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Electrochemical biosensors for food safety

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Resum. La convergència de tecnologies com la nanotecnologia, la biotecnologia i les tecnologies de la informació estan obrint nous camins en el camp dels biosensors electroquímics. La integració de materials microestructurats i nanoestructurats en dispositius biosensors (com micropartícules de grafit, bioreceptors, nanopartícules d'or o micropartícules o nanopartícules magnètiques) proporciona característiques excel·lents per a l'anàlisi de residus alimentaris (plaguicides o antibiòtics), additius alimentaris (àcid fòlic), al·lergògens alimentaris (gliadina) i patògens transmesos pels aliments. Una de les contribucions clau en el camp de la biodetecció electroquímica es basa en el disseny de transductors nous, no solament amb propietats transductores realçades, sinó també amb la immobilització millorada de biomolècules, que retenen l' activitat biològica. La integració de partícules magnètiques és un avenç molt clar en la detecció de residus alimentaris en matrius complexes, que millora les respostes electroquímiques perquè augmenta l'àrea activa transductora. En aquesta contribució es descriuen sistemes immunosensors i genosensors electroquímics desenvolupats recentment en el nostre laboratori com a mètodes de detecció, d'anàlisi i de diagnòstic ràpids, sensibles i automatitzables per als contaminants alimentaris, principalment residus alimentaris i patògens.

Paraules clau: tecnologies convergents · immunosensors · biosensors de DNA · patògens bacterians · residus alimentaris · plaguicides · antibiòtics · al·lergògens · partícules magnètiques · nanopartícules d'or · micropartícules de grafit

Summary. The convergence of new technologies, including nanotechnology, biotechnology, and information technology, has opened new horizons in electrochemical biosensors. The integration of micro- and nanostructured materials within biosensing devices (graphite microparticles, bioreceptors, gold nanoparticles, and magnetic micro- or nanoparticles) has provided excellent analytical performances in the detection of food residues (pesticides, antibiotics), food additives (folic acid) and allergens (gliadin), and food-borne pathogens. One of the key contributions in the electrochemical biosensing field relies on the design of novel transducers, not only with enhanced transducing features but also with improved immobilization of biomolecules while preserving their biological activity. The integration of magnetic particles provides further improvements in the detection of food residues in a complex matrix, achieving better electrochemical response due to the increased size of the transducing active area. Electrochemical immunosensors and genosensors systems recently developed in our laboratories are described in this contribution. These sensitive, hand-held, user-friendly devices provide new analytical approaches for the in-field detection of food contaminants, mainly food residues and pathogens.

Keywords: converging technologies · immunosensors · DNA biosensors · pathogenic bacteria · food residues · pesticides · antibiotics · allergens · magnetic beads · gold nanoparticles · graphite microparticles

Introduction

Agents affecting food safety. In recent years, a number of high-profile food-safety emergencies have shaken consumer confidence in the production of food, focusing attention on how food is produced, processed, and marketed [24]. The European Commission has identified food safety as one of its top priorities and has established plans for a proactive new food policy—modernizing legislation into a coherent and transparent set of rules, reinforcing controls from the farm to the table, and increasing the capability of the scientific advisory system—so as to guarantee a high level of human health and consumer protection. An effective food safety policy requires assessment and monitoring of the risks to consumer health associated with contaminants in raw materials, farming practices, and food processing activities.

Contaminants in food can be microbiological (bacteria, virus, parasites), exogenous matter (biological, chemical, physical), natural toxins (seafood toxins, mycotoxins), other chemical compounds (pesticides, toxic metals, veterinary drug residues, undesirable fermentation products), and packing materials. Most of the agents found in food are natural contaminants from environmental sources, but some are deliberate chemical compounds added during food processing [34]. Many consumers are concerned about the long-term impacts

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of mixtures of chemical additives (pesticides, toxic metals, veterinary drug residues, flavorings, and colors) and their chronic as well as acute effects, especially on children [35]. While such additives have been a major concern, microbiological issues are currently the most important, especially the presence of food pathogens (the most common being *Salmonella*, *Escherichia coli*, and *Listeria*), followed by pesticide and animal drug residues as well as antimicrobial drug resistance [41].

Food regulatory agencies have thus established control programs to prevent food contaminants from entering the food supply. Food quality and safety can only be insured through the enforcement of quality-control systems throughout the entire food chain. They must be implemented at the farm level through: (i) the use of good agricultural and veterinary practices at the production level, (ii) good manufacturing practices at the processing level, and (iii) good hygienic practices at the retail and catering levels. One of the most effective ways for the food sector to protect public health is to base their food management programs on hazard analysis critical control point (HACCP). This systematic approach to the control of potential hazards within a food operation aims to identify problems before they occur [35].

Culture and instrumental methods for food pathogen detection. Conventional bacterial identification methods usually include morphological evaluation of the microorganism as well as tests of its ability to grow in various media under a variety of conditions. This is accomplished by: (i) pre-enrichment, (ii) selective enrichment, (iii) biochemical screening, and (iv) serological confirmation [40]. However, these growth and enrichment steps are relatively time-consuming, with a total assay time of up to 1 week for certain food pathogens [14]. Although classical culture methods can be sensitive, they are greatly restricted by the assay time. As long as 96 h may be needed to obtain a negative result if the entire culture process has to be applied, while presumptive positive results may be obtained within 48–96 h. Accordingly, many alternative methods have been introduced in recent years to reduce analytical and staff time as well as media requirements. These rapid methods are designed to avoid the need for selective culturing and serological/biochemical identification.

The new instrumental methods developed using various principles of detection are chromatography, infrared or fluorescence spectroscopy, bioluminescence, flow cytometry, and other techniques based on electrical conductance and impedance. However, these methods are centralized in large stationary laboratories as they require complex instrumentation and highly qualified technical staff [14].

During the past decade, immunological detection has become more sensitive and reliable, stimulated by technological improvements that have resulted in the rapid and inexpensive production of polyclonal antibodies. Consequently, many commercial immunoassays are now available for the detection of a wide range of bacteria in food. These test kits include immunodiffusion, enzyme linked immunosorbent assays (ELISA), and the use of specific antibodies to "capture and concentrate" the organism [23].

Nucleic-acid-based detection coupled with polymerase chain reaction (PCR) has distinct advantages over culture and other standard methods for the detection of microbial pathogens, such as specificity, sensitivity, rapidity, accuracy, and the capacity to detect small amounts of target nucleic acid in a sample [42]. In addition, multiple primers can be used to detect different pathogens in a single multiplex reaction [16]. The use of nucleic-acid recognition layers represents a new and exciting area in analytical chemistry but requires further study before it can be commercially applied. Besides classical methodologies to detect DNA, novel approaches have been designed, such as DNA chips [5] and "lab-on-a-chip" based on microfluid techniques [36]. These technologies are still beyond the scope of the food industry, which requires simple, inexpensive, and user-friendly analytical devices.

Detection methods for food residues. As pesticides and many drugs produce undesirable residues, their use has been regulated by various national and international authorities, which have set maximum residue levels (MRLs) for food [24]. The MRL is the maximum concentration of a food residue and/or its toxic metabolites that is legally permitted in food commodities and animal feed. Authorities have also introduced a definition for residues of veterinary medicinal drugs, which, according to EU Council Regulation 2377/90, states: "All pharmacologically active substances whether active principles, excipients or degradation products, and their metabolites which remain in food stuffs obtained from animals to which the veterinary medicinal product in question has been administered." Monitoring of such residues in foods is often at the microgram per kilogram level.

Multi-residue analysis has been carried out using conventional chromatographic methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). GC has proved to be an excellent technique for the detection of volatile pesticides and drug residues. Previously, thermal conductivity, flame ionization, and, in certain applications, electron capture and nitrogen phosphorus (NPD) detectors were commonly used in GC analysis. In current GC-based residue detection methods, mass spectrometry(MS) in combination with electron-impact ionization (EI) is by far preferred due to the universality, selectivity, and specificity of this technique. HPLC is increasingly being employed in the determination of pesticide and drug residues, as it is especially suited to the analysis of non-volatile, polar, and thermally labile residues that are difficult to analyze using GC. Besides physicochemical methods, microbiological growth-inhibition assays have long been used to test meat and milk for the presence of antibiotics residues [3].

Immunochemical methods rapidly screen for the presence of an individual residue or a group of closely related residues. They require little or no sample clean-up or expensive instrumentation and are suitable for field use. Antibodies against almost all major food residue compounds are currently available and are among the many recent developments in immunoassays aimed at the detection of food components and contaminants [6]. Classical immunochemical methods, such as

immunodiffusion and agglutination methods, for food analyses generally do not involve labeled antigen or antibody and remain in use. Moreover, the scope of immunoassays has been widened by radioimmunoassay (RIA) techniques while non-radioactively labeled markers form the basis of numerous immunological detection methods, including fluorescence immunoassay (FIA), time-resolved FIA, FIA polarization immunoassay, enzyme immunoassay (EIA), luminescent immunoassay (LIA), metalloimmunoassay (MIA), and viroimmunoassay (VIA). EIA is a general term for immunoassays in which an enzyme is used as a marker for the detection of immunocomplex formation. In the most common type of EIA, the ELISA, the assay is performed on solid supports, such as microtiter plates, cellulose, nylon beads/tubes, nitrocellulose membrane, polystyrene tubes/balls, and modified magnetic beads. In some cases, the solid surface is coated with staphylococcal protein A or protein G, entrapping the antibody for subsequent analysis. ELISAs may be competitive, in which both hapten and macromolecule can be analyzed, or of the noncompetitive sandwich-type, whose use is restricted to the detection of divalent and multivalent antigens.

There are a number of quick screening tests based on the ELISA principle described above. For example, microtiter plate ELISA assays can be completed in less than 20 min. Other approaches involve immobilizing the antibody on a paper disk or other membrane that is mounted on a plastic card, in a plastic cup, or on the top of a plastic tube. In the “dipstick” assay, antibody or antigen is coated on a stick that is then dipped in various reagents and the reactions monitored. Most of these screening tests are very simple, easily performed, and are designed to provide semiquantitative information at defined cut-off concentrations for the substance of interest. Immunoscreening tests have gained wide application for monitoring residues in foods, with versatile assay kits commercially available.

Biosensing strategies for food safety

The demand for rapid, real-time, simple, selective and low-cost techniques for the detection of food residues has led to rapid advancements in biosensors [16]. These compact analytical devices incorporate a biological sensing element either closely connected to or integrated within a transducer system. The combination of biological receptor compounds (antibody, enzyme, nucleic acid) with the transducers allows, in most cases, real-time observation of a specific biological event (e.g., antibody-antigen interaction) [9]. Depending on the method of signal transduction, biosensors can also be electrochemical, optical, thermometric, piezoelectric, or magnetic [39]. They enable the detection of a broad spectrum of analytes in complex sample matrices and have shown great promise in areas such as clinical diagnostics, food analysis, bioprocessing, and environmental monitoring. The sensitivity of a particular sensor system varies depending on the transducer’s properties and the biological recognizing elements. An ideal biosensing device for the rapid detection of food contaminants should be fully automated, inexpensive, and able to be used routinely in the field as well as in the laboratory. Optical transducers are particularly attractive since they allow direct label-free and real-time detection, but they lack sensitivity. Surface plasmon resonance (SPR) has shown good biosensing potential, with many commercial SPR systems now available. Among these, the Pharmacia BIAcore (a commercial SPR system), which is based on optical transducing, is by far the most frequently reported method for biosensing food residues in food. However, as analytical systems, electrochemically based transduction devices are more robust, easy to use, portable, and inexpensive.

Electrochemical immunosensors and genosensors are capable of meeting the demands of food monitoring in addition to being of considerable promise in terms of providing information in a faster, simpler, and less expensive manner than is the case with traditional methods. Such devices have great potential for applications ranging from decentralized clinical testing to environmental monitoring, food safety, and forensic investigations.

Electrochemical biosensing based on graphite-epoxy composites. Rigid conducting graphite-epoxy composites (GEC) based on graphite microparticles have been extensively used in our laboratories and shown to be suitable for electrochemical (bio)sensing due to their unique physical and electrochemical properties [1]. Carbon composites result from the combination of carbon with one or more dissimilar materials. Each component maintains its original characteristics while conferring upon the composite distinctive chemical, mechanical, and physical properties. The user’s ability to integrate various materials is one of their main advantages.

An ideal material for electrochemical biosensing should allow the effective immobilization of bioreceptor on its surface, a robust biological reaction between the target and the bioreceptor, a negligible non-specific adsorption of the label, and a sensitive detection of the biological event. GECs fulfill all these requirements. Other advantages of GEC-based biosensing devices over more traditional carbon-based materials are: higher sensitivity, robustness, and rigidity in addition to greater simplicity of preparation. Additionally, the GEC surface can be regenerated by a simple polishing procedure. Unlike carbon paste and glassy carbon, the malleability of the GEC material before the curing step permits different configurations with respect to shape and size which are then fixed after the curing step. Moreover, the surface of the composite can be easily modified by dry and wet adsorption of the bioreceptor (DNAs, oligonucleotides, proteins, antibodies), yielding a reproducible and stable layer of bioreceptor on the transducer surface [31] (Fig. 1-A1, -A2) that can be used in electrochemical detection (Fig. 1D).

The direct adsorption of DNA and proteins on solid supports has the main advantages of simplicity as well as applicability to almost any type of macromolecule or solid support, since no reagents or reactive functions are required. However, the main disadvantage is that the biomolecule is not oriented because it is bound to the surface at multiple sites [31]. Based on its dual nature, with hydrophobic and hydrophilic domains, the GEC

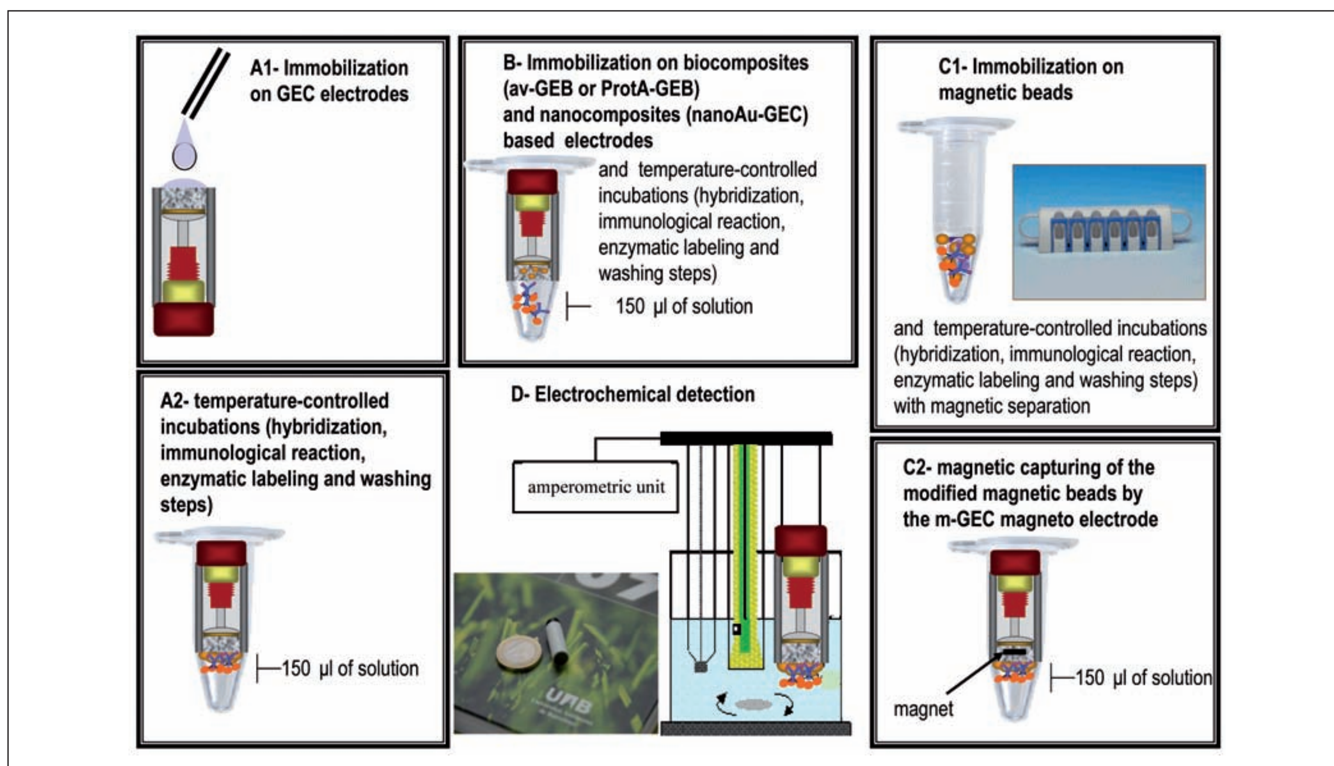

Fig. 1. Schematic representation of the procedures based on GEC biosensors using different strategies for food analysis. (**A1**) Immobilization and (**A2**) modification of graphite-epoxy composite (GEC) based biosensors. (**B**) Immobilization and modification of graphite-epoxy biocomposite (Av-GEB and ProtA-GEB) and gold nanoparticles graphite-epoxy nanocomposite (AuNP-GEC) based biosensors. (**C1**) Immobilization and (**C2**) modification of graphite-epoxy magnetocomposite (m-GEC) combined with magnetic beads or magnetic nanoparticles. (**D**) Electrochemical detection.

platform has demonstrated excellent food-safety features with respect to the stable adsorption of different biomolecules (mainly DNA and proteins) and without a loss in biorecognition capacity, at least for DNA biosensors (Fig. 2-A2) and immunosensors (Fig. 2-A3).

Electrochemical biosensing based on graphite-epoxy biocomposite. An additional interesting property of GECs is their biocompatibility. This feature allows not only adsorption but also integration of the bioreceptor into the bulk of the GEC without subsequent loss of the receptor's biological properties, thus generating a rigid and renewable transducing material for biosensing, namely, a graphite-epoxy biocomposite (GEB) (Fig. 2-B1–B3). With the bioreceptor integrated within its bulk, the biocomposite acts as a reservoir for the biomolecule while retaining all the interesting electrochemical and physical features previously described for GECs. The main advantage of GEBs is that they can be easily prepared by adding the bioreceptor to the composite formulation using dry-chemistry techniques, thereby avoiding tedious, expensive, and time-consuming surface immobilization procedures. Moreover, the surface of GEB electrodes can be easily modified (Fig. 1B) with DNA, oligonucleotides, proteins, antibodies for electrochemical detection, etc. (Fig. 1D).

The use of affinity proteins such as avidin, protein A or protein G, in the biocomposite provides a robust platform for the oriented immobilization of DNA (Fig. 2-B2) or immunoreagents (Fig. 2-B3) that improves the performance of the electrochemical biosensing devices by ensuring exposure of the bioreceptor to the complementary sites of the target molecule.

With advancements in the knowledge about avidin–biotin interactions, this system has become an extremely versatile tool. Strept(avidin) can be considered as a universal affinity biomolecule based on its ability to link not only biotinylated DNA and oligonucleotides but also biotinylated enzymes or antibodies. The extremely specific and high affinity reaction between biotin and the glycoprotein avidin (association constant K_a = 10^{15} M) leads to strong associations, similar to the formation of a covalent bond [15]. Protein A, produced by *Staphylococcus aureus,* is a highly stable receptor capable of binding to the Fc region of immunoglobulins, such as IgG, from a large number of species [38]. When the antibodies are immobilized through their Fc fragment to protein A (or G), their Fab binding sites are mostly oriented away from the solid phase. As protein A is able to link the Fc region of different antibodies, there is no need to previously modify the antibody. A rigid and renewable transducing material for electrochemical immunosensing or genosensing, based on bulk-modified GEBs, can be easily prepared by adding a small amount (2%) of an affinity protein, such as avidin, protein A or protein G, to the formulation of the composite to obtain, in this case, Av-GEB, ProtA-GEB, or ProtG-GEB respectively [33,44]. After its use, the electrode surface can be renewed by a simple polishing procedure, thus allowing multiple uses—a further advantage of these materials with respect to surface-modified approaches such as classical biosensors and other common biological assays. These transducers therefore represent excellent alternatives for biosensing in food safety.

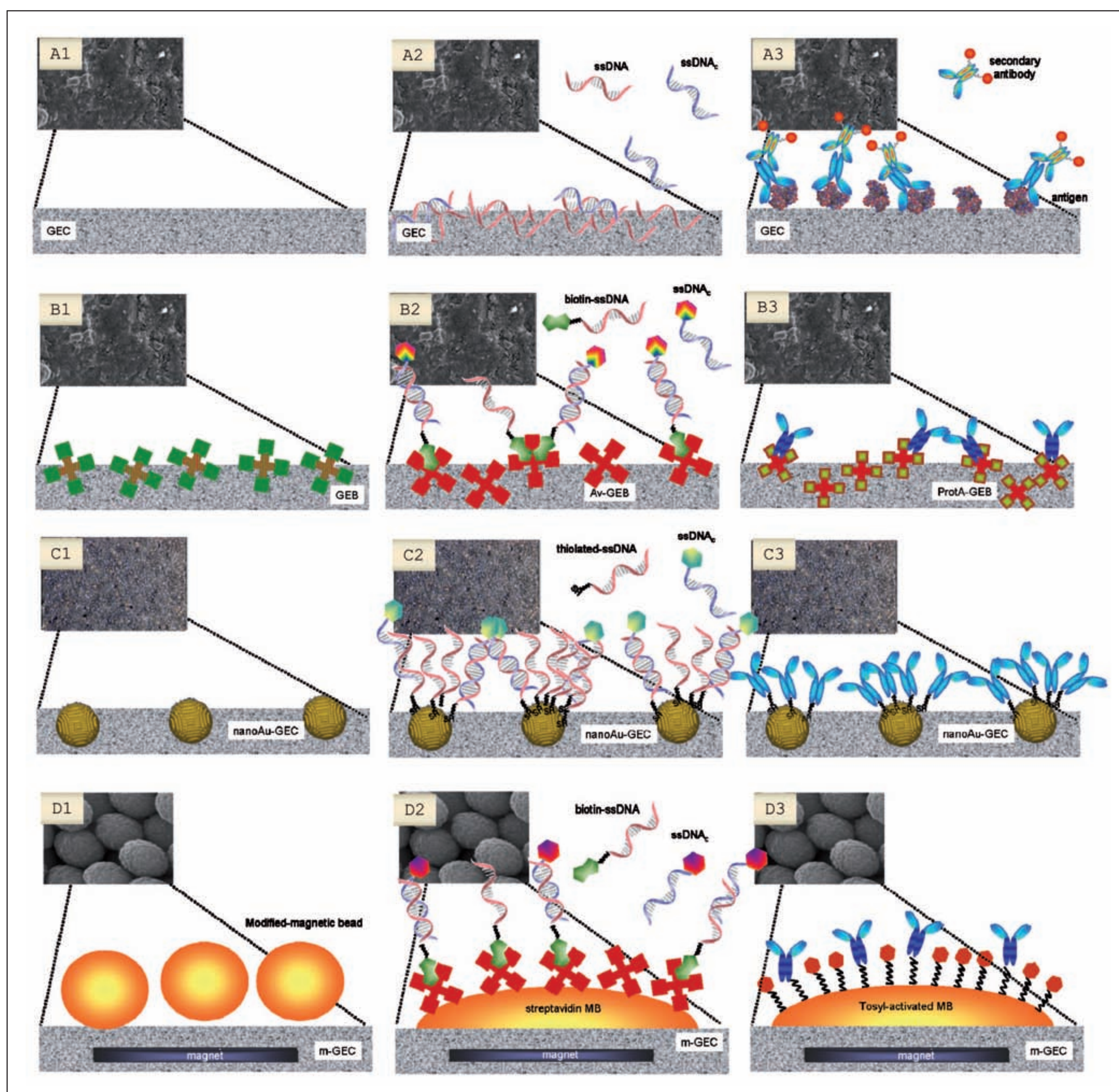

Fig. 2. Schematic representation of the different biosensing strategies based on (**A**) graphite-epoxy composite (GEC), (**B**) graphite-epoxy biocomposite (Av-GEB and ProtA-GEB), (**C**) gold nanoparticles graphite-epoxy nanocomposite (AuNP-GEC), (**D**) graphite-epoxy magnetocomposite (m-GEC) combined with magnetic beads or magnetic nanoparticles. DNA biosensing as well as immunosensing strategies are schematically outlined for each biosensing transducer.

Electrochemical biosensing based on graphite-epoxy nanocomposites. Due to their unique size effects and enhanced chemical and physical properties, nanosized gold particles have been the focus of recent interest due to their potential applications in physics, chemistry, biology, medicine, and material science [12].

Gold nanoparticles (AuNP) are nanostructured materials that bridge the gap between "bottom-up" synthetic methods and "top-down" fabrication [37]. The ability of AuNP to provide a suitable environment for the immobilization of biomolecules while preserving the biological activity of the latter has led to their intensive use in the construction of biosensors with improved performances [27].

Chemisorption based on self-assembled monolayers (SAMs) is a single-point immobilization strategy that allows the oriented attachment of a wide range of biomolecules on gold-based transducer surfaces. One of the main drawbacks of SAMs is their highly-ordered compact layer, which dramatically reduces the diffusion of electroactive species towards the transducer's surface. Moreover, the tightly packed layer may also produce steric hindrance and, as a consequence, a lower rate of reaction between the probe and the DNA target. The use of AuNPs in a graphite-epoxy composite (AuNP-GEC) has been proposed as an alternative to continuous gold surfaces films as this strategy avoids the need for stringent control of surface coverage parameters during immobilization of thiolated

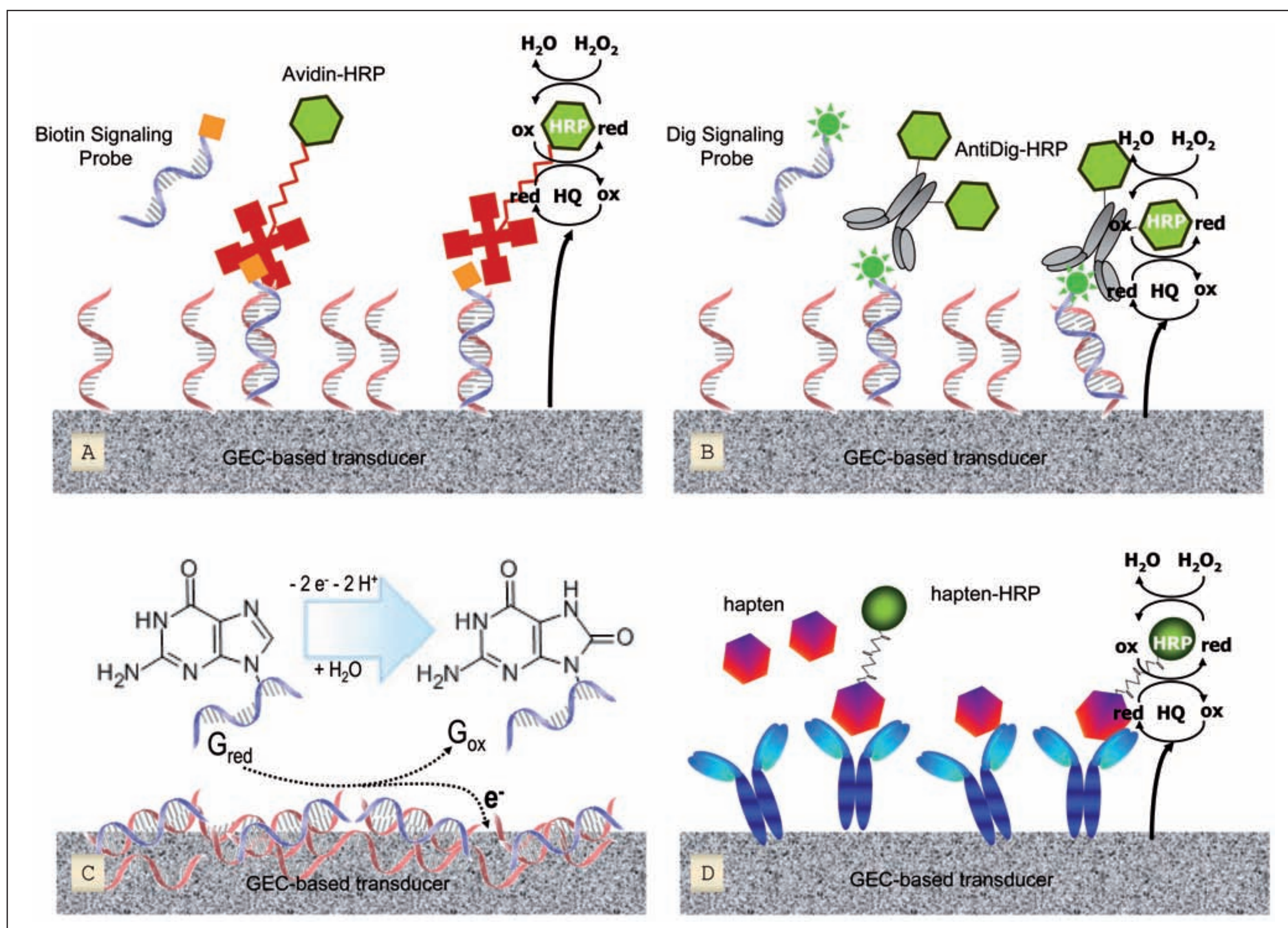

Fig. 3. Schematic representation of the different electrochemical detection strategies for electrochemical biosensors in food safety. (**A** and **B**) Electrochemical genosensing based on enzymatic tags (avidin-HRP and antiDIG-HRP, respectively. (**C**) Electrochemical genosensing based on the intrinsic oxidation signal of DNA. (**D**) Electrochemical competitive immunosensor based on enzymatic tags, in this case the hapten-HRP conjugate.

oligos or antibodies, as outlined in Fig. 2-C1–C3. In this novel transducer, islands of chemisorbing material (AuNPs) surrounded by a rigid, non-chemisorbing, conducting GEC are obtained [25]. The spatial resolution of the immobilized thiolated DNA can be easily controlled by varying the percentage of AuNPs in the composition of the composite.

The electrode surface is easily renewed, as described above. This favors the use of these transducers over continuous gold transducers, as the latter require complex surface pre-treatment. Moreover, as with GEBs, the surface of AuNP-GEC electrodes can be easily modified (Fig. 1B) with DNAs, oligonucleotides, proteins, antibodies, etc., for electrochemical detection (Fig. 1D). These transducers can thus be easily adapted for electrochemical genosensing of food pathogens.

Electrochemical biosensing based on magnetic particles coupled with a magneto electrode. One of the most promising materials in bioanalysis is biologically modified magnetic beads, the use of which is based on the concept of magnetic bioseparation. Magnetic beads offer several novel attractive possibilities in biomedicine and bioanalysis since they can be coated with biological molecules and manipulated by an external magnetic field gradient. As such, the biomaterial, i.e., specific cells, proteins, or DNA, can be selectively bound to the magnetic beads and then separated from its biological matrix by applying an external magnetic field (as outlined in Fig. 2-D1–D3). Moreover, magnetic beads of a variety of materials and sizes—even nanosized magnetic particles—and modified with a wide variety of surface functional groups are now commercially available. The integration of magnetic beads and electrochemical biosensing strategies improves analytical performance. Instead of direct modification of the electrode surface, both the biological reactions (immobilization, hybridization, enzymatic labeling, or affinity reactions) and the washing steps can be successfully performed on magnetic beads (Fig. 1-C1). After the modifications (Fig. 1-C1), the beads are easily captured by applying a magnetic field onto the surface of the GEC electrodes, which contain a small magnet (m-GEC) designed in our laboratories (Fig. 1-C2). This procedure constitutes a versatile platform for electrochemical biosensing (both DNA biosensors and immunosensors) in complex food matrices.

Electrochemical detection strategies in genosensors and immunosensors. Figure 3 shows the different strategies used in electrochemical genosensing (Fig. 3 A-C) and immunosensing (Fig. 3D) as applied to food safety. In most cases, the desired electrochemical signal is due to an enzymatic tag (usu-

ally horseradish peroxidase, HRP), as this approach is be more sensitive than others. In electrochemical genosensing, the DNA duplex can be labeled with either strept(avidin)-HRP (Fig. 3A) or antiDIG-HRP (Fig. 3B) conjugates, depending on the tag of the DNA signaling probe (biotin or digoxigenin, respectively). In electrochemical immunosensing, the enzymatic tag depends on the format of the immunoassay. In competitive immunoassays for small haptenic molecules, it is usually a conjugate obtained with HRP and the hapten (Fig. 3D). In other immunological formats, such as in sandwich assays or indirect approaches, the enzymatic label is typically a conjugate obtained with HRP covalently linked to the Fc part of the specific antibody.

In all cases, amperometric determination is finally based on HRP activity following the addition of H_2O_2 and using hydroquinone as mediator. The modified electrode is immersed in the electrochemical cell (Fig. 1D) containing hydroquinone and, under continuous magnetic stirring, a potential of –0.100 V vs. Ag/AgCl is applied. When a stable baseline is reached, H_2O_2 is added into the electrochemical cell (to a concentration able to saturate the total amount of enzyme employed in the labeling procedure) and the current is measured until steady state is reached (normally after 1 min of H_2O_2 addition). Figure 3C depicts the electrochemical determination of DNA based on its intrinsic oxidation signal. Here, electrochemical determination is performed by differential pulse voltammetry (DPV), in which the oxidation signal of guanine (or adenine) is measured by scanning from +0.30 to +1.20V at a pulse amplitude of 100 mV and a scan rate of 15 mV/s.

Electrochemical immunosensing of agents affecting food safety: food residues, additives, allergens, and bacteria

Pesticides in bottled water and orange juice. Pesticides are designed to prevent, destroy, repel, or reduce pests (animal, plant, and microbial). They are categorized according to their mode of action: insecticides, herbicides, fungicides, acaricides, nematicides, and rodenticides. Pesticides are also used as plant-growth regulators and for public-health purposes. Some are selective, impacting only the target organism, whereas others have broad-range toxicity. However, pesticide residues may remain in fresh produce and in processed foods. Atrazine, a triazine herbicide used to control broadleaf and grassy weeds in corn, sorghum, soybeans, sugarcane, pineapple, and other crops, is of low toxicity but animal studies have suggested its potential for endocrine disruption and carcinogenicity [41]. Moreover, it was found to be a persistent environmental contaminant because of its polarity [26]. Triazine herbicides are usually not absorbed by the soil but percolate through it, causing the contamination of groundwater and surface waters. The design of an immunosensor able to detect the small organic molecule atrazine involves a competitive strategy, one that is based on immunological reaction of the pesticide with its specific antibody, immobilized on a solid support, and competition with the enzymatic tracer. Immobilization of the anti-atrazine antibody on a proper electrochemical transducer is a critical issue in this assay. Different strategies have been reported for the immobilization of anti-atrazine polyclonal antibodies to GEC-based transducers, as schematically outlined in Fig. 4 [45,47]. For example, the non-modified anti-atrazine antibodies are easily immobilized on GEC electrodes by dry adsorption (Fig. 4A). Although this is the simplest and most readily automated procedure, it does not provide a well-oriented layer of specific antibodies, which, instead, are bound through multiple sites to the GEC surface. Anti-atrazine antibodies also can be directly included in the formulation of a biocomposite, yielding an Ab(anti-atrazine)-GEB (Fig. 4B). Although the anti-atrazine antibody seems to be available for immunological reaction when included in the immunocomposite formulation, proper orientation of the antibody can be an issue. These limitations have led to the development of alternative approaches for coupling antibodies in a controlled and oriented manner, ensuring their exposure to both the aqueous environment and the complementary sites of the target molecule. The antibody can be easily oriented by means of affinity proteins, i.e., avidin and protein A, integrated in GEBs (Fig. 4C and Fig. 4D, respectively). Biotinylated anti-atrazine antibodies are immobilized on the surface of the avidin-modified transducer (Av-GEB) through the avidin–biotin reaction, since they can be readily linked to biotin without serious effects on their biological, chemical, or physical properties (Fig. 4C). An additional improvement of the strategy for electrochemical immunosensing based on biocomposites is achieved by using ProtA-GEB electrodes (Fig. 4D). In this case, native, non-modified anti-atrazine antibodies are immobilized on the surface of the protein-A-modified transducer, thus exposing the Fab region. This approach has clear advantages [47].

As suggested in the results comparatively shown in Fig. 4, the oriented immobilization of antibodies provided by the Av-GEB and ProtA-GEB transducers promotes a better immunological reaction in terms of signal-to-non-specific adsorption ratios. Even better results are achieved when the anti-atrazine antibodies are attached to magnetic beads (Fig. 4E). Although these antibodies can be successfully attached to different types of magnetic beads (–COOH modified magnetic nanoparticles, protein A magnetic beads), better attachment performance is achieved using tosylactivated magnetic beads [45].

Two types of competitive immunosensing assays for the determination of atrazine in orange juice and bottled water are compared in Fig. 5. Briefly, the experimental procedure based on ProtA-GEB electrodes (Fig. 5A) consists of: (i) competitive immunological reaction between atrazine and the atrazine-HRP enzymatic tracer for anti-atrazine antibody immobilized on the ProtA-GEB electrodes and (ii) amperometric determination based on enzyme activity following the addition of H_2O_2 and using hydroquinone as mediator. The immunological determination of atrazine in orange juice samples can also be performed successfully with anti-atrazine specific antibodies immobilized on magnetic beads combined with m-GEC magneto electrodes (Fig. 5B). The protocol consists, briefly, of the following steps: (i) competitive immunological reaction between

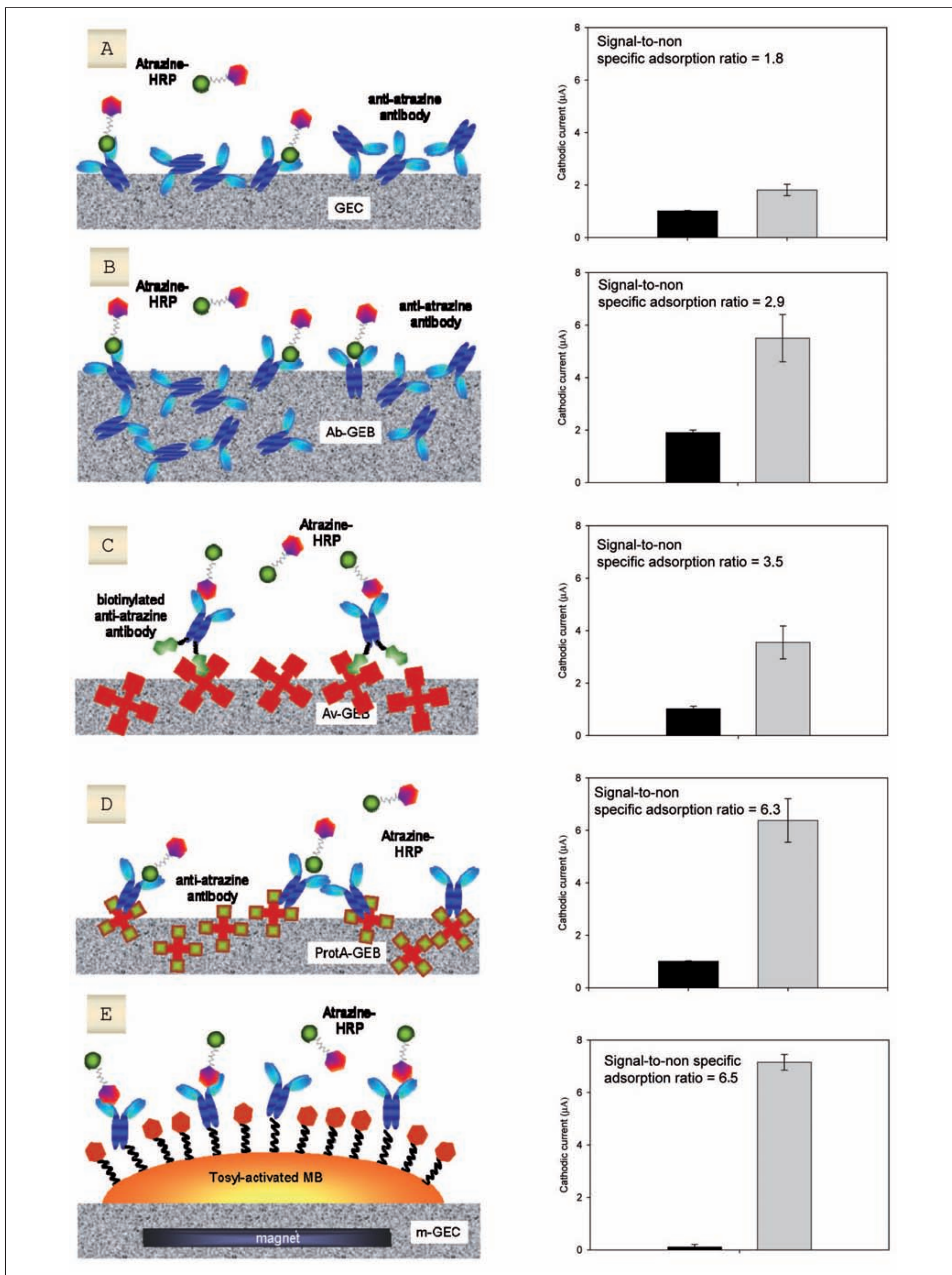

Fig. 4. Comparative results for the immunological reaction of atrazine-HRP tracer with anti-atrazine polyclonal antibody (Ab) immobilized on graphite-epoxy platforms through different strategies based on (**A**) dry adsorption on GEC; (**B**) biocomposite Ab-GEB; (**C**) biocomposite Av-GEB; (**D**) biocomposite ProtA-GEB; (**E**) m-GEC combined with magnetic beads. In all cases, negative controls (black bars) were assayed with pre-immune serum. Experimental conditions are detailed in [45] and [47].

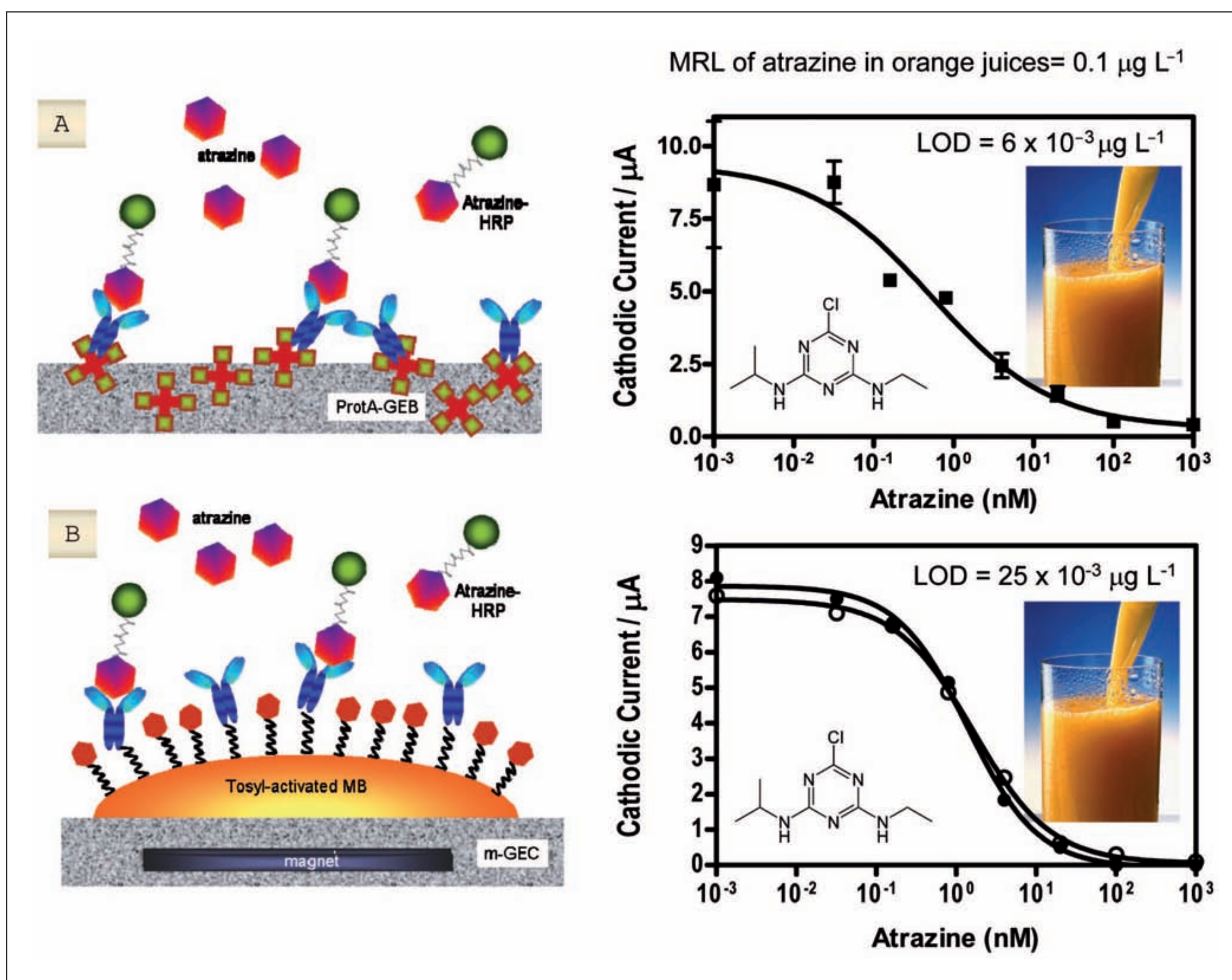

Fig. 5. Competitive immunoassays for the determination of atrazine in spiked samples of orange juice and bottled water, based on (**A**) electrochemical immunosensing with ProtA GEB electrodes and (**B**) electrochemical magneto immunosensing with m-GEC magneto electrodes. Experimental conditions are detailed in [47] and [45], respectively.

atrazine and the HRP-atrazine marker for anti-atrazine antibodies immobilized on the magnetic beads; (ii) magnetic capture of the modified magnetic particles; and (iii) amperometric determination based on enzyme activity following the addition of H_2O_2 and using hydroquinone as mediator.

In both cases, the competitive electrochemical immunosensing strategy can easily detect the MRL established by European Community directives, i.e., 0.1 μg/l for atrazine in potable water and orange juice, with very simple sample pre-treatment, with limits of detection (LODs) of 6×10^{-3} μg/l and 25×10^{-3} μg/l, respectively. Orange juice samples spiked with atrazine are diluted only five times with phosphate-buffered saline/Tween (PBST) and filtered through 0.22-μm filters before measurement.

Similar results were obtained with the competitive electrochemical immunosensing strategies based on ProtA-GEB electrodes and on magnetic beads coupled with magneto electrodes m-GEC, showing that the immunological competitive reaction is achieved with similar rates obtained over the ProtA-GEB biocomposite as on the surface of the magnetic beads.

Antibiotic residues in milk. Over the past few decades, the use of antibiotics and chemotherapeutics in animal husbandry has increased considerably [43]. Antibiotics are added to reduce disease and improve the growth of farm animals and aquaculture fish. The chemical effects of these compounds are of less concern than their ability to increase antimicrobial resistance in strains that might subsequently infect humans. There is evidence that antimicrobial resistance is increasing worldwide but particularly in developing countries. The human effect of consuming foods containing antibiotics is still being debated, but many countries refuse to accept products derived from animals given these drugs. The withdrawal times of antibiotics are critical to insure that the residues in food are as low as possible.

The presence of certain antimicrobial agent residuals in milk constitutes a potential hazard for the consumer and may cause allergic reactions, interference in the intestinal microbiota, and resistance in populations of bacteria, thereby rendering subsequent treatment with the antibiotic ineffective. Furthermore, important economic losses are related to the inhibition of bacterial processes involved in the elaboration of cheese and cultured milk products.

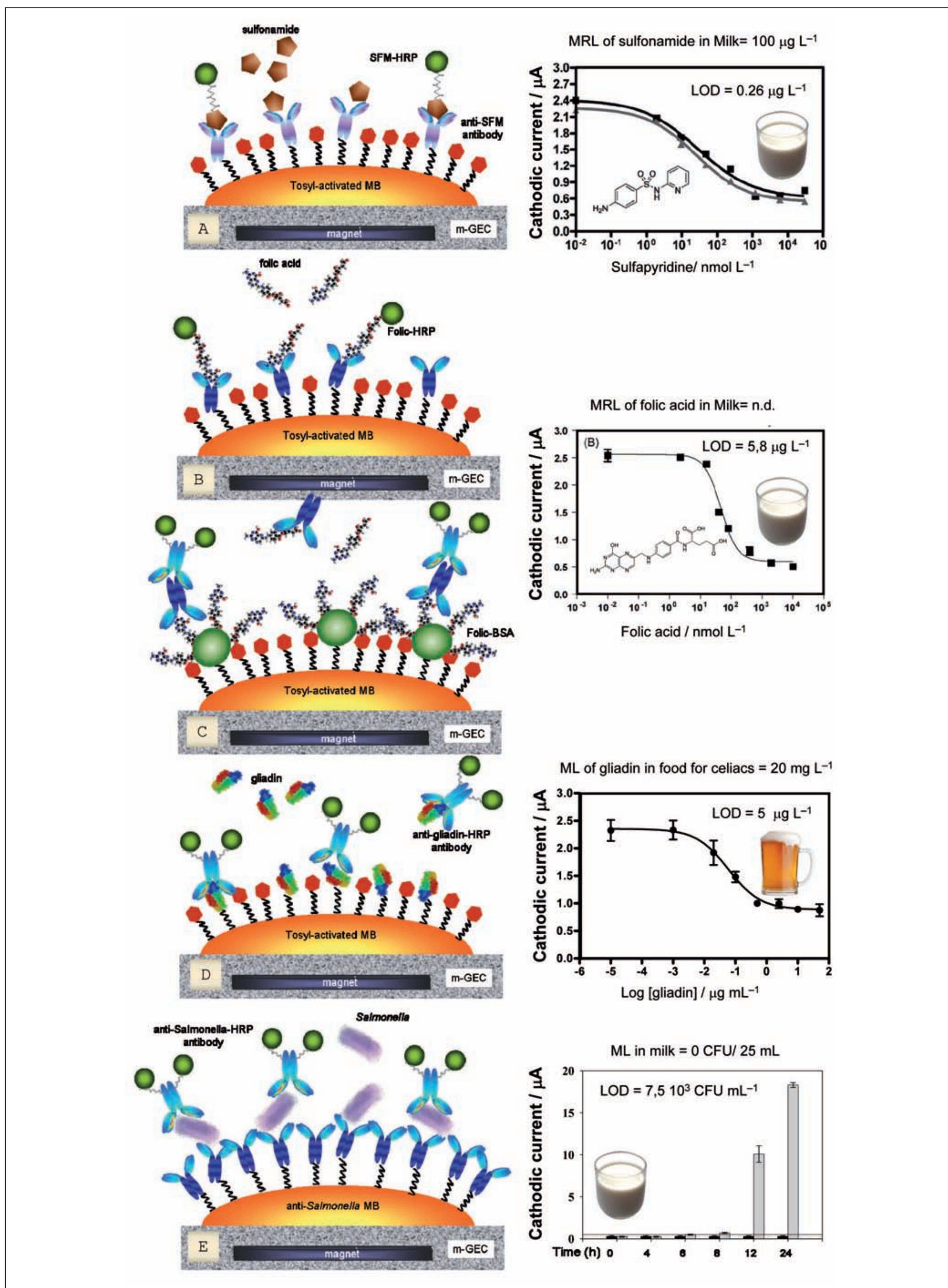

Fig. 6 Electrochemical magneto immunosensing of different agents affecting food safety. (**A**) Sulfonamides detection based on a direct competitive immunoassay. (**B**) Folic acid determination based on direct and (**C**) indirect competitive immunoassays. (**D**) Gliadin detection based on direct competitive immunoassay. (**E**) *Salmonella* detection based on a sandwich immunoassay. Experimental conditions are detailed in [46], [19], and [21].

The electrochemical magneto immunosensing of sulfonamides can be successfully performed in raw full cream as well as in all varieties of UHT (ultra-high temperature) milk, including full cream (~3.25% fat), semi-skimmed (~1.5–1.8% fat), and skimmed (0.1% fat), by using class-specific anti-sulfonamide (anti-SFM) antibodies immobilized on magnetic beads and an SFM-HRP tracer for electrochemical detection [46]. Specificity studies have shown that at least eleven sulfonamide antibiotics can be sensitively detected using a class-specific anti-SFM antibody. This strategy is schematically outlined in Fig. 6A. Although, as in the case of atrazine determination, the anti-sulfonamide antibodies can be successfully attached on different types of magnetic beads (–COOH modified magnetic nanoparticles, protein A magnetic beads), the best attachment performance was again achieved with tosylactivated magnetic beads. Raw full-cream samples spiked with sulfapyridine were diluted five times with PBST, while all UHT samples are processed without treatment [46]. As European legislation has set the MRL of sulfonamide at 100 μg/kg, the detection limit of the electrochemical magneto immunosensing strategy (0.26 μg/l) is sufficient to assay any type of milk according to the requirements of the EC legislation.

Folic acid in vitamin-fortified food. Folates, including various forms of tetrahydrofolate (THF), are part of the vitamin B group. In order to prevent folate deficiency in individuals, folic acid is added to many food products. Supplementation is particularly important in pregnant women, as folic acid insufficiency can cause neural-tube defects in the developing fetus. Moreover, folate deficiency is the most common cause of anemia after iron deficiency. Although folic acid is not thought to be toxic, it may contribute to the potential masking of pernicious anemia in the elderly, as well as interfering with anticonvulsive therapy or cancer treatment with antifolates. In addition, a rapid assay for folic acid may be mandatory to control the practice of "overage" during food preparation [2].

The electrochemical magneto immunosensing of folic acid in vitamin-fortified milk samples can be successfully performed, as shown in Fig. 6 [19]. In the direct assay, the enzyme tracer conjugated with folic acid (FA-HRP) competes with folic acid (FA) for the binding sites of a specific antibody immobilized on the solid support (Fig. 6B). In the indirect assay, the protein conjugate (BSA-FA) is immobilized on the solid support and competition for the specific antibody is established between the FA in the sample and immobilized BSA-FA (Fig. 6C). In this case, the amount of specific antibody specifically bound on the solid support was determined using a secondary antibody conjugated with HRP as enzyme label (anti-IgG-HRP). As seen in Fig. 6, the indirect assay achieved a better performance in terms of LOD [2], which for skimmed milk was 5.8 μg/l. Commercial vitamin-fortified milk samples were also evaluated, obtaining good accuracy in the results [19].

Allergens in milk and beer. Gliadin is the toxic protein fraction of gluten responsible for the intolerance underlying celiac disease. Its detection is of great interest for the food safety of celiac patients, for whom the only treatment currently known is a lifelong dietary avoidance of this cereal protein [13]. As a result, gluten content has been included in food regulations, as mandated by the recent statement that foodstuffs labeled "gluten-free" may not exceed a gluten content of 20 ppm. Therefore, an easy, rapid, and reliable method of analysis is essential to control gliadin content in gluten-free foods and to permit the on-site monitoring of food materials during industrial processing.

Gliadin detection in safe, gluten-free food is also successfully performed with an electrochemical magneto immunosensing strategy (Fig. 6D) on micro and nanostructured magnetic beads as solid supports, with the antigen, in this example gliadin, covalently immobilized on the activated surfaces. In all cases, the biorecognition strategy is based on a direct competitive assay using an anti-gliadin antibody-peroxidase (HRP) conjugate as the enzymatic label. Subsequent detection is achieved through an appropriate substrate and mediator for the HRP enzyme. Excellent LODs (on the order of 5 μg/l) have been achieved, consistent with the requirements for gluten-free products. The matrix effect on different samples (milk, beer) as well as the assay's performance have been successfully evaluated using spiked-food samples, with good recovery values obtained in the results.

Food pathogens in milk. *Salmonella* is one of the most frequently occurring foodborne pathogens affecting the microbial safety of foods [7]. Official agencies charged with ensuring food safety, such as the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA), the Association of Official Analytical Communities International (AOACI), and the International Organization of Standardization (ISO), recommend classical culture methods for recovering *Salmonella* spp. from food. However, the development of new methodologies, with advantages of rapid response, sensitivity, and ease of multiplexing, is a challenge for food hygiene inspection aimed at screening-out negative samples.

A very simple and rapid method for the detection of *Salmonella* in milk is performed by electrochemical magneto immunosensing with m-GEC electrodes (Fig. 6E) [22]. In this approach, the bacteria are captured and pre-concentrated from milk samples with magnetic beads by immunological reaction with a specific antibody against *Salmonella*. A second polyclonal antibody labeled with peroxidase is used as serological confirmation, with electrochemical detection based on a magneto-electrode. Among the different procedures, better performances have been obtained using one-step immunological reactions. The "immunomagnetic separation step (IMS)/m-GEC electrochemical immunosensing" approach was employed, for the first time, in the detection of *Salmonella* artificially inoculated into skimmed-milk samples. A limit of detection of 7.5×10^3 CFU/ml in milk was obtained in 50 min without any pre-treatment. If the skimmed-milk is pre-enriched for 6 h, the method can detect as low as 1.4 CFU/ml, while following pre-enrichment for 8 h as few as 0.108 CFU/ml (2.7 CFU in 25 g of milk) are detected, thus complying with legislative criteria (Fig. 6E). Magnetic immunoseparation and detection with a second specific antibody can effectively replace "selective enrichment/differential plating" and "biochemical/serological testing" assays,

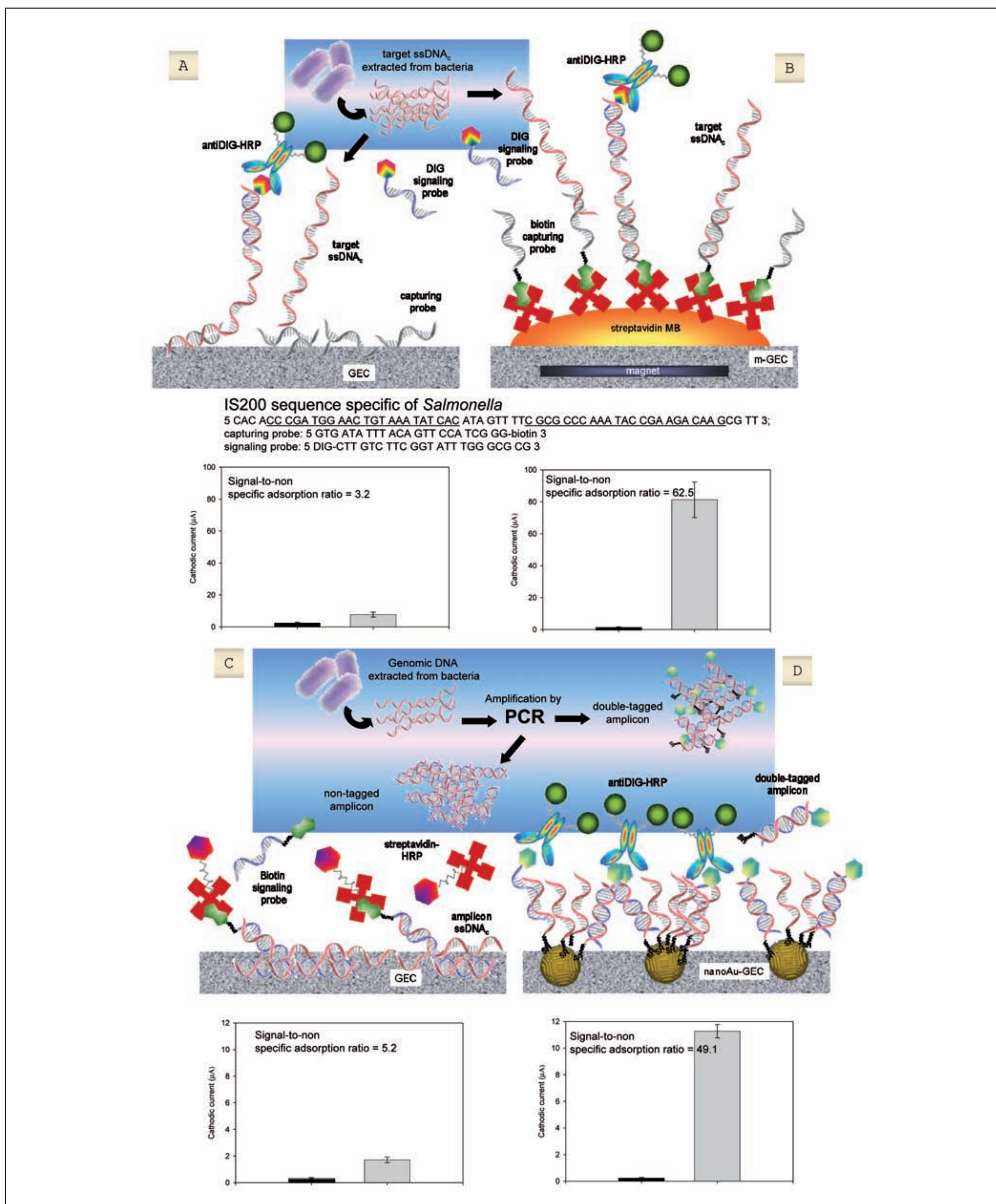

Fig. 7. Comparative results for electrochemical genosensing of *Salmonella enterica* serovar Typhimurium ATCC 14028 using different strategies and transducers. (**A** and **B**) Electrochemical DNA sequence-specific detection in capture format for the *Salmonella* IS200 element. (**C** and **D**) PCR-amplified IS200 element of *Salmonella*. (**A**) DNA biosensing strategy in capture format based on GEC. DNA IS200 target specific for *Salmonella* 22 pmol, capturing probe 30 pmol, DIG signaling probe 30 pmol, AntiDig-HRP 60 µg. (**B**) DNA biosensing strategy in capture format based on streptavidin magnetic beads integrated in m-GEC. DNA IS200 target specific for *Salmonella* 22 pmol, biotin capturing probe 200 pmol, DIG signaling probe 225 pmol, AntiDig-HRP 60 µg, magnetic beads 1.6×10^6. (**C**) PCR amplification followed by dry adsorption of the amplicon (2.9 pmol) on GEC transducer, hybridization with 68.2 pmol of biotinylated probe, and detection with 9.0 µg of streptavidin-HRP conjugate. (**D**) Double-tagging PCR amplification to obtain a double-tagged amplicon with thiol and digoxigenin moieties (2.7 pmol) chemisorbed on AuNP-GEC transducer and detection with 60.0 µg of AntiDig-HRP. In all cases, gray bars show the specific signal, and black bars the corresponding non-specific adsorption omitting either the DNA target (**A** and **B**) or the DNA template (**C** and **D**) during PCR. Error bars indicate the standard deviation ($n = 3$). Other experimental conditions are detailed in [32], [17], [30], and [25], respectively.

respectively. Moreover, the assay time is considerably reduced, from 4–5 days to 50 min.

Electrochemical genosensing of foodborne pathogens

***Salmonella*.** The multisite physical adsorption of DNA extracted from pathogenic bacteria on GEC has the main advantage of simplicity, since no reagent or reactive function is required. Once immobilized on the GEC, genomic DNA preserves its unique hybridization properties, which can be revealed using different strategies: (i) enzymatic labeling based on HRP conjugates (Fig. 3A,B) [28,30] and (ii) intrinsic signal coming from DNA oxidation (Fig. 3C) [10,32].The detection of the *Salmonella* IS*200* element by a DNA biosensing strategy in capture format based on a GEC is shown in Fig. 7A. Briefly, the protocol consists of the following steps: (i) capturing probe immobilization by dry adsorption; (ii) hybridization of the DNA target in one step with the complementary DIG signaling probe; (iii) enzyme labeling of the DNA duplex with antiDIG-HRP; and (iv) amperometric determination based on enzyme activity following the addition of H_2O_2 and using hydroquinone as mediator. As low as 22 pmol of DNA target—with a signal-to-non-specific adsorption of 3.2—can be easily and cost effectively detected with this strategy, which was also successfully applied to the detection of a determinant of β-lactamase resistance in *Staphylococcus aureus* [32].

Moreover, to increase the assay's sensitivity, amplification of the bacterial genome by PCR can be coupled with a DNA electrochemical approach. In this case, the amplicon is directly adsorbed on the GEC transducer. As the amplicon is double-stranded, a denaturing alkaline procedure is mandatory to break the hydrogen bonds for further hybridization with the complementary signaling probe [30]. Briefly, the protocol consists of the following steps, as schematically outlined in Fig. 7C: (i) amplicon immobilization by dry adsorption and alkaline treatment; (ii) hybridization with the complementary biotin signaling probe; (iii) enzyme labeling with streptavidin-HRP conjugate; and (iv) amperometric determination based on the enzyme. As low as 2.9 pmol of amplicon—with a signal-to-non-specific adsorption of 5.2—can be easily and cost effectively detected with this strategy.

As shown in Fig.7A,C, and although tightly adsorbed on the GEC electrode, DNA preserves its unique hybridization properties in GEC platforms, suggesting that DNA bases are not fully committed in the adsorption mechanism but mostly available for hybridization [29]. The results comparatively presented in Fig. 7 suggest that the oriented single-point immobilization of DNA achieved by different strategies, such as the integration of magnetic beads in m-GEC electrodes (Fig. 7B) and AuNPs in graphite-epoxy nanocomposites (AuNP-GEC) (Fig. 7D), provides, in all cases, improved results in terms of signal-to-non-specific adsorption, as discussed below.

Streptavidin-modified magnetic beads are useful platforms for DNA biosensing, when combined with a biotinylated capturing probe complementary to the DNA target (Fig. 7B). The *Salmonella* IS*200* element can be easily detected in a one-step capture format, as schematically outlined in Fig. 7B. The procedure consists of the following steps: (i) one-step immobilization/hybridization procedure, in which the biotin-labeled capturing probe is immobilized on streptavidin magnetic beads, while hybridization with both the target and a second complementary probe—in this case, labeled with digoxigenin—occurs simultaneously; (ii) enzymatic labeling using the antibody anti-DIG-HRP as enzyme label; (iii) magnetic capture of the modified magnetic particles on the m-GEC electrode; and (iv) amperometric determination based on enzyme activity following the addition of H_2O_2 and using hydroquinone as mediator [17].

This approach (streptavidin magnetic beads integrated within m-GEC electrodes) clearly provides better analytical performances in terms of signal-to-non-specific adsorption than achieved with other strategies involving adsorption on GEC (Fig. 7A). The strategy exploits the advantages of magnetic beads, such as improved and more effective biological reactions, washing procedures, and magnetic separation after each step. This assay also benefits from the increased size of the active area due to the integration of magnetic beads within the m-GEC transducer. In addition to electrochemical detection based on the enzyme label, the DNA target immobilized on the magnetic beads can be successfully detected by the intrinsic DNA oxidation signal coming from the guanine moieties (Fig. 3C) [11].

To further increase the sensitivity of detection of foodborne pathogens, a double-tagged PCR strategy is coupled to an electrochemical magneto genosensing approach, based on streptavidin-magnetic beads integrated within m-GEC electrodes [17]. Rapid electrochemical verification of the amplicon derived from the *Salmonella* IS*200* element is performed by double-labeling the amplicon during PCR with a set of two labeled PCR primers—one with biotin and the other with digoxigenin. During PCR, not only amplification of the bacterial genome is achieved but also double-labeling of the amplicon ends with: (i) the biotinylated capture primer, to achieve immobilization on the streptavidin-modified magnetic bead, and (ii) the digoxigenin signaling primer, to achieve electrochemical detection. The procedure briefly consists of the following steps [17]: (i) DNA amplification of the bacterial genome and double-labeling; (ii) immobilization of the doubly-labeled amplicon in which the biotin end of the dsDNA amplicon is immobilized on the streptavidin magnetic beads; (iii) enzymatic labeling using the antibody antiDIG-HRP as an enzyme label capable of binding the other labeled end of the dsDNA amplicon; (iv) magnetic capture of the modified magnetic particles; and (v) amperometric determination. Rapid and sensitive verification of the *Salmonella*-related PCR amplicon can be achieved with 2.8 fmol of amplified product [17]. Interestingly, the PCR also can be performed directly on the magnetic beads by using a magnetic primer, thus allowing real-time electrochemical detection of the bacteria [17].

The detection of the *Salmonella* IS*200* element is also possible using other strategies based on graphite-epoxy nanocomposites (AuNP-GEC). In these materials, isolated AuNPs generate bioactive chemisorbing islands for the immobilization

of thiolated DNA probes (Fig. 2-C2). Less compact layers are thus achieved, favoring the biological reaction on biosensing devices. Hybridization efficiency is expected to be higher on the edge of the AuNPs surrounded by nonreactive GEC, as shown in Fig. 2C. Briefly, the procedure consists of the following steps [25]: (i) thiolated probe immobilization by chemisorption; (ii) hybridization with the complementary probe modified with digoxigenin; (iii) enzyme labeling of the DNA duplex using antiDIG-HRP; and (iv) amperometric determination based on enzyme activity following the addition of H_2O_2 and using hydroquinone as mediator. The chemisorbing ability of AuNPs in the AuNP-GEC was shown to have an excellent LOD (9 fmol/60 pM of ssDNA) in hybridization studies aimed at the detection of DNA from *Salmonella* [25]. Moreover, and for the first time, a double-tagging PCR strategy was performed using a thiolated primer for the detection of *Salmonella* sp. [25]. The results are shown in Fig. 7D.

The rapid electrochemical verification of the amplicon coming from the genome of pathogenic *Salmonella*, as performed by PCR using a set of two labeled primers, readily allows thiolation of the PCR product [25]. The thiolated end facilitates immobilization of the amplicon on the AuNP-GEC electrode (Fig. 7D). The procedure consists of the following steps [25]: (i) DNA amplification and double-labeling of the *Salmonella* IS*200* insertion sequence; (ii) immobilization of the doubly-labeled amplicon, with the –SH end of the dsDNA amplicon immobilized on the AuNP-GEC nanocomposites by chemisorption; (iii) enzymatic labeling using as enzyme label the antibody antiDIG-HRP, capable of binding the other labeled end of the dsDNA amplicon; and (iv) amperometric determination. Detection using this strategy is as low as 200 fmol, with an electrochemical signal of almost 3 μA [25].

Figure 7 compared the results obtained for the detection of 2.7 pmol of amplicon using AuNP-GEC sensors (Fig. 7D) and

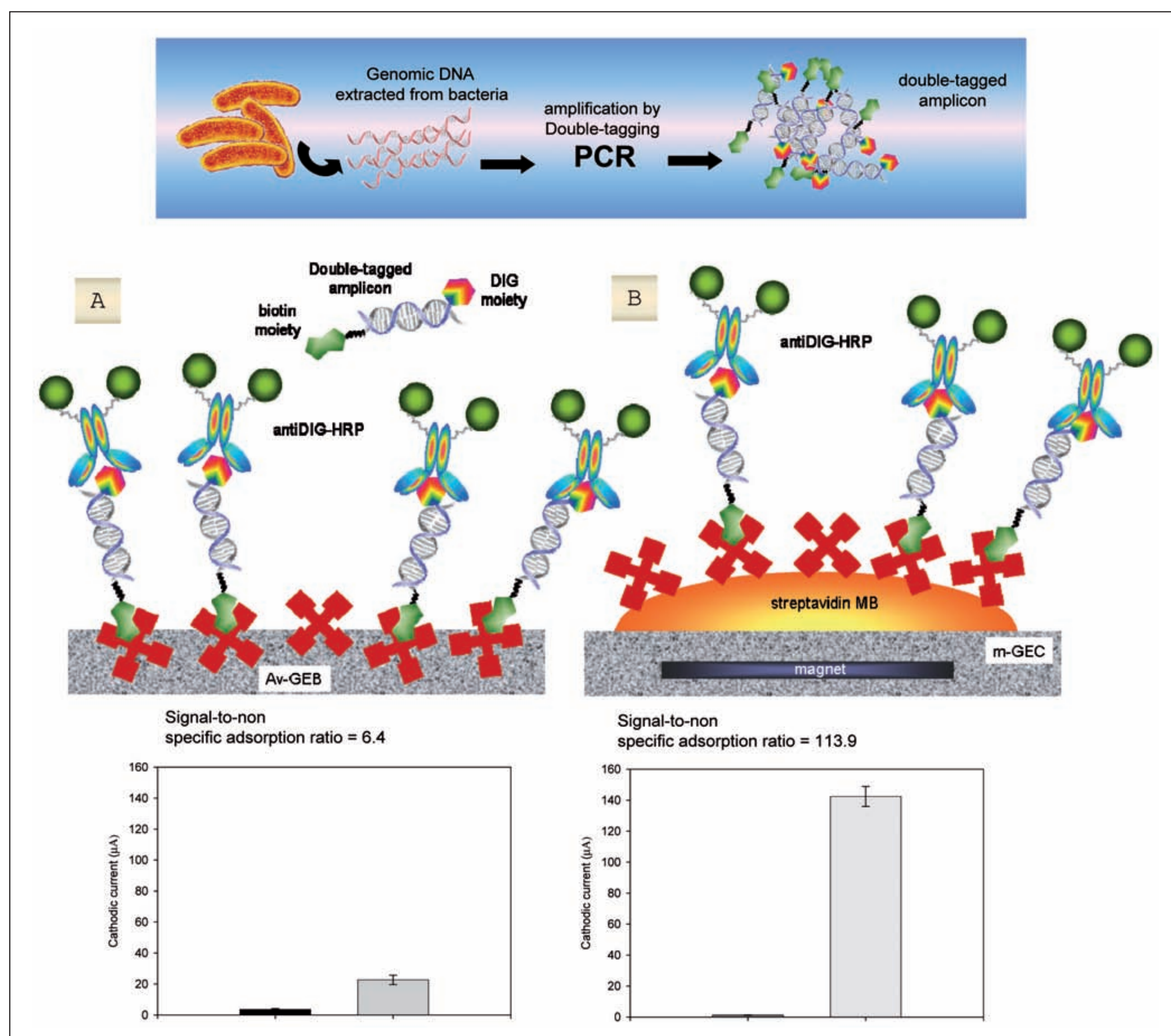

Fig. 8. Electrochemical detection of 2 ng/μl DNA template of *E. coli* by double-tagging PCR followed by electrochemical genosensing based on Av-GEB (**A**) and electrochemical magneto genosensing based on m-GEC (**B**). In all cases, 60 μg AntiDig-HRP was used. Other experimental conditions are detailed in [18].

GEC electrodes (Fig. 7C). The oriented single-point immobilization of DNA achieved by chemisorption on AuNP-GEC clearly provides improved results in terms of signal-to-non-specific adsorption. This double-tagging PCR strategy opens new routes not only for immobilization purposes but also as an easy strategy for labeling with gold or quantum dots during PCR. Moreover, the AuNP-GEC material shows interesting properties for electrochemical genosensing in hybridization experiments and very promising features for electrochemical biosensing of a wide range of biomolecules, such as double-stranded DNA, PCR products, affinity proteins, antibodies, and enzymes.

***E. coli* O157:H7.** Like *Salmonella*, *Escherichia coli* is one of the most frequent pathogens implicated in human bacterial infections. Enterohemorrhagic *E. coli* (EHEC) O157:H7 is one of the most dangerous serotypes of the bacterium, causing hemorrhagic colitis and severe hemolytic uremic syndrome, either of which may result in death due to acute or chronic renal failure. Outbreaks of EHEC O157:H7 infections have been associated with contaminated food products, such as ground beef and raw milk [8].

The rapid electrochemical verification of the amplicon coming from the *eaeA* gene of *E. coli* O157:H7 is performed by double-tagging the amplicon during PCR with a set of labeled PCR primers—one with biotin and the other with digoxigenin [18] (Fig. 8A). During PCR, not only is amplification of *E. coli* achieved but also double-labeling of the amplicon ends with: (i) the biotinylated capturing primer, resulting in immobilization on a biosensor based on a bulk-modified avidin biocomposite (Av-GEB), and (ii) the digoxigenin signaling primer, enabling electrochemical detection. The procedure consists of the following steps: (i) DNA amplification and double-tagging of *eaeA*, the gene associated with the pathogenic activity of *E .coli* O157:H7; (ii) immobilization of the double-tagged amplicon, in which the biotin end of the dsDNA amplicon is immobilized on the Av-GEB biosensor; (iii) enzymatic labeling with antiDIG-HRP, which is capable of binding the other labeled end of the dsDNA amplicon; and (iv) amperometric determination [18].

As shown in Fig. 8A, the assay is very sensitive, detecting as little as 2 ng DNA template of *E. coli* per μl in 13 cycles—with a signal-to-non-specific adsorption of 6.4. Moreover, 4.5 ng of the original bacterial genome per μl can be feasibility detected after only 10 cycles of PCR amplification [18]. DNA biosensors based on Av-GEB for amplicon detection are more sensitive than Q-PCR strategies based on fluorescent labels such as TaqMan probes. In addition, this strategy can be used for the electrochemical real-time quantification of amplicon since a linear relationship with the amount of amplified product is obtained [18].

To increase the sensitivity of *E. coli* detection, a double-tagged PCR strategy is coupled to an electrochemical magneto genosensing approach, based on streptavidin-magnetic beads integrated within m-GEC electrodes. Rapid electrochemical verification of the amplicon coming from the *eaeA* gene is performed by double-labeling the amplicon during PCR with a set of two labeled PCR primers—one with biotin and the other with digoxigenin (Fig. 8B), as previously explained. The procedure consists of the following steps [18]: (i) DNA amplification of the bacterial genome and double-labeling; (ii) immobilization of the doubly-labeled amplicon, in which the biotin end of the dsDNA amplicon is immobilized on the streptavidin magnetic beads; (iii) enzymatic labeling using as enzyme label the antibody antiDIG-HRP, capable of binding the other labeled end of the dsDNA amplicon; (iv) magnetic capture of the modified magnetic particles; and (v) amperometric determination. The rapid and sensitive verification of the PCR amplicon derived from *E. coli* allows the detection of 0.45 ng of the original bacterial genome per μl after only 10 cycles of PCR amplification [18] Moreover, as above, electrochemical strategies for amplicon detection have proven to be more sensitive than Q-PCR strategies based on fluorescent labels such as TaqMan probes. Electrochemical magneto genosensing of the double-tagged amplicon clearly provides better analytical performance in terms of signal-to-non-specifc adsorption than obtained with electrochemical genosensing based on Av-GEB (Fig. 8), when both are carried out with a similar single-point-oriented DNA attachment though avidin-biotin linkage. This strategy can be used for the electrochemical real-time quantification of amplicon since the relationship with the amount of amplified product is linear [18]. However, this strategy is useful only when a unique and specific band is observed by gel electrophoresis, because of the high specificity of the set of primers used in PCR amplification of the bacterial genome. If the primer set amplifies not only the sequence of interest but also other, non-specific fragments, it is necessary to confirm the internal sequence of the amplicon by a second hybridization using a digoxigenin signaling probe [18].

***Mycobacterium bovis* in raw contaminated milk on dairy farms.** Tuberculosis (TB) in humans and other mammals is usually caused by *Mycobacterium tuberculosis* or *Mycobacterium bovis*. While, worldwide, *M. tuberculosis* is the single greatest cause of infectious disease in humans, *M. bovis* affects the largest number of animals. In humans, the global prevalence of TB infection involves about one-third of the world's population, a number that is expected to grow steadily [4]. *M. bovis* causes bovine tuberculosis, which is easily transmitted between farm animals. This disease is also an important zoonosis, targeting not only workers on dairy farms but also the general public following the consumption of contaminated dairy products.

A very sensitive assay for the rapid screening-out of TB based on electrochemical genosensing can be performed by specific amplification and double-tagging of the IS6110 fragment, highly related to *M. bovis*, followed by electrochemical detection of the amplified product. PCR amplification is performed using a labeled set of primers, yielding a double-tagged amplicon with biotin and digoxigenin at the respective ends, as explained above for other pathogens. Two different electrochemical platforms for the detection of double-tagged amplicon can be used [20]: (i) an avidin biocomposite (Av-GEB); and (ii) a magneto sensor (m-GEC) combined with streptavidin magnetic beads. In both cases, immobilization of the double-tagged amplicon is achieved through the bioti-

nylated end of the amplicon, and electrochemical detection through the digoxigenin end by using an antiDIG-HRP conjugate. The assay has proven to be very sensitive, as it is able to detect 620 and 10 fmol of PCR amplicon for Av-GEB and m-GEC strategies, respectively. Compared with inter-laboratory PCR assays and the "gold standard" tuberculin skin test, the m-GEC assay has shown promising features for the detection of TB on dairy farms, though the determination of *M. bovis* DNA in milk samples.

Electrochemical genosensing combined with immunomagnetic separation for the sensitive detection of foodborne pathogens in milk

Electrochemical magneto genosensing of a double-tagged amplicon can be combined with an IMS of the bacteria to improve the LOD of pathogenic bacteria. The procedure consists of the following steps [21]: (i) immunomagnetic separation of the bacteria from food samples (Fig. 9A); (ii) lysis of the bacteria and DNA release; (iii) DNA amplification and double-labeling of the *Salmonella* IS*200* insertion sequence; (iv) immobilization of the doubly-labeled amplicon, in which the biotin end of the dsDNA amplicon is immobilized on streptavidin magnetic beads; (v) enzymatic labeling using as enzyme label the antibody antiDIG-HRP, which is capable of binding the other labeled end of the dsDNA amplicon; (vi) magnetic capture of the modified magnetic particles; and (vii) amperometric determination.

In this approach, the bacteria are captured and pre-concentrated from food samples with magnetic beads through an immunological reaction with a specific antibody against *Salmonella*, as shown in Fig. 9A. After lysis of the captured bacteria, the genetic material is PCR-amplified with a double-tagging set of primers to confirm the identity of the bacteria (Fig. 9C). Both steps are rapid alternatives to the time-consuming classical selective enrichment and biochemical/serological tests. The double-tagged amplicon is then detected by electrochemical magneto genosensing using m-GEC electrodes. The "IMS/doubletagging PCR/m-GEC electrochemical genosensing" approach can be used for the sensitive detection of *Salmonella* artificially inoculated into skim milk samples. A limit of detection of 1 CFU/ml is obtained in 3.5 h without any pretreatment, in Luria-Bertani (LB) broth and in milk diluted 1/10

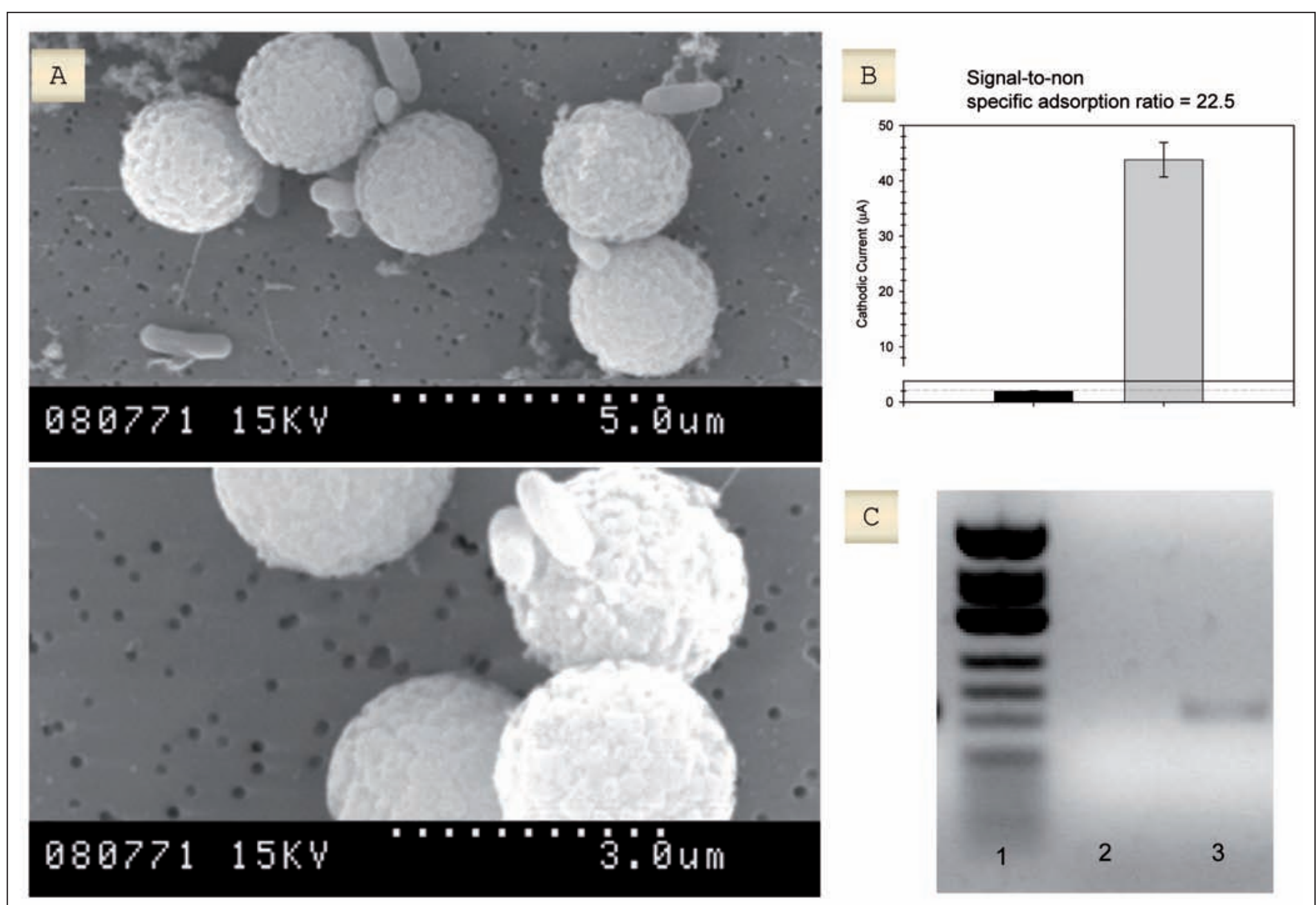

Fig. 9. (**A**) Scanning electron microscopy images of the immunomagnetic separation of 10^4 CFU/ml of *Salmonella*. The images show *Salmonella* cells attached to the magnetic beads. In all cases, 15 KV acceleration voltages were used. (**B**) Electrochemical signals for the IMS/double-tagging PCR/m-GEC electrochemical genosensing approach, with a pre-enrichment step of 6 h for artificially inoculated skim milk (0.04 CFU/ml or 1 CFU in 25 g of milk) (gray bar) and for negative controls (black bar). In all cases, n = 4. (**C**) Corresponding agarose gel electrophoresis of double-tagged PCR amplicon obtained with the "IMS/double-tagging PCR/electrophoresis" approach with pre-enrichment steps of 6 h (0.04 CFU/ml or 1 CFU in 25 g of milk, lane 3). A negative control (0 CFU/ml, lane 2) and molecular mass markers (ΦX174-Hinf I genome, lane 1) are also shown. Other experimental conditions are detailed in [21].

in LB [21]. When the skim milk is pre-enriched for 6 h, as few as 0.04 CFU/ml (1 CFU in 25 g of milk) are detected, complying with current legislation, and with a signal-to-background ratio of 22.5 [47] (Fig. 9B).

Final remarks

As food regulatory agencies have established strict control programs aimed at preventing contaminants from entering the food supply, official laboratories must be able to efficiently process a high number of samples. This has led to a demand for routine, rapid, and efficient food control procedures. Consequently, there is an urgent need to develop rapid, cost-effective, sensitive, high sample throughput, and on-site analytical strategies that can be used as an "alarm" to rapidly detect the risk of contamination by food pathogens in a wide variety of food matrices, especially since standard methods do not meet these detection requirements. As demonstrated herein for many agents affecting food safety, the use of electrochemical biosensing approaches based on graphite-epoxy composite electrodes fulfills the need for in-field, low-cost, and user-friendly detection methods to ensure food safety.

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