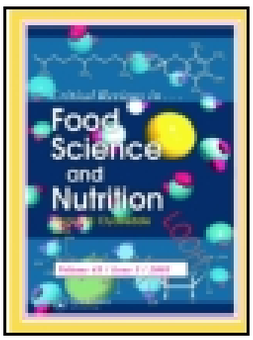

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Electrochemical Genosensors in Food Safety Assessment

Begoña Martín-Fernández [a], C. Lorena Manzanares-Palenzuela [a], Marta Sánchez-Paniagua López [a], Noemí de-los-Santos-Álvarez [b], Beatriz López-Ruiz [a,*]

[a] Sección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense de Madrid, Pza. Ramon y Cajal s/n, 28040 Madrid, Spain

[b] Departamento de Química Física y Analítica, Universidad de Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain

*Corresponding autor e-mail: bealopru@ucm.es (B. López-Ruiz).

Abstract

The main goal of food safety assessment is to provide reliable information on the identity and composition of food and reduce the presence of harmful components. Nowadays, there are many countries where rather than the presence of pathogens, common public concerns are focused on the presence of hidden allergens, fraudulent practices and genetic modifications in food. Accordingly, food regulations attempt to offer a high level of protection and to guarantee transparent information to the consumers. The availability of analytical methods is essential to comply these requirements. Protein-based strategies are usually employed for this purpose, but present some limitations. Because DNA is a more stable molecule, present in most tissues, and can be amplified, there has been an increasing interest in developing DNA-based approaches (polymerase chain reaction, microarrays and genosensors). In this regard, electrochemical genosensors may play a major role in fulfilling the needs of food industry, such as reliable, portable and affordable devices. This work review the achievements of this technology applied to allergen detection, species identification and genetically modified organisms testing. We

summarized the legislative framework, current design strategies in sensor development, their analytical characteristics and future prospects.

Keywords

Allergen; Food Authenticity; Species Identification; Genetically modified organisms; Electrochemical DNA sensor; Food Safety

1. INTRODUCTION

During the past few decades, food quality and food safety have become a topic of public awareness. Guaranteeing food safety is nowadays the main focus of authorities in the food supply chain. The importance of food safety has grown, not only due to foodborne diseases, but also to the concern raised by allergic consumers inadvertently exposed to allergen containing foods (Monaci and Visconti, 2010), as well as various food-related crises undermining public confidence, such as species adulteration, e.g. undeclared horsemeat. Additionally, there has been an increased alarm regarding the introduction of GMOs into the food and feed market due to their controversial safety for human consumption and loss of biodiversity.

In this sense, the main objective is to provide safe food that consumers can trust. Public opinion, along with scientific research and the opinion of experts are continually engaged into promoting careful evaluation of all aspects related to food (Figure 1). Authorities are pushing the food and the feed industry to restructure the food inspection system and try to enhance consumer information to regain consumers trust in food (Röhr et al., 2005). For health, economical and religious reasons, of the promotion of the consumer protection has fuelled the need of legal regulations enforcing the labeling of all food products containing potentially harmful (allergens) or controversial ingredients (GMOs). Species identification, also known as food authenticity, allows the consumer to prevent fraud by substitution of products by others of lesser value for economic purposes. One of the key challenges to ensure food safety is the availability of fast, sensitive and reliable analytical methods to identify specific hazards associated to food before they become a health problem (Amaya-González et al., 2013).

The methodology for testing allergens, authenticity and GMOs are well-known topics in food science since many years. Food products can be characterized through classical approaches such as fatty acid and protein composition profiling. However, for safety-related purposes, proteins and nucleic acids are highly discriminating targets. ELISA methods are the common approaches to detect proteins, though their low thermal stability and the availability of specific antibodies are the main obstacles for the general application of protein-based methods. This is especially true for transgenic proteins, where the absence of antibodies is notorious and also for allergenic proteins, whose sequence is conserved among different species complicating the discrimination ability of the antibodies. Consequently, since the introduction of the PCR technique, DNA has been the target of choice due to its relatively high stability in processed products (Mafra et al., 2008a). Also this target presents a remarkable advantage, it is self-amplifying and, thus, extremely low amounts of DNA can be detected. Specific recognition of unique sequences of DNA from samples can provide the basis of highly specific assays for allergen-coding sequences, species identification and GMO testing.

The limitations of existing methods for DNA detection have encouraged the development of new alternative technologies, especially to comply with current desirable analytical features, such as low-cost equipment, portability, automation and robustness. Electrochemical genosensors can play a major role in addressing these needs. Genosensors are DNA biosensors that rely on a hybridization recognition reaction between two complementary strands, the target and a recognition element, called probe. These devices use an electrode-based platform as transducer and, in order to convert this highly specific hybridization reaction into a measurable signal, a reporter molecule (electroactive molecules or enzymes) is usually incorporated. In the past

decades, many of these electrochemical sensors have been developed for food safety assessment. In Figure 2, we have summarized the quantity of publications in the last 15 years regarding DNA sensors for general food safety. An increased interest on these devices is clearly observed especially in the last years, with a minimum of 20 papers reported per year. The main focus has been pathogen detection in food, which has been a widely reviewed topic (Alonso-Lomillo et al., 2010; Arora et al., 2011; Pividori and Alegret, 2010; Poltronieri et al., 2014; Sforza et al., 2011; Van Dorst et al., 2010). When the literature analysis was refined to another type of food control, namely "non-pathogenic", including the terms "genetically modified OR allergen OR gluten OR authenticity", the number of papers were considerably less, yet the chart reflects an increasing number of publications in the past year. This rise may be in response to the enforcement of food regulations and the growing interest of consumers in being more informed about food composition. Moreover, when the search included the keyword "electrochemical", the chart indicates that most "non-pathogenic" devices were based on electroanalytical techniques. As expected, electrochemical devices have gained more notice as an alternative to conventional optical or mass approaches for DNA detection, keeping in thought that electrochemical instrumentation bears great advantages; portability, automation and low cost being the most important ones.

The aim of this review is to collect and discuss the electrochemical DNA sensors reported so far for food authentication (meat and fish), allergens (hazelnut, peanut and gluten) and recent advances achieved in GMO assessment. We will focus on these specifics not only because they are paramount to the industry and to the consumers, but also because there has been a growing trend in pursuing this type of food assessment by researchers in the biosensor field. Our interest

was driven into carefully reviewing this topic of up-to-date prominence, considering that it has not been thoroughly addressed yet. This work summarizes the requirements imposed by the legislative framework regarding food, the current knowledge about electrochemical genosensors development, the possible gaps and how they should be addressed. Thus, the main progresses to date are also presented, highlighting potential prospects for the future and emphasizing aspects that deserve special attention for improvement of these devices.

2. LEGISLATION

The increasing use of packaged foods has forced governments to subject the food industry to more and more stringent labeling of their products. Labeling helps consumers to choose the product and identify its contents, allowing the consumer to exercise freedom of choice (Premanandh, 2011). Accordingly, one of the main objectives of the governments is to achieve a high level of protection and to guarantee their right to information, ensuring that consumers are appropriately informed in regards to the food they consume (Regulation (EU) 1169/2011).

Legal framework related to GMOs, authenticity and allergen in foodstuffs is wide and comprises different goals. It covers issues ranging from personal choice (GMOs), the prevention of food fraud (food authentication), and to certify the safety of food to allergic or celiac individuals (allergen issue).

2.1. Allergen legislation

Labeling rules generally only cover deliberately added ingredients in pre-packed foods, but supply chain and manufacturing processes are extremely complex, resulting in the adventitious presence of small amounts of allergens (traces) in many products and restricted variety of food products for allergenic people (Crevel et al., 2008). Besides, recently the use of a precautionary

label "may contain" in food products has risen to become overused, thus leading to unnecessary limitation of consumer choice and devaluation of the allergen labeling information (van Hengel, 2007). Because of that, allergic people can find avoidance of specific food very difficult.

To date, most regulatory authorities have focused on ingredient labeling irrespective of the amount in the food. Presently, the European Union (EU) ruled through the Regulation (EU) 1169/2011 the mandatory labeling of fourteen groups of certain substances or products that are known that cause allergies or intolerances (annex II) (Regulation (EU) 1169/2011). It is required to highlight these ingredients from the rest of the ingredients enumerated in processed food, regardless of their quantity. The list includes crustaceans, eggs, fish, peanuts, soybean, milk, nuts, gluten containing cereals, celery, mustard, sesame, sulphur dioxide, lupin and molluscs. The United States (US) also implemented legislation to improve the protection for allergic consumers, with the Food Allergen Labeling and Consumer Protection Act (FALCPA). The US lay down 8 groups of allergenic ingredients, while the legal basis for food allergies imposed by the EU includes a list of 14 groups, thus being the most complete legislation framework so far. Other countries such as Canada (11 groups), Australia and New Zealand (10 groups), and Japan (6 groups) also establish restrictive legislation to protect the allergic patients. In spite of the advances achieved in labeling requirements throughout the world (Gendel, 2012), there is a lack of consensus regarding the establishment of threshold values for allergen labeling (Taylor et al., 2002).

Unlike most allergens, gluten is the only one identified with a clear and explicit threshold at least in the EU. Although universal safe levels for consumption of gluten has not yet been defined, literature suggests that small amounts of gluten could be sufficient to cause damage to the celiac

intestinal mucosa over time (Catassi et al., 2007) and an increased risk of autoimmune disorders (Ventura et al., 1999). Therefore, mandatory labeling of celiac harmful food ingredients has been established to inform the consumer about the presence of gluten in food products. The Codex Alimentarius and the Regulation (EC) 41/2009 endorse a maximum gluten contamination of 20 mg/kg in gluten free labeled products as a safe threshold (CODEX-STAN118, 1979; Regulation (EC) 41/2009). However, these regulations do not specify an analytical methodology for gluten analysis.

2.2. Authenticity legislation

One of the risks gaining attention from industry, governments, and standards-setting organizations is fraud conducted for economic gain by food producers, manufacturers, processors, distributors, or retailers (Moore et al., 2012). It is essential to establish that species of high commercial value declared are not replaced, partially or entirely, with other lower value species (Mafra et al., 2008a). Religious reasons also play an important role in this subject. Labeling legislation has to ensure that food is properly described, and it has to protect consumers from being sold a product with a fake description (Dennis, 1998). According to Regulation (EC) 178/2002 and 1169/2011, it is a general principle of food law to provide a basis for consumers to make informed choices in relation to food they consume and to prevent any practices that may mislead the consumer (Regulation (EC) 1169/2011). In agreement with these rules it is necessary to provide comprehensible and trustworthy information on the identity and composition of the food.

2.3. GMOs legislation

According to the European legislation, the presence of genetically modified (GM) material in food and feed is governed by Regulation (EC) 1829/2003, which insists on the mandatory labeling of all products consisting of, produced or containing authorized GMO above 0.9%. Below this limit, GMO labeling is not required when its presence is considered adventitious or technically unavoidable. For non-authorized GM ingredients, the threshold is set at 0.5% (Regulation (EC) 1829/2003). Unlike the European legislation, the labeling of transgenic products is voluntary in the United States.

Since the correct labeling of foods is the only effective way of gain the consumers' confidence and protecting the allergic patients, legislation in terms of food assurance has undoubtedly been improved in the last decades. However, the issue of adventitious contamination and cross-contact during manufacturing is not addressed yet, even though substantial incorporation of undeclared constituents can occur (Crevel et al., 2008). Differences not only in food regulation but also in the different threshold levels set for labeling in many countries, as well as the complexity and variety of food matrices, make extremely difficult the development of only one analytical method universally applicable to assess food safety. This is why there is a compendium of existing methods, some of which are implemented on a routine basis and others that are more difficult to apply in every research or industry facility. The number and diversity of available methods to detect species, allergen-coding sequences and GM DNA, keeps growing. The common goal is to develop robust and reliable analytical methods, cost-effective and easy to implement in food control practices.

3. ALLERGEN DETECTION

Food allergy is an adverse, abnormal immune-mediated reaction to a certain food or food ingredient that occurs in susceptible individuals who often require a strict avoidance of the offended component. Food sensitivity also includes food intolerance, which is clinically undistinguishable from allergy but it is a non-immune mediated reaction (Koppelman and Hefle, 2006). Up to 1-2% of the total human population suffers from clinically proven food allergies (Sicherer and Sampson, 2006). Prevalence among children is even higher, about 5-8% (Branum and Lukacs, 2009; Gupta et al., 2011). Food allergy has thus evolved from being a restricted problem for the food-allergic population to an emerging public health issue.

Allergens determination in foodstuffs is a major concern for both the food industry and food-allergic consumers. Ultrasensitive analytical approaches are demanded to detect even traces of allergens. Thus biosensors have been applied to numerous kinds of allergen targets as reviewed recently (Alves et al., 2015). Even though the target itself is the allergenic protein, there is a very important limitation regarding protein-targeting methods, such as ELISA, i.e., food thermal processing can promote degradation of food constituents, modifications, or interactions with other components of the food matrix and therefore thermal processing has a potential to modify allergenic properties of proteins (Sathe et al., 2005; van Boekel et al., 2010). Given the difficulty of detecting allergenic proteins under certain circumstances (e.g. with ELISA assays), DNA-based methods are also used for screening or confirmation, relying on the high stability of DNA upon food processing when compared with proteins (Mafra et al., 2008a). Since DNA detection methods are completely orthogonal to ELISA, a positive result with both assays gives almost irrefutable proof that the allergen is present (Janssen, 2006).

Usually, DNA target sequences are selected among genes coding for allergenic proteins or simply on a taxon-specific region of the allergenic species genome. Most of electrochemical genosensors rely on the determination of DNA sequences that encode allergenic proteins (Table 1). Various types of designs have been reported, using different sorts of immobilization strategies and electrochemical methods. Most methods were applied to real sample analysis.

3.1. Hazelnut

Hazelnut, *Corylus avellana*, is one of the most commonly consumed tree nuts being virtually found in a wide range of processed foods (Costa et al., 2012). It has been regarded as a food with potential heart-protective benefits (FDA, 2003), but also as a source of allergens capable of inducing mild to severe allergic reactions in sensitized individuals. Hazelnut allergy is one of the most global incidence, with an estimated prevalence in Europe of 0.1–4% of the population (Zuidmeer et al., 2008). Several allergenic proteins have been identified and characterized: Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12 and Cor a 13 and Cor a 14 (Akkerdaas et al., 2006; Breiteneder et al., 1993; Garino et al., 2010; Iniesto et al., 2013; Lüttkopf et al., 2002; Pastorello et al., 2002)

Bettazzi et al. (2008) reported an electrochemical genosensor for the detection Cor a 1.04 and of the isoform Cor a 1.03. To achieve the desired sensitivity, genosensors generally require previous DNA extraction and subsequent amplification; being PCR the most frequent amplification procedure coupled to biosensing protocols. In this work, the amplification of these two hazelnut sequences by PCR rendered long amplicons. Because these long strands present a peculiar hybridization behavior with immobilized probes, compared to the predictable hybridization kinetics of synthetic oligonucleotides, this work also included a careful evaluation

of the hybridization efficiency with PCR products. The high steric hindrance of long amplicons and the presence of the sister strand in solution competing with the immobilized short capture probe, make difficult the hybridization on the surface. To enhance hybridization efficiency and sensor performance, sandwich hybridization was implemented using a biotinylated signaling probe. The sandwich hybridization is often recommended with long target sequences and involves two probes: a capture probe partially complementary to the target and anchored to the electrode, and a labeled signaling probe that hybridizes with the rest of the target. The relative hybridization position of probes in a sandwich assay is of main importance, as it has been previously reported (Del Giallo et al., 2005; Holzhauser et al., 2000), since higher hybridization efficiencies are obtained when one of the termini of the amplicon was involved. In this work, Bettazzi et al. demonstrated that the signaling probe should be designed in order to be contiguous to the capture probe sequence to get the optimal hybridization yield and therefore the highest electrochemical response. On the contrary, when fringe single-stranded regions between the probes exist, intramolecular folding may occur reducing the availability of the target strand, and lowering the hybridization efficiency at the electrode surface. Enzymatic signal amplification using streptavidin–alkaline phosphatase with DPV was performed. After optimizing the device, an extensive analysis of real samples without precedent was conducted. DNA was extracted from several hazelnut-containing commercial samples (including chocolates and lecithin-based products) with a commercial kit. PCR amplicons were analyzed by gel electrophoresis, purified and then quantified with PicoGreen. These pretreated samples were diluted and analyzed with the genosensor. The obtained data correlated well with those obtained with an ELISA method. Despite all efforts, only qualitative data was reported in nanomolar detection levels.

Another work reported the detection of the same hazelnut DNA sequence Cor 1.04, coupled to a microfluidic system (Berti et al., 2009). Capture probes were immobilized to streptavidin-coated paramagnetic micro-beads. The complementary sequence was then recognized via sandwich hybridization with a capture probe and a biotinylated signaling probe. After labeling the biotinylated hybrid with a streptavidin–alkaline phosphatase conjugate, the particles were introduced in a disposable cartridge composed of eight parallel microchannels etched in a polyimide substrate. The modified particles were trapped with a magnet addressing each microchannel individually. The presence of one microelectrode in each channel allowed direct electrochemical detection of the enzymatic product within the microchannel. The main advantage of these systems is their portability and the multi-target assessment, which together with the lower propensity to nonspecific signals of the magnetic particles, resulted in improved detection limits for diluted PCR amplicons.

3.2. Peanut

Another highly frequent food allergy is caused by peanut consumption. Their symptoms range from mild oral allergy syndrome to anaphylactic reactions and even death (Bock et al., 2001). Up to now, there are 13 listed allergens that account for peanut allergy, including 11 allergens named Ara h 1 (*Arachis hypogaea* allergy 1) to Ara h 11, and two recently identified allergens, agglutinin and 18 kDa oleosin (Mari et al., 2006). The major peanut allergens are Ara h 1−3 that are recognized by over 90% of peanut-allergic adults (Koppelman and Hefle, 2006).

Ara h 1 is a 7S vicillin-like globulin also known as cupin, which affects 35-95% of peanut-allergic patients. The group of Li reported different sensors for Ara h 1 detection, with a variety of immobilization and detection strategies (Sun et al., 2012; Sun et al., 2015a; Sun et al., 2015b).

Initially, they reported the detection of a 125 bp sequence of Ara h 1 peanut DNA (Sun et al., 2012). The device relied on the formation of a SAM of a thiolated hairpin capture probe and MCH onto gold electrodes. A signal-off detection was employed, based on the decrease of the electron transfer of ferrocyanide/ferricyanide ($[Fe(CN)_6]^{3-/4}$) redox probe after the hybridization with the target measured by EIS . They obtained femtomolar sensitivity for synthetic samples, and detected a concentration level of 0.32 pM of peanut DNA extracted from one sample of peanut milk.

Later, the same group reported another device based on the previous design, but using a multilayer graphene-gold nanocomposite onto a GCE electrode for the immobilization of the hairpin capture probe. Monitoring of $[Fe(CN)_6]^{3-/4}$ by DPV provided a lower detection limit for synthetic oligonucleotides (sub-femtomolar) (Sun et al., 2015a). They also reported the detection of DNA extracted from a peanut milk beverage sample. When known amounts of target DNA were added to the extract, adequate recoveries were obtained.

Another genosensor for Ara h 1 detection involved a mixture of chitosan and multiwalled carbon nanotubes and a spongy gold film, employed to coat a glassy carbon electrode (Sun et al., 2015b). The adsorption of a dually-tethered hairpin capture probe was carried out. Its 5’ end was functionalized with a thiol group and its 3’ end with a biotin tag. Streptavidin–horseradish peroxidase was used as enzymatic label with CA detection. They also reached a femtomolar quantification level for synthetic samples. In addition, quantification of one real sample of DNA extracted from peanut milk was reported, showing a good correlation with the results obtained with real-time PCR.

All of the previous sensors detected Ara h 1, which represents about 20% of the total peanut proteins. However, Ara h 2 (conglutin family, 2S albumin) constitutes the most frequently recognized allergen in children (Flinterman et al., 2007; Nicolaou et al., 2010). An electrochemical sensor for detection of an 86-mer DNA sequence encoding part of the allergenic protein Ara h 2 was developed (Sánchez-Paniagua López et al., 2014). The detection system of synthetic oligonucleotides was based on a sandwich hybridization assay and enzymatic amplification. The study was focused on the optimization of the sensing phase, a mixed SAM of thiolated capture probe and MCH, using the Design of Experiments approach, to improve the performance of the device. The use of Response Surface for desirability function generated the overlay contour plots for each response, signal and blank, which allowed finding the optimum experimental area with maximum signal for target and minimal for blank. As a result, picomolar sensitivity was achieved.

3.3. Gluten

Celiac disease is defined as a common autoimmune inflammatory disease with both genetic and environmental components. The celiac disease is one of the most frequent chronic health illness, being estimated to affect as much as 1% of the general population, although this number could be underestimated owing to difficulties in diagnosing this autoimmune disorder (Green and Cellier, 2007; Kagnoff, 2007). Clinical manifestations of celiac disease are highly variable, including both gastrointestinal and non-gastrointestinal features, which may result in systemic manifestations (Briani et al., 2008). The major environmental trigger is the consumption of gluten, which corresponds to a significant portion of protein fraction in cereals, namely wheat, barley and rye (Fasano and Catassi, 2012). In the celiac disease, one of the most well studied

peptides is the so-called 33-mer peptide, which is the immunodominant fragment of the alpha2-gliadin playing a major role in the immunogenicity (Shan et al., 2002). Mutant peptides lacking this fragment lose their immunotoxicity. The immunogenicity of the referred peptide was attributed to its resistance to breakdown in the human intestine, mainly due to its high content of proline residues as well as high susceptibility to transglutaminase deamidation (Qiao et al., 2004). Nowadays, the only effective treatment consists on the avoidance of gluten- containing food.

So far, most of the methods employed in the detection of gluten rely on protein-based approaches, namely ELISA method (Diaz-Amigo and Popping, 2013; Janssen, 2006). However, false negative results, poor reproducibility and cross-reactions are frequently considered as the major drawbacks of these methods (Haraszi et al., 2011; Mujico et al., 2011). Hence, DNA-based technologies have arisen as an alternative strategy for gluten detection. The enormous complexity of the wheat genome (probably one of the most complicated genomes among domesticated plant species), along with its high genetic variability, make the detection of wheat-related DNA sequences both a challenging but stimulating task. For these reasons, only two electrochemical sensors have been reported to detect wheat DNA in food samples (Martín-Fernández et al., 2015; Martin-Fernandez et al., 2014). In both works, a 99 nucleotide DNA sequence from wheat was selected as target to develop a sandwich type DNA genosensor. This sequence is a highly structured, long and repetitive target fragment that encodes the alpha2-gliadin 33-mer allergenic protein. The high content of guanine in this fragment and in its complementary probes (capture and signaling probes) might favor the formation of G-quartets that provide additional stable 3D conformations that further prevent the strands hybridization. In

this work it was shown that a rational design of the capture and tagged-signaling probes along with a two-hour hybridization time coupled to a heating shock can be considered a general strategy to overcome these problems. PCR was also optimized to be applied to DNA extracted from cereal flour samples. The genosensor developed allowed the selective detection of this fragment in the presence of both DNA spurious products and PCR reagents without the need of amplicon purification. The successful detection of gliadin DNA verifies the suitability of the described PCR-coupled genosensor to complement the immunochemical methods to detect gluten for celiac disease patients' safety.

Although the EU Regulations establishes gluten labeling regardless of the cereal species, in other countries the identification of the specific gluten-containing cereal origin is compulsory (Diaz-Amigo and Popping, 2012). In this sense, it is important to have highly discriminatory methods for selective detection of wheat, to meet these standards. In order to improve the selectivity of the previously described genosensor against wheat DNA sequences, and to provide the highest discrimination ability, the former design was modified by incorporating a structured capture probe instead of a linear one (Figure 3) (Martín-Fernández et al., 2015). Usually, the selectivity of the hybridization reaction is based on mismatches that cause a destabilization of double-stranded DNA. Therefore, structured oligonucleotides, hairpin or stem-loop structures, provide a competition reaction with probe-target hybridization that increases probe specificity and prevents the hybridization of undesired sequences (with a few mismatches). The hairpin-based genosensor provided slightly inferior sensitivity than that obtained with linear probes due to energetic penalty associated to the need for hairpin opening, but exhibited complete specificity against wheat with respect to closely-related species (barley and rye).

4. SPECIES IDENTIFICATION

Food and feedstuffs are generally of plant or animal origin. The identity of ingredients in processed or composite mixtures is not always readily apparent and verification that the components are authentic and belong to sources acceptable for human consumption is needed.

The reliable identification of the species is a key issue for food authenticity and should preferably be based on parameters that do not undergo too many alterations during food processing, e.g., the taxonomic identification of fish species based on morphological characteristics is a difficult task because most species are available commercially after the removal of external features (viscera, skin, head, etc.) (Ward et al., 2009). Routinely traditional analysis involves detection of species-specific proteins by means of electrophoretic and immunological methods (Gallardo et al., 1995; Piñeiro et al., 1999). As mentioned before, heating and processing treatments are prone to alter proteins and modify the results (Lockley and Bardsley, 2000). DNA is more thermostable, and is present in the majority of the cells of an organism, enabling the identification regardless of the tissue of origin. For these reasons, in recent years mitochondrial DNA has been used as target for fish and meat species identification.

Fish species can only be visually identified while the fish remains in its whole state. The matter becomes further complicated as fish is processed where mincing, battering, crumbing and frying operations take place. Consequently, in this type of food, substitution for toxic or low valued fish species represents a risk (Asensio Gil, 2007). One example of the need of ensuring authenticity of fish species comes from the dangerous consumption of members of the *Tetraodontidae* family (pufferfish). Pufferfish poisoning is one of the most dangerous intoxications from marine species. In Japan and in some parts of the world, pufferfish are prepared by highly trained cooks,

and are considered a delicacy, regardless of the fact that they contain a deadly toxin (Lau et al., 1995). Del Giallo et al. (2005) developed a genosensor for differentiation of pufferfish (*Takifugu niphobles*, Tetraodontidae family) from anglerfish (*Lophius budegassa*, Lophiidae family), considering that the latter lacks from toxicity. Long (~600 bp) sequences of mitochondrial DNA of both were amplified using primers that are common to both species. Purification and quantification (with Picogreen) steps of the amplified target were required prior to analysis. The amplified sequences were hybridized with a thiol-thethered capture probe, immobilized to SPEAu, and different biotinylated signaling probes, forming a dendritic-like assay (Figure 4). Afterwards, labeling and detection were done by means of an enzymatic reaction using ALP-streptavidin. They demonstrated that both the surface coverage by the probe and its relative position on the target strand are important to control the hybridization efficiency. Providing the long target sequence, they proposed a multiple signal amplification design, anchoring several enzymes to multiple sites on the target strand. The high steric hindrance limited the use of more than three closely located enzymes. An unusual bell-shaped relationship between the amplicon concentration and the electrochemical response was found. It was attributed to the renaturation of the two strands of the extremely long target sequence, favored over the formation of the surface-immobilized capture probe amplicon complex. This work is worthy of mention because it was the only genosensor for fish identification, and one of the first works involving authenticity assessment.

On the other hand, meat species adulteration represents a relatively frequent issue in the food industry, as it is the case of the addition of pork to beef products for economic gain. This type of adulteration is especially important in some religions, where pork meat consumption is forbidden

(Soares et al., 2010). There is also an increased concern over undeclared meat species due to the relatively recent outbreaks of bovine spongiform encephalopathy, commonly known as Mad-Cow Disease, a fatal, neurodegenerative disease in cattle. The potential for transferring this disease to humans in the form of variant Cruetzfeld Jacobs Disease has increased the need for traceability of meat, meat products and feedstuffs (Mafra et al., 2008b). Similarly, consumers may be unwilling to ingest meat because of the risk of avian influenza virus and foot and mouth disease, which are associated with the consumption of chicken and swine meats, respectively.

One of the first reports in regards of meat species identification with genosensors was carried out by Mascini et al. (2005). They designed two different genosensors for the detection of bovine and sheep samples targeting satellites of DNA (~250 bp and 430 bp, respectively), which are highly repetitive oligonucleotide sequences. A label-free approach was employed, based on the square-wave voltammetry detection of guanine oxidation when the target is present. To avoid background signals given by the probe itself, inosine-modified (guanine-free) DNA probes of ~25 bases were designed. The probe was designed to hybridize with the peripheral zone of the DNA satellite fragment to avoid steric hindrance effects (Figure 5). Bovine genomic DNA from extracted samples was analyzed with the genosensors without PCR, after performing restriction enzyme digestion to reduce the size of the genomic fragments, consequently reducing the steric hindrance. The bovine genomic DNA was applied to both bovine and sheep genosensors. As expected, it only provided a positive response in the former, confirming the selectivity of the designed device. So, they reported the detection of 3 µg/mL of synthetic target, and a clear discrimination of the mammalian species, bovine and sheep, in real samples with a detection limit lower than 30 µg/ml of total genomic bovine DNA.

Chaumpluk et al. (2006) reported an electrochemical platform for bovine DNA detection and semi-quantification in feedstuff using amplification by PCR and LSV detection. This work used Hoeschst 33258, H33258, a DNA minor groove binder used as a fluorescent dye to stain DNA. Its electrochemical activity was exploited by mixing it with the PCR-amplified double-stranded DNA and measuring the change in the anodic peak current of free and DNA-bound H33258. In the absence of DNA, the highest diffusion rate of the free H33258 resulted in a higher signal on screen-printed carbon electrodes. On the contrary, lower oxidation currents were measured in the presence of amplicons due to binding of dye to DNA.

The PCR amplification was designed to target two sequences, one bovine-specific (bovine parathyroid, Pth) and the other for an eukaryote common gene (12S rRNA). Qualitative (discrimination of bovine-containing products) and quantitative determination of the bovine content as percentage were reported. For the quantitative analysis, the authors introduced a plasmid reference system to build standard calibration curves with both genes. Cloned portions of Pth and 12S rRNA were used as known DNA copies and serial dilutions were performed to obtain a wide concentration interval. Animal blood and bone meals for feedstuffs production and commercial pet foods were extracted and purified with commercial kits. By performing 30 cycles of PCR amplification (before saturation), semi-logarithmic relationships between the anodic current and copy number of the Pth gene and the 12S rRNA gene were found. Bovine composition was expressed as a ratio between the Pth gene copy number and total copy number of 12S rRNA. Only semi-quantitative data was obtained because 12S rRNA could not be estimated accurately in mixed animal and plant ingredients, making it impossible to achieve a full quantitative analysis.

Later, Ahmed et al. (2010) developed a faster and simpler method for bovine, chicken and swine meat identification, using a combination of isothermal amplification of DNA and electrochemical detection on disposable carbon-based electrochemical printed chips (Figure 6). This work also used H33258 to measure DNA products by LSV. Mitochondrial sequences were amplified in DNA extracted from raw meats and processed food, by means of LAMP, instead of the most widely employed temperature-dependent PCR amplification. In this type of isothermal amplification, four loop primers for species-specific detection were used, where six distinct regions are targeted and amplified without the use of a thermal cycler. It is considered to be more specific than PCR as it uses a greater number of primers to delimit the region to be amplified, which may diminish the occurrence of false-positive results. The amplified products were diluted and then mixed with H33258. The mixture was placed onto the chip surface for each measurement. The limits of detection were 20.33 ng/μL, 78.68 pg/μL and 23.63 pg/μL for pork, chicken and bovine species, respectively. The results from this method were compared to a multiplex-PCR and capillary gel electrophoresis system. The authors claimed that their LAMP-based electrochemical genosensor was more specific, reduced the cross-reactivity and avoided unspecific amplicon formation in raw and processed meats, compared to the PCR-based method.

5. GMO TESTING

Genetically modified foods are those produced from organisms that have undergone specific changes into their DNA using genetic engineering methods in order to introduce new traits (Figure 7) to improve crop production (herbicide or insect resistance) or to enhance nutritional properties. Shortly after their release to the food and feed market, many genosensors have been reported as alternative methods towards their detection (Arugula et al., 2014) Target selection is

relatively easy to undertake because the transgenic inserts and specific sequences are fully described in open databases and in validated PCR methods when they have been authorized. Some of these sequences serve as "generic" targets for all GMO-containing products, such as 35S or PEP promoters and NOS terminator (van den Eede et al., 2004), so the methods that target such sequences are called "screening methods" and they assist in preliminary analysis. As it has been recently reviewed by Manzanares-Palenzuela et al. (2015b), most electrochemical devices have been reported for screening purposes, followed by the group of genosensors that target gene-specific sequences, i.e. part of the gene that encodes the protein conferring herbicide resistance or insect resistance in crops. However, the highest level of specificity is given by event-specific methods, in which the targets are the unique junction, characteristic of each transgenic event, found at the integration locus between the inserted DNA and the recipient genome. Only few works have been developed for detecting event-specific sequences. Taxon-specific detection is required for GMO quantification and for species identification in preliminary screening analysis but this is not usually accomplished when developing electrochemical methods. This fact seriously limits the applicability of the methods so far reported that remain restricted to simple identification or quantitation of transgenic events in an absolute fashion that is not acceptable to establish compliance with legislation, which requires relative quantitation (content expressed in percentage).

In this section we review the most recent and relevant electrochemical genosensors reported for GMO detection.

5.1. Screening methods

As it is shown in Figure 7, many genosensors have been reported as screening methods (Carpini et al., 2004; Kerman et al., 2006; Lien et al., 2010; Ligaj et al., 2003; Lucarelli et al., 2005; Meric et al., 2004; Sun et al., 2008; Sun et al., 2007; Tichoniuk et al., 2008; Ulianas et al., 2014; Wang et al., 2009; Wang et al., 2008; Xie et al., 2008; Xu et al., 2006; Yang et al., 2012b; Zhu et al., 2008). The group of Mascini (Lucarelli et al., 2005; Meric et al., 2004) pioneered the development of electrochemical sensing platforms for GMO screening. Their contribution is worthy of mention due to the high sensitivity achieved by their proposed device and the application to reference materials (see Figure 7, screening methods). They reported an EIS-based sensor using mixed SAMs as immobilization method and sandwich hybridization with enzymatic labeling. The amplification of the signal was mediated by the addition of ALP-streptavidin conjugate that binds to biotin-signaling probe (Lucarelli et al., 2005). The enzyme transforms 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium mixture in an insoluble and insulating product which precipitates on the sensing interface. Electrochemical transduction of the hybridization process was performed by EIS in presence of $[Fe(CN)_6]^{3-/4-}$. The signal increases after hybridization due to the precipitation of the enzymatic product onto the surface. A remarkable sensitivity was achieved, with a LOD of 1.2 pM. However, this impedimetric genosensor was not able to differentiate between two different concentrations of both GM soy and maize powders reference materials, amplified by PCR. Therefore, the authors concluded that this method could only work as a “yes or no” system, until an adaptation for quantitative determination of GMOs could be done. Lucarelli’s work is one of the most sensitive sensors reported up to this date.

Recently, Ulianas et al. (2014) reported the use of a composite based on acrylic microspheres and gold nanoparticles onto screen-printed carbon electrodes, achieving sub-femtomolar levels of detection, which is considered to be a remarkable contribution to the field. The nanoparticles, used as electron transfer material, were suspended in ethanol and placed onto the electrode surface. Subsequently, acrylic microspheres were deposited onto the modified electrode for DNA probes immobilization via covalent bonding. The hybridization process was monitored by DPV of anthraquinone-2-sulfonic acid monohydrate sodium salt as hybridization redox indicator, because of its intercalation capacity in the double helix, increasing the signal when hybridization occurs. The biosensor was used for the determination of GMO extracted from GM soybean samples. They reported a wide linear range of six orders of magnitude with a sub-femtomolar detection limit (0.779 fM) with synthetic oligonucleotides. Such sensitivity was attributed to a robust covalent bond of the DNA probe onto the enhanced large area given by the acrylic microspheres/gold nanoparticles composite.

5.2. Gene-specific methods

Many genosensors have been reported as gene-specific methods (Bonanni et al., 2009; Feng et al., 2008; Jiang et al., 2008; Jiang et al., 2011; Ligaj et al., 2006; Ma et al., 2008; Ren et al., 2005; Yang et al., 2007a; Yang et al., 2012a; Yang et al., 2007b; Yang et al., 2008; Yang et al., 2009; Zhang et al., 2008; Zhou et al., 2009), accounting for more than 30% of the reported GMO sensors so far (Manzanares-Palenzuela et al., 2015b). One of the commonly inserted gene in genetically engineered plants is the Bt gene obtained from *Bacillus thuringiensis*, which encodes an insecticidal protein in plants. Jiang et al. (2011) presented an electrochemical DNA biosensor for a 30-mer Bt fragment. A MCH-mixed SAM was used to immobilize a 15-mer capture probe

in a gold electrode (Figure 8). The authors proposed a sandwich hybridization format, with a signaling probe modified with biotinylated Ag nanoparticles. The modified electrode was incubated with streptavidin and subsequently more biotinylated Ag nanoparticles were added in order to form aggregates that amplified the response. Detection of Ag nanoparticles was carried out using solid-state Ag/AgCl voltammetric process in which the Ag was oxidized to Ag^+ and the electrogenerated Ag^+ formed insoluble AgCl on the electrode surface in the presence of Cl^-. The solid AgCl on the electrode was then reduced to Ag. By measuring this reduction current, a detection limit of 10 fM was obtained, three orders of magnitude less than single Ag nanoparticle label detection.

Other frequently reported target for gene-specific genosensor analysis is the PAT gene, which encodes an herbicide resistant protein, phosphinothricin acetyltransferase. Herein, we limit to discuss the most sensitive approaches for PAT detection. Yang et al. (2009) reported an EIS-based genosensor based on a carbon paste electrode modified by a multi-walled-nanotubes/polyaniline nanofibers (MWCNT/nano-PANI) composite dispersed in chitosan (Figure 9.I). A mixture of all these components was dripped onto the fresh surface of the electrode, followed by the adsorption of the DNA probe. The combination of these materials resulted in the synergistic effect between nano-PANI and MWCNT nanocomposites in chitosan film, giving a highly enhanced conductivity and an increase of the surface area that consequently increases the load of the DNA probe. Quantification of PAT gene was performed and a LOD of 27 fM was estimated. In the same article PCR amplification of the NOS terminator sequence, extracted from a sample of genetically modified soybean, was performed and qualitative results were reported. Another strategy reported by the same group involved the electrodeposition of L-

tyrosine on a carbon electrode surface, followed by the addition of PANI-ZrO_2 nanoparticles (Yang et al., 2012a). The resulting PANI-nanoZrO_2 composite displayed an interconnected dendritic structure (Figure 9.II), which provided high specific surface area for the electron transfer and for the immobilization of the capture probe. The limit of detection for PAT sequence was 26.8 fM using EIS as electrochemical technique.

5.3. Event-specific methods

Event-specific genosensors have been the least reported group compared with other levels of GMO specificity (Barroso et al., 2015; Duwensee et al., 2009; Liao et al., 2013; Manzanares-Palenzuela et al., 2015a; Mix et al., 2012; Sun et al., 2014; Sun et al., 2013). Among these, two works based on an event-specific genosensor for a transgenic variety of maize (MON810) and its application in maize flour samples are worthy of mention because they address important issues in GMO monitoring: multi-target detection and applicability to real samples. The genosensor relied on a direct assay, where the capture probe was immobilized by mixed SAMs with MCH onto gold electrodes. The complex [OsO_4(bipy)] was utilized as an electrochemically reversible redox probe covalently bound to DNA. It reacts only with the pyrimidine bases of ssDNA. The reaction involves the oxidation of the C=C double bond in the pyrimidine ring giving a diester of osmic (VI) acid. Four different targets, two from wild type maize and two from genetically modified maize were simultaneously labeled and detected using four working electrodes, each modified with a specific capture probe. The sequences were labeled simultaneously with the osmium complex and afterwards, detection was achieved with square wave voltammetry and the signal increased proportionally to the target concentration in the nanomolar range (Duwensee et al., 2009).

The application of the previous device to detect MON810 in maize flour samples employed an asymmetric PCR, that is, a PCR that preferentially amplifies one DNA strand in a double-stranded DNA template using a great excess of one of the primers. Results showed that it was possible to discriminate between maize and GM maize in real samples from 0.6 % of GMO content. No significant detection of MON810 was possible when only 0.5% of transgenic maize was present in the sample, most likely due to insufficient amplification of the template DNA (Mix et al., 2012).

Considering the great contributions of multiplexing in GMO detection, given the increasing number of commercialized GMOs, it is important to mention the work of Liao et al. (2013). They reported a genosensor that provides a multiplex GMO analysis in a gold 16-array format. The proposed device implemented a biomolecular computational assay based on the logic gates principle. This consists on logic operations from dual or multiple inputs to produce a single logic output. Exploiting this principle, GMO-related target sequences were used as inputs. The output was the electrochemical signal obtained if hybridization of the targets with specific probes occurred. A monolayer-modified Au electrode surface was prepared with a thiolated capture probe and 3-mercaptopropionic acid. Target DNA was incubated with two signaling probes (functionalized with FITC, or biotin) to ensure homogeneous hybridization. These hybrids were loaded over the DNA capture probe of Au surfaces. A mixture of anti-FITC-HRP and avidin-glucose oxidase solution was added followed by the addition of a mixture of enzyme substrates (glucose and TMB). When all the probes (capture and signaling probes) recognized the target, an enzymatic cascade reaction occurred in which GOx catalyzed the oxidation of glucose to produce H_2O_2, which was immediately reduced by HRP along with the resulting TMBox. The

latter was finally detected by chronoamperometry. Different target sequences were designed corresponding to different GM events. Each sequence was composed by fragments of three GMO regions: a promoter, a trait gene and an endogenous gene. A specific probe recognized each region: the capture probe was complementary to the promoter region and the FITC-labeled and biotinylated signaling probes were complementary to the trait and endogenous genes, respectively. According to the authors, the number of oligonucleotide bases separating the target sequences, especially trait genes from endogenous genes, may play a role in the efficiency of the cascade enzymatic reaction, which requires further attention to meet sequence diversity in various GMOs.

The system was tested with DNA extracted with a commercial kit from soybean powder containing known GM levels. The assay was able to detect concentrations as low as 25 nM of total DNA, when measuring a sample containing 0.9 % GMO. Accordingly, the LOD corresponds to 225 pM of GM DNA level. This device was capable to integrate screening and event-specific methods, and a multiplex detection toward different GM events in one analysis.

Most recently, a multiplex electrochemical platform was reported for Roundup Ready soybean quantification (Manzanares-Palenzuela et al., 2015a). The work consisted on the simultaneous detection of two DNA sequences, event and taxon-specific, via sandwich hybridization onto magnetic microparticles. Both biotinylated capture probes were anchored to the surface of streptavidin-modified magnetic beads. The target sequences were hybridized with their specific signaling probes, each one end-labeled with FITC and digoxigenin, complementary to the transgenic and taxon sequences, respectively. Dual enzymatic labeling was performed with HRP and ALP, both conjugated to Fab fragments of anti-FITC and anti-digoxigenin, using TMB and

1-naphthyl phosphate as enzymatic substrates, respectively. This dual labeling system permitted to obtain two distinct electrochemical readouts in parallel measurements, using DPV and chronoamperometry for ALP and HRP, respectively. The LOD values obtained were 0.65 pM and 0.19 pM for the event-specific and taxon-specific targets. The transgenic-taxon ratio approach was proposed with the aim of quantifying GMOs according to the European Recommendation 2004/787/EC. To calculate the percentage of GMO in a sample, the accepted criterion is to normalize the amount of the GMO specific sequences against the amount of a plant specific gene. Based on this recommendation, the suitability of the proposed assay was assessed with synthetic mixtures of both sequences containing 0.8% and 2% of GMO. The obtained results were closed to the theoretical ones, giving low relative error values (6.3 and 5%, respectively), claiming that the assay is adequate for GMO quantification. This is the first attempt to quantify GMOs without the use of real-time PCR in terms of percentage as required by legislation.

6. KEY FEATURES IN FOOD SAFETY ASSESSMENT BY GENOSENSOR TECHNOLOGY

In recent years consumers have become very critical about food safety and food quality, especially due to modern controversies related to the hidden presence of allergens in some foods, genetically engineered crops and species authentication (Figure 10). Legal framework covers issues ranging from personal choice (GMOs), the prevention of food fraud and substitution of some food for others of lesser value (food adulteration), and to certify the safety of food to allergic or celiac individuals (allergen issue). It is clear from this review that each one of these topics deserves unique perspectives regarding legislation and analytical monitoring.

In general, food assessment related to GMOs, allergens and species authentication has been based in two analytical methodologies, immunoassays and DNA-based methods. The superior stability of DNA against food processing technologies compared to proteins, holds a major benefit for food-related applications. Among DNA methods, electrochemical genosensors are still considered as an emerging technology under ongoing and continuous research, comprising all kinds of innovative designs for DNA immobilization, labeling and detection. We have reviewed a compendium of genosensors described for specific analytical applications in food control. Detection levels as low as femto- and attomolar have been achieved with GMO and allergen sensors, respectively. Sensitivity of genosensors is mainly conditioned by the electrochemical technique and by the material used as immobilization matrices for DNA capture probes. Among electrochemical detection methods, EIS-based methods showed the best sensitivity and, in relation with the immobilization strategies, nanomaterial combinations provided best limits of detection. Also, the sensitivity is superior when the DNA sequence selected as target is a multicopy gene (Tosar et al., 2010).

It is important to highlight that the results obtained with synthetic sequences, usually used for preliminary optimization of the genosensor, and DNA from real samples, are often very different (Tosar et al., 2010). Several authors (Bettazzi et al., 2008; Del Giallo et al., 2005; Martin-Fernandez et al., 2014) have studied this difference in sensor performances and they have concluded that sometimes it is required to redesign the target-complementary probes in order to obtain adequate analytical characteristics.

However, depending on the intended application of the sensor, analytical requirements can vary, e.g. the need of obtaining very low detection limits. For instance, allergens require labeling

regardless its quantity and severe reactions can be induced by very low amounts, so ultrasensitive methods are needed for allergen detection. On the contrary, in GMO analysis, sensitivity is not an issue according to the detectability required by legislation and that obtained with some electrochemical approaches. However, real quantitation aimed at evaluating compliance with legislation requires the implementation of two measurements in parallel, the taxon-specific and event-specific targets, which is rarely addressed. First trials recently have shown that this goal is achievable and deserves further development to bring genosensors and genoassays to the marketplace.

Among the three topics revised in this work, allergen detection may be the most challenging task for DNA-methods, due to the fact that they are based on its indirect detection. DNA quantity is difficult to correlate with protein content in a given sample. This, together with the high sensitivity required to detect the lowest possible quantity of the allergenic species, makes it a defiant work. Additionally, the absence of reference materials hampers the development of quantitative methods to assess some allergens, e.g. gluten. The development of these materials would undoubtedly assist on the validation of genosensors towards this aim, raising their potential for their application in real samples.

On the other hand, genosensor applicability is often conditioned by extensive pre-treatment steps. Ideally, the genosensing technology should provide simple, direct and quick analysis of untreated samples. However, given that DNA is within cells, its concentration is usually very low in food matrices, and there are many interfering agents that prevent the direct application of genosensors to this type of samples, extraction, amplification and sometimes purification are required prior to final detection. An example regarding the low quantity of DNA in food samples

is given by one of the reviewed genosensors, in which a peanut allergen-related sequence was detected with a linear response in the range of 10^{-17} to 10^{-15} M with synthetic oligonucleotides; after extracting genomic DNA from a commercial peanut sample and performing PCR amplification, a content of 10^{-11} M of the peanut sequence was quantified (Sun et al., 2015b). Considering that PCR amplifies billions of copies of DNA in a sample, the initial quantity of DNA was extremely low to be detected without PCR, especially in the case of allergens as it was previously mentioned, in which case the sensitivity needs to be higher.

From this point of view, more merit should be given to those DNA sensors capable of analyzing complex food samples, using as few sample pretreatment steps as possible. Overviewing the works reported so far, there are significant differences in relation to the complexity of analyzed samples. Some devices required post-PCR purification of amplicons, increasing the time and cost required to perform the method. Thus, great efforts should be attained in this regard, since most electrochemical sensors described to this point require, at a minimum, a pretreatment step consisting in the extraction of DNA and subsequent amplification (PCR or LAMP). Few of the reviewed works detected DNA from real food matrices without prior amplification (Liao et al., 2013; Mascini et al., 2005). In Mascini's work, the fact that the target was part of a multicopy gene makes it highly available for analysis without the need of amplification. This is why the pretreatment only was assisted by digestion with restriction enzymes, in order to cut the DNA fragment prior to the biosensing protocol. These steps, DNA extraction, amplification and/or cleavage, are time-consuming and require expensive reagents, but today are unavoidable. In the case of Liao et al., high amounts of raw material were required to detect threshold levels of GMOs. Only a few electrochemical sensors applied to other types of targets, such as clinical

markers and pathogenic targets, have been able to analyze DNA samples without prior processing (Tosar et al., 2010). However, current research aimed at finding ultrasensitive detection methods with minimized nonspecific electrochemical signals, will make it possible to provide quick and simple analysis, avoiding sample pretreatment steps in the near future.

Food safety is a greatly debated topic nowadays. We have evaluated how this has been reflected in the growing number of publications regarding novel technologies for food assessment. Genosensors can fulfill desirable characteristics for food testing, such as low cost, miniaturized and portable equipment, execution simplicity and robust results, and that is why many devices have been reported for this aim. Yet, there is still much work to do for routine application, because many drawbacks have not been overcome, especially when practical applicability is intended. However, the high sensitivity and versatility of the reviewed papers demonstrate the remarkable potential of this promising technology.

ABBREVIATIONS

ALP Alkaline Phosphatase

Biot Biotin

bp Base pair

Bt *Bacillus thuringiensis* gene

CA Chronoamperometry

DPV Differential Pulse Voltammetry

EIS Electrochemical Impedance Spectroscopy

ELISA Enzyme Linked Immunosorbent Assay

EU European Union

FALCPA Food Allergen Labeling and Consumer Protection Act

FITC Fluorescein Isothiocyanate

GCE Glassy carbon electrodes

GM Genetically Modified

GMO Genetically Modified Organism

HRP Horseradish Peroxidase

LAMP Loop-mediated isothermal amplification

LOD Limit of Detection

LSV Linear Sweep Voltammetry

MCH Mercaptohexanol

MWCNT Multiwall Carbon Nanotubes

NOS nopaline synthase (GMO terminator sequence)

PAT Phosphinothricin Acetyltransferase

PCR Polymerase Chain Reaction

PEP Phosphoenolpyruvate carboxylase promoter

SAMs Self-Assembled Monolayers

SPEAu Screen-printed gold electrode

SPEC Screen-printed carbon electrode

Strp Streptavidin

TMB Tetramethylbenzidine

US United States

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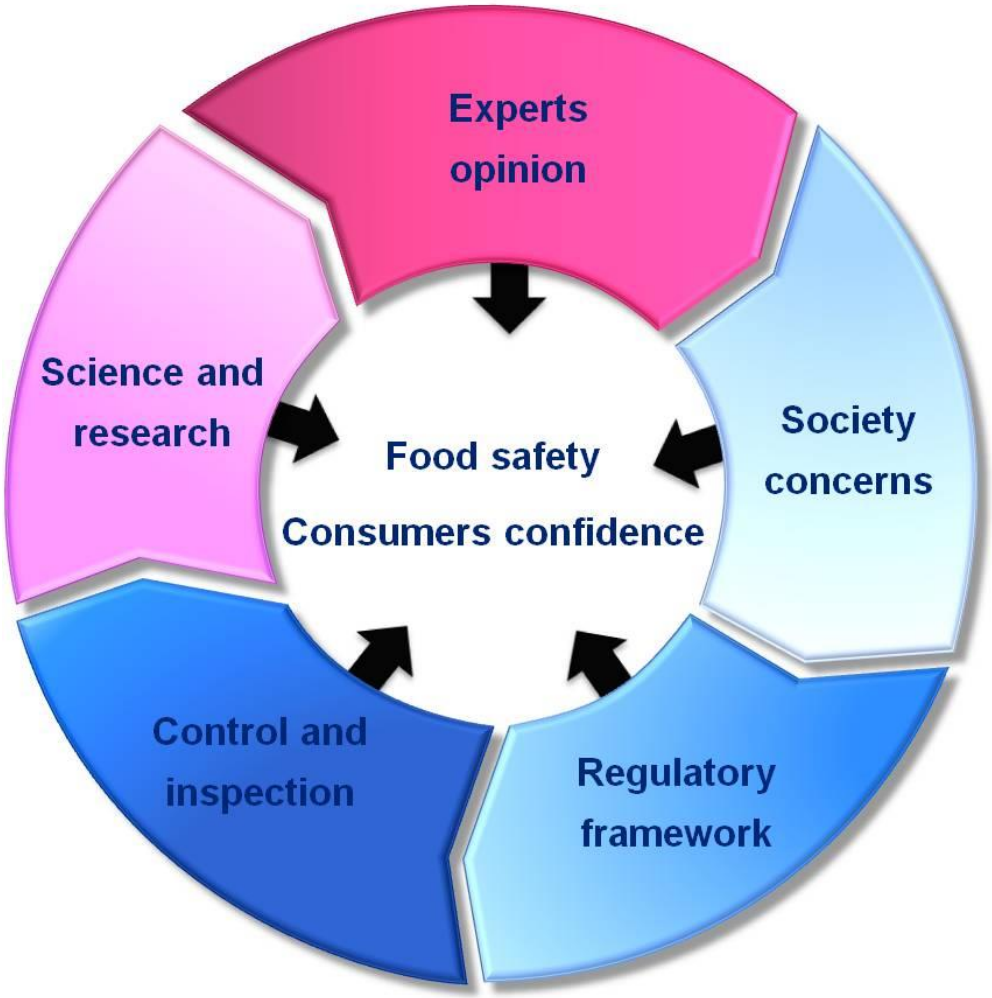


Figure 1 Representation of the main agents that improve food safety and quality and increasing consumer confidence.

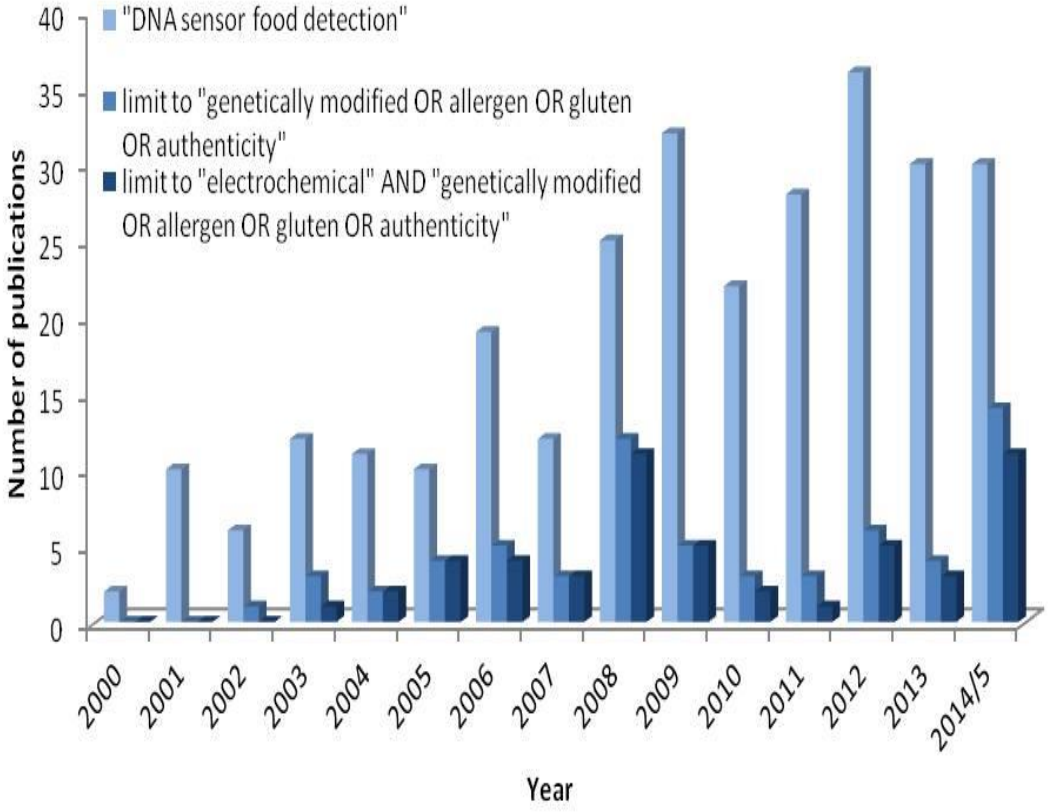


Figure 2 Number of publications reporting DNA sensors since 2000 till 2014/5. The publications were retrieved from ISI Web of Knowledge and Scopus and analyzed.

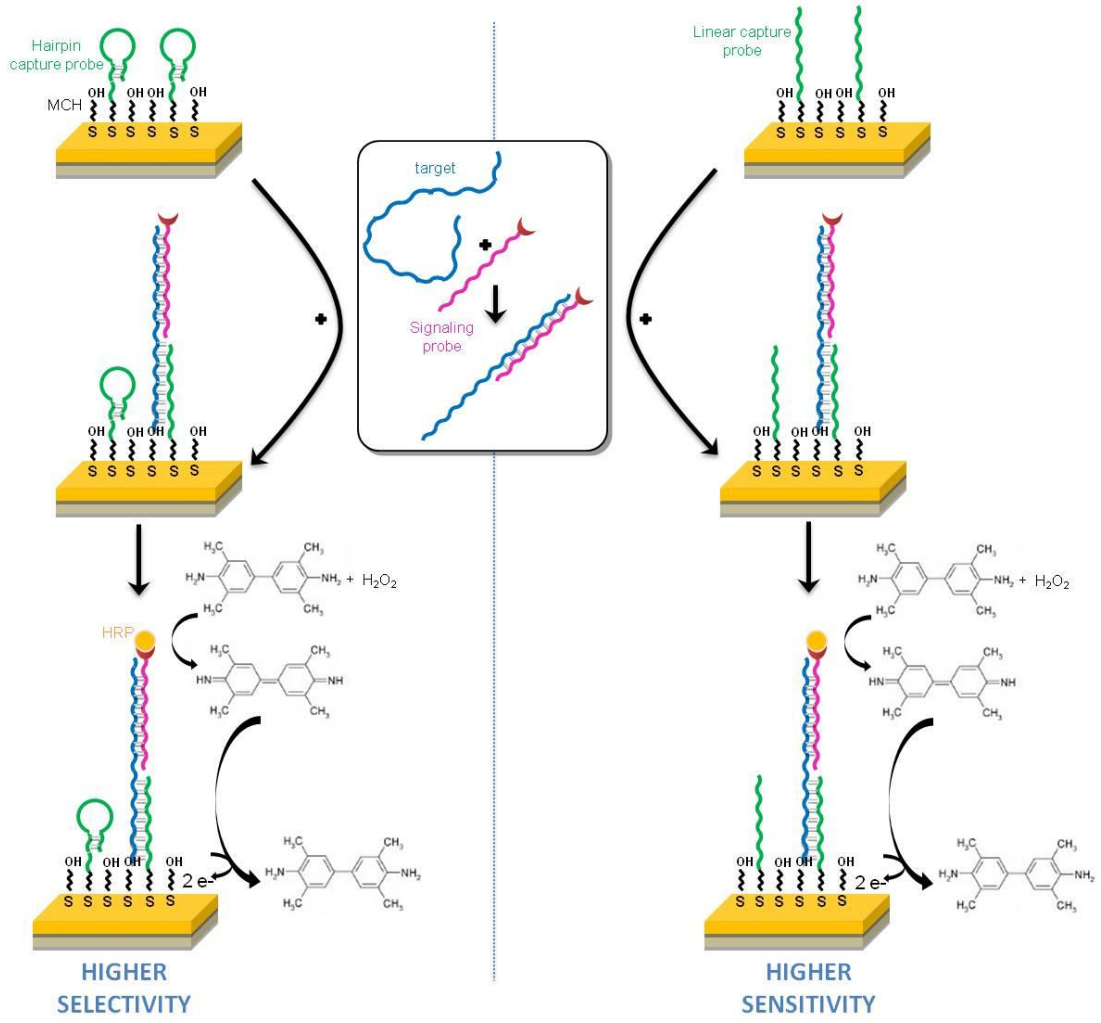


Figure 3 Schematic illustration of the sensing phase and the assay steps of the genosensors reported for gluten detection.

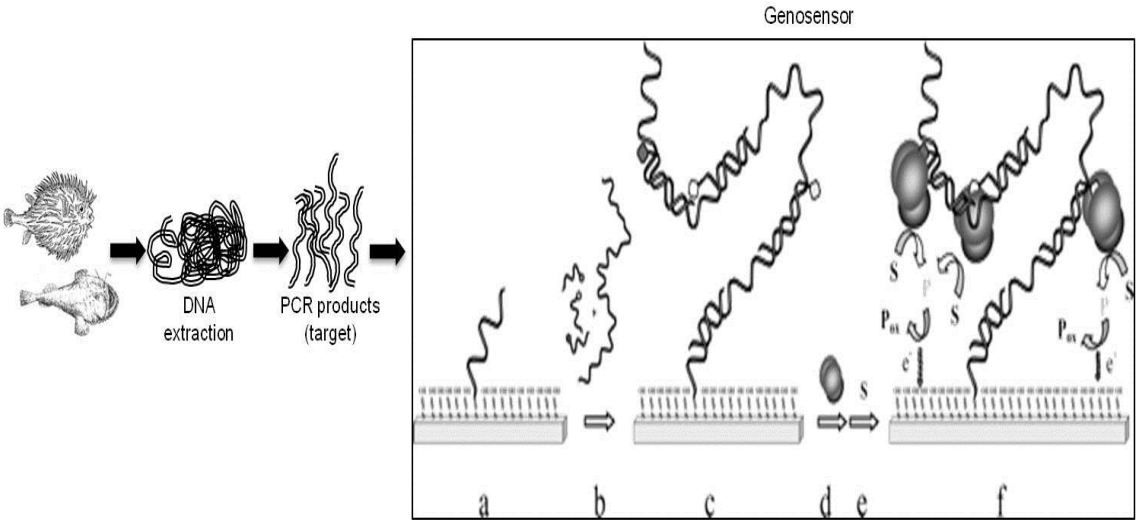


Figure 4 Schematic representation of the dendritic-like signal amplification path. Unmodified PCR products (b) were captured at the sensor interface (a) via sandwich hybridization with the surface-tethered probe and up to three biotinylated signaling probes. The polybiotinylated hybrid (c) was then coupled with streptavidin-alkaline phosphatase conjugates (d) and exposed to the enzyme substrate solution (e). DPV was finally used to detect the oxidation signal of the product of enzymatic hydrolysis (f). Reprinted with permission from (Del Giallo et al., 2005) Copyright (2005) American Chemical Society.

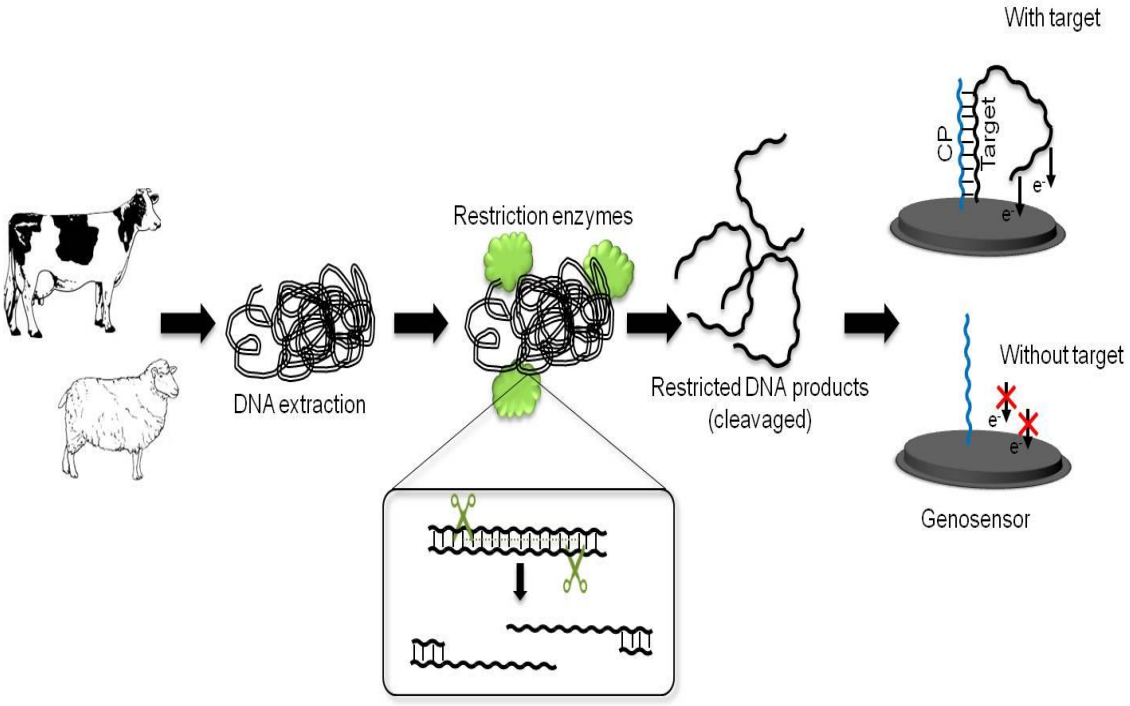


Figure 5 Meat species identification with genosensors with previous genomic DNA cleavage by restriction enzyme digestion

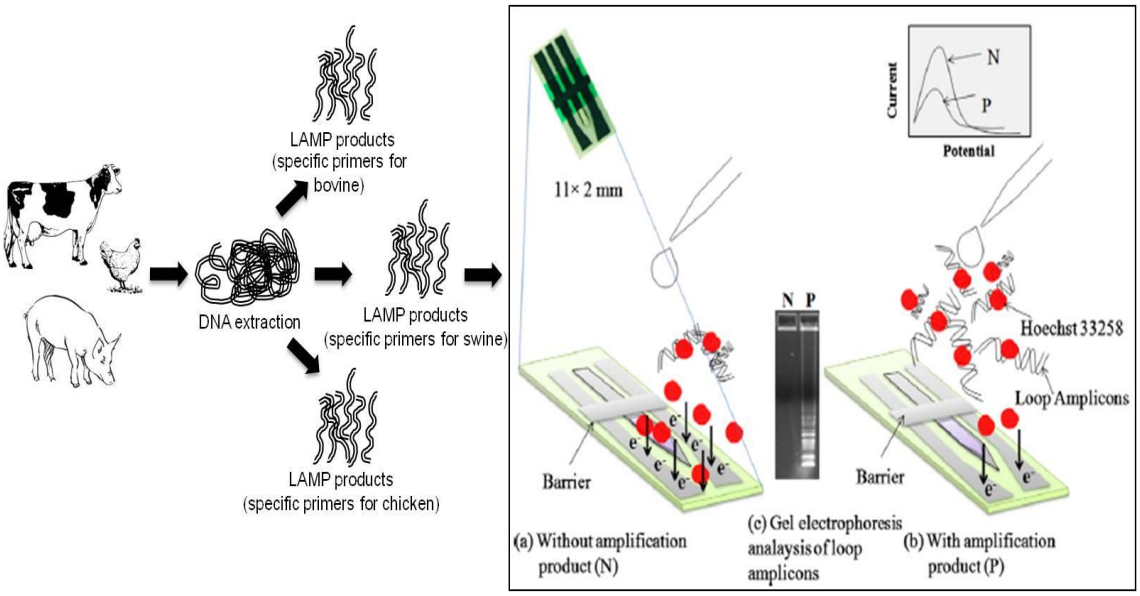
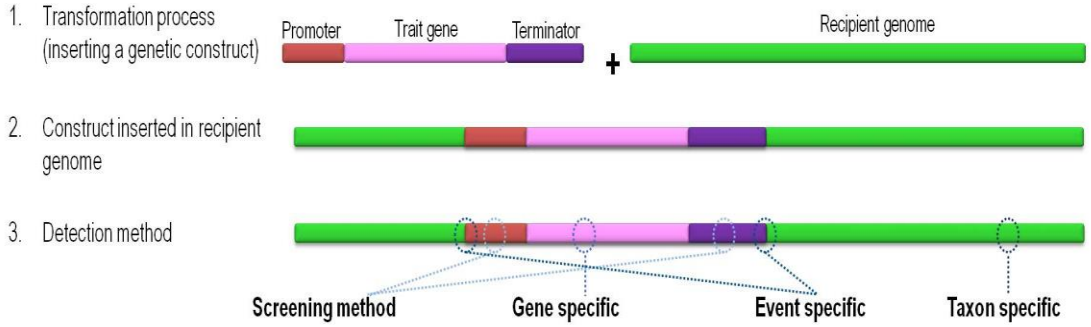


Figure 6 Species-specific identification and detection of loop amplicons based on DNA–H33258 interaction using the disposable carbon-based electrochemical printed chip. A carbon barrier is used to prevent the reaction mixture to adhere with the chip connector. (a) Higher rate of diffusion and oxidation of H33258 molecules on the electrode surface in the absence of DNA. (b) Lower oxidation observed due to DNA–H33258 interaction and for the low amount of unbound/free H33258 molecule. (c) Gel electrophoresis analysis of negative sample, N and positive sample, P are shown. On the right top inset, the illustration is showing the electrochemical response. Adapted with permission from Elsevier (Ahmed et al., 2010).



Target	LOD (M)	Ref.
35S	$2.5 \cdot 10^{-10}$	(Carpini et al. 2004)
	$1.2 \cdot 10^{-12}$	(Lucarelli et al. 2005)
	Not reported	(Xu et al. 2006)
	Not reported	(Kerman et al. 2006)
	$1 \cdot 10^{-9}$	(Wang et al. 2008)
	$4.38 \cdot 10^{-12}$	(Sun et al. 2008)
	$1.6 \cdot 10^{-11}$	(Xie et al. 2008)
	Not reported	(Lien et al. 2010)
	$7.79 \cdot 10^{-16}$	(Ulianas et al. 2014)
35S and NOS	Not reported	(Tichoniuk et al. 2008)
NOS	$2.4 \cdot 10^{-6}$	(Meric et al. 2004)
	$2.75 \cdot 10^{-12}$	(Sun et al. 2007)
	$3.6 \cdot 10^{-8}$	(Zhu et al. 2008)
nptII	Not reported	(Ligaj et al. 2003)
	$1 \cdot 10^{-10}$	(Wang et al. 2009)
PEP gene	$3.6 \cdot 10^{-13}$	(Yang et al. 2012b)

Target	LOD (M)	Ref.
Bar	Not reported	(Ligaj et al. 2006)
Bar and CP4 EPSPS amplicons	$2.25 \cdot 10^{-8}$ (Bar)	(Ren et al. 2005)
Bt	$1 \cdot 10^{-14}$	(Jiang et al. 2011)
	$72 \cdot 10^{-15}$	(Bonanni et al. 2009)
PAT	$3.1 \cdot 10^{-13}$	(Feng et al. 2008)
	$1.5 \cdot 10^{-12}$	(Ma et al. 2008)
	$2.4 \cdot 10^{-11}$	(Yang et al. 2007b)
	$3.1 \cdot 10^{-11}$	(Zhang et al. 2008)
	$2.68 \cdot 10^{-14}$	(Yang et al. 2012a)
PAT and NOS amplicons	$1.4 \cdot 10^{-12}$ (PAT)	(Yang et al. 2007a)
	$2.6 \cdot 10^{-12}$ (PAT)	(Yang et al. 2008)
	$3.1 \cdot 10^{-13}$ (PAT)	(Jiang et al. 2008)
	$2.7 \cdot 10^{-14}$ (PAT)	(Yang et al. 2009)
	$5.6 \cdot 10^{-13}$ (PAT)	(Zhou et al. 2009)

Target	LOD (M)	Ref.
SSIIb ivrp cryIa/b event MON810	Not reported	(Duwensee et al. 2009)
	0.6 %	(Mix et al. 2012)
35S G1b1 CP4-EPSPS PAT cordapaA lectin SSIIb	$2.25 \cdot 10^{-10}$	(Liao et al. 2013)
A2704-12 soybean	$2.9 \cdot 10^{-13}$	(Sun et al. 2013)
MON810	$4.52 \cdot 10^{-12}$	(Sun et al. 2014)
	$4.8 \cdot 10^{-10}$	(Barroso et al. 2015)
Lectin RR Soybean	$1.9 \cdot 10^{-13}$	(Manzanares et al. 2015a)

Target	LOD (M)	Ref.
SSIIb ivrp	Not reported	(Duwensee et al. 2009)
		Mix et al. (2012)
Lectin	$2.25 \cdot 10^{-10}$	(Liao et al. 2013)
	$6.5 \cdot 10^{-13}$	(Manzanares et al. 2015a)

Figure 7 Scheme of the transformation process in GMOs. Levels of specificity of analytical methods targeting DNA sequences and electrochemical genosensors described for each level, highlighting their sensitivity and the detected target. LOD obtained with synthetic oligonucleotides.

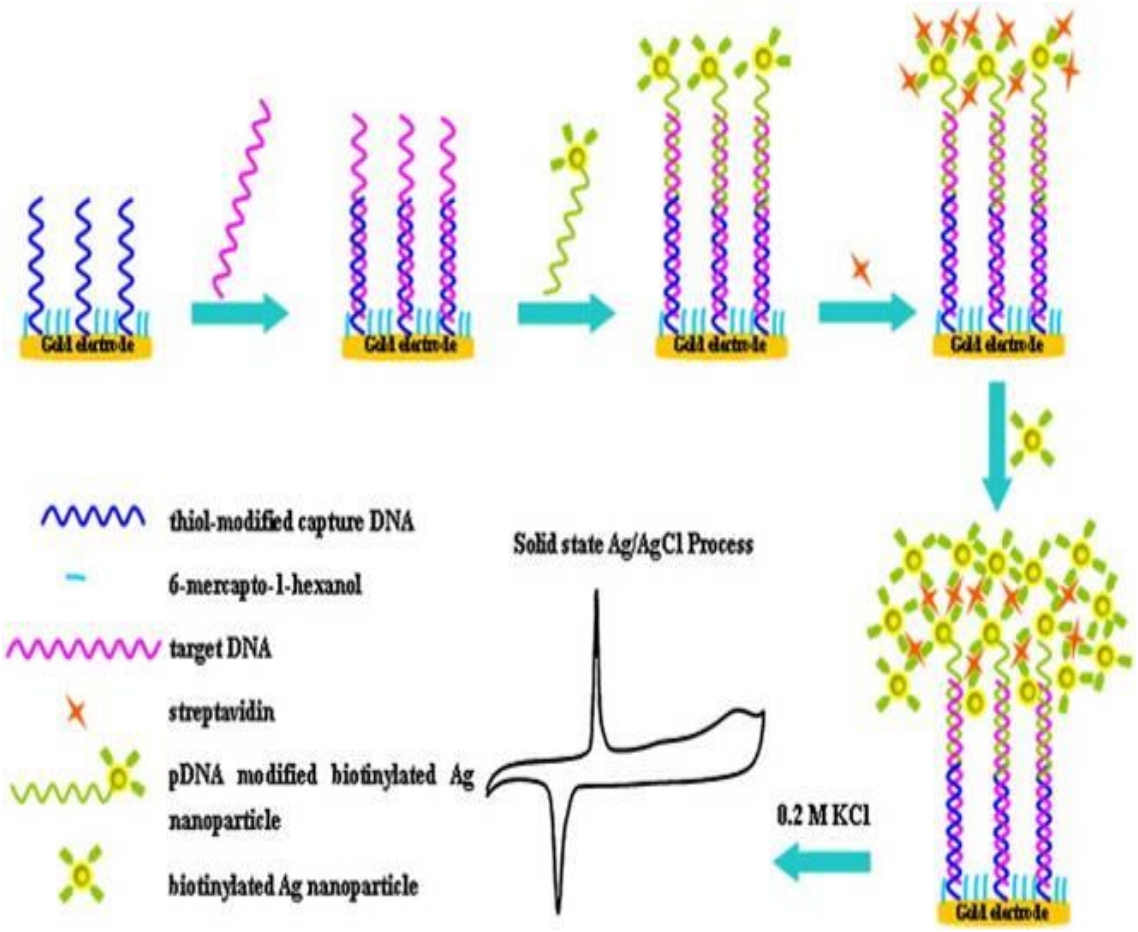


Figure 8 Schematic image of the DNA sensing strategy. Reproduced with permission from Elsevier (Jiang et al., 2011).

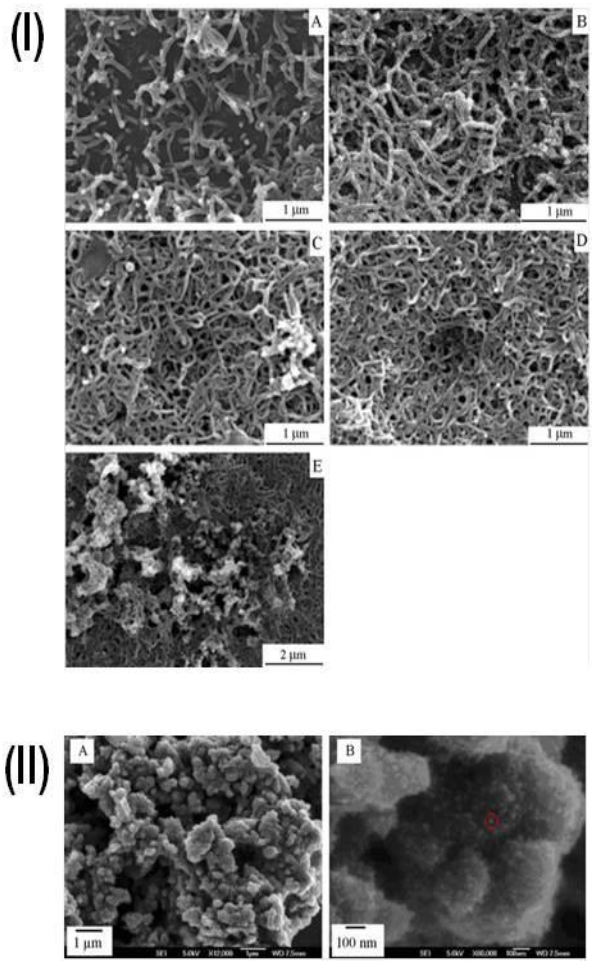


Figure 9 I) SEM images of PANI nanomaterials (A) and PANI-MWCNT nanocomposites film under different ratios of PANI to MWCNT as 1:0.01 (B), 1:0.1 (C), 1:1 (D) and 1:2 (E). Reproduced with permission from Elsevier (Yang et al., 2009). II) The SEM images at low magnification (A) and high magnification (B) of PAN-nanoZrO_2 Reproduced with permission from Elsevier (Yang et al., 2012a).

Figure 10 Hot topics of public concern related to food products