubation

step was added with a secondary antibody labelled with peroxidase. The substrate used in the peroxidase catalysed reaction, 3-amino-9-ethylcarbazole, formed a precipitate as result of the enzymatic reaction. The electrochemical impedance spectrum was acquired and by fitting the impedance data spectrum at two frequencies, the R_{CT} was found to increase proportionally with antigliadin antibody concentration in the range from 10^{-8} M to 10^{-6} M [58].

Multiplexing and inclusion of nanomaterials such as carbon nanotubes and gold nanoparticles, together with enzyme-labelled secondary antibodies for amplifying the analytical signal are current trends in the biosensors field that were also explored for sensors for the diagnostic of celiac disease [44,59].

Lysozyme

Lysozyme from egg-white is a 14 kDa allergen protein that is in the same time an enzyme, catalysing the hydrolysis of glycosidic bonds in the peptidoglycan, in the walls of Gram-positive bacteria. Based on this property, the use of lysozyme was allowed in the fabrication processes of wine and cheese. In wines, it is used as a fining agent and to control the malolactic fermentation, and the maximum admissible amount being 500 mg/L. As per Regulation (EU) No. 579/2012, wines for which lysozyme was used during the fabrication process must have appropriate allergen labelling.

In the case of some types of cheese such as Gran Padano, lysozyme could be used at levels around 50 mg kg^{-1} to control the growth of *Clostridium tyrobutiricum* during manufacturing [60]. The International Organisation of Vine and Wine has established two standard analysis methods for lysozyme, based on high performance liquid chromatography (HPLC) and capillary electrophoresis respectively. Nowadays, ELISA is the currently used method-together with HPLC- for assessing the content of lysozyme in food matrices. As faster and more affordable alternatives to these methods, biosensors for lysozyme have been developed based on optical and mostly, electrochemical methods.

A number of impedimetric biosensors for lysozyme have been described based on aptamers as biorecognition elements, which allowed to explore a couple of detection strategies (*e.g.* charge switching, target-induced aptamer displacement etc.). The current trend in the field of biosensing consisting in amplification strategies *for improving sensing by using nanomaterials* manifested also with regards to lysozyme detection. Some of the most illustrative examples are presented hereinafter.

One strategy with aptamer biosensors refers to *charge switching*. In a representative study, an indium tin oxide (ITO) electrode was used as transducer and modified with streptavidin, then a biotinilated lysozyme aptamer was confined to the surface by taking advantage of the high affinity streptavidin-biotin interaction [62]. When introduced in a ferricyanide/ferrocyanide solution, the electron transfer from the mediator to the ITO electrodes was hindered due to the repulsion between the negatively charged DNA phosphate backbone and the ions from the $[Fe(CN)_6]^{3-/4-}$, which was observed experimentally as a high R_{CT} in EIS determinations. After the incubation of the aptamer-modified electrode in lysozyme, the electron transfer was facilitated by the positively charged protein introduced (lysozyme has a pI = 11). The charge electron transfer was found to decrease with increasing lysozyme concentrations in the range from 5 to 25 µg mL^{-1} and the biosensor was characterised by a detection limit of 0.2 µg mL^{-1}.

Target-induced aptamer displacement is based on a duplex formed by an aptamer and a partial complementary DNA sequence, that is confined (*e.g.* by self-assembling [63]) on an electrode. After the introduction of the target probe – in this case lysozyme – the aptamer strand is displaced from the duplex into the solution. This displacement is translated into a decrease in the electron transfer measured by EIS with a typical redox probe (Fig. 6). Detection limit as low as 0.07 nM lysozyme was achieved by this strategy [63].
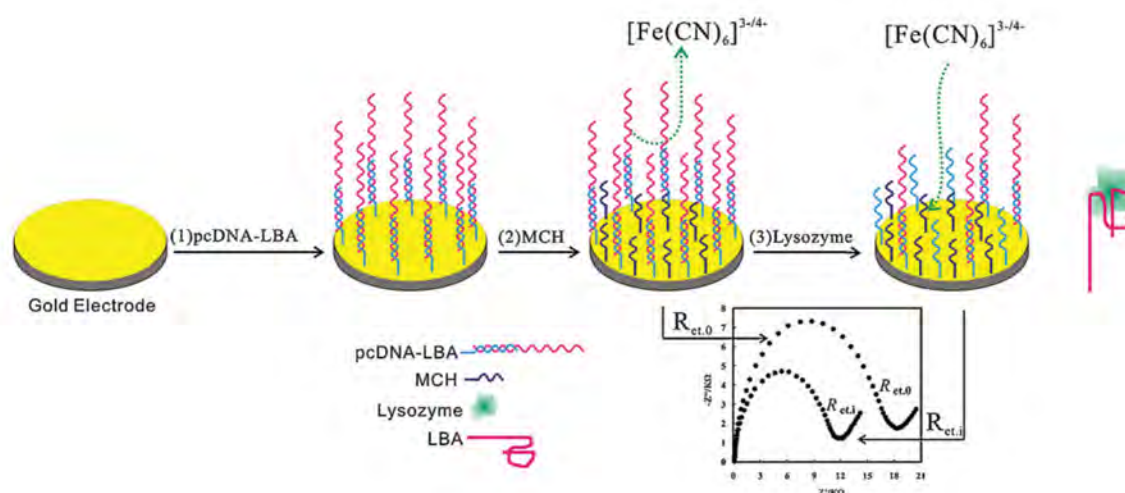

Fig. 6: *Schematic representation of the FIS aptasensor with fabrication and performance steps.* Reprinted with permission from [63]. Copyright (2009) Elsevier.

In a *"signal-off"* architecture [64,65], the electrode surface (typically gold) is modified with a DNA duplex formed by the aptamer strand (covalently bound to the surface) and a partial complementary strand tagged with a redox mediator (*e.g.* ferrocene – Fc). The electrochemical signal due to mediator oxidation, recorded by square wave voltammetry (SWV) is strong since the mediator is situated in the immediate proximity of the surface. Upon incubation with the target lysozyme, the binding with the aptamer takes place and the Fc-tagged strand is released in solution (Fig. 7). Consequently, the electrochemical signal decreases. The limit of detection achieved by this approach was 0.45 nM and the fabrication steps of the aptasensor were characterised by impedance measurements [64].
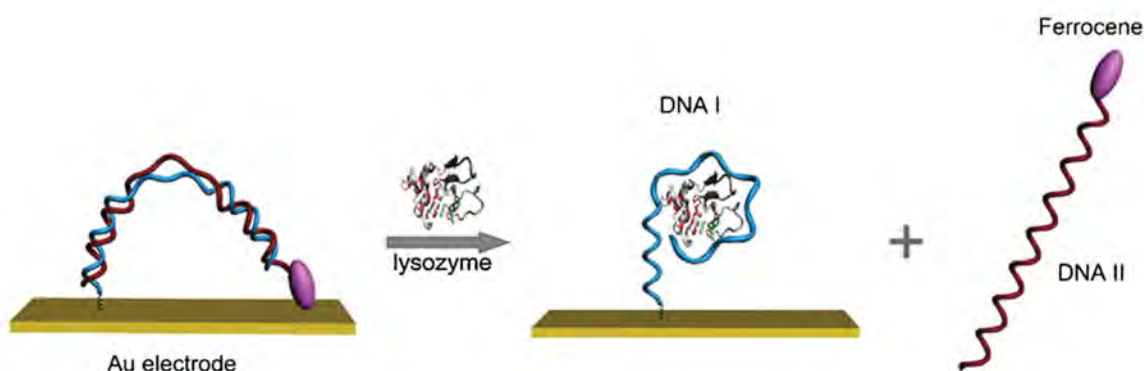

Fig. 7: *The proposed mechanism of the signal-off architecture for electrochemical aptasensor.* Reprinted with permission from [64]. Copyright (2013) Elsevier.

Two of the concepts presented above, *signal off* and *target induced aptamer displacement* were combined in a "three way junction" (TWJ) aptasensor [66]. In this study, the TWJ/DNA was formed by hibridization of a lysozyme aptamer with a partial complementary strand containing A2 bulges ("cDNA"), tagged with the electrochemical mediator ferrocene. The DNA structure thus obtained stabilises upon folding as a three-way helical junction with two coaxial helices and a third one at an angle (Fig. 8). The sensing platform was built by immobilising the TWJ/DNA onto a glassy carbon electrode (GCE) previously modified with Au, through Au-S bonds. In the initial ''eT on'' state, the TWJ provide a good conductivity as the space between the Fc-tagged cDNA and electrode is small and a good signal for ferrocene oxidation is observed by SWV.
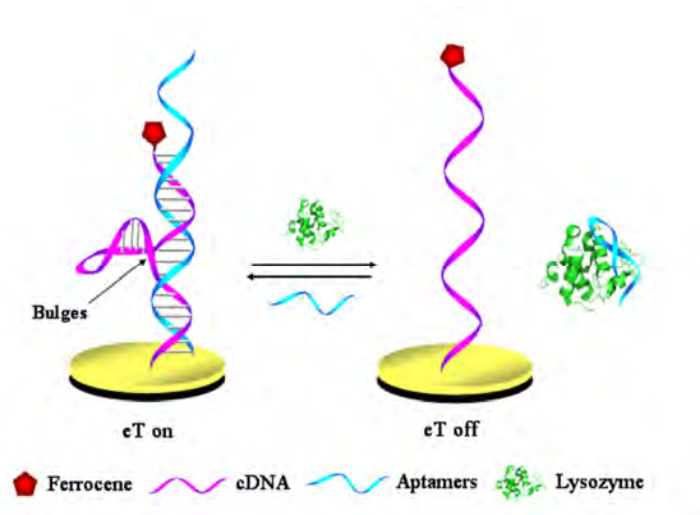
Fig. 8: *Schematic representation of the aptasensor for detection of lysozyme and regeneration procedure.* Reprinted with permission from [66]. Copyright (2013) Elsevier.

The binding between the TWJ/DNA and lysozyme leads to a signal decrease, the interaction lysozyme-aptamer being much stronger compared with the interaction aptamer – cDNA. The aptamer-lysozyme complex is released in solution. This is traduced in less conductivity due to the dissociation of the aptamer from the cDNA into solution, correlated with the increase of the distance between the tagged cDNA and the electrode, the so-called "eT off" state [66]. EIS measurements helped to verify the fabrication steps of the biosensor (Fig. 9). A detection limit of 0.2 nM was reached by this analytical strategy [66].
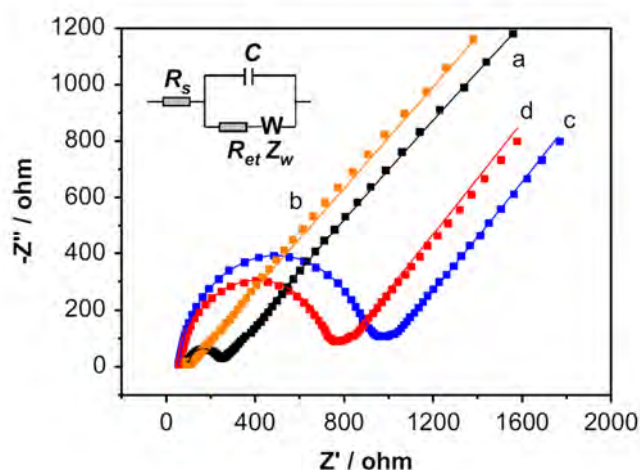

Fig. 9: *EIS (Nyquist plots) of (a) bare GCE, (b) Au/GCE, (c) TWJ-DNA/Au/GCE and(d) the denatured cDNA1/Au/GCE.* The data were obtained in 10 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M PBS (pH 7.4). The biased potential was 0.19 V. The frequency was from 100 kHz to 0.01 Hz and the amplitude was 5 mV. The inset shows the equivalent circuit applied to fit the EIS. Fitted data (solid line). Reprinted with permission from [66]. Copyright (2013) Elsevier.

Improving Sensing with Nanomaterials

Due to some extraordinary electrical, magnetic, optical, thermal, and mechanical properties compared to their macroscopic counterparts nanomaterials have attracted a great attention and interest in the last decade in the field of biosensors. The scientific literature has practically "exploded" with studies based on nanomaterials such as gold nanoparticles (AuNPs), carbon nanotubes (CNT) and more recently graphene. Several reviews summarizing recent progress in the field are available in literature [33,67-69]. A few representative applications in the construction of impedimetric biosensors for lysozyme are summarized below.

Gold Nanoparticles. Owing to their high specific area, good conductivity and biocompatibility, gold nanoparticles were widely used in electrochemical biosensors. Electrochemical deposition of AuNPs onto the electrode surface is straightforward and the amount of the immobilized biospecific receptor on the electrode can be significantly increased compared to an unmodified one, without losing their biological activity [70,71].

Starting from this point of view, Chen and co-workers [72] explored the advantage of AuNPs for increasing the sensitivity of a lysozyme aptasensor. A lysozyme aptamer was immobilised directly on a gold electrode or on AuNPs previously electrodeposited on the gold electrode. EIS measurements with the AuPN-based aptasensor emphasized an increase of the charge-transfer resistance with the lysozyme concentration in the linear range 0.1-500 pM. The detection limit was improved by more than an order of magnitude compared to the bare Au-based sensor, reaching 0.01 pM and 3 pM, respectively [72].

Carbon Nanotubes (CNTs). The entrance of CNT into the world of (bio)sensing was received with high enthusiasm due to their remarkable electronic, mechanic and thermic properties. They are also easy to functionalise, which prompted an avalanche of studies where CNTs were included in sensors either to increase the electron transfer rate or to immobilize higher amounts of bioreceptors. One recent example is an aptasensor for lysozyme based on screen-printed electrodes modified with multi-wall carbon nanotubes (MWCNT–SPEs), with an amino-ended aptamer covalently bound to sensor surface [73].
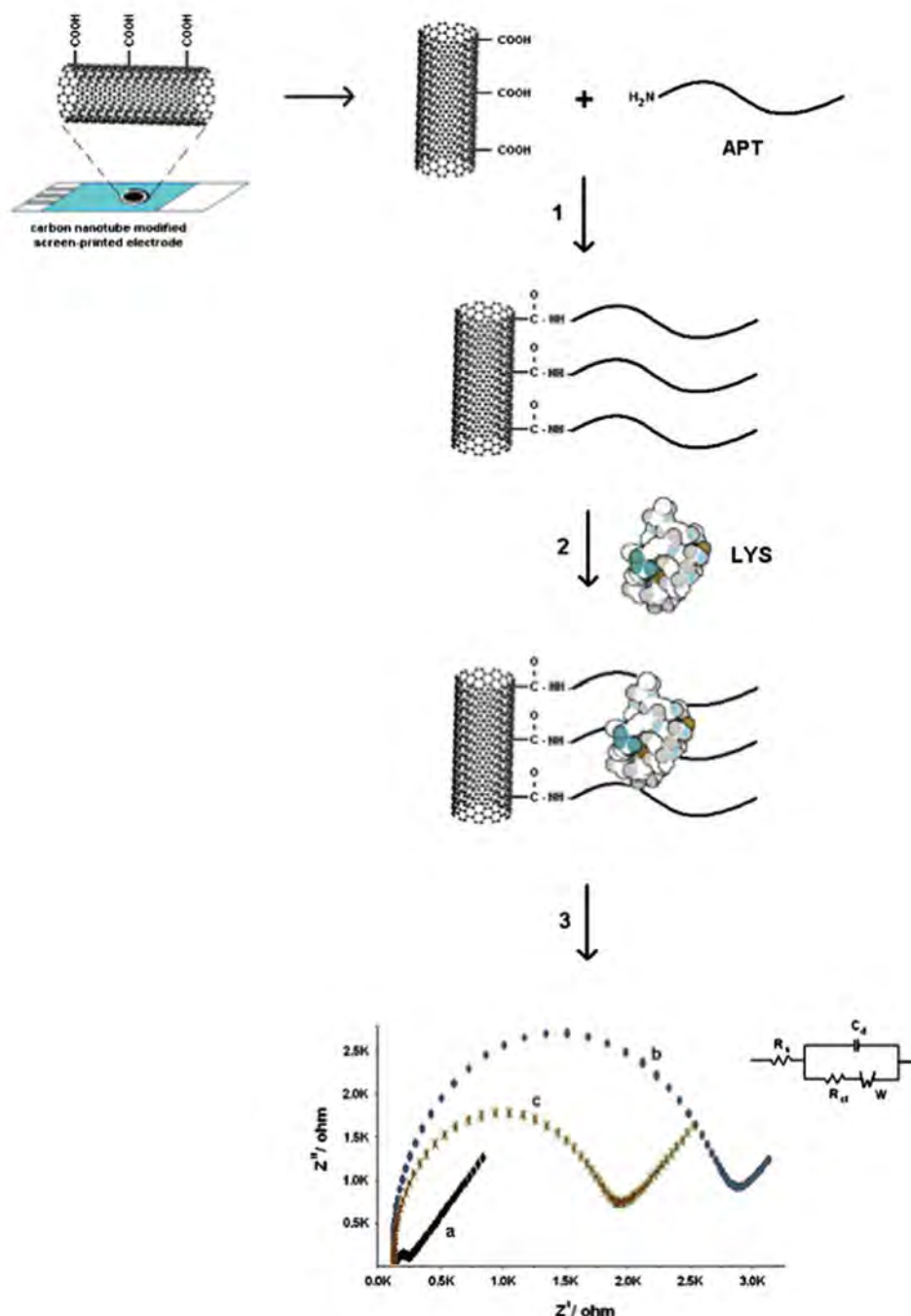
Scheme 3: *Before (1) and after (2) immobilization of amino-linked DNA aptamer onto the surface of MWCNT–SPEs and (3) the interaction of LYS with APT measured by using EIS.* Representative Nyquist diagrams show the EIS measurements for (a) bare (unmodified) electrode, (b) APT-modified electrode, and (c) APT+LYS-modified MWCNT–SPEs. Reprinted with permission from [73]. Copyright (2012) Elsevier.

The modification of the SPE with MWCNT leads to a significant improvement of the impedimetric signal. The charge-transfer resistance decreased with the increase of the lysozyme concentration in the range 0-400 µg mL^{-1}, then levelled off (Fig. 10). A detection limit of 12.09 µg mL^{-1} was obtained [73].
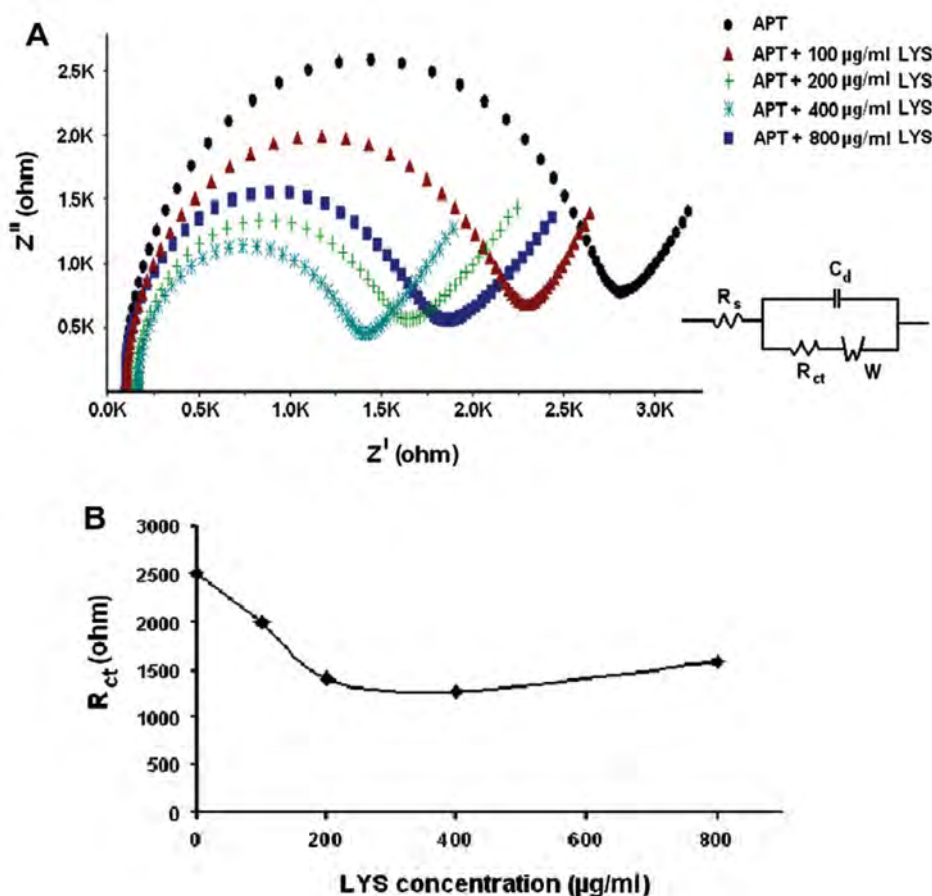
Fig. 10: *(A) Nyquist diagrams recorded with supporting electrolyte solution 2.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) containing 0.1 M KCl by using MWCNT–SPEs with immobilized 200 µg mL^{-1} aptamer for different LYS concentrations such as 0, 100, 200, 400, and 800 µg mL^{-1}.* The inset shows the equivalent circuit model used to fit the impedance data, the parameters of which are listed in the text; R_1 is the solution resistance. The constant phase element C_d is then related to the space charge capacitance at the DNA/electrolyte interface. R_2 is related to the charge transfer resistance (R_{ct}) at the DNA/electrolyte interface. The constant phase element W is the Warburg impedance due to mass transfer to the electrode surface. *(B) Line graph for relation between LYS concentration and R_{ct} values.* Reprinted with permission from [73]. Copyright (2012) Elsevier.

Graphene. Graphene, an one-atom thick layer of sp^2 carbon forming a hexagonal lattice, shows great promise in the field of biosensors due to its excellent electrical conductivity, large surface area and modifiable surface chemistry [74,75]. Typically, for use in electrochemical biosensors, graphene oxide is obtained by chemical exfoliation of graphite using strong oxidising agents. Graphene oxide is further reduced either chemically (*e.g.* using hydrazine) or electrochemically to increase its conductivity and decrease the solubility in water.

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Sensitive detection of lysozyme (detection limit 6 fmol L^{-1}) was reported with a graphene-based, aptamer modified glassy carbon electrode. The charge-transfer resistance determined by EIS increased linearly with the concentration of lysozyme in the range from 0.01 to 0.5 pmol L^{-1} [12].

Another recently described impedimetric aptasensor for lysozyme relies on a pencil graphite electrode (PEG) that is modified with chitosan/graphene oxide and an amino aptamer. The charge-transfer resistance decreased with the increase of lysozyme in the range 0.25-1 µg mL^{-1}, with a detection limit of 0.38 µg mL^{-1}. The non-specific response is a potential problem in graphene-based sensors due to the high adsorption capacity of this nanomaterial. Non-specific adsorption was examined in lysozyme solutions containing also bovine serum albumin and thrombin and was found to be non-significant [76].

An original impedimetric aptasensor was also described, allowing mediator-free detection of lysozyme [77]. By modifying a GCE with reduced graphene oxide (GR) and Fe_2O_3, the electrochemically active area increased from 0.04 to 0.15 cm^2 for GCE and Fe_2O_3/GR/GCE, respectively. Mediator-free impedimetric measurements performed in PBS buffer pH 7 at open circuit potential allowed to observe significant differences between the response with the aptamer and that without the aptamer at high frequencies (Fig. 11). The aptasensor presented a detection limit of 0.16 ng mL^{-1} and a linear range between 0.5 ng mL^{-1} and 5 µg mL^{-1} [77].
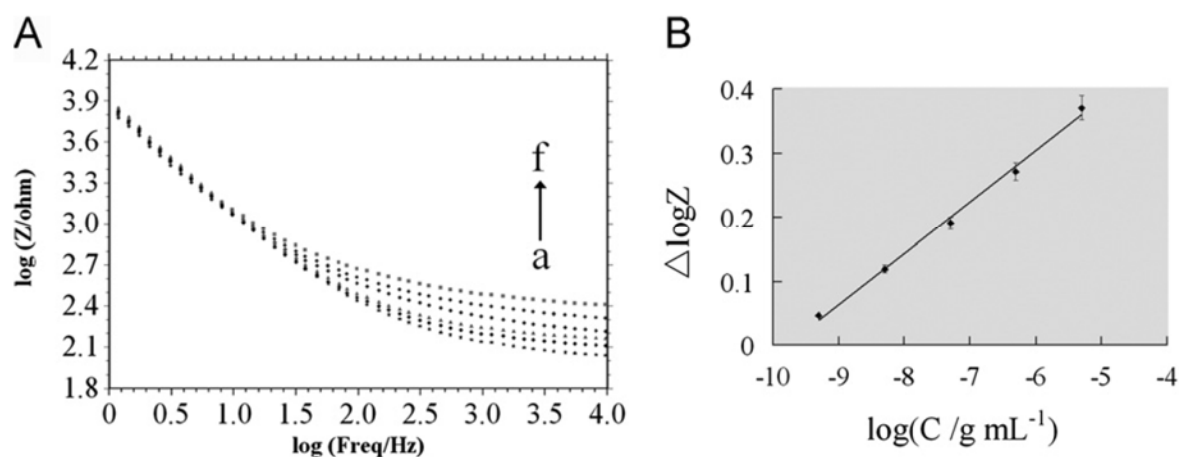
Fig. 11: *(A) Bode plots in PBS (pH 7.0) at LBA/Fe₂O₃/graphene/GCE for different concentrations of lysozyme:* (a) 0, (b) 0.5, (c) 5, (d) 50, (e) 500, and (f) 5000 ng mL^{-1}. *(B) The plot of Δlog Z vs. the logarithm of lysozyme concentrations.* Reprinted with permission from [77]. Copyright (2013) Elsevier.

Analysis of Food Samples with Impedimetric Biosensors

Despite the development of various impedimetric biosensors for allergens, their application for the analysis of food samples remains very limited. Only a few examples are known to date, including detection of Ara h1 in canned food [47,78], detection of gliadin in bakery products [10] and detection of lysozyme in chicken egg white [72]. The challenges posed by complex matrices reffer mainly to non-specific adsorption. EIS is very sensitive to changes in the electric properties at the interface due to non-specific adsorption phenomena.

In some cases when the difference between the specific and non-specific response could not be captured by EIS, a solution was found by employing a different electrochemical method [10]. Additionally, many methods make use of conformational changes of the biorecognition element (aptamer) which is highly influenced also by ions presented in solution [79]. For example, extraction procedures employing high amounts of salt [80] would have to be reconciled with the best experimental conditions for electrochemical measurements-in many cases this means low ionic strength. Sample clean-up or use of highly diluted sample solutions are some possible strategies to overcome this problem.

Conclusions

Electrochemical impedance spectroscopy proved to be a very powerful technique for characterizing the fabrication of biosensors and also in some cases for sensitive quantitative detection. Incorporation of nanomaterials and aptamers into recently described biosensors lead to a wealth of possible sensor configurations, some of high originality and complexity. Additionally, this made possible to reach extraordinary detection limits, which is extremely appealing for the detection of allergens, where the ability to determine minute amounts of protein in sometimes highly processed foods is desired. However, the number of applications of impedimetric biosensors in the analysis of real samples remains rather small, the main challenges being related to problems of non-specific response. More investigations with real food matrices and validation in comparison with current reference methods are needed and will undoubtedly follow in next years.

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