Electrochemical Affinity Biosensors in Food Safety

Susana Campuzano, Paloma Yáñez-Sedeño * and José Manuel Pingarrón

Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid,
28040 Madrid, Spain; susanacr@quim.ucm.es (S.C.); pingarro@quim.ucm.es (J.M.P.)
* Correspondence: yseo@quim.ucm.es

Abstract: Safety and quality are key issues of today's food industry. Since the food chain is becoming more and more complex, powerful analytical methods are required to verify the performance of food safety and quality systems. Indeed, such methods require high sensitivity, selectivity, ability for rapid implementation and capability of automatic screening. Electroanalytical chemistry has, for decades, played a relevant role in food safety and quality assessment, taking more and more significance over time in the solution of analytical problems. At present, the implementation of electrochemical methods in the food is evident. This is in a large part due to the relevant results obtained by combining the attractive advantages of electrochemical transduction strategies (in terms of relatively simple hardware, versatility, interface with automatic logging and feasibility of application outside the laboratory environment) with those from biosensors technology. Important examples of enzyme electrochemical biosensors are those dedicated to the determination of glucose, alcohol or cholesterol are important examples. In addition, other types of different electrochemical biosensing approaches have emerged strongly in the last years. Among these, the strategies involving affinity interactions have been shown to possess a large number of applications. Therefore, electrochemical immunosensors and DNA-based biosensors have been widely used to determine major and minor components in foodstuffs, providing sufficient data to evaluate food freshness, the quality of raw materials, or the origin of samples, as well as to determine a variety of compounds at trace levels related to food safety such as micotoxins, allergens, drugs residues or pathogen microorganisms. This review discusses some critical examples of the latest advances in this area, pointing out relevant methodologies related to the measurement techniques, including the use of nanostructured electrodes and strategies for signal amplification.

Keywords: food; safety; electrochemical biosensors; immunosensors; DNA-sensors

1. Introduction

Innovation and development in the food industry basically relies on the concepts of food safety and quality [1]. The food industry implements quality and management systems that include increased testing to verify performance in order to give adequate answers to the consumers about both concepts with the main purpose of boosting wellbeing and preventing future diseases [2]. The assessment of food safety is one key area for the modern food industry. Contaminants may enter the food chain during various stages of production. They can also accumulate in food during storage, form in the food by interaction of chemical compounds, or may be concentrated from the natural food components [3]. Food regulatory agencies have established control programs for the food industry. A preventative approach to food safety is the HACCP (Hazard Analysis Critical Control Point), which attempts to avoid the entering of unwanted substances into the food chain [4]. Allergens, drug residues, or pathogen microorganisms are some of the main targets concerning food safety.

For decades, electrochemical techniques have been demonstrated to be useful tools for food analysis. As a consequence, there has been a large extension of the implantation of sensors and biosensors in recent years. In fact, the combination of (bio)sensing strategies with efficient electrochemical transduction techniques has allowed the implementation of rapid and reliable detection methods for many compounds of concern in a wide variety of food samples. Electrochemical sensing facilitates signal transduction and automated data logging. Significant advances in miniaturization, multi-detection, automation and easy handling of electroanalytical devices and instruments, together with the relatively low costs of these techniques, make them an important alternative to meet the increasingly demanding requirements of the food industry [5]. Moreover, the high sensitivity that inherently characterizes the electrochemical methods, especially when using the modern voltammetric techniques and schemes for signal amplification, and the improvements in the analytical characteristics provided by the use of nanomaterials have definitely demonstrated their utility to ensure food quality and safety.

More recent applications of electrochemical biosensors in the field of food analysis encompass the use of immunosensors and nucleic acid-based biosensors for the detection and determination of toxins, allergens, microorganisms, genetically modified organisms (GMOs), species associated with celiac disease, and pesticide or drug residues.

The main affinity bioreceptors used in these biosensing approaches include antibodies, aptamers and DNA strands (both single and double-stranded, linear and hairpin probes). As it will be illustrated through several examples, the selection between these particular bioreceptors depends on several factors including commercial availability, molecular level of the analyte to be detected, size of the analyte and endogenous level of the analyte in the sample to be analyzed. Electrochemical immunosensors have been extensively used for food safety because of the interesting advantages they offer in terms of inherent high sensitivity and selectivity, great precision and accuracy, low cost, minimum sample requirement, simplicity of operation and possible integration in compact analytical devices as well as demonstrated suitability for food analysis [6]. Electrochemical nucleic acid-based sensors have been widely used for detecting food safety related analytes or specific genes associated with their presence. Electrochemical DNA biosensors comprise a nucleic acid recognition layer that is immobilized onto an electrochemical transducer that detects the changes that occur in the DNA structure during interaction with other complementary oligonucleotides or with small molecules [7–13]. Different strategies have been employed for electrochemical detection of DNA-target analyte interaction: direct electrochemical detection of the target analyte itself, if it is an electroactive molecule, or measuring changes in the electron transfer resistance as a consequence of the binding and indirect strategies involving the electrochemical detection of external redox probes used both to label the affinity reagents or in solution. Aptamers are artificial ss-RNA or DNA oligonucleotides screened from synthetic DNA/RNA libraries which typically fold into a three-dimensional structure, whose conformation is changing upon ligand binding [14,15]. Possibly the most important advantages of aptamers from an analytical point of view are the high affinity and binding specificity for their targets, ease to be produced and labeled with different reporter molecules, and their relatively low production cost, which make them very attractive bioreceptors [14,16]. Moreover, they are more stable to denaturation and degradation than antibodies [17,18]. Although aptamer applications are dominated by clinical or medical diagnostics market so far, first steps have already been taken for the application of aptamers to ensure food safety. In order to improve the performance of electrochemical affinity biosensors for food safety different nanomaterials such asMWCNTs, graphene, AuNPs, PtNPs have been used as electrode modifiers and signal enhancers (mesoporous carbon-enriched palladium nanostructures, Fe_3O_4 nanoclusters, nCe particles, MnO_2 nanoparticles, CNTs) [19–24].

To provide an overview of the state of the art in the use of electroanalytical approaches in the fields of food quality and safety, we discuss in this review some selected examples of the latest advances in this area related with the use of immunosensors and nucleic acid-based sensors many of them involving the use of nanomaterials and pointing out relevant methodologies related to the measurement techniques, and the use of signal amplification strategies. For a better understanding,

the relevant data of the selected configurations discussed, including the fundamentals and the analytical characteristics of the resulting methodologies, are summarized in Tables 1–5. In the next sections, some particularly relevant aspects are also critically discussed.

2. Electrochemical Affinity Biosensors for Toxins

Natural toxins are poisonous substances naturally produced by and derived from microorganisms, plants and animals that are harmful to another organism. These compounds cause a significant number of food poisoning cases. Recently, Reverte et al. [25] revised the advances in electrochemical biosensors for the detection of toxins emphasizing on the use of nanomaterials, magnetic beads and microfluidic systems. Important examples of these compounds are neurotoxins produced by microalgae species which can contaminate shellfish and produce human poisoning with effects that can even lead to death [26]. Saxitoxin (STX) and its analogous are the causing agents of paralytic shellfish poisoning (PSPs). They are water-soluble and thermostable tetrahydropurines that block sodium transport through sodium-channel receptors present in nerves, muscle fibers and cardiac fiber cells in mammals. Symptoms of respiratory muscle paralysis appear in humans within few minutes after ingestion of PSP-contaminated shellfish [27]. Maximum permitted level of STX at which fisheries are closed has been regulated to be 80 μg STX equivalents per 100 g shellfish in most countries [28,29]. A label-free electrochemical aptasensor for STX has been described recently involving target-induced conformational change of an STX-specific aptamer binding the toxin. The preparation of the biosensor implied the deposition of an octadecanethiol monolayer on a gold electrode and further coating with multiwalled carbon nanotubes (MWCNTs) to which the aptamer was covalently conjugated. Methylene blue was electrostatically anchored on carboxylated MWCNTs and used as the electrochemical indicator that produced a strong differential pulse voltammetric signal in the absence of target STX. However, when STX binds the aptamer, the aptamer changes its conformation dramatically, preventing the exposure of the guanine bases to which MB is able to bind and results in the establishment of a barrier for heterogeneous electron transfer, which results in a lowered electrochemical signal of methylene blue. The oxidation peak current of methylene blue measured at -0.27 V (vs. Ag/AgCl) linearly decreased with increasing STX concentrations in the 0.9–30 nM range and the detection limit was 0.38 nM. This aptasensor was applied to the determination in mussel samples spiked with two different STX concentrations (1.12 and 7.44 $\mu g \cdot kg^{-1}$) obtaining recoveries ranging from 63% to 121% which indicated an acceptable accuracy and reproducibility [28].

Brevetoxins (BTXs) are potent cyclic polyether neurotoxins naturally produced by the marine "red tide" dinoflagellates Karenia brevis. They exhibit toxicity towards humans whose exposure to BTXs can occur through consumption of contaminated shellfish as well as aerosol exposure in coastal areas [30]. The legal limit of BTXs is also 80 μg of toxin per 100 g of shellfish tissue [31,32]. An interesting electrochemical aptasensing platform for BTX-2 has been reported using a competitive approach electrochemical impedance spectroscopy (EIS) transduction. The competition was established between BTX-2 immobilized on a cysteamine-modified gold electrode (Cys/Au) activated with 1,4-phenylenediisocyanate(PDIC) and free BTX-2 in solution in the presence of a fixed amount of the aptamer. Change in R_{CT} of $[Fe(CN)_6]^{4-/3-}$ measured by EIS was taken as analytical signal. As concentration of BTX-2 in solution increased, less free aptamer amount was available for binding to the immobilized BTX-2 on the gold surface which resulted in lower R_{CT} change. The resulting label-free competitive impedimetric biosensor allowed a detection limit of 106 $pg \cdot mL^{-1}$ to be achieved with good recoveries (102–110%) in the analysis of shellfish extracts spiked at three different BTX-2 concentrations (0.1, 10 y 100 $ng \cdot mL^{-1}$) [33]. With the aim of improving sensitivity, a signal amplification strategy in an enzyme-free electrochemical immunoassay of brevetoxin B (BTB) has been proposed. A mesoporous carbon-enriched palladium nanostructure (MSC-PdNS) was synthesized and employed as the signal-transduction tag after labeling with anti-BTB and competitive assay onto a BSA-BTB-functionalized electrode (Figure 1). The catalytic effect of the doped palladium nanostructure into MSC-PdNS toward reduction of H_2O_2 and the highly efficient immunoassay

allowed a range for BTB detection between 0.01 and 10 ng·mL^{-1} with a LOD of 5.0 pg·mL^{-1} [31]. The immunosensor was successfully applied to seafood extracts spiked with BTB at concentration levels well below the permitted values.
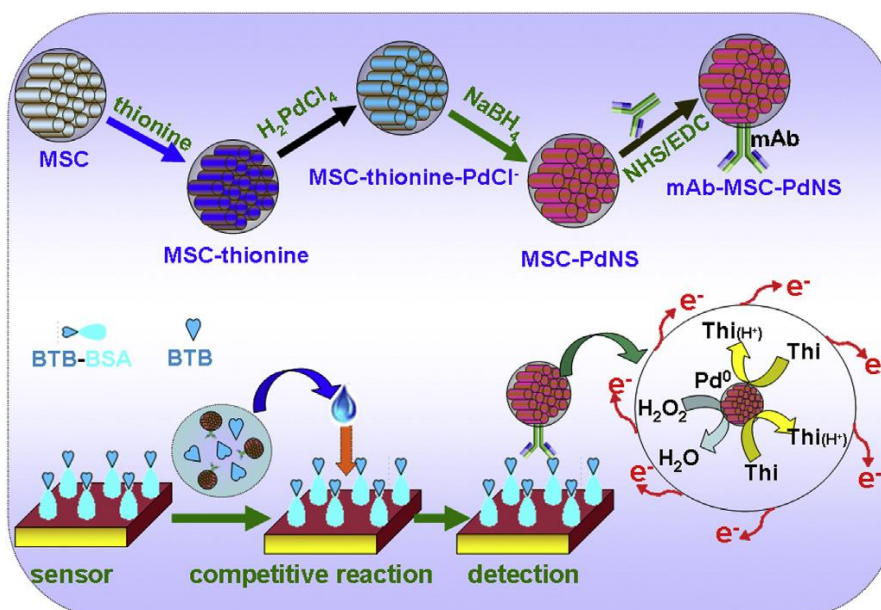

Figure 1. Scheme of the preparation of an enzyme-free electrochemical immunosensor for brevetoxin B using mesoporous carbon-enriched palladium nanostructures as the signal-transduction tag for catalytic reduction of H$_2$O$_2$. Reprinted from [31] with permission.

Okadaic acid (OA) is a lipophilic marine biotoxin produced by dinoflagellates. OA intoxication is considered as the most of concern diarrhetic shellfish poisoning (DSP) for human health [34]. The European Commission (EC) has implemented regulation on the concentration of OA with maximum permitted levels of 160 µg·kg^{-1} of mussels (EC No 853/200415). Furthermore, the European Food Safety Authority (EFSA) has suggested to decrease the maximum permitted level to 45 µg·kg^{-1} of mussel [35]. The need for developing high performing methods for OA analysis is an important challenge for shellfish industries worldwide. Some recent electroanalytical methods using affinity sensors are summarized in Table 1. An illustrative example is the use of protein G-functionalized magnetic beads modified gold electrodes to immobilize anti-OA monoclonal antibodies and electrochemical detection in ferri/ferrocyanide solutions. The analytical readout was the decrease in current produced as the concentration of OA conjugate to the immobilized anti-OA antibody increased. The limit of detection obtained was 0.5 µg·L^{-1} [36]. As an application, recovery studies were performed with good results by analyzing mussel extracts spiked with OA at three different levels: 185, 43 and 10 µg·kg^{-1}. The same group prepared an interesting flow-based electrochemical immunosensor for the determination of OA in mussels. OA-modified magnetic beads were injected onto screen-printed carbon electrodes (SPCEs) in the flow system and a competitive assay between the analyte and immobilized OA to bind anti-OA antibody was established. The electrochemical detection was performed using a secondary antibody labeled with the enzyme alkaline phosphatase (AP) and injection of 1-naphthyl phosphate. The limit of detection was 0.15 µg·L^{-1} [34]. Eissa et al. [37] developed a voltammetric immunosensor for OA by applying a simple and efficient electrografting method to functionalize graphene-modified SPCEs followed by covalent immobilization of OA-antibody on the resulting modified electrodes. A competitive assay between OA and a fixed concentration of OA–ovalbumin conjugate for the immobilized antibodies was employed for OA detection by measuring the decrease in current of [Fe(CN)$_6$]$^{3-/4-}$ as increased OA concentration using square wave voltammetry. A linear concentration range was found up to 5,000 ng·L^{-1} with a LOD of 19 ng·L^{-1}. Moreover, once studied the matrix

effect with spiked shellfish tissue extracts, the method was validated with certified reference mussel samples obtaining high recoveries and acceptable RSD values.

A method for the simultaneous monitoring of BTX-2 and dinophysistoxin-1 (DTX-1) marine toxins was reported based on distinguishable metal nanocluster-labeled molecular tags. Bi-functionalized magnetic capture probes were prepared by immobilization of both the specific anti-BTX-2 and anti-DTX-1 mouse antibodies onto a magnetic bead. Cadmium and copper nanoclusters from the corresponding Cd(0) and Cu(0) nanoparticles were synthesized and labeled with artificial peptides with amino acid sequence CCCYYY conjugated with BSA-BTX-2 and BSA–DTX-1, respectively. A competitive-type immunoassay was established for the online simultaneous monitoring of BTX-2 and DTX-1 using a homemade flow-through magnetic detection cell by applying square wave anodic stripping voltammetry to measure the labeled Cd and Cu nanoclusters in wide working ranges of 0.005–5 ng·mL^{-1} for the two marine toxins [38].

Microcystins are a kind of hepatotoxins which can accumulate in aquatic organisms and transfer to higher trophic levels, causing functional and structural disturbances of the liver [39]. Among them, microcystin-LR (MC-LR) is one of the most toxic. A guideline value of 1.0 µg·L^{-1} for MC-LR in drinking water has been set by the World Health Organization (WHO) [40]. A variety of electrochemical biosensors has been proposed for the detection of MC-LR at the required concentration levels [41–44]. However, they have been mostly applied to environmental samples but not to food samples. Therefore, only biosensors related with drinking water or seafood-derived samples have been summarized in Table 1. A representative example is the graphene-based immunosensor fabricated by Zhao et al. [45] using an electrode platform involving graphene and chitosan (Chit) as immobilization materials, and a multienzyme HRP functionalized carbon nanospheres–antibody system for signal amplification. The method provides a linear range from 0.05 to 15 µg·L^{-1} MC-LR with a detection limit of 0.016 µg·L^{-1}. The developed method was validated by application to real water samples which contained trace levels of MC-LR ranging between 0.29 ± 0.11 µg·L^{-1} (tap water) and 1.13 ± 0.01 µg·L^{-1} (river water), and comparison of the results with those obtained by high-performance liquid chromatography.

Carbon nanotubes/cobalt silicate core–shell nanocomposites were synthesized and used to immobilize the antigen for the preparation of another immunosensor for MC-LR. In this configuration, Fe_3O_4 nanoclusters/polydopamine/gold nanoparticles Fe_3O_4@PDA-AuNPs–shell magnetic nanocomposites were employed as the label carrier to conjugate the detection antibody (Ab_2) and HRP (Figure 2). Both the large surface area of the three-dimensional structure in CNT@Co silicate and the high electrochemical signals provided by Fe_3O_4@PDA–AuNPs-HRP-Ab_2 conjugate by CV in the presence of hydroquinone (HQ), allowed a linear response to MC-LR in the 0.005–50 µg·L^{-1} range with a detection limit of 0.004 µg·L^{-1} [46].

A multiwalled carbon nanotube (MWCNT)-based electrochemical biosensor was developed for monitoring MC-LRin sources of drinking water supplies. The modified electrode substrate consisted of vertically well-aligned, dense and millimeter-long MWCNT arrays grown onto Si substrates. MC-LR was linked to the oxygen-containing functional groups on the nanotubes surface, and addition of the specific anti-MC-LR antibodies made possible the construction of the immunosensor by incubation in the sample solution. Detection of changes in the electron-transfer resistance related with MC-LR concentration demonstrated a linear dependence in the range of 0.05 to 20 µg·L^{-1}, enabling cyanotoxin detection below the World Health Organization (WHO) provisional concentration limit of 1 µg·L^{-1} in drinking water [47,48].

An aptasensor for MC-LR was also prepared using graphene-modified screen-printed carbon electrodes where the aptamer was physically adsorbed onto graphene via π-π stacking interactions with DNA nucleobases. SWV of $[Fe(CN)_6]^{4-/3-}$ as probe redox couple was used to detect current changes related with MC-LR concentration. As expected, the peak current decreased after aptamer assembly. However, the presence of MC-LR provoked an increase in the peak current value depending on the toxin concentration which was attributed to the conformational changes of the aptamer in

the presence of the analyte. A linear range of 0.1 pM to 1.0 nM was obtained with a LOD of 1.9 pM. The aptasensor was applied to the analysis of spiked tap water and fish extracts [49].
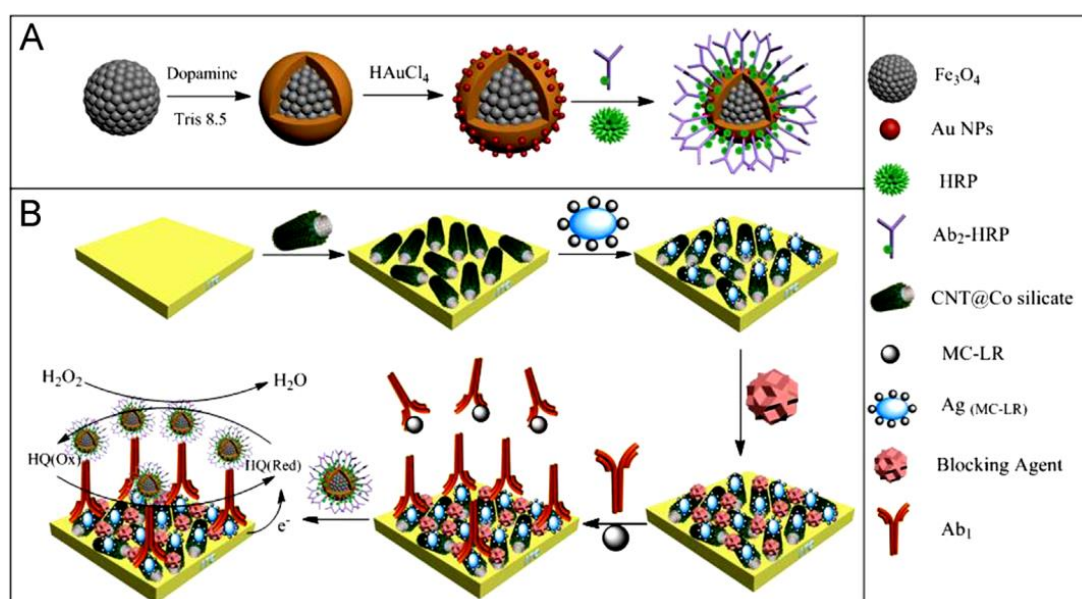

Figure 2. Schematic illustration of the preparation of multi-horseradish peroxidase (HRP)–(Fe$_3$O$_4$@PDA–Au)–Ab$_2$ conjugate (**A**) and the construction of the microcystin (MC)-LR electrochemical immunosensorbased on the antigen immobilization on (CNT@Co silicate) core–shell nanocomposites and the use of (Fe$_3$O$_4$@PDA–Au) core–shell magnetic nanocomposites as label carriers of the second antibody (Ab$_2$) and HRP (**B**). Reprinted from [46] with permission.

Mycotoxins are a structurally diverse group of mostly small molecular weight compounds naturally occurring as secondary metabolites produced by fungi or molds [50]. Contamination of feed and food is responsible for mycotoxicosis causing harmful effects on animal and human health. To ensure food safety and prevent risks in agro-food sector, maximum permitted levels for most of mycotoxins in foods were established by the European Commision [51]. Therefore, for mycotoxins monitoring at the required concentrations, a variety of electrochemical affinity biosensors using different analyzing techniques have been reported. Vidal et al. [52] reviewed the use of electrochemical affinity biosensors for detection of mycotoxins. More recently, electrochemical immunosensing strategies for mycotoxins were also reviewed by Catanante et al. [53].

Aflatoxins, produced mainly by Aspergillus flavus and A. parasiticus possess an extremely high toxicity and carcinogenicity. Aflatoxin B1 (AFB1) is regulated by legislation in the European Union (EU) at 2 µg·kg^{-1} and the sum of aflatoxins B1, B2, G1, and G2 at 4 µg·kg^{-1} in foodstuff for direct human consumption [51]. The Food and Drug Administration (FDA) of USA has mandated the maximum limit of 20 µg·kg^{-1} for aflatoxins in all foods [54,55]. Various electrochemical immunosensors using nanoparticles have been reported for these compounds [56]. Table 1 summarizes the analytical characteristics of recent immunosensors constructed for aflatoxin B1 (AFB1) [57–60]. A relevant example is the strategy involving a Chit-AuNPs nanocomposite electrodeposited onto the surface of a disk-ring gold microelectrode to prepare a label-free impedimetric immunosensor for AFB1 in wheat samples [57]. The specific antibody was immobilized onto the modified electrode and the sample was incubated for 15 min followed by CV measurement in the presence of [Fe(CN)$_6$]$^{3-/4-}$. The linear range extended between 0.2 and 30 ng·mL^{-1}. In another recent report, Linting et al., [58] electrodeposited graphene and AuNPs onto a gold electrode to improve the electron transfer and used the conducting film for anti-AFB1 immobilization and construction of a label-free impedimetric immunosensor. The method displayed a very high sensitivity with a LOD value of 1.0 fM and a long-term stability of

over 26 weeks. Moreover, the usefulness of the immunosensor was demonstrated by application to the analysis of a variety of food samples containing AFB1. As examples, peanut or soybean with toxin contents of 0.13 and 1.0 ng·g^{-1}, respectively, were analyzed with good results when compared with the results provided by HPLC.

Zhang et al. [59] immobilized AFB1 onto a glassy carbon electrode (GCE) modified with SWCNTs and Chit to fabricate a competitive indirect-type voltammetric immunosensor. After incubating anti-AFB1 with AFB1 solutions prepared at different concentrations (or the sample), a small volume (10 μL) of the resulting solution was dropped onto AFB1-Chit/SWCNTs/GCE. Next, an IgG secondary antibody labeled with AP was bound through reacting with primary antibodies on the electrode surface and the electrochemical measurements were carried out by DPV after addition of 1-naphthylphosphate as the enzyme substrate. This method could quantitatively detect AFB1 from 0.01 to 100 ng·mL^{-1} with a detection limit of 3.5 pg·mL^{-1} and was successfully applied to determine AFB1 in corn powder. Furthermore, another impedimetric immunosensor was proposed for the detection of AFB1 in olive oil. A composite prepared by mixing MWCNTs and the liquid ionic 1-butyl-3-methyl-imidazolium hexafluorophosphate ([BMIM]PF$_6$) was dropped onto a GCE and used as a medium to achieve fast electron transfer and a favorable microenvironment for antibody immobilization. The resulting anti-AFB1/IL/MWCNTs/GCE provided a LOD value of 0.03 ng·mL^{-1} and made possible the aflatoxin determination in real samples after extraction with methanol/water [60].

OchratoxinA (OTA) mycotoxin is produced mainly by *Aspergillus ochraceous*, *A. carbonarius* and *Penicillium verrucosum*. It has been identified as contaminant in a variety of food or food-related materials such as cereals, spices, coffee, beer, wine or beans. Also, due to its long lifetime, it has been found in human blood and breast milk of individuals exposed to contaminated products [36]. The European Committee Regulations (ECR) has established the maximum limits of OTA in cereals at 5 μg·kg^{-1} (raw cereals grains) and 3 μg·kg^{-1} in processed cereals products for direct human consumption (EC) No 123-2005. Some methods based on electrochemical biosensors for the determination of OTA in different matrices are summarized in Table 1 [61–64]. A particularly appealing enzymatic-free design was developed by Bulbul et al. [62] for OTA determination involving the synergistic contribution of the catalase activity of a nanoceria (nCe) tag and the peroxidase like activity of graphene oxide (GO) used as electrode modifier for amplification purposes. In this strategy, the NH$_2$-terminated aptamer was immobilized covalently using EDC/NHS on the surface of a GO-modified SPCE and a direct competitive assay between the target analyte and the nCe labeled target for the immobilized aptamer was implemented (see Figure 3). The electrochemical signal was generated by monitoring by CV the electrocatalysis of H$_2$O$_2$ oxidation by the nCe particles. The aptasensor provided a linear response to OTA in the 0.15–180 nM range and a LOD of 0.1 nM. In addition, it was successfully applied to the determination of in cereal samples.

An original impedimetric immunosensor for the fast determination of OTA in food samples was recently developed using signal tags by co-immobilization of anti-OTA antibody and amine-terminated dendrimer (PAMAM) onto graphene oxide nanosheets and also combining manganese ion. Upon target OTA introduction, a competitive-type immunoassay was implemented involving the analyte and BSA-OTA immobilized onto a GCE for the capture of anti-OTA antibody immobilized onto GO-PAMAM-Mn^{2+}. Once BSA-OTA-anti-OTA-GO-PAMAM-Mn^{2+} conjugate was formed, the in situ formation of MnO$_2$ was induced via the redox reaction between Mn^{2+} and KMnO$_4$ on the immunosensing platform (Figure 4). The generated MnO$_2$ nanoparticles acted as efficient catalyst for the 4-chloro-1-naphthol oxidation producing an insoluble precipitate. As a result, the R$_{TC}$ of the electrode (given by the diameter of the semicircle as indicated in Figure 4), monitored upon immersing the immunosensor into a [Fe(CN)$_6$]$^{3-/4-}$ solution, decreased with increasing OTA concentration within a dynamic range of 0.1 pg·mL^{-1}–30 ng·mL^{-1}. The developed method was successfully used for the determination of OTA in red wine samples where the maximum permitted level of toxin is 2 μg·kg^{-1} [65]. Good recoveries were obtained in the analysis of samples spiked with OTA at different concentration levels ranging between 5 pg·mL^{-1} and 10 ng·mL^{-1} [63].
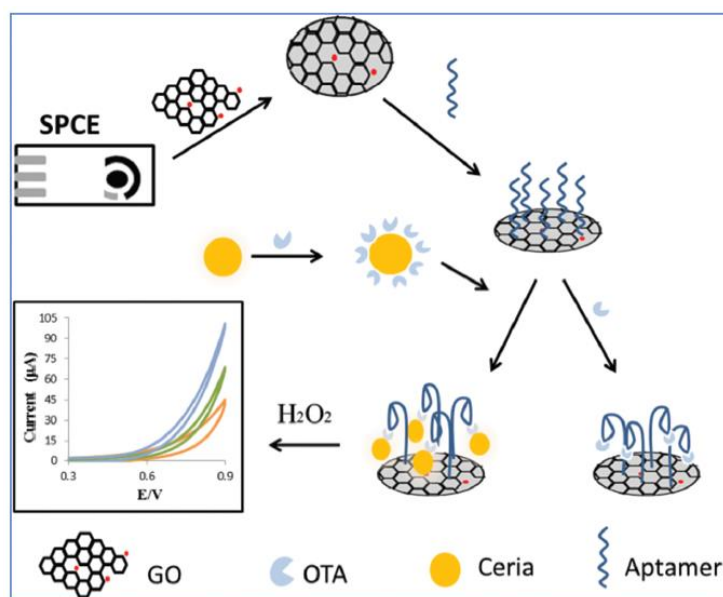
Figure 3. Electrochemical aptasensor developed for Ochratoxin A (OTA) determination involving the use of nanoceria (nCe) particles and graphene oxide (GO) for amplification purposes. Reprinted from [62] with permission.
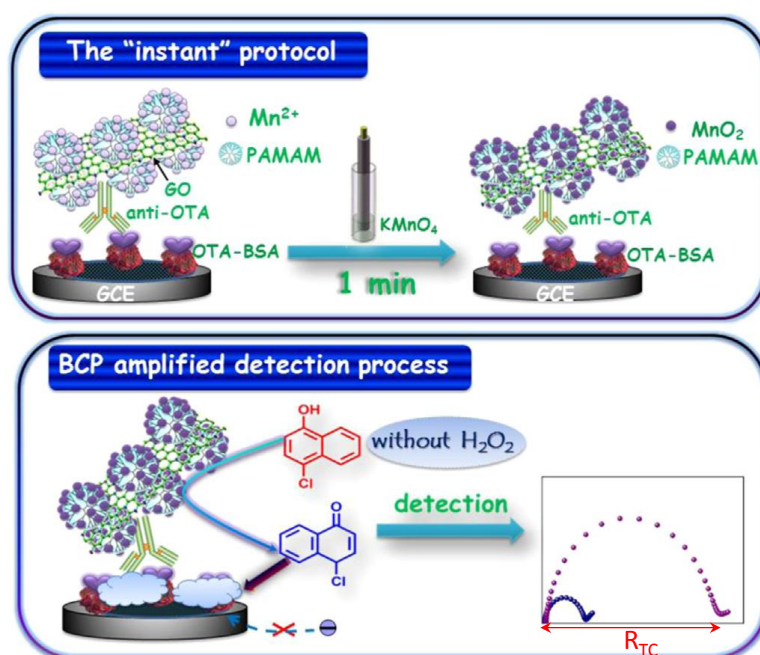

Figure 4. Schematic illustration of the preparation protocol and the catalyst promoted precipitation process of MnO_2 nanoparticles for the amplified impedimetric detection of OTA. Reprinted from [63] with permission.

A disposable electrochemical magneto-immunosensor for OTA was developed using SPCEs and protein G-functionalized magnetic beads for the oriented immobilization of anti-OTA antibody. A direct competitive immunoassay was established between OTA and OTA–HRP, and the immunocomplex formation was evaluated, after the addition of H_2O_2, by amperometry in the presence of hydroquinone as the redox mediator. The method was used for monitoring OTA contamination in coffee samples [61]. Protein-A/G covalently linked to a self-assembled monolayer (SAM) of 4-mercaptobenzoic acid (MBA) onto a gold electrode was also used for anti-OTA immobilization, and the resulting anti-OTA-protein

A/G-MBA-AuE oriented immunosensor was compared with the non-oriented anti-OTA-MBA-AuE configuration for implementing impedimetric immunosensors. In the non-oriented configuration, the capture antibodies were covalent immobilized onto the MBA SAM previously activated with EDC/NHS but omitting the addition of Protein A/G. In this case the capture antibodies were covalently immobilized on the gold electrodes but oriented binding of antibodies through their Fc region provided by Protein A/G leaving their Fab region exposed to the solution and free to bind the antigen is missing. A lower limit of detection (5 pg·mL^{-1}) for the non-oriented immobilization was found, but the oriented anti-OTA configuration exhibited a longer linear range (0.01–5 ng·mL^{-1} OTA) and a higher sensitivity, with a slope value of 26.45 kΩ·mL·ng^{-1}. These results agreed with the more uniform and homogeneous antibody layer in the oriented immunosensor that favors higher antigen-binding capacity and sensitivity. Consequently, the oriented design was used for application to real samples, and was specifically validated by analyzing spiked cocoa bean samples [64].

As it is known, aptamers are capable of specifically binding providing highly sensitive techniques for on-site detections. An aptamer-based biosensor was also developed for the determination of OTA in wheat samples. The system involved thionine (THI)-labeled OTA aptamers which were attached to the surface of graphene oxide (GO) nanosheets through the strong non covalent binding of GO with nucleobases and aromatic compounds. Initially, THI-OTA-aptamer/GO composites were suspended in the detection solution far away from the electrode. Then, upon addition of target OTA, the analyte reacted with the aptamer and caused the dissociation of THI–aptamer from GO nanosheets (Figure 5). The formed THI-aptamer/OTA could be cleaved by deoxyribonuclease (DNaseI), an endonuclease that digests double and single stranded DNA into oligo and mononucleotides, releasing OTA which could retrigger THI-aptamer/GO nanocomposite with target recycling to generate numerous free THI molecules. These molecules were captured by negatively charged SPCE producing an electrochemical signal at the applied potential. The electrochemical responses of THI at the SPCE were used as the analytical signals to be related with OTA concentrations from values as low as 5.6 pg·mL^{-1} [66].

Bacterial toxins, toxic substances produced and released by bacteria to target other bacterial or host cells, have also been determined using electrochemical affinity biosensors [67]. For example, a sandwich-type electrochemical immunoassay for ultrasensitive detection of *Staphylococcal enterotoxin* B (SEB) in food was developed using HRP-nanosilica-doped multiwalled carbon nanotubes (HRPSiCNTs) for signal amplification [68]. Rabbit polyclonal anti-SEB antibodies immobilized on the screen-printed carbon electrode (SPCE) and covalently bound to the HRPSiCNTs were used as capture antibodies and detection antibodies, respectively. The electrochemical transduction is based on the DPV measurement on the enzymatic reduction of H_2O_2 in the presence of thionine. This approach exhibited a dynamic range of 0.05–15 ng·mL^{-1} with a LODof 10 pg·mL^{-1} SEB and provided results comparable to a commercial ELISA kit in the analysis of different spiked samples (watermelon juice, soymilk, apple juice, and pork food). Other immunosensor was developed for the determination of *Staphylococcal enterotoxin* A (SEA) in cheese by assembling a cysteamine SAM onto a gold electrode followed by covalent immobilization of protein A and the specific capture antibody. The captured SEA was sandwiched with an HRP-labeled detector antibody and amperometric transduction in the presence of H_2O_2 and HQ was used to follow the immunoreactions. This amperometric immunosensor, which provided a linear calibration curve from 0.016 to 0.150 μg·mL^{-1} and a LOD of 33.9 ng·mL^{-1} was able to discriminate between SEA contaminated and non-contaminated cheese samples [69]. Wu et al. [70] developed an electrochemical immunosensor to detect staphylococcal enterotoxin B (SEB) based on the use of bio-magnetosomes, polyaniline nano-gold composite and 1,2-dimethyl-3-butylimidazolium hexafluorophosphate [D(n-C4)Im][PF6] ionic liquid. In this approach the SEB immuno-magnetosomes were deposited on a gold electrode previously modified with a polyaniline nano-gold composite (PANI/Au) and the ionic liquid. The immunoreaction between the immuno-magnetosomes and the SEB was followed by the increase in RTC measured by EIS in the presence of $Fe(CN)_6^{3-/4-}$. This immunosensor showed good linear response in the range from 0.05 to 5 ng·mL^{-1}, a LOD of 0.017 ng·mL^{-1} and high recoveries in spiked milk samples (81%–118%).

Table 1. Electrochemical affinity biosensors for food toxins.

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
MWCNTs/ODT/AuE	Aptasensor/covalent immobilization of aptamer; direct detection of target-induced conformational change with methylene blue as indicator	saxitoxin/mussels	DPV	0.9–30 nM	0.38 nM	[28]
Cys-PDIC/AuE	Aptasensor/ competitive indirect with BTX-2 immobilized and BTX-10 aptamer mixed with BTX-2 analyte	BTX-2/shellfish extracts	EIS	0.1–100 ng·mL^{-1}	106 pg·mL^{-1}	[33]
AuNPs/GCE	Immunosensor/competitive indirect/immobilized BSA-BTB; anti-BTB/MSC/THI/PdNS as signal tag	BTB/asian mussel	DPV	0.01–10 ng·mL^{-1}	5.0 pg·mL^{-1}	[31]
SPCE	Immunosensor/competitive indirect/automated flow; OA-MBs/SPCE and anti-OA mixed with OA analyte; addition of AP-anti-OA/1-NPP	okadaic acid/mussels	FI-amperometry	0.19–25 μg·L^{-1}	0.15 μg·L^{-1}	[34]
AuE	Immunosensor/ direct with anti-OA-MBs and [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	okadaic acid/mussel	DPV	10–100 ng·mL^{-1}	0.5 μg·L^{-1}	[36]
graphene/SPCE	Immunosensor/covalent immobilization of anti-OA; competitive assay between OA and OA-OVA; [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	okadaic acid/spiked shellfish tissue, mussel	SWV	up to 5000 ng·L^{-1}	19 ng·L^{-1}	[37]
ITO	Multiplex immunosensor/ anti-BTX-2 and anti-DTX-1 co-immob. on MB; competitive assay CdNC/BSA/BTX-2 and CuNC/BSA/DTX-1	BTX-2 and DTX-1/seafood	SWASV	0.005–5.0 ng·mL^{-1}	1.8 pg·mL^{-1} (BTX-2) 2.2 pg·mL^{-1} (DTX-1)	[38]
graphene/Chit/GCE	Immunosensor/competitive between immobilized MC-LR and MC-LR conjugated to anti-MCLR- HRP-CNS-Ab2	MC-LR/water	DPV	0.05–15 μg·L^{-1}	0.016 μg·L^{-1}	[45]
CNT@Co/ITO	Immunosensor/ competitive indirect/ immobilized MC-LR and signal amplification with Ab$_2$-HRP/Fe$_3$O$_4$@PDA-AuNPs	MC-LR/water	CV	0.05–50 μg·L^{-1}	0.004 μg·L^{-1}	[46]
MWCNTs/Si	Immunosensor/ competitive indirect/ immobilized MC-LR and MC-LR conjugated to anti-MCLR	MC-LR/water	EIS	0.05–20 μg·L^{-1}	0.04 μg·L^{-1}	[48]
graphene/SPCE	Aptasensor/immobilization of aptamer; direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	MC-LR/fish extracts, water	SWV	1 nM–10 nM	1.9 pM	[49]
Chit/AuNPs/AuE	Immunosensor/ immobilization of anti-AFB; direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	AFB1/wheat	CV	0.2–2 ng·mL^{-1} 2–30 ng·mL^{-1}	0.12 ng·mL^{-1}	[57]
AuNPs/DPB/GO/AuE	Immunosensor/ anti-AFB immobilization, addition of IL and Chit	AFB1/peanut, rice, soybean, milk, flour	EIS	3.2 fM–0.32 pM	1 fM	[58]

Table 1. *Cont.*

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
SWCNTs/Chit/GCE	Immunosensor/competitive indirect between free AFB1 and AFB1-BSA. Addition of AP-IgG and 1-NPP substrate	AFB1/corn powder	DPV	0.01–100 ng·mL^{-1}	3.5 pg·mL^{-1}	[59]
MWCNTs/IL	Immunosensor/ Anti-AFB1 immobilization. Direct detection with $[Fe(CN)_6]^{3-/4-}$ as the redox probe	AFB1/oil	EIS	0.1–10 ng·mL^{-1}	0.03 ng·mL^{-1}	[60]
GO-modified electrode	Aptameric sensor/direct competitive between OTA and OTA labeled with a nanoceria (nCe) tag	OTA/Spiked corn samples	CV (H_2O_2 oxidation)	0.15–180 nM	0.1 nM	[62]
GCE	Immunosensor/competitive. OTA-BSA immobilized; anti-OTA/GO/PAMAM/Mn^{2+} as signal label. MnO$_2$ generation and 4-CN precipitation	OTA/wine	EIS	0.1 pg·mL^{-1}– 30 ng·mL^{-1}	0.55 pg·mL^{-1}	[63]
SPCEs	Immunosensor/immobilization of anti-OTA onto protein G-MBs and competitive assay between OTA and HRP-OTA	OTA/coffee	amperometry	1.3–153.8 µg·mL^{-1}	0.32 µg·mL^{-1}	[61]
Protein A/G-MBA-AuE	Immunosensor/immobilization of anti-OTA. Direct detection with $[Fe(CN)_6]^{3-/4-}$ as the redox probe	OTA/cocoa beans	EIS	0.01–5 ng·mL^{-1}	0.01 ng·mL^{-1}	[64]
SPCE	Aptasensor/dissociation of THI-OTA aptamer-GO in the presence of OTA; cleavage by DNaseI of THI-aptamer OTA; free THI detection	OTA/wheat	DPV	0.01–50 ng·mL^{-1}	5.6 pg·mL^{-1}	[65]
SPCE	Immunosensor/sandwich type using antibody conjugated with HRP-nanosilica-doped MWCNTs (HRPSiCNTs) as advanced labels	SEB/watermelon juice, soymilk, apple juice, and pork food	DPV	0.05–15 ng·mL^{-1}	10 pg·mL^{-1}	[68]
ProteinA/Cyst/AuE	Immunosensor/sandwich type immunoassay by immobilization of capture antibody and conjugation with HRP-anti-SEA	SEA/cheese	Amperometry	16–150 µg·mL^{-1}	33.9 ng·mL^{-1}	[69]
[D(n-C4)Im][PF6]/ (PANI/Au)-AuE	Immunosensor/direct immunoassay by immobilization of SEB immuno-magnetosomes	SEB/milk	EIS	0.05 to 5 ng·mL^{-1}	0.017 ng·mL^{-1}	[70]

Key: AFB: aflatoxinB; ASV: anodicstrippingvoltammetry; AuE: goldelectrode; AuNPs: goldnanoparticles; BTB: brevetoxin B; BTX: brevetoxin; Chit: chitosan; CV: cyclic voltammetry; Cys: cysteamine; DPB: 2,5-di-(2-thienyl)-1-pyrrole-1-(p-benzoic acid); [D(n-C4)Im][PF6]: 1,2-dimethyl-3-butylimidazolium hexafluoro phosphate; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; FI: flow injection; HRPSiCNTs: HRP-nanosilica-doped multi-walled carbon nanotubes; IL, ionic liquid; ITO: indium-tin oxide electrode; LSV: linear sweepvoltammetry; MBA: 4-mercaptobenzoic acid; MBs: magneticbeads; m-GEC: magneticgraphite-epoxycomposite; MSC: mesoporouscarbon; MWCNTs: multi-walledcarbonnanotubes; NPP: naphthylphosphate; OA: okadaicacid; ODT: octanedecanethiol; OTA: ochratoxin; OVA: ovalbumin; PANI: polyaniline; PDA: polydopamine; PDCI: 1,4-phenylene diisocyanate; QD: quantum dots; SEA: Staphylococcal enterotoxin A; SEB: Staphylococcal enterotoxin B; SPCE: screen-printedcarbonelectrode; dSPCE: dualscreen-printedcarbonelectrodes; SWV: square-wave voltammetry; SWASV: square-wave anodic stripping voltammetry; THI, thionine.
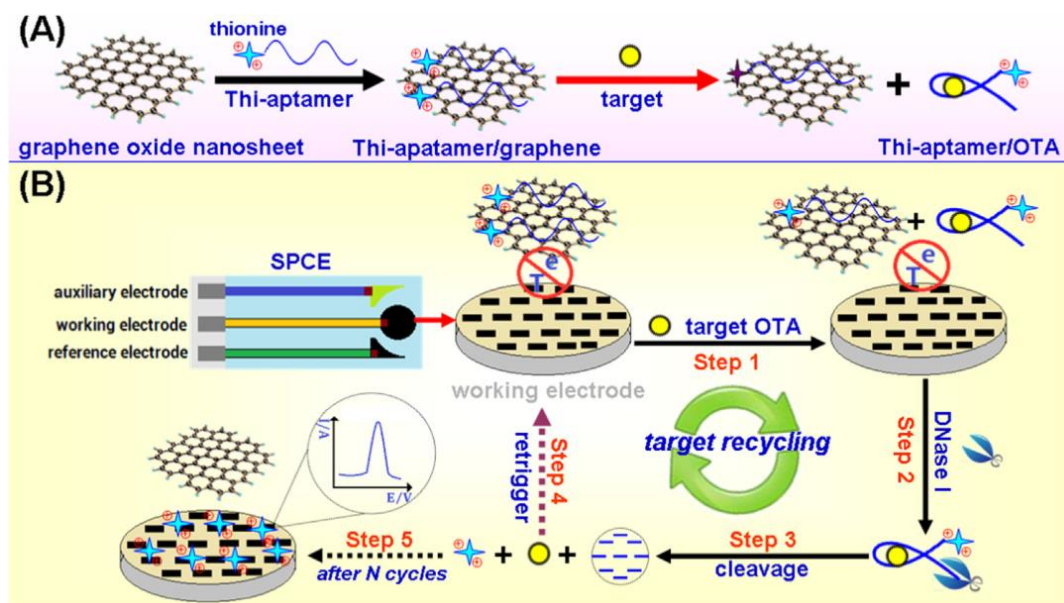
Figure 5. Scheme of GO nanosheet-based homogeneous aptasensing system for detection of OTA on negatively charged SPCEs by coupling with DNaseI-based target recycling reaction and thionine-labeled OTA aptamer: target-induced dissociation of thionine–aptamer from GO nanosheet (**A**) and DNaseI-triggered target recycling detection (**B**). Reprinted from [66] with permission.

3. Electrochemical Affinity Biosensors for Pesticides

Contamination by pesticides, which are nowadays an integral part of intensive agriculture, may negatively affects food safety [71]. Pesticide residues in foods and organic produce adversely affect human health because of their high biological activity and inherent toxicity. Most countries have establishedmaximum residue levels (MRLs) of pesticides in food and animal feed (http://www.ecpa.eu/faq/what-maximum-residue-level-mrl-andhow-are-they-set). According to the status list of active substances available commercially in the EU, more than 1100 pesticides are currently registered. Increasing public concern in recent years about the possible health risk of pesticide residues in the diet has profoundly modified crop-protection strategies, with emphasis on food quality and safety. Widespread concern for the health of society has led to the strict regulation of maximum residue limits (MRL) of pesticide residues in food [72]. The guidelines reported by the European Commission for the 2050 food system comprise not only the development of effective integrated approaches to establish, promote and support a sustainable food chain, but also including measures to ensure integrity in terms of food safety and quality [73]. Electrochemical biosensors constitute a valuable tool for achieving this goal as evidenced by the high number of affinity configurations developed so far. Some relevant examples have been summarized in Table 2 [74–84].

Organophosphorous insecticides are characterized by the high activity, the relatively low persistence, and the high toxicity. The need to determine these compounds at low concentrations in a variety of food samples has led to the development of several electrochemical immunosensors. For example, Wei et al. [74] fabricated a disposable amperometric immunosensor for detection of chlorpyrifos-methyl, a persistent insecticide widely used in farming and horticulture. A PtNPs-silica sol-gel hybrid matrix was prepared onto SPCEs surface and a competitive immunoassay was established by immobilizing the antigen onto the modified electrode to effectively capture the unbound HRP-labeled antibody in the sample solution. The determination was performed by DPV after adding H_2O_2 in the presence of o-phenylenediamine. A linear response to chlorpyrifos-methyl concentration ranging from 0.4 to 20 ng·mL^{-1} was reported and recoveries from 96.4% to 109.3% were obtained in the analysis ofgrape and soils samples spiked with three different concentrations of the insecticide. This same insecticide

was determined by Jia et al. [75] with an impedimetric immunosensor using a microfluidic chip. A gold interdigitated microelectrode array modified with poly(diallyl-dimethyl-ammonium) (PDDA) and AuNPs was embedded in the microchannel of the microfluidic chip, and protein A was employed to immobilize an antibody's fragment for chlorpyrifos. The immunocomplex formation was monitored by the change in the relative impedance in the presence of $[Fe(CN)_6]^{3-/4-}$ solution. The developed method, with a limit of detection of 0.5 ng·mL^{-1}, was applied to the determination of the insecticide in vegetables.

　　Coumaphos is another organophosphorus pesticide used worldwide to control pests although it has been associated with some types of cancer. As also occur for other analytes, the small size of coumaphos and the difficulty to produce two distinct epitopes that can be bound simultaneously by antibodies, immunosensors for this compound mostly involve a competitive format. However, these types of immunoassays usually exhibit low sensitivity which, in the case of pesticide residues, is not enough for determination in food samples. Taking this into account, Dai et al. [76] prepared and efficient approach for the sensitive determination of coumaphos by means of an electrochemical displacement immunoassay coupled with oligonucleotide sensing. A gold substrate was used to immobilize guanine-rich single strand DNA-labeled monoclonal antibody (mAb) specific for coumaphos and the coating antigen (Figure 6). Since the binding affinity of coating antigen to antibody is 1346-fold lower than that of coumaphos, the anchored guanine-rich single strand DNA-labeled antibody was displaced rapidly and efficiently after the addition of the analyte by the formation of a displacement complex. An ITO chip electrode modified with capture ssDNA was used to hybridize ssDNA from the displacement complex, and for coumaphos detection by the enhanced catalytic cycle of guanine-Ru(bpy)$_3^{2+}$ which provided amperometric currents related to insecticide concentrations in the 0.5–80 ng·L^{-1} range, with a limit ofdetection down to 0.18 ng·L^{-1} (4.96×10^{-13} M). The method was applied to the determination of coumaphos in milk samples with good recoveries.
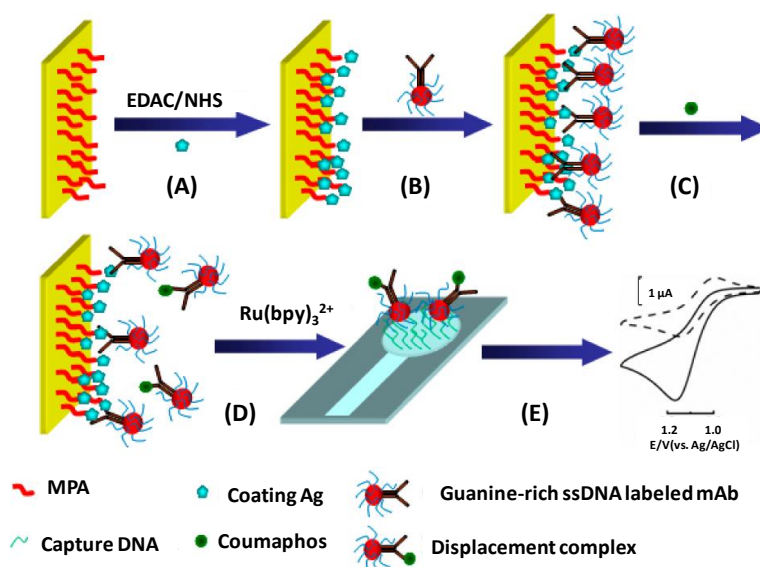

Figure 6. Steps involved in the determination of coumaphos by electrochemical displacement immunoassay coupled with oligonucleotide sensing: (**A**) immobilization of coating antigen through a 3-mercaptopropionic acid self-assembled monolayer (MPA SAM); (**B**) addition of guanine-rich ssDNA-labeled mAb to form the immunosubstrate; (**C**) addition of coumaphos and displacement of guanine-rich ssDNA-labeled mAb to form the displacement complex; (**D**) addition of Ru(bpy)$_3^{2+}$ and hybridization of the displacement complex to the ssDNA/ITO; (**E**) CV electrochemical detection. Reprinted and adapted from [76] with permission.

Table 2. Electrochemical affinity biosensors for pesticides.

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
PtNPs/SiO$_2$ SPCE	Immunosensor/competitive indirect; BSA-CM immobilization and capture of unbound anti-CM-HRP	chlorpyrifos-methyl (CM) /grapes	DPV	0.4–20 ng·mL^{-1}	22.6 ng·L^{-1}	[74]
Protein A/AuNPs /PDDA/IDAM	Immunosensor/microfluidic chip. Immobilization of capture antibody. Direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	chlorpyrifos/ cucumber, lettuce	EIS	0.5–500 ng·mL^{-1}	0.5 ng·mL^{-1}	[75]
MPA/ITO chip	Immunosensor/electrochemical displacement immunoassay and oligonucleotide sensing; antigen immobilization + ssDNA-Ab + antigen + displacement + Ru(bpy)$_3$$^{2+}$ and hybridization	coumaphos/milk	amperometry	0.5 to 80 ng·L^{-1}	<0.18 ng·L^{-1}	[76]
FDMA- or PQQ-SWCNTs/GCE	Immunosensor/immobilization of the respective antigens and antibodies. Direct detection	endosulfan/water paraoxon/water	SWV	0.05–100 ng·mL^{-1} 2–2500 ng·mL^{-1}	0.05 ng·mL^{-1} 2 ng·mL^{-1}	[77]
(DpAu/DMDPSE)n/AuE	Immunosensor/anti-carbofuran immobilization and direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	carbofuran/lettuce, strawberries, tomatoes	DPV	0.1–10^6 ng·mL^{-1}	0.06 ng·mL^{-1}	[78]
FDMA-SWCNTs/GCE	Immunosensor/ immobilization of endosulfan and antibody. Direct detection	endosulfan/water	SWV	0.01–20 ng·mL^{-1}	0.01 ng·mL^{-1}	[79]
AuNPs/AuE	Aptasensor/ immobilization of aptamer and direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	acetamiprid/tomatoes	EIS	5–600 nM	1 nM	[80]
AuNPs/MWCNT/rGONR	Aptasensor/ immobilization of aptamer and direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	acetamiprid/water	EIS	5×10^{-10}–10^{-5} M	7×10^{-14} M	[81]
AuNPs/AuE	Immunosensor/immobilization of antibody and direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	atrazine/maize	DPV	0.05–0.5 ng·mL^{-1}	0.016 ng·mL^{-1}	[82]
GA/Chit/GCE	Immunosensor/immobilization of antibody and direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	fenvalerate/tea	EIS	1.0–10^4 µg·L^{-1}	0.80 µg·L^{-1}	[83]
GEC	Immunosensor/immobilization of CdS-labeled-anti-PQ and PQ-MBs. Cd dissolution and detection of Cd^{2+} by current reduction	paraquat (PQ)/potatoes	Culombimetry	3.08–67.76 µg·kg^{-1}	1.4 µg·kg^{-1}	[84]

Key: Chit: chitosan; DpAu: deposited Au nanocrystals; DMSPSE: 4,4´-thiobisbenzenethiol; FDMA: ferrocenedimethylamine; GA: glutaraldehyde; GEC: graphite composite electrode; IDAM: gold interdigitated microelectrode array; ITO: indium-tin oxide electrode; MPA: 3-mercaptopropanoic acid; PDDA: poly(diallyl-dimethyl-ammonium); PQQ: pyrroloquinoline quinone; rGONR: reduced graphene oxide nanoribbon.

A forest of SWCNTs patterned onto GCE was fabricated for the simultaneous detection of endosulfan and paraoxon. Ferrocene dimethylamine (FDMA) and pyrroloquinoline quinone (PQQ) were attached to the SWCNTs, followed by the immobilization of the respective antigens and antibodies. Based on the different electrochemistry signals from the two redox probes, modulated by the binding events, the changes in the SWV peak currents at +0.3 V (FDMA) and −0.2 V (PQQ) provided calibration plots for endosulfan and paraoxonon the 0.05–100 ng·mL^{-1} and 2–2500 ng·mL^{-1} concentration ranges, respectively [77]. A label-free amperometric immunosensor for the determination of carbofuran residues in vegetables and fruits was constructed by modifying a gold electrode with gold nanocrystals/4,4′-thiobisbenzenethiol multilayers by layer-by-layer self-assembly. The immunoreaction between anti-carbofuran antibody and carbofuran was followed by DPV in the presence of [Fe(CN)$_6$]$^{3-/4-}$. The peak current measured by DPV decreased with increasing carbofuran concentrations due to more carbofuran binding to the immobilized antibodies, which act as a barrier for the electron transfer. Under the optimized conditions, a linear relationship between the relative change in DPV peak current and the logarithm of carbofuran solution was obtained in the 0.1 to 1.0–10^6 ng·mL^{-1} range, with a detection limit of 0.06 ng·mL^{-1}. These characteristics allowed the application of the method to the analysis of lettuces, tomatoes, or strawberries among other samples [78].

Liu et al. [79] also prepared a GCE modified by electrografting with a mixed layer of 4-aminophenyl and phenyl for further anchor SWCNTs vertically. Ferrocene-dimethylamine (FDMA) was subsequently attached to the ends of nanotubes (Figure 7) followed by incorporation of endosulfan hapten for antibody binding. SWV was applied to detect changes in the ferrocene voltammetric responses related with the interaction of the sensing interface with the antibody.
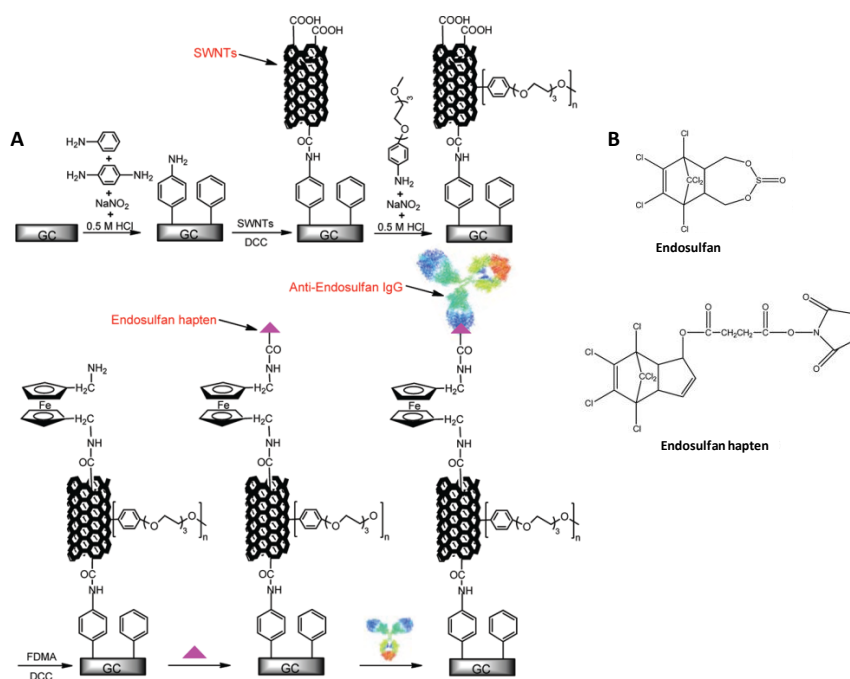

Figure 7. (**A**) Scheme of the steps involved in the preparation of single-walled carbon nanotubes (SWCNTs)-modified biosensing platform for the detection of endosulfan; (**B**) Structures of one isomer of endosulfan and its hapten. Reprinted from [79] with permission.

As it can be deduced for data summarized in Table 2, several recently reported immunosensors and aptasensors for pesticides make use of electrochemical impedance spectroscopy as electrochemical transduction technique. For example, Fan et al. [80] developed an aptasensor for the detection of acetamiprid, a new neonicotinoid systemic broad-spectrum insecticide used to replace organophosphorous and other insecticides. A gold electrode modified with AuNPs was used to immobilize the aptamer and EIS was employed to measure the changes in the electron transfer resistance related with complexation

with different analyte concentrations. The method was applied to determine acetamiprid in real samples such as tomatoes where the MRL value for this active substance is 0.5 mg·kg^{-1} (Part A of Annex I of Regulation (EC) No 396/2005). The characteristics of other methods based on electrochemical affinity biosensors for the detection of acetamiprid [81], atrazine [82], fenvalerate [83] or paraquat [84] are also summarized in Table 2.

4. Electrochemical Affinity Biosensors for Allergens

Food allergy, a type of adverse reaction to food, dependent on the susceptibility of the host, is produced by an immunological mechanism resulting from ingestion, contact or inhalation of certain substances presented in food known as allergens (mainly proteins from nuts and tree nuts, fish, shellfish, wheat, soy, eggs or milk [85]). Since the contact with food processing surfaces or sharing food utensils could constitute inadvertent routes of oral exposure to allergens, it is highly recommended to perform allergen screening, apart from food, in sanitation activities. Nowadays, food allergy is considered a problem of public-health relevance with the main concern being the unintentional exposure of allergic consumers to the offending ingredient through allergen containing food. It affects 2%–3% of adults and up to 10% of children in industrialized countries with the majority of allergies persisting through lifetime [85]. Since there is no cure for allergies yet, the strict elimination of allergens from the diet and the availability of reliable and fast methods for detection and quantification of food allergens are the only options to ensure compliance with food labeling and improve consumer protection [86]. The "food allergy" working group of the German Society of Allergology and Clinical Immunology and the Association of German Allergologists proposed limits of 10–100 mg·kg^{-1} of the allergenic food or 1–10 mg·kg^{-1} of the protein fraction of the allergenic food (depending on its allergenicity) to protect most sensitized consumers downloaded by from severe allergic reactions. Lower values could be applied to highly allergenic foods (e.g., peanut) [87].

At present, the main analytical techniques used to detect food allergens can be classified into protein-based or DNA encoding allergen proteins-based assays. The former detect either a specific target food allergen, mainly using enzyme-linked immunosorbent assays (ELISAs), or total soluble allergenic proteins. On the other hand, DNA-based techniques detect the presence of allergens by using a specific DNA fragment of the encoding gene for the target allergenic protein which is in many cases amplified through polymerase chain reaction (PCR) [88]. However, false positive results (due to cross-reactivity by co-existing proteins), the large variability in quantitative results found between available ELISA kits and the high number of replicates for samples or an external standard required by all the PCR methods to minimize the potential tube-to-tube variations and the variability in nucleic acids extraction in the case of complex multi-compound matrices such as food products which may result due to the exponential nature of DNA amplification in significant variation between replicates and various sample types [89], have hindered the application of these methods to processed foods or complex food matrices. Additional limitations of ELISAs for food allergens detection include the possibility to evade detection in some ELISA formats since some proteins may be degraded during food processing steps implying heating or fermentation [90,91].

Furthermore, even though some level of automation has been achieved in the recent years, ELISAs remain laborious, time consuming and expensive, particularly when multiple targets need to be screened [86]. An additional limitation of DNA-based methods consists of the likelihood to find residual DNA encoding for the allergenic protein in the final processed food, with the PCR sensitivity strongly depending on the amount and the quality of the isolated DNA, particularly challenging in case of complex multi-compound matrices such as food products [88,89].

Therefore, there is an urgent need to improve the robustness of the available analytical methods and to develop new standardized methods which must be fast, more sensitive, more accurate and more specific in order to achieve better reliability to allow unambiguous identification of the allergens [91]. Among these, nowadays electrochemical biosensors have been well established as interesting alternatives to conventional methods for detection of allergens [86,92].

Table 3 summarizes the relevant analytical characteristics of the electrochemical affinity biosensors reported in the last 5 years for allergens determination. They are classified according to the type of used bioreceptor. The bioreceptors most commonly used are specific antibodies [93–104], single-stranded DNA molecules [105–108], aptamers [109–112], and other affinity receptors like lectins [113] and peptides [114]. The affinity reaction between these bioreceptors, which are immobilized onto electrodes or solid supports like magnetic beads (MBs), and the target allergen has been monitored using (chrono)amperometry [68,94,97,99,101–104,109], voltammetry [93,95,96,98,102,103,106,107,112–114] and electrochemical impedance spectroscopy [105,110,111].

Regarding immunosensors, particularly useful biosensors involving the use of functionalized MBs have been described for the determination of gliadin [94], β-lactoglobulin [97], ovalbumin [98], Ara h 1 [99,103], Ara h 2 [103] and α-lactalbumin [104]. Most of the approaches employ sandwich-type configurations implemented onto carboxylic acid-modified magnetic beads (HOOC-MBs) and SPEs to perform an amperometric transduction [97–99,103,104]. These approaches demonstrated very attractive performance and successful applicability to the analysis of real samples such as spiked skimmed milk and beer [94], milk [97,99], infant formulations [104] food extracts [99,103] and saliva [100]. Among them it is worth to highlight the immunosensor reported recently by Ruiz-Valdepeñas Montiel et al. [103] for the rapid and simultaneous detection of Ara h 1 and Ara h 2 (the two major peanut allergenic proteins). It involves a sandwich immunoassay using selective capture and detector antibodies, HOOC–MBs and amperometric detection at dual screen-printed carbon electrodes (SPdCEs) by using the H_2O_2/hydroquinone (HQ) system. The method exhibited high sensitivity and selectivity for the target proteins providing detection limits of 18.0 and 0.07 ng·mL^{-1} for Ara h 1 and Ara h 2, respectively, in 2 h. It also showed successful applicability for the determination of the endogenous levels of both allergenic proteins in different food extracts, with similar results to those obtained by means of commercial spectrophotometric ELISA kits. The developed methodology was also able to carry out the unequivocal identification of wheat flour samples spiked with only 0.0001% (*w/w*) of peanuts.

Integrated immunosensors in connection with the use of nanomaterials as modifiers of electrode surfaces for amplification purposes have been used for allergens determination. A GCE functionalized with AuNPs and poly(l-Arginine)/multi-walled carbon nanotubes composite film was reported by Cao et al. [93] to develop an immunosensor applicable to the determination of casein(together with β-lactoglobulin one of the two main allergens involved in allergy to cow's milk)in cheese samples. Monitoring of the antigen-antibody interaction using DPV in the presence of $[Fe(CN)_6]^{4-/3-}$, allowed a LOD of 5×10^{-8} g·mL^{-1} to be achieved. Immunosensors for β-lactoglobulin [94] and OVA [96] were developed by electro-grafting an aryl diazonium salt organic film on graphene-modified SPCEs. The capture antibody was covalently immobilized using EDC/NHS chemistry or glutaraldehyde on the carboxyphenyl [96] or nitrophenyl [95] groups of the film grafted onto the graphene electrodes by electrochemical reduction of the in situ generated diazonium salt. The decrease in the $[Fe(CN)_6]^{3-/4-}$ reduction peak current, measured by DPV after the immunological reaction, was used for the target allergen quantification. Both immunosensors provided LODs in the low pg·mL^{-1} level (0.85 and 0.83 pg·mL^{-1} for β-lactoglobulin and OVA, respectively) and demonstrated applicability to the analysis in real food samples (cake, cheese snacks and sweet biscuits). More recently, Alves et al. developed sandwich immunosensors for Ara h 1 [100] and Ara h 6 [101] determination onto AuNPs-SPCEs monitoring electrochemically the antibody–antigen interaction through stripping analysis of enzymatically (using alkaline phosphatase) deposited silver. These methods provided LODs of 0.27 and 3.8 ng·mL^{-1} for Ara h 6 and Ara h 1, respectively, and were successfully applied to complex food matrices.

Regarding DNA sensors, all the reported works use specific thiolated probes self-assembled on gold electrodes [105] or AuNPs-modified electrodes [106–108]. While linear DNA probes were used for the implementation of sandwich hybridization assays for the determination of Ara h 2 [107] and α2-gliadin [108], direct assays were implemented for Ara h 1 determination using dual-labeled

stem-loop probes [105,106]. The strategy developed by Martin-Fernández et al. [108] is based on sandwich hybridization of target DNA with a thiolated capture probe immobilized together with MCH onto Au-SPEs and a fluorescein isothiocyanate (FITC)-modified detector probe. After labeling the FITC-modified sandwich duplex with an antiFITC-HRP conjugate, the hybridization event was electrochemically monitored by chronoamperometry upon addition of tetramethylbenzidine (TMB) and H_2O_2. This methodology, applied to the determination of a PCR amplified fragment encoding the immunodominant peptide of α2-gliadin, was able to detect selectively as low as 0.001% (*w*/*w*) of wheat flour (0.9 ppm of gluten), with a sensitivity similar to that provided by real-time PCR methodologies, and to perform the determination in highly processed food samples.

A hairpin probe dually labeled with thiol and biotin was used both as capture and signaling probe by Sun et al. [106] for determination of Ara h 1 gene. The stem-loop probe was assembled through the thiol moiety onto a multilayer graphene–gold nanocomposite, able to amplify the electrochemical signal measured by DPV in the presence of $[Fe(CN)_6]^{4-/3-}$ due to its high electron-transfer efficiency, electrodeposited onto a GCE. The hairpin probe was initially immobilized in a folded state with the biotin unit in proximity to the electrode surface, but changed its conformation to an open state upon target DNA hybridization and, accordingly, an "on−off" change of the electrochemical signal was observed as a consequence of the increased distance between the biotin moiety and the electrode surface, which hindered the electron transfer. The biosensor exhibited a linear range for the target DNA between 10^{-16} and 10^{-13} M, a LOD of 0.041 fM, an excellent selectivity even to discriminate a single base mismatch and successfully applicability to the determination in a peanut milk beverage.

Furthermore, some attractive aptamer-based sensors have been reported for allergen determination. Amaya-González et al. developed a MBs-based aptameric method for the determination of gliadin [109]. This protein is present in wheat and other cereals and, as a component of gluten, is involved in celiac disease. EU regulations allow labeling as "gluten free" those products whose gluten concentration does not exceed 20 ppm [110]. In the proposed method, a direct competitive strategy between specific biotinylated peptide immobilized on the surface of Strep-MBs and a free biotinylated-aptamer for the target gliadin was established. In the presence of increasing concentrations of gliadin, the amount of free biotinylated aptamer available to bind the peptide immobilized on the MBs surface decreased resulting in a smaller analytical signal, measured by chronoamperometry using TMB/H_2O_2 after labeling the biotinylated aptamer attached to the MBs with Strep-HRP. The method allows the determination of as low as 0.5 ppb gliadin standard (0.5 ppm of gluten). Moreover, a direct approach for the impedimetric determination of Ara h 1 was developed by self-assembling a thiolated-80-base DNA aptamer on a gold-disk electrode [111]. This approach provided a relatively narrow linear range (0.1–1.8 μM) and a C50 value of 1.8 μM. Aptamer sensors, using direct and sandwich formats, were also developed for lysozyme determination [112,113]. Among other applications, lysozyme from egg-white is widely used to mitigate or prevent heterolactic fermentation in wines, the maximum concentration allowed being regulated by the International Organization of Vine and Wine (OIV) in 500 mg·L^{-1} (~35 μM) [114]. Covalent immobilization of the specific aptamer onto SPCEs using diazonium salt chemistry was used for direct and sandwich assays. The direct assay provided a linear range between 0.025 and 0.8 mM, and a LOD of 25 nM using impedimetric detection [112]. A more sensitive strategy was achieved by using a sandwich format and enzymatic amplification [113]. In this case, the target protein was sandwiched between the NH_2-terminated capture aptamer covalently immobilized onto SPCE via diazonium salt chemistry and a biotinylated detector antibody. After labeling with avidin-alkaline phosphatase (AP), the oxidation signal of 1-naphthol generated by AP hydrolysis of the 1-naphthyl phosphate substrate was measured by DPV. This aptasensor offered a wide linear range (5 fM–5 nM) and a very low LOD (4.3 fM). Both approaches demonstrated successful application to the analysis of spiked wine samples.

Less common affinity bioreceptors have been also used to develop electrochemical biosensors for the determination of food allergens. So, an electrochemical biosensor for the direct detection of chicken ovomucoid (CHOM) was reported by using Concanavalin A as recognition element and

ZnO quantum dots (QDs) bioconjugates synthetized by self-assembling of CHOM onto ZnO QDs through electrostatic interaction [115]. The prepared ZnO-QD/CHOM bioconjugates were captured by the Concanavalin A (Con A) immobilized on silicon substrates, the extent of the biorecognition being monitored after acidic dissolution by means of the zinc square wave voltammetric (SWV) peaks of captured QDs at a GCE (Figure 8). The voltammetric peak current was linear with the CHOM concentration in the 1–140 ng·mL^{-1} range and provided a LOD of 0.1 ng·mL^{-1}.
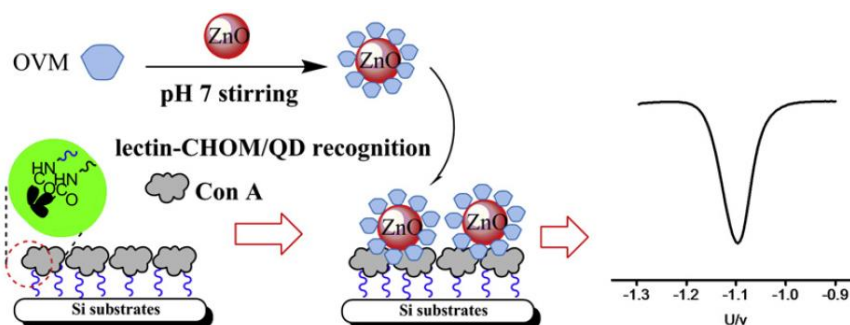

Figure 8. Direct determination of chicken ovomucoid (CHOM) using ZnO-quantum dots (QD)/CHOM bioconjugates, ConA-modified silicon substrates and SWV detection at a glassy carbon electrode (GCE). Reprinted from [115] with permission.

Recently, Sugawara et al. [116] developed an electrochemical biosensor to detect ovalbumin (OVA) by adsorbing a specific peptide probe (peptide-1) conjugated with the electroactive daunomycin on a GCE. In the presence of OVA, the peptide probe was released from the electrode surface and the DPV peak current of the daunomycin moiety decreased. This method provided a calibration curve with a linear range up to 3.0×10^{-10} M and a LOD of 1.5×10^{-11} M OVA and was applied to the determination in egg whites and spiked fetal bovine serum.

The highlighted approaches summarized in Table 3 show that although immunosensors are the electrochemical biosensors most commonly used, electrochemical aptasensors are becoming an attractive alternative for the development of more powerful, cleaner and cheaper analytical methods for the detection of allergens. Moreover, unlike DNA sensors, aptasensors are direct indicators of the potential presence of the targeted allergen [117]. Particularly relevant is the progress in the determination of gluten content in food, ensuring the celiac disease sensitized population safety [109]. In this sense, it is worth to mention that although quantification of food allergens using genosensors is a challenging task which has been rarely addressed probably because results in terms of DNA concentration are meaningless for consumers which need often yes/no tools, a genosensor that allows for the first time to quantify the gluten content in food products has been described recently [108].

In addition, given the sensitivity required for some allergens detection, many biosensor devices with improved sensitivity based on the use of nanomaterials, such as QDs [115], AuNPs [93,100,101,106–108], MWCNTs [93] and graphene [95,96,106] have been developed. Worth to mention also the increasing tendency to fabricate these electrochemical biosensors onto screen-printed electrodes (SPEs) [85,95–104,107,109,112,113] since they are suitable to be fabricated with different materials, to be drawn in diverse geometries and multiplexed formats and characterized by the possibility for mass production and low fabrication cost. MBs-based biosensors have also been described for food allergens determination [94,97,99,103,104] making use of the advantages of these magnetic carriers in the final performance of electrochemical biosensors in terms of simplicity, sensitivity, reduced assay time and minimization of matrix effects, essential for the analysis of complex food extracts. Regarding the assay formats, competitive formats [94,102,109] have been scarcely employed and direct [93,95,96,105,106,111,112] and sandwich [97–101,103,104,107,108,112] configurations have been the most commonly used.

Table 3. Electrochemical affinity biosensors for food allergens.

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
GCE functionalized with (AuNPs) and (P-L-Arg/MWCNT) composite	Immunosensor/direct detection with $[Fe(CN)_6]^{3-/4-}$ as the redox probe	Casein/Cheese	DPV	1×10^{-7}–1×10^{-5} g·mL^{-1}	5×10^{-8} g·mL^{-1}	[93]
m-GEC	Tosyl-MBs-based immunosensor/indirect competitive with H_2O_2 in the presence of HQ	gliadin or small gliadin fragments/spiked gluten-free foodstuffs (skimmed milk and beer)	Amperometry	12.5–329.3 ng·mL^{-1}	12.5–329.3 ng·mL^{-1}	[94]
Aryl diazonium salt organic film electrographted on graphene modified-SPCE	Immunosensor/direct detection with $[Fe(CN)_6]^{3-/4-}$ as the redox probe	β-lactoglobulin/several milk-containing food products (cake, cheese, snacks and biscuits)	DPV	1 pg·mL^{-1}–100 ng·mL^{-1}	0.85 pg·mL^{-1}	[95]
Aryl diazonium salt organic film electrographted on graphene modified-SPCE	Immunosensor/direct detection with $[Fe(CN)_6]^{3-/4-}$ as the redox probe	OVA/spiked cake extracts	DPV	1pg·mL^{-1}–0.5 µg·mL^{-1}	0.83 pg·mL^{-1}	[96]
Pt-SPE	Immunosensor/sandwich configuration onto HOOC-MBs; addition of H_2O_2 with THI as the redox mediatorruiz	Ovalbumin/°	LSV	11–222 nM	5 nM	[98]
SPCEs	Immunosensor/sandwich configuration onto HOOC-MBs; addition of H_2O_2 with HQ as the redox mediator	β-lactoglobulin/Milk samples	Amperometry	2.8–100 ng·mL^{-1}	0.8 ng·mL^{-1}	[97]
SPCEs	Immunosensor/sandwich configuration onto HOOC-MBs; addition of H_2O_2 with HQ as the redox mediator	Ara h 1/Food extracts and saliva samples	Amperometry	20.8–1000.0 ng·mL^{-1}	6.3 ng·mL^{-1}	[99]
AuNPs-SPCEs	Immunosensor/sandwich; addition of 3-IP and Ag$^+$; Ag° stripping	Ara h 1/food matrices	ASV	12.6–2000 ng·mL^{-1}	3.8 ng·mL^{-1}	[100]
AuNPs-SPCEs	Immunosensor/sandwich; addition of 3-IP and Ag$^+$; Ag° stripping	Ara h 6/food matrices	ASV	1–100 ng·mL^{-1}	0.27 ng·mL^{-1}	[101]
8 carbon working electrodes screen printed on alumina	Immunosensor/indirect competitive; addition of H_2O_2 with HQ as the redox mediator	β-casein	Amperometry	0–10 ppm	—	[102]
SPdCEs	Immunosensors/sandwich configurations onto HOOC-MBs; addition of H_2O_2 with HQ as the redox mediator	Ara h 1 and Ara h 2/food extracts	Amperometry	60–1000 ng·mL^{-1} (Ara h 1); 0.25–5 (Ara h 2)	18.0 ng·mL^{-1} (Ara h 1); 0.07 ng·mL^{-1} (Ara h 2)	[103]
SPCEs	Immunosensor/sandwich configuration onto HOOC-MBs; addition of H_2O_2 with HQ as the redox mediator	α-lactalbumin/milk samples and infant formulations	Amperometry	37.0–5000 pg·mL^{-1}	11.0 pg·mL^{-1}	[104]

Table 3. *Cont.*

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
Gold electrode	DNA/direct, dually-labeled stem-loop probe (thiol and biotin tags)	Ara h 1/peanut-milk beverage	EIS	10^{-15}–10^{-10} M	0.35 fM	[105]
multilayer graphene–gold nanocomposite prepared on a GCE	DNA/direct dually-labeled stem-loop probe (thiol and biotin tags); Direct detection with $[Fe(CN)_6]^{3-/4-}$ as the redox probe	Ara h 1/peanut-milk beverage	DPV	10^{-16} to 10^{-13} M	0.041 fM	[106]
Au-SPCEs	DNA sensor/sandwich hybridization; addition of 1-NPP in the presence of AP-Strept	Ara h 2/—	DPV	5×10^{-11}–5×10^{-8} M	10 pM	[107]
Au-SPCEs	DNA sensor/sandwich hybridization; addition of anti-FITC and TMB/H_2O_2	α2-gliadin/commercial food products (gluten-containing and gluten-free)	Chronoamperometry	—	0.001% (*w/w*) of wheat flour in rice flour	[108]
SPCE	Aptameric sensor/competitive assay between Biotin-peptide immobilized on Strep-MBs and free biotin-aptamer for gliadin	Gliadin/heated and hydrolyzed foods	Chronoamperometry	—	0.5 ppm	[109]
Gold disk electrode	Aptameric sensor/direct assay using athiolated aptamer	Ara h 1	EIE	—	—	[111]
SPCE	Aptameric sensor/direct assay by aptamer immobilization on a SPCE by covalent binding via diazonium salt chemistry	Lysozyme/spiked wine samples	EIE	0.025 to 0.8 μM	25 nM	[112]
SPCE	Aptameric sensor/sandwich assay by aptamer immobilization on a SPCE by covalent binding via diazonium salt chemistry	Lysozyme	DPV	5 fM to 5 nM	4.3 fM	[113]
GCE	Lectins-based sensors/direct assay using ZnO QDs/CHOM bio-conjugatesand Con A-modified Si substrates	CHOM/—	SWV	1–140 ng·mL^{-1}	0.1 ng·mL^{-1}	[115]
GCE	Peptide-based sensors/direct assay specific peptide probe (peptide-1) conjugated with daunomycin adsorbed on a GCE	OVA/chickens and quail eggs and spiked fetal bovine serum	DPV	1.5×10^{-11}–3.0×10^{-10} M	—	[116]

Key: AuNPs: gold nanoparticles; ASV: anodic stripping voltammetry; CHOM: chicken ovomucoid; Con A: Concanavalin A; CV: cyclic voltammetry; GCE: glassy carbon electrode; DPV: differential pulse voltammetry; EIS: electrochemical impedance spectroscopy; LSV: linear sweep voltammetry; MBs: magnetic beads; m-GEC: magnetic graphite-epoxy composite; OTA: ochratoxin; OVA: ovalbumin; A P-L-Arg: poly(l-Arginine); MWCNTs: multi-walled carbon nanotubes; QDs: quantum dots; SPCE: screen-printed carbon electrode; SPdCE: screen-printed dual carbon electrodes.

5. Electrochemical Affinity Biosensors for Microorganisms

Foodborne illnesses caused by pathogenic bacteria constitute an important threat to the health of people. Therefore, a growing demand exists for sensitive, accurate and fast analytical methods for the detection of microorganisms, as a whole or through target analytes indicative of their presence (toxins, specific cell membrane proteins, etc.), which is of key importance in food and water quality monitoring. Table 4 summarizes relevant information regarding recent reports on electrochemical affinity biosensors for microorganisms [118–126] and some important features of selected configurations are commented below.

Wu et al. [127] demonstrated that the use of ternary SAMs-modified transducers for the construction of DNA biosensors was an efficient strategy to push down the detection limits for various pathogens. They implemented a DNA recognition system using a ternary surface monolayer consisting of mercaptohexanol and dithiothreitol co-assembled with a thiolated capture probe, coupled with HRP/tetramethylbenzidine (TMB) enzyme-based chronoamperometric transduction. Detection limits as low as 40 zmol (in 4-μL sample volume) of the synthetic DNA and just 1 colony forming unit (cfu) of *Escherichia coli* (LOD of 250 cfu·mL^{-1}) were attained with no need for signal amplification steps. This appropriate sensitivity for urinary tract infection (UTI) diagnosis [128] was attributed to the favorable spacing between the assembled thiolated capture probes which improved significantly the hybridization efficiency and to the noticeable resistance of this rationally designed surface to nonspecific adsorptions. A method based on target-induced aptamer displacement for direct detection of *E. coli* O111 was developed by Luo et al. [118]. The aptamer was immobilized on a goldelectrode by hybridization with a capture probe linked to the electrode surface by Au-thiol binding. In the presence of *E. coli* O111, the aptamer was dissociated from the capture probe–aptamer duplex, and the single-strand capture probe, which remained immobilized on the electrode, hybridized with a biotinylated detection probe tagged with streptavidin–alkaline phosphatase. Using this strategy, a detection limit of 305 cfu·mL^{-1} in milk was obtained.

Shiga toxin-producing *E. coli* O157:H7 is the responsible of food-borne illness causing various disorders such as severe bloody diarrhea. Development of methods other than those traditional based on plate counting for determination of *E. coli* O157:H7 are subject of concern in the field of food analysis. Electrochemical biosensors for rapid detection of *E. coli* O157:H7 have been reviewed very recently by Xu et al. [129]. An illustrative example is the electrochemical immunosensor prepared by layer-by-layer assembly involving the formation of 11-amino-1-undecanethiol SAM onto a gold electrode and the immobilization of AuNPs followed by the incorporation of Chit-MWCNTs–SiO$_2$/thionine nanocomposites and AuNPs multilayer films. Finally, anti-*E. coli* O157:H7 antibodies were covalently bound and electrochemical impedance spectroscopy was used to obtain a calibration curve for heat-killed *E. coli* O157:H7 by measuring the increase in the charge transfer resistance as the antigen concentration increased. The working range was 4.12×10^2–4.12×10^5 cfu·mL^{-1}. Moreover, the immunosensor was applied to analyze inoculated milk and water samples [119]. The qualitative results in milk samples were in agreement with those provided by the ELISA method while quantitative results in water samples were successfully compared with those provided by the count plate method. More recently, a method for the determination of *E. coli* O157:H7 in meat and water samples was reported relying on bacteria pre-concentration onto MBs followed by gold nanoparticles adsorption. Bacteria were captured from the sample using MBs-anti-*E. coli* conjugates and sandwiched with AuNPs modified with secondary anti-*E. coli* antibodies. Chronoamperometric detection at SPCEs allowed a broad detection range between 10^2–10^5 cfu·mL^{-1} taking advantage of the electrocatalytic properties of AuNPs towards hydrogen evolution reaction [120]. On the other hand, an impedimetric immunosensor using also MBs and a screen-printed interdigitated microelectrode was proposed for the rapid detection of *E. coli* O157:H7 and *Salmonella typhimurium* in foods [88]. The corresponding biotinylated antibodies were immobilized onto Streptavidin-coated MBs and glucose oxidase(GOx)–Ab conjugates were employed to label the MBs–Ab–cell complexes (Figure 9). In the presence of glucose, gluconic acid was produced thus increasing the ion strength of the solution

and decreasing the impedance of the solution measured with the microelectrode. The developed immunosensor specifically detected *E. coli* O157:H7 and *Salmonella typhimurium* (*S. typhimurium*) within the 10^2–10^6 cfu·mL^{-1} range in pure culture samples as well as in ground beef and chicken rinse water, respectively. The limits of detection for the two bacteria in foods were 2.05×10^3 cfu·g^{-1} and 1.04×10^3 cfu·mL^{-1}, respectively, higher values than those obtained in pure culture samples.
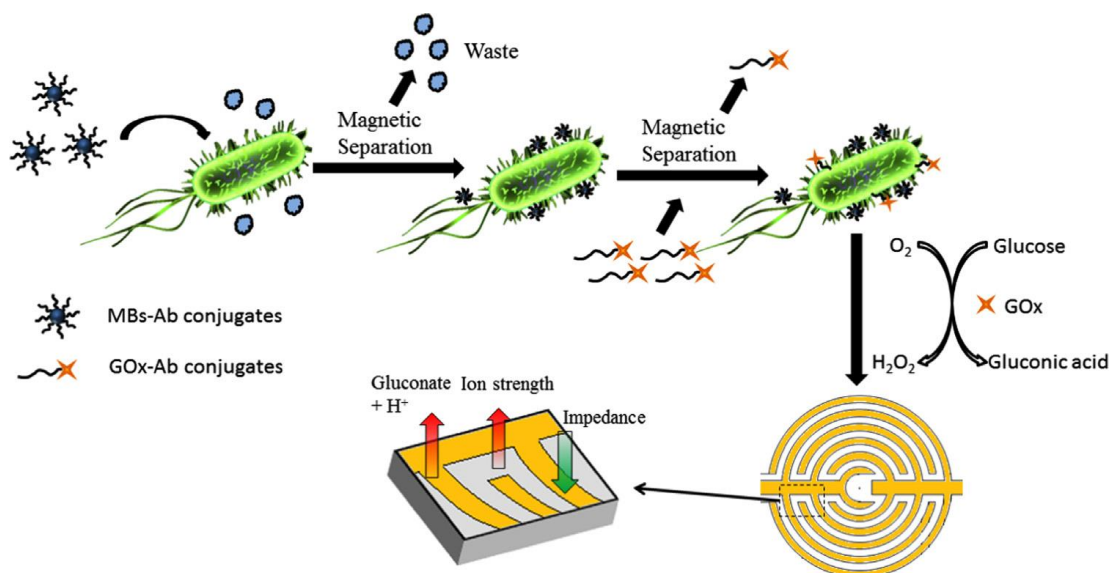
Figure 9. Scheme of the preparation and fundamentals of an impedimetric immunosensor for *E. coli* O157:H7 and *S. typhimurium* using MBs and a screen-printed interdigitated microelectrode. Reprinted from [121] with permission.

The wide incidence of salmonellosis intestinal infection has led to the preparation of a variety of biosensors for the detection of the causative bacteria. Electrochemical immunosensors, genosensors and phagosensors for this particular application were reviewed by Liébana et al. [130]. More recently, Araújo et al. also reviewed the reported designs of electrochemical immunosensors for Salmonella detection in food [131].

With the objective of attaining a sensitive detection of *S. typhimurium*, a label-free electrochemical impedance immunosensor was developed by immobilizing anti-Salmonella antibodies onto AuNPs/PAMAM-MWCNTs-Chit/GCE. The binding of Salmonella cells to the specific antibodies attached to the modified electrode increased the electron transfer resistance measured by EIS using [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe. A linear relationship was obtained between 1.0×10^3 to 1.0×10^7 cfu·mL^{-1} with a detection limit of 5.0×10^2 cfu·mL^{-1}. The method was successfully applied to determine *S. typhimurium* content in milk samples [122]. A label-free impedimetric aptamer-based biosensor for *S. typhimurium* was also fabricated by grafting a diazonium-supporting layer onto SPCEs followed by immobilization of an aminated-aptamer. This strategy allowed obtaining a dense aptamer layer, which resulted in a high sensitivity with a LOD of 8 cfu·mL^{-1} [123]. Also, a specific surface antigen, OmpD, was recently reported as a surface biomarker for detecting *S. typhimurium*. The extraction of OmpD was done from *S. typhimurium* serovars under the optimized growth conditions for its expression. Anti-OmpD antibodies were also generated and used as detector probe in an impedimetric immunosensor using graphene-graphene oxide-modified SPCEs. The developed method was able to specifically detect *S. typhimurium* in spiked water and juice samples with a sensitivity up to 10 cfu·mL^{-1} [124].

Table 4. Electrochemical affinity biosensors for microorganisms.

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
AuE	Aptasensor/aptamer immob; dissociation by *E. coli*; hybridization with biotin-detection probe, tagging with Strept-AP and 1-NPP	*E. coli* O111/milk	DPV	2×10^2–2×10^6 CFU·mL^{-1}	112 CFU·mL^{-1}	[118]
(Chit-MWCNTs-SiO$_2$@THI)$_n$/AuNPs/AUT/AuE	Immunosensor/antibody immobilization. Direct detection of electrochemical response from THI	*E. coli* O157:H7/milk, water	CV	4.12×10^2–4.12×10^5 CFU·mL^{-1}	250 CFU·mL^{-1}	[119]
SPCEs	Immunosensor/sandwich-type immunoassay with Ab1-MBs and AuNPs-Ab$_2$	*E. coli* O157:H7/meat, water	amperometry	10^3–10^4 CFU·mL^{-1}	148 CFU·mL^{-1}	[120]
SPIDMEs	Immunosensor/sandwich; immobilization of antibodies onto Strept-MBs and conjugation with GOs-Ab$_2$. Addition of glucose triggers the enzymatic reaction	*E. coli* O157:H7/chicken rinse water; *S. typhimurium*/ground beef	EIS	10^2–10^6 CFU·mL^{-1}	2.41×10^3 CFU·mL^{-1} 1.04×10^3 CFU·mL^{-1}	[121]
AuNPs/PAMAM/MWCNTs/Chit/GCE	Immunosensor/immobilization of capture antibody and direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	*S. typhimurium*/milk	EIS	1.0×10^3–1.0×10^7 CFU·mL^{-1}	5×10^2 CFU·mL^{-1}	[122]
SPCEs	Aptasensor/immobilization of aminated aptamer by diazonium-grafting; direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	*S. typhimurium*/apple juice	EIS	10–10^8 CFU·mL^{-1}	6 CFU·mL^{-1}	[123]
graphene/GO/SPCEs	Immunosensor/Immobilization of capture antibody and direct detection with [Fe(CN)$_6$]$^{4-}$ as the redox probe	*S. typhimurium* (OmpD) water, apple juice	EIS	10–10^5 CFU·mL^{-1}	10 CFU·mL^{-1}	[124]
MPA/AuE	Immunosensor/sandwich type immunoassay by immobilization of capture antibody and conjugation with HRP-anti-LM	*Listeria monocytogenes* (LM)/milk	amperometry	10^2–10^6 CFU·mL^{-1}	-	[125]
AuNPs/[BMIM]PF$_6$/ERGO/SPCEs	Immunosensor/immobilization of HRP-anti-*E. sakazakii*; direct detection with THI as the redox probe	*Enterobacter sakazakii*	CV	10^3–10^9 CFU·mL^{-1}	1.19×10^2 CFU·mL^{-1}	[126]

Key: AUT: amine-terminated alkanethiol 11-amino-1-undecanethiol; Chit: chitosan; Cyst: cysteamine; ERGO: electrochemically reduced graphene oxide; GO: graphene oxide; MPA: 3-mercaptopropionic acid; 1-NPP, 1-naphthyl phosphate; OmpD: specific surface antigen of *S. typhimurium*; PAMAM: polyamidoamine; SPIDMEs: screen printed interdigitated microelectrode; THI, thionine.

Other pathogenic bacteria have also been analyzed using electrochemical affinity biosensors. *Listeria monocytogenes* (LM), a gram-positive food-borne pathogen, could be detected with a configuration where a 3-mercaptopropionic acid (MPA) SAM was prepared onto a gold electrode for covalent immobilization of the specific antibody. Then, a sandwich-type format was implemented using HRP-anti-LM conjugate. In this case, HRP catalyzes, in the presence of H_2O_2, the oxidation of thionine (THI), whose further electrochemical reduction was amperometrically detected. A linear concentration range from 10^2 to 10^6 cfu·mL^{-1} was obtained which was adequate to detect the bacteria in milk [125]. Another interesting approach was reported by Hu et al. [126] for *Enterobacter sakazakii*. This consisted on an immunosensor fabricated on a SPCE modified with electrochemically reduced graphene oxide and AuNPs mixed with the liquid ionic [BMIM]PF$_6$ and using HRP-anti-*E. sakazakii* conjugate. In this case, the decrease in the CV cathodic peak current of THI redox probe in the presence of the bacteria was monitored, this being proportional to *E. sakazakii* concentration throughout a linear range from 10^3 to 10^9 cfu·mL^{-1} with a detection limit of 1.19×10^2 cfu·mL^{-1}.

It is worth to mention also interesting capabilities offered by potentiometric nucleic acid-based sensors for immediate determination of living microorganisms in complex samples. Zelada-Guillén et al. [132] developed apotentiometric aptasensor based on the use of single-walled carbon nanotubes (SWCNTs) as excellent ion-to-electron transducers with a specific aptamer covalently immobilized to selectively determine a particular strain of *E. coli* (*E. coli* CECT 675 cells). This biosensor, of easy fabrication and regeneration, allowed the label-free determination of living bacteriawith both interspecies and interstrain selectivity and in real samples in a couple of minutes and in a direct, simple, and selective way at concentration levels as low as 6 CFU·mL^{-1} in complex matrices such as milk or 26 CFU·mL^{-1} in apple juice using extremely easy sample treatments.

6. Electrochemical Affinity Biosensors for Drug Residues

Residues of drugs used in veterinary medicine pose a significant risk for human health. This is particularly important in the case of antibiotics, since they are used as growth promotors and to treat animals for preventive and curative purposes, but can cause antibiotic resistance affecting other infectious diseases [133]. Wu et al. [134] reviewed recent progress on nanomaterial-based biosensors for veterinary drug residues in animal-derived food. A review dealing with advances in biosensor development for the screening of antibiotic residues in food products was also published recently by Gaudin [133].

Recently reported electrochemical immunosensors and DNA sensors for the detection of drug residues in foods are summarized in Table 5. As it can be observed, a relatively large number of configurations for tetracyclines (TCs) have been developed in the last years, most likely because TCs have widespread use in animal breeding industry as antibiotic and growth promoters. Therefore, the presence of TCs residues in foods of animal origin has become one of the most noticeable problems for food safety [135]. The European Union has stated the MRLs of TCs in edible products at 100 µg·kg^{-1} in milk and muscle, 200 µg·kg^{-1} in eggs, and 300 µg·kg^{-1} in liver [136]. Que et al. [137] prepared an electrochemical platform consisting of a gold electrode modified with glutaraldehyde and Chit for immobilizing anti-TC antibody, and performed a competitive immunoassay with non-enzymatic signal amplification by means of the platinum-catalyzed hydrogen evolution (HER) reaction. Separately, PtNPs/graphene nanosheets were synthesized and labeled with TC-BSA conjugates to produce bio-PtGN (see Figure 10). TC standards or the samples were incubated with a suspension of bio-PtGN and the incubation solution was dropped onto the immunosensor which was immersed into a solution of PtCl$_2$ in the presence of formate and Tween 80. The voltammetric response from PtNPs was measured by LSV in hydrochloric medium. The method allowed a LOD value down to 6 pg·mL^{-1} (13 pM) TC.
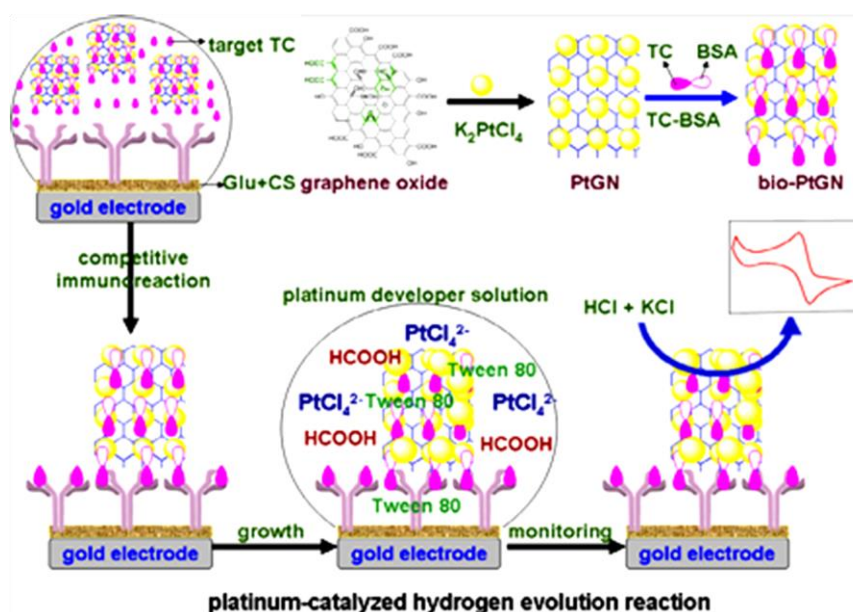
Figure 10. Scheme of the competitive-type electrochemical immunoassay between target tetracycline (TC) and the labeled tetracycline-bovine serum albumin conjugates (TC-BSA) on the platinum/ graphene nanosheets (PtGN) with immobilized anti-TC onto gold electrode, and platinum-catalyzed HER with platinum-mediated seed growth method for signal amplification (Inset: fabricationprocess of bio-PtGN). Reprinted from [137] with permission.

A disposable amperometric magneto-immunosensor involving the use of SPCEs and immobilization of the capture antibody onto protein G-functionalized magnetic beads (ProtG-MBs) was reported for the determination of TC residues in milk. A direct competitive immunoassay using a tracer with horseradish peroxidase (HRP) for the enzymatic labeling was implemented. The amperometric response was measured at the SPCE upon the addition of H_2O_2 in the presence of HQ as the redox mediator. Limits of detection were in the low ppb level for four TC antibiotics in untreated milk samples. The analytical usefulness of the magneto-immunosensor was demonstrated by analyzing spiked UHT whole milk and a reference milk containing a certified oxytetracycline (OTC) content [138].

Aptasensors have been also employed for the determination of TCs. Shen et al. [139] prepared a GCE modified with Prussian blue, Chit and glutaraldehyde, and immobilized TC aptamer onto AuNPs. The decrease in DPV current at the electrode surface caused by the interaction with the analyte was used to quantify the content of TCs in milk samples. Another configuration involving the immobilization of the aptamer onto a MWCNTs/GCE and measurement of the decrease in the DPV response of $[Fe(CN)_6]^{3-/4-}$ after complexation was reported [140]. Chen et al. [135] constructed an impedimetric aptasensor by immobilizing an amino-modified aptamer on a gold electrode. In this case, the presence of the aptamer increased dramatically the charge transfer resistance of the electrode when used $[Fe(CN)_6]^{3-/4-}$ since the redox probe was rejected by the negative charge of the aptamer on the electrode surface. However, in the presence of TC, the drug molecules were inserted into the aptamer structure which changes from lying to upright, this resulting in a linear decrease of Rct values with the drug concentration.

The interaction of the antibiotic with DNA has been also the basis of some electrochemical approach for TC determination in milk samples. For example, Gholivand et al. proposed the measured of electrochemical oxidation of tetracycline (TC) by DPV at ct-dsDNA immobilized on a GCE by UV-irradiation (UV-DNA-GCE) [141] for the determination of this antibiotic. This UV-irradiated DNA film was, apart from water-insoluble, also resistant to nuclease hydrolysis and maintained the B-form structure of DNA in water, so the base pairs were vertical for the axis of double helix. This electrode,

which showed a linear dynamic range of 0.30–90.00 μM and a LOD of 0.27 μM, was used for the analysis of spiked pharmaceutical formulations and milk.

The construction of a disposable amperometric immunosensor for the specific determination of sulfonamide residues in milk using a selective capture antibody and SPCEs has been also reported. The antibody was covalently immobilized onto 4-aminobenzoic acid (4-ABA) grafted onto the electrode, and a direct competitive immunoassay was implemented using a HRP tracer for detection upon addition of H_2O_2 in the presence of HQ as the redox mediator. The developed methodology showed very low limits of detection (in the low ppb level) for 6 sulfonamide antibiotics in untreated milk samples [142] where MRL have been established at 100 mg·kg^{-1} [136]. The same group also developed an immunosensor for the multiplexed determination of sulfonamide and TC antibiotics residues in milk (Figure 11). A similar strategy was employed to modify a dual SPCE with grafted 4-ABA followed by covalent immobilization of protein G. Then, after incubation with the respective antibodies, a direct competitive immunoassay using the same approach commented above was performed [143].
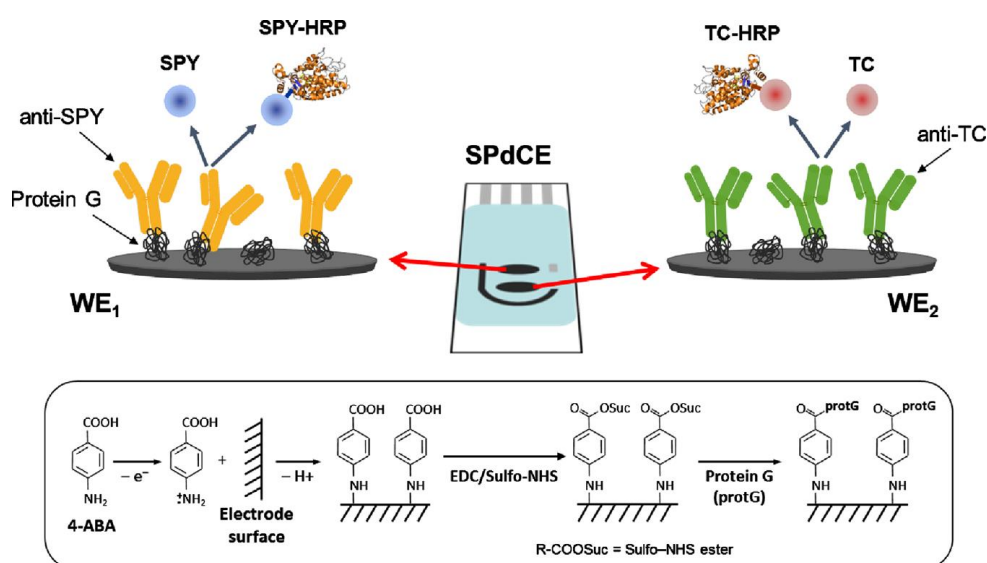

Figure 11. Schematic display of the disposable dual immunosensor prepared for the simultaneous determination of sulfapyrine (SPY) and TC antibiotics in milk. Bottom inset: details of the surface chemistry involved in the modification of dual SPCE including 4-ABA film grafting and covalent binding of protein G using EDC and Sulfo-NHS. Reprinted from [143] with permission.

Streptomycin is a broad-spectrum antibiotic used in human and veterinary medicine whose uncontrolled application can result in the presence of drug residues in foodstuffs causing serious side effects on human health [144]. According to the European Commission, maximum permitted level of this antibiotic in milk is 200 mg·kg^{-1} (Commission Regulation (EEC) 2377/90, 1990). An electrochemical aptasensor for the sensitive and selective detection of streptomycin in milk and serum has been reported involving the use of exonuclease I (ExoI), complementary strand of aptamer (CS), arch-shape structure of a mer (Apt)-CS conjugate, and a gold electrode. Arch-shape structure of Apt-CS conjugate acts as a gate and barrier for the access of redox probe to the electrode surface and ExoI operates as an enzyme digesting selectively the 3′-end of ssDNA. A weak DP voltammetric response was measured in the absence of streptomycin since the gate remained closed. However, upon addition of streptomycin, the Apt leaves the CS binding to streptomycin and the Arch-shape structure is disassembled. Then, ExoI addition leads to a strong electrochemical signal. This behavior allows obtaining a calibration plot for streptomycin with a linear range between 30 and 1500 nM and a limit of detection as low as 11.4 nM [145].

An interesting approach reported by Zang et al. [146] relies on the preparation of a competitive enzyme-amplified immunosensor for the detection of ofloxacin (OFL). This is one of the new

generation fluorinated quinolones widely used in the treatment and prevention of veterinary diseases in food-producing animals, and even as growth-promoting [147]. A polypyrrole film-Au nanocluster matrix fabricated on a GCE was used as the sensor platform where OFL-OVA conjugate was immobilized. Furthermore, gold nanorod loading HRP and HRP-labeled secondary antibody were used as detection labels. Competitive immunoassay between free OFL and captured antigen provided a sensitive response to OFL in the range from 0.08 to 410 ng·mL^{-1} with a detection limit of 0.03 ng·mL^{-1}.

Kanamycin is widely used as a second line antibiotic both for human and veterinary medicine [148]. It can be accumulated in the animal body and transferred into the food chain and, thus, the presence of kanamycin in animal derived foods is potentially hazardous to human health. Accordingly, the animal derived food suspected of contamination by kanamycin is controlled in most countries. For example, the European Union (EU) established maximum residue limits (MRLs) for kanamycin of 100 μg·kg^{-1} in meat and 150 μg·kg^{-1} in milk [149]. A label-free amperometric immunosensor involving the use of graphene sheets (GS), Nafion (Nf), thionine (THI), and PtNPs allowed the ultrasensitive detection of kanamycin. A GCE modified with GS/NF/THI/PtNPs was used to immobilize the anti-kanamycin antibody through electrostatic adsorption. In this configuration, THI acted as an electron transfer mediator, and its electrochemical behavior was improved by the synergistic effects in the presence of GSand PtNPs due to their good electron-transfer ability. The decrease in the electrochemical response of THI measured by CV decreased due to the antibody–antigen immunocomplex formed on the electrode surface being used to determine kanamycin concentration. The immunosensor showed a detection limit of 5.74 pg·mL^{-1} and a wide linear range between 0.01 and 12.0 ng·mL^{-1}. Furthermore, the immunosensor was applied to the determination of kanamycin in spiked chicken liver with satisfactory results (recoveries from 99.4% to 106%) [150].

An aptasensor was also proposed by Zhu et al. [151] for the determination of kanamycin. A 22-bases DNA aptamer showing a high binding affinity to kanamycin wascovalently immobilized onto AuNPs modified with poly-[2,5-di-(2-thienyl)-1H-pyrrole-1-(p-benzoic acid)]. The antibiotic determination was performed by measuring the characteristic LSV reduction peak of kanamycin captured by the aptameric probe at the electrode. The calibration plot showed a linear range from 0.05 to 9.0 mM with a detection limit of 9.4 nM. Recoveries between 94% and 97% were provided by this aptasensor in the analysis of spiked 5-times diluted milk samples. Daprà et al. [14] reported an all-polymer electrochemical microfluidic biosensor for simultaneous determination of kanamycin and ampicillin using a conductive bilayer consisted of tosylate-doped poly(3,4-ethylenedioxythiophene) (PEDOT-TsO) and its hydroxymethyl derivative as electrode material covalently functionalized with two aptamer probes with affinity to each antibiotic. Using EIS, ampicillin could be determined in a concentration range from 100 pM to 1 μM, and kanamycin from 10 nM to 1 μM. Moreover, this approach demonstrated feasibility to determine ampicillin in milk samples below the MRL.

Chloramphenicol (CAP) antibiotic is widely used in animals for the treatment of several infectious diseases. CAP can produce harmful effects on humans such as leukemia, a plastic anemia or gray baby syndrome. This has led to its banning in various countries for treatment of food-producing animals. Despite this, CAP is still found in foods like honey, due to its effectiveness on the control of bees' infection and the increase in honey production. An electrochemical aptasensor for sensitive detection of CAP in honey was developed based on target-induced strand release (TISR). CAP aptamer was immobilized on a gold electrode and hybridized with the complementary biotinylated detection probe to form aptamer/DNA duplex (Figure 12). In the presence of CAP, the TISR resulted in the dissociation of biotinylated detection probe from the electrode followed by binding of streptavidin–alkaline phosphatase (strept–AP) to the remaining biotinylated detection probe. The electrochemical response was obtained by addition of α-naphthyl phosphate (αNP) as the enzyme substrate. SWV detection of the α-naphthol oxidation current led to enzyme-amplified electrochemical signal, which decreased linearly with increasing CAP concentration in the 1 to 1000 nM range showing a LOD value of 0.29 nM. In addition, the designed strategy allowed the direct analysis of real honey samples and the results were in good agreement with those obtained by LC–MS/MS method [152].

Table 5. Electrochemical affinity biosensors for drugs residues.

Electrode	Type of Biosensor/Format	Analyte/Sample	Electrochemical Technique	L.R.	LOD	Reference
Chit/GA/AuE	Immunosensor/competitive; immobilization of anti-TC. Signal amplification based on HER with PtNPs/graphene-TC-BSA	tetracycline (TC)/ honey, milk, peanut	LSV	0.05–100 ng·mL^{-1}	6 pg·mL^{-1}	[137]
SPCE	Immunosensor/competitive; immobilization of anti-TC onto protein G-MBs and use of HRP-TC; addition of H$_2$O$_2$ in the presence of HQ	oxytetracycliine/milk	amperometry	12.5–676.2 ng·mL^{-1}	3.9 ng·mL^{-1}	[138]
AuNPs/GA/Chit/PB/GCE	Aptasensor/immobilization of TC-aptamer; direct detection with PB as the redox probe	TCs/milk	DPV	10^{-9}–10^{-5} M	3.2×10^{-10} M	[139]
MWCNTs/GCE	Aptasensor/immobilization of aptamer; direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as the redox probe	tetracycline/milk	DPV	10^{-8}–5×10^{-5} M	5×10^{-9} M	[140]
AuE	Aptasensor/immobilization of an amino-modified aptamer; direct detection	tetracycline/milk	EIS	5.0–5.0×10^3 ng·mL^{-1}	1.0 ng·mL^{-1}	[135]
GCE	Interaction of TC with UV-ct-dsDNA-GCE; direct electrochemical detection of TC	TC/milk	DPV	0.30–90.00 μM	0.27 μM	[141]
SPCE	Immunosensor/competitive; covalent immobilization of capture antibody and use of Ag-HRP conjugate; addition of H$_2$O$_2$ in the presence of HQ	sulfonamides/milk	amperometry	0.6–64.2 ng·mL^{-1}	0.15 ng·mL^{-1}	[142]
ProteinG-dual SPCE	Immunosensor; competitive; immobilization of capture antibodies and use of Ag-HRP conjugates; addition of H$_2$O$_2$ in the presence of HQ	sulfonamides tetracyclines	amperometry	1.92–454 nM; 6.40–385 nM	0.39 ± 0.01 nM 1.9 ± 0.01 nM	[143]
AuE	Aptasensor/immobilization of Apt-CS conjugate; addition of Exo I and streptomycin. Direct detection with [Fe(CN)$_6$]$^{3-/4-}$ as redox probe	streptomycin/milk	DPV	30–1500 nM	11.4 nM	[145]
pPy/AuNC/GCE	Immunosensor/immobilization of OFL-OVA conjugate; competitive immunoassay with gold nanorod-HRP and HRP-Ab2using H$_2$O$_2$ in the presence of HQ	ofloxacin (OFL)	CV	0.08–410 ng·mL^{-1}	0.03 ng·mL^{-1}	[146]
PtNPs/THI/Nf/GS/GCE	Immunosensor/immobilization of anti-kanamycin; direct detection with THI as the redox probe	kanamycin/chicken liver	amperometry	0.01–12.0 ng·mL^{-1}	5.74 pg·mL^{-1}	[150]
AuNPs/poly-DBP/SPE	Aptasensor/immobilization of aptamer; direct detection of kanamycin	kanamycin/milk	LSV	0.05–9.0 μM	9.4 ± 0.4 nM	[151]
PEDOT-TsO chip	Microfluidic aptasensor/ immobilization of aptamers; direct detection	kanamycin ampicillin/milk	EIS	10 nM–1 μM 100 pM–1 μM	–	[14]
AuE	Aptasensor/aptamer immobilization; formation of aptamer/DNA duplex; TISR and binding to Strept-AP; addition of αNP	chloramphenicol/honey	SWV	1–1000 nM	0.29 nM	[152]

Key: AuNC: Au nanocluster; Chit: chitosan; GA: glutaraldehyde; GCE: glassy carbon electrode; GS: graphenesheets; HER: hydrogenevolutionreaction; HQ: hydroquinone; Nf: Nafion; αNP: α-naphthylphosphate; PB: Prussianblue; PEDOT: polyethylenedioxythiophene; poly-DBP: poly-[2,5-di-(2-thienyl)-1H-pyrrole-1-(p-benzoicacid)](poly-DPB); pPy: polypyrrole; TCs: tetracyclines; THI, thionine; TISR: target-inducedstrandrelease; TsO: tosylate.
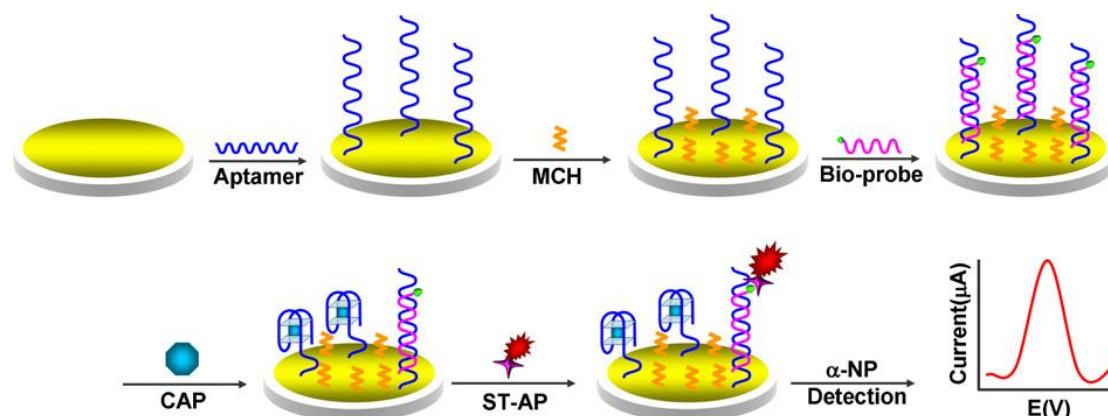
Figure 12. Schematic representation of the electrochemical aptasensor based on target-induced strand release (TISR) for the determination of chloramphenicol (CAP) in honey. Reprinted from [152] with permission.

7. Conclusions and Future Perspectives

Food quality and safety is of paramount importance from health and economic points of view. In particular, the fast and reliable detection and determination of toxins, pesticides, allergens, microorganisms and drug residues is an upcoming field, since standard techniques are not able to fulfill current standards in their determination. This paper reviewed the most recent literature (over the past 5 years) on electrochemical affinity biosensors developed for these food quality and safely-related compounds management using a bioreceptor-based classification, highlighting also the most important achievements and the new research trends.

It is clear from the discussed literature that electrochemical biosensors can play a very helpful role in assuring food safety and facilitate to take quicker preventive actions when required. Featuring high speed of execution, good sensitivity and selectivity, robustness, ease of use and high degree of automation and portability for in-field applications, they have demonstrated their suitability for direct, real-time, on-line, monitoring of toxins, pesticides, allergens, microorganism and drug residues along the production chain. These features made electrochemical affinity biosensors being advantageously compared with conventional methods used (ELISA, HPLC, etc.) in terms of simplicity, cost, assay time and portability of the required instrumentation, characteristics that make them analytical tools especially attractive for the implementation of devices of affordable cost for routine determinations which ensure Food quality and consumer protection. However, despite the great efforts performed in the last years, in order to exploit all the attractive features and great potential of electrochemical biosensors for food quality and safety there are still some important challenges that should be faced such as off-line measurements, and non-specific binding from matrix components. Some described protocols involve the use of sensitive reagents and multiple-step procedures which increase measurement time and cost, making their translation into the food safety and regulatory field very complicated. Moreover, most of the described assays have addressed target analyte quantification just in aqueous solutions or synthetic samples (prepared by adding the contaminant in an intermediate step or even at the end of sample preparation) and only a few faces the analysis of real samples. There are two relevant issues associated with real sample analysis: possible electrochemical interferences and efficient extraction of target analyte from the complex food matrix. To avoid electrochemical interferences, surface chemistry and type of affinity bioreceptor need to be carefully optimized, in conjunction with sample pre-treatment and cleanup. Sample preparation and efficient extraction of the target analytes (involving complex and time demanding protocols particularly for solid samples) remain as the limiting factors for the total analysis time and final performance of the electrochemical biosensor. Moreover, more rigorous validation studies of these electrochemical biosensors should be performed by participating in proficiency tests, performing comparative studies using well-established

analytical techniques and analyzing certified reference materials. Apart from demonstrating their robustness using statistically-relevant sample numbers and performing inter-laboratory studies, the storage and operational stability of the electrochemical biodevices under field conditions and the potential toxicity of involved nanomaterials should be also evaluated before their introduction into the marketplace. Additional work should be performed also in the application of these electrochemical affinity biosensors to the analysis of food processed samples in order to evaluate the impact of food processing processes on their detection capability.

Furthermore, although it is unquestionable that the progress in the preparation, modification and bioconjugation of NPs and nanostructures, used both as electrode modifiers and labels for amplification purposes, will play a major role in the development of electrochemical affinity biosensors with improved performance for food safety, the stability of the electrochemical sensors using nanomaterials is still a vital issue which limits the application of these analytical devices with commercialization purposes [23].

Apart from facing all these critical steps for achieving industry acceptance and AOAC/regulatory approvals, recent trends in the development of electrochemical biosensor for food quality and safety include the use of paper-based platforms [153–157] the development of portable multiplexed biosensing platforms able to be integrated in single lab-on-a-chip microfluidics devicescomprising sample extraction, separation and sensing units, which would greatly improve portability for field use, and the exploration of new nanomanufacturing techniques, such as 3D printing, in their fabrication.

Finally, given the on-going demand for devices that can be used to assess the safety and quality of foods on-site, we expect a steep increase in the number of electrochemical biosensors developed for this purpose and their transfer to the market where the resistance of the agri-food sector, a conservative sector not prone to emerging technologies, will be overcome by the superseding capacities offered by electrochemical biosensors in food safety. Indeed, the future integration of these sensors into food packaging to indicate contamination/risk, highly valuable for on-line control and safety of processed and stored food, will be very useful to improve their acceptance.

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

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