Magnetic Beads-Based Electrochemical Sensors Applied to the Detection and Quantification of Bioterrorism/Biohazard Agents

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Abstract
Nowadays, detecting the presence of bioterrorism and biohazard agents in environmental and food samples is of great concern, due to their toxicity, and because many of them are prone to be used in terrorism attacks. The use of functionalized magnetic beads (MBs) in the development of electrochemical immuno- and genosensors has resulted in innovative and powerful detection strategies that may be applied to environmental, food and clinical analysis. This review describes current research on the combination of functionalized MBs with electrochemical detection for the development of magnetobiosensors applied to rapid, sensitive and specific detection of bioterrorism and biohazard agents.

Keywords: Magnetic beads, Bioterrorism/biohazards, Electrochemical sensors

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1 Introduction

The development and application of magnetic beads (MBs) in separation and detection methodologies has attracted strong interest in the last years. This is mainly due to the versatility, high surface area, chemical and physical stability, low toxicity and high biocompatibility exhibited by MBs [1]. Their size, similar to that of molecules in nature, range from nm to a few μm, and the particles-linked molecules can quickly agglomerate and be separated from a matrix or resuspended in an appropriate working medium without retaining any residual magnetism as a consequence of a change in an external magnetic force [2,3].

MBs consist of a paramagnetic or superparamagnetic core surrounded by a polymeric outer layer suitable for the immobilization of biomolecules [4]. The magnetic core is readily available in different iron oxide forms, among which magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3, ferrimagnetic) stand out since their compatibility has been proven in bio-labelling and bio-separation [3,5,6]. The development of coatings for the magnetic core of MBs was necessary to address their limitations including (a) high surface energies which lead to aggregation, (b) high chemical activity leading to their oxidation, loss of magnetic properties and dispersibility when exposed to air, and (c) biodegradation with subsequent changes in magnetic properties. Consequently, protective shells, mainly composed of agarose, cellulose, silica, silicone, porous glass, mica or polystyrene [7], were developed to protect and preserve the stability of iron oxide MBs. These shells also allowed further functionalization, thus promoting MBs performance as recognition elements in sensing and (bio)chemical arrays [3]. Moreover, nowadays there are commercially available MBs modified with biomolecules that allow their use in different types of bioassays. So, MBs functionalized with: (a) streptavidin suitable for capturing biotinylated nucleic acids, aptamers, peptides, proteins, etc.; (b) protein A (protA) or protein G (protG), which specifically bind antibodies; (c) oligonucleotides; or (d) affinity ligands for specific capture of tagged recombinant proteins, etc. [7] can be purchased from different companies. MBs modification has resulted in important practical advantages from an analytical point of view, including (a) shorter reaction times between dissolved species and biomolecules immobilized on the surface of the beads which is also favoured by the easy dispersion of MBs into solution with only gentle shaking, (b) readily miniaturization of the assay system by using MBs as a mobile solid phase, (c) reduction of the required volumes of reagents and produced waste, and (d) obtaining lower detection limits with shorter assay times [1].

Owing to all these properties, MBs constitute an attractive platform for the design of electrochemical biosensors which also add their inherent advantages such as system miniaturization, low cost and ease of operation [2]. In fact, electrochemical enzyme-based biosensors, immunosensors and nucleic acid hybridization-based sensors have been described in literature and cited in several reviews [1–5,7–9].

Nowadays, there is a great concern on detecting the presence of toxic and infectious agents in environmental and food samples, not only due to their toxicity to human

beings, animals or plants, but also because many of them are prone to be used in terrorism attacks. These agents can be classified in two main groups: one is constituted by chemical agents while the second one involves complex bioengineered microorganisms and pathogens.

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Among chemical agents, insecticides and organophosphorous (OP) nerve gases can be highlighted. OP nerve gases are the most harmful chemical agents. They act in a similar way as insecticides, irreversibly inhibiting the catalytic active site of acetylcholinesterase (AchE), the enzyme that catalyzes the hydrolysis of the neurotransmitter acetylcholine. However, their mammalian toxicity is much higher [10]. Examples include sarin, soman, fonofos and VX agent. The most widely used insecticides belong essentially to two families: organophosphorus (such as paraoxon, parathion, chlorpyriphos, ethyl oxon, or malathion) and carbamate (such as carbofuran, or carbaryl). Vesicants (mustards, lewisite) and pulmonary agents (chlorine, phosgene) are also used as chemical toxic agents.

Unlike a chemical agent attack, biological attacks do not cause an immediate reaction. However, biological agents are much more deadly than chemical ones [11]. Bioterrorism agents are pathogenic organisms (bacteria, viruses) or biological toxins used to produce death and disease in humans, animals or plants for terrorist purposes. Although these agents are typically microorganisms found in nature, they can be modified to increase their virulence, to make them resistant to current antibiotics or vaccines, or to enhance the ability of these agents to be disseminated throughout the environment. While any germ, bacteria or virus could potentially be utilized by terrorists, there are a number of biological agents that have been recognized as being more likely to be used. This is due to their availability to terrorists and the ease by which these agents can be disseminated. Table 1 lists the main biological warfare agents (BWAs) together with the diseases they cause. The presence of microorganisms in food is also a natural and unavoidable occurrence. Cooking generally destroys most harmful bacteria, but undercooked foods, processed ready-to-eat foods, and minimally processed foods can contain harmful bacteria that are serious health threats.

To minimize the effects of natural outbreaks or deliberate attacks, near real-time detection of infectious agents is an essential first step in mounting an appropriate response.

These infectious agents were traditionally detected and identified using standard microbiological and biochemical assays that are accurate but time-consuming. These methods require isolation and/or culturing of large quantities of the infectious agents, and therefore the analysis requires several days to be completed. More recently, molecular approaches, such as the polymerase chain reaction (PCR) amplification and analysis of unique DNA sequences and/or rRNA, have supplanted traditional microbiological methods because they are highly accurate and sensitive, and take less time [12]. However, these assays require specialized instruments and still take several hours to be performed. In addition, DNA-based molecular techniques are limited to the detection of whole organisms and cannot detect toxins and other extracellular products or infectious agents.

Table 1. Main BWAs and diseases they can cause.

	BWA	Caused disease
Bacterial agents	*Bacillus anthracis*	Anthrax
	Yersinia pestis	Plague
	Francisella tularensis	Tularemia
	Brucella species	Brucellosis
	Coxiella burnetii	Q fever
	Salmonella species	Food poisoning
	Escherichia coli O157:H7	Food poisoning
	Shigella	Food poisoning
Viral agents	*Variolla major*	Smallpox
	Alphaviruses and flaviviruses	Viral encephalitis
	Filoviruses and arenaviruses	Viral hemorrhagic fevers
	Influenza virus	Influenza
	Hepatitis B virus	Hepatitis B
	Human immunodeficiency virus (HIV)	AIDS
	SARS coronavirus (SARS-CoV)	SARS
Biological toxins	Staphylococcal enterotoxin B	Food poisoning
	Ricinus communis (castor beans)	Ricin toxin poisoning
	Clostridium botulinum toxin	Botulism
	Mycotoxins	Mycotoxicoses

Biosensor technology brings together the accuracy and sensitivity of standard approaches with improvement in rapidity of detection. Electrochemical sensors, in particular, offer several advantages compared to PCR and other molecular detection approaches, including a broad range of target analytes, minimal sample preparation, ability to analyze complex body fluids, high sensitivity, miniaturization, and compatibility with compact instrumentation. Biosensors also offer the possibility of continuous and real-time monitoring of the environment for the presence of infectious agents to allow timely implementation of preventive and protective measurements. In fact, the majority of existing technologies for detecting biological agents rely on either antibodies as the recognition molecules, which bind with a surface feature of the BWA, or on the recognition of a nucleic acid sequence known to be found in the BWA being tested. A few other types of recognition molecules such as peptides, glycolipids and aptamers have also been used with similar transduction schemes [13]. All these recognition elements can also be employed in the development of electrochemical biosensors.

The combination of functionalized MBs with electrochemical detection constitutes a powerful and efficient strategy for the development of magnetobiosensors which can be applied to the rapid and sensitive detection of many agents of environmental and clinical significance. This review focuses on current research regarding the use of MBs in the electrochemical sensing of bioterrorism and biohazard agents. Strategies involving methods based on the use of immuno- and nucleic acid-MB-based sensors by means of different amplification systems to achieve sensitive and specific electrochemical detection of these agents are discussed.

2 MBs-Based Electrochemical Immunosensors

Several advantages have been claimed for immunoassays making use of MBs over conventional ones. They can be summarized as: (1) high reaction kinetics in a small volume, high surface area and high dispersion capability of MBs leading to an increase in surface-to-volume ratio, together with a decrease of matrix effects and, consequently, to a substantial shortening of the immunoreaction times; (2) versatile manipulation and optimization of experimental conditions, as a consequence of the separation between the electrochemical detection and the immunoreaction steps, as it will be illustrated below; (3) less time consuming due to decreased coating, competition and blocking times; (4) modification of MBs in numerous ways allowing different immobilization strategies; and (5) the immunocomplexes formed on the MBs surface can be easily detected without preconcentration or purification steps, which are normally required for standard immunoassays [1,14,15]. In summary, the good analytical performance of the immunodevices using functionalized MBs can be attributed to the increased surface area and reactivity, high effectiveness of blocking reagents, and improved washing and separation steps.

2.1 Detection of Chemical Agents

Direct competitive immunoassay schemes, where the analyte and enzyme-analyte conjugates (using mainly alkaline phosphatase (AP) and horseradish peroxidase (HRP) as enzyme labels) compete for a limited amount of antibodies-coated MBs have been used to develop magnetoimmunosensors to detect 2,4-dichlorophenoxiacetic acid (2,4-D) [16], polychlorinated biphenyls (PCBs) [17–19], atrazine [20,21], and the insecticide biomarker trichloropyridinol (TCP) [22]. As an example, the schematic pro-

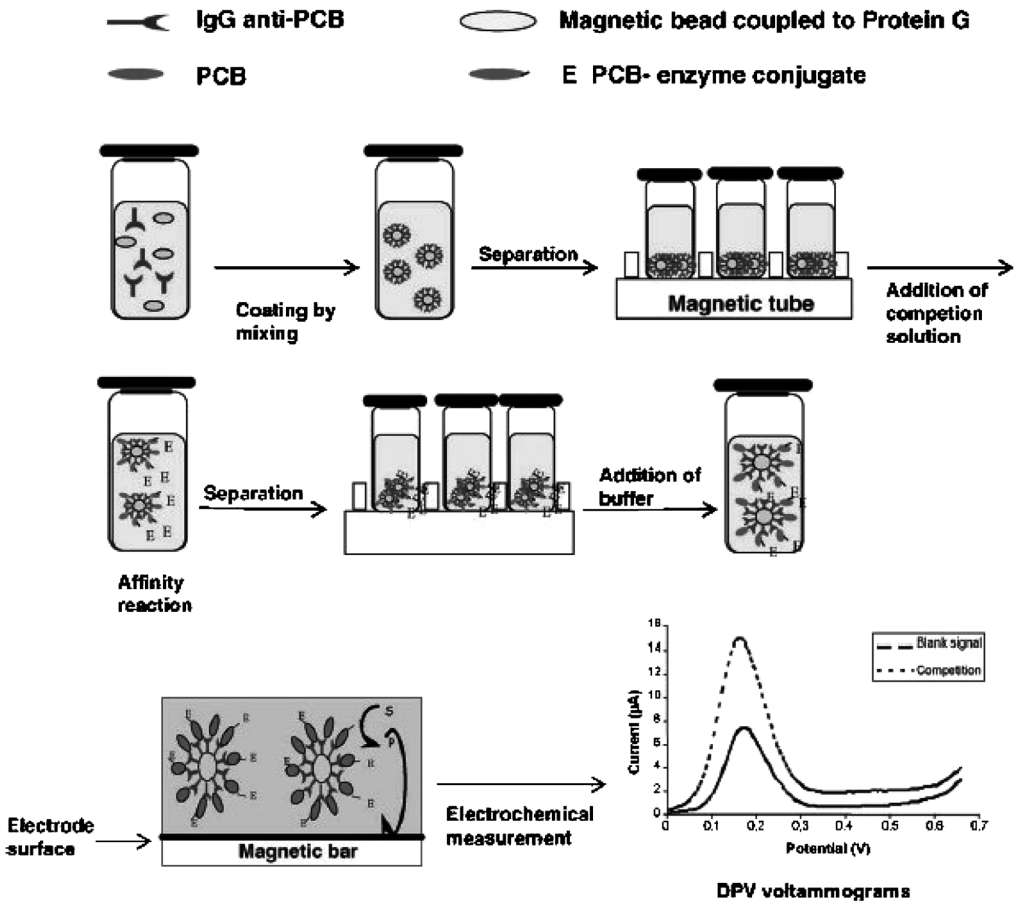

Fig. 1. Schematic display of the experimental procedure used by Centi et al. for the determination of PCBs using MBs-based immunosensors. Reprinted from [17] with permission. Copyright 2005 Elsevier.

cedure for the determination of PCBs used by Centi et al. is displayed in Figure 1 [17]. Moreover, the characteristics and performance of these magnetoimmunosensors are summarized in Table 2. As can be seen, the performance of some of these immunosensing systems was successfully evaluated using spiked environmental samples (soils, marine sediment extracts, foods, and river and bottled water samples). The obtained results indicate that these convenient and sensitive methodologies offer great promise for decentralized environmental applications. In fact, they constitute interesting approaches which can be further used in the development of easy-to-use inexpensive tests for preliminary sample screening directly in-field. Moreover, these strategies can be readily transferred to the detection of other environmental contaminants through the development of specific antibodies against these contaminants and are expected to open new opportunities for environmental monitoring and public health.

Table 2. Analytical characteristics for the detection of chemical agents using electrochemical magnetoimmunosensors.

Target	Label	Detection technique	Detection limit	Real sample	Ref.
2,4-D	AP	CV	0.01 $\mu g L^{-1}$	River water	[16]
PCBs	AP	DPV	0.4 $ng mL^{-1}$	Marine sediments, soil	[17]
Atrazine	–	EIS	$< 50\ ng mL^{-1}$	–	[20]
Atrazine	HRP	Amp.	$6 \times 10^{-3}\ \mu g L^{-1}$	Orange juice	[21]
TCP	HRP	SWV	5 $ng L^{-1}$	River water	[22]
PCBs	AP	DPV	0.4–0.8 $ng mL^{-1}$	Marine sediments	[18]
PCBs	HRP	SWV	10 $pg mL^{-1}$	River water	[19]

Amp.: amperometry; AP: alkaline phosphatase; CV: cyclic voltammetry; 2,4-D: 2,4-dichlorophenoxiacetic acid; DPV: differential pulse voltammetry; HRP: horseradish peroxidase; EIS: electrochemical impedance spectroscopy; PCBs: polychlorinated biphenyls; SWV: square wave voltammetry; TCP: trichloropyridinol.

2.2 Detection of Viral, Bacterial and Antibacterial Agents

The development of magnetoimmunosensors for the detection of pathogen bacteria was firstly described by Kradtap et al. [23] and Che et al. [24]. Kradtap et al. used an artificial microorganism called "Bugbead" as stimulant for the development of microbial sensors without introducing potential experimental hazards or other variables coming in when using real microorganisms [23]. The applicability of these bugbeads in the development of immunosensors was demonstrated by the analysis of extracts containing serotype antigens of *Escherichia coli* O157:H7 (*E. coli* O157:H7). However, to our knowledge, these beads have never been used again in the development of electrochemical immunosensors. On the other hand, Che et al. developed an immunomagnetic separation (IMS) procedure to isolate the enteropathogen *Campylobacter jejuni* (*C. jejuni*) cells from the sample solution [24]. The developed sensor that made use of a tyrosinase-modified electrode was applied to the analysis of pure culture and poultry samples. The potential application of this immunosensor in the poultry industry was demonstrated by the rapidity of the assay (2.5 h), the detection limit at the lowest concentration level expected in these samples, and the simple system setup. Table 3 summarizes the main characteristics of these sensors.

A fully-automated fluidic system implying a bead-based sandwich immunoassay with electrochemical detection was described for bacteriophage MS2 and ovalbumin (OVA) detection [25]. The sandwich immunosensor was constructed by attaching a biotinylated antibody to streptavidin-coated beads, capturing the antigen, and exposing the conjugate to a β-galactosidase-labelled antibody. The detection limits for MS2 and OVA were 990 and 470 $ng\,mL^{-1}$, respectively.

Another magnetoimmunosensing sandwich approach was developed for the detection of *Salmonella* allowing, in 50 min and without any pretreatment, a detection limit of 7.5×10^3 colony forming units (cfu) mL^{-1} to be obtained in 10 times diluted milk samples [26]. In this approach, the bacteria were captured and preconcentrated from milk samples with MBs through an immunological reaction. A polyclonal anti-Salmonella antibody labelled with HRP was used as serological confirmation with electrochemical detection at a magnetoelectrode.

A disposable magnetoimmunosensor for the detection of *Streptococcus pneumoniae* (*S. pneumoniae*) was reported using a similar sandwich format where protA-coated MBs were employed as a solid phase to immobilize specific *S. pneumoniae* capture antibodies. As can be seen in Figure 2, the same antibody but conjugated to HRP as enzyme label was used as the detection antibody to recognize the captured *S. pneumoniae* cells. The electrochemical detection of the enzyme reaction product was accomplished at disposable gold screen-printed electrodes (Au/SPEs) modified with tetrathiafulvalene (TTF) as electron transfer mediator and using H_2O_2 as the enzyme substrate [27]. The developed methodology was shown to be suitable for detecting 1.0×10^4 cfu of *S. pneumoniae* in inoculated urine samples without any sample pretreatment.

Some strategies have also been developed for the quantification of human antibodies against some bacterial agents or their specific toxins. Interesting examples of these approaches are the electrochemical magnetoimmunosensing assays developed for the rapid, selective and sensitive quantification of anti-*Helicobacter pylori* [28], anti-*Clostridium tetani* [14], and anti-hepatitis B virus [29] IgG antibodies. The detection of these antibodies in serum was carried out by indirect antibody capture assays based on the use of purified antigens immobilized on magnetic microspheres. The IgG antibodies present in the serum samples were allowed to react immunologically with the immobilized antigens, and the bound antibodies were quantified by enzyme-labelled [14,28] or gold nanoparticles (AuNPs)-conjugated [29] secondary antibodies specific to human IgG. These original approaches may be extended to on site applications dealing with the screening of antibody levels in complex samples.

Some novel strategies for the detection of viruses and bacteria based on magnetoimmunosensors have been described recently. A magnetic gold electrode was fabricated for the direct sensing of Japanese encephalitis virus (JEV) [30]. Gold-coated MBs were employed as the platforms for the immobilization and immunoreaction processes, and HRP was chosen as an enzymatic tracer. After the immunoreactions, multiwalled carbon nanotubes (MWCNTs) were mixed with the immunocomplex coated Au-coated MBs to improve the sensitivity of the assay. A detection limit two orders of magnitude lower than that of immunochromatographic strip and similar to that obtained by reverse transcriptase polymerase chain reaction (RT-PCR) was obtained (Table 3). The method was successfully applied in clinical samples (brain tissue of swine, mosquito and cerebrospinal fluid of human patients).

Also, novel magnetoimmunoassay-based strategies for the detection of *Plasmodium falciparum* histidine-rich protein 2 (HRP2), a malaria parasite biomarker, have been described. Two different kinds of magnetic supports were employed for this purpose: 1 μm tosyl-activated MBs, and 300 nm active magnetic nanoparticles [31]. This is the first report of a procedure based on a sandwich magnetoimmunoassay between the protein in the sample and two commercially available monoclonal antibodies that recognize two different epitopes of the antigen, one of them covalently coupled to MBs and the other labelled with the enzyme HRP. The electrochemical magnetoimunosensor showed better analytical performance than other methods in terms of limit of detection (see Table 3). Due to the high sensitivity achieved, this strategy offers great promise to implement rapid, simple, inexpensive and user-friendly analytical methods for on-site detection of *falciparum* malaria disease in patients, and also to screen out at-risk blood samples for prevention of trans-

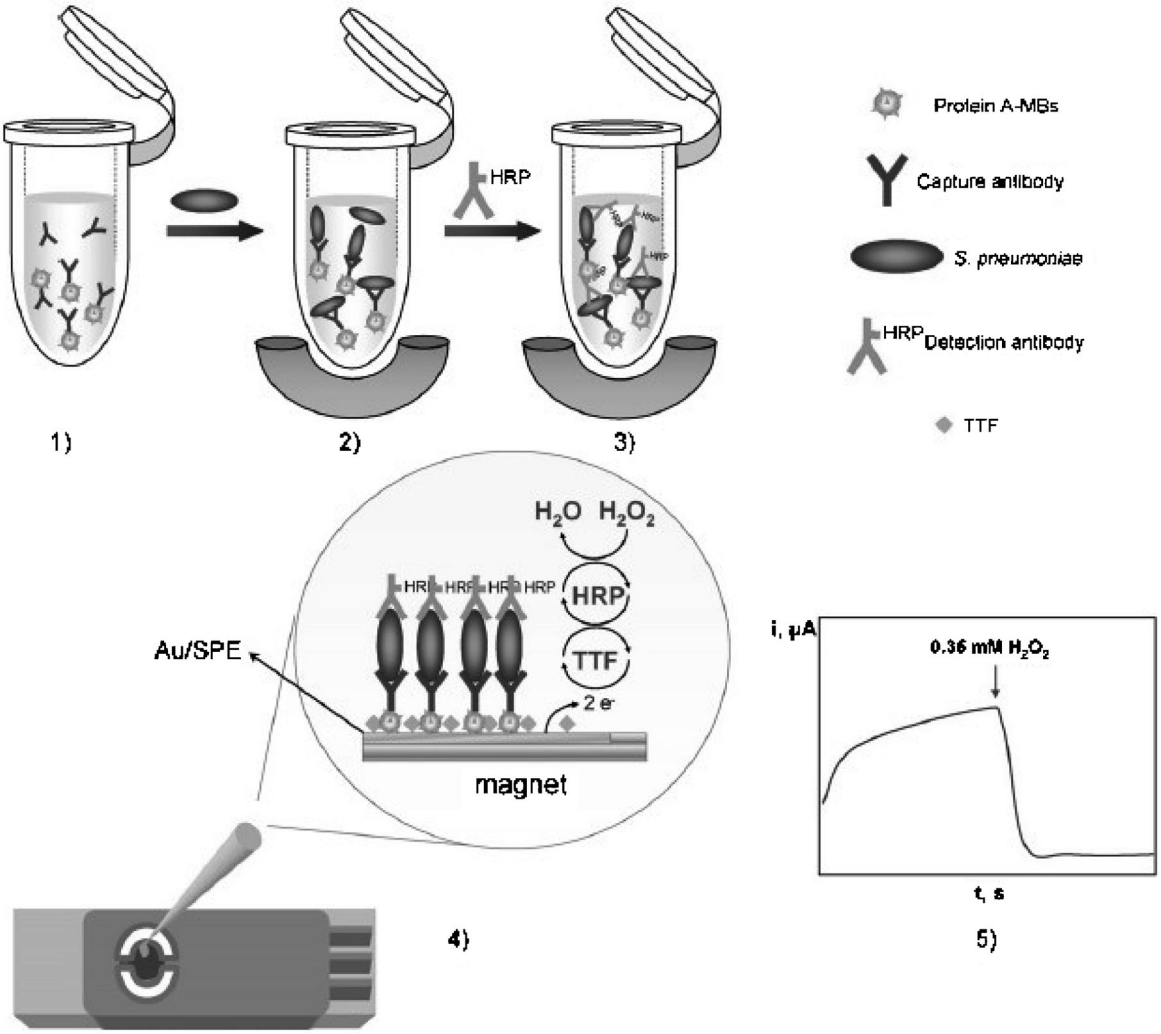

Fig. 2. Immunosensor for the determination of *S. pneumoniae*: 1) immobilization of capture antibodies on protA-modified MBs; 2) MB-antibody–bacteria complexes separation and removing excess antibody; 3) incubation with labelled antibody; 4) MBs capture on TTF-Au/SPEs; 5) amperometric detection of the mediated reduction of H_2O_2 with TTF. Reprinted from [27] with permission. Copyright 2010 Elsevier.

fusion-transmitted malaria, as well as for in vitro drug sensitivity testing.

Moreover, a novel electrochemical immunosensing assay for bacterial detection that combines a one-step sandwich immunoassay, MBs target preconcentration, microfluidic technology, and amperometric detection using HRP as the label enzyme has recently been described [32]. The enzymatic reaction takes place in an incubation micro-chamber where the MBs are confined, upstream from the working electrode. The enzyme product is then pumped along a microchannel, where it is amperometrically detected by a set of microelectrodes. The system provided a significant improvement in terms of limit of detection and assay time (1 h) compared to classical ELISA detection. Also, it provides the possibility of addressing relatively complex sample matrices so that it could be potentially applicable to the food, pharmaceutical and medical fields if preconcentration and/or preenrichment strategies were implemented.

Table 3 summarizes the analytical characteristics of the reported methods for the detection of viral, bacterial and anti-bacterial agents using electrochemical magnetoimmunosensors.

2.3 Detection of Biological Toxins

Competitive magnetoimmunoassays have been described for the determination of several biological toxins. An electrochemical immunoassay for the determination of aflatoxin B_1 (AFB_1) in food has been reported. The approach was based on the use of multifunctional MBs as probes, which comprised magnetic $CoFe_2O_4$ and Prussian Blue nanoparticles and were used as an affinity support for the immobilization of the AFB_1-bovine serum albumin conjugate (AFB_1-BSA) [33]. The concentrations of

Table 3. Analytical characteristics for the detection of viral, bacterial and antibacterial agents using electrochemical magnetoimmunosensors.

Target	Label	Detection technique	Detection limit	Sample	Ref.
E. coli O157:H7	AP	Amp.	–	–	[23]
C. jejuni	Tyr	Amp.	2.1×10^4 CFU mL^{-1}	Pure culture, poultry	[24]
MS2 OVA	β-gal	Amp.	990 and 470 ng mL^{-1}	–	[25]
Salmonella	HRP	Amp.	7.5×10^3 CFU mL^{-1}	Milk	[26]
S. pneumoniae	HRP	Amp.	1.0×10^4 CFU	Urine	[27]
Anti-*H. pylori*	AP	Amp.	0.37 U mL^{-1}	–	[28]
Anti-*C. tetani*	HRP	Amp.	0.0046 IU mL^{-1}	Guinea pig serum	[14]
Anti-hepatitis B	AuNPs	Chronoamp.	3 mIU mL^{-1}	Human serum	[29]
JEV	HRP	DPV	0.056 ng mL^{-1}	Clinical	[30]
HRP2	HRP	Amp.	0.36 ng mL^{-1}	Human serum	[31]
E. coli	HRP	Amp.	55 cells mL^{-1}	Milk	[32]

AP: alkaline phosphatase; AuNPs: gold nanoparticles; *C. jejuni*: *Campylobacter jejuni*; *C. tetani*: *Clostridium tetani*; DPV: differential pulse voltammetry; *E. coli*: *Escherichia coli*; β-gal: β-galactosidase; *H. pylori*: *Helicobacter pylori*; HRP: horseradish peroxidase; HRP2: *Plasmodium falciparum* histidine-rich protein 2; JEV: Japanese encephalitis virus; OVA: ovalbumin; Tyr: tyrosinase.

AFB_1 were determined by means of a competitive-type immunoassay using AuNPs modified with HRP-labelled anti-AFB_1 as detection antibodies for the amplification of the electrochemical signal (Figure 3). This strategy gave rise to a high sensitivity, enhanced by the use of AuNPs, together with avoiding the need of adding an electron mediator in the detection solution due to the presence of Prussian Blue NPs, thus providing a convenient platform for clinical testing and drug screening.

A related approach has been described for the detection of deoxynivalenol (DON), a mycotoxin produced by *Fusarium* fungi which represents a serious threat for the

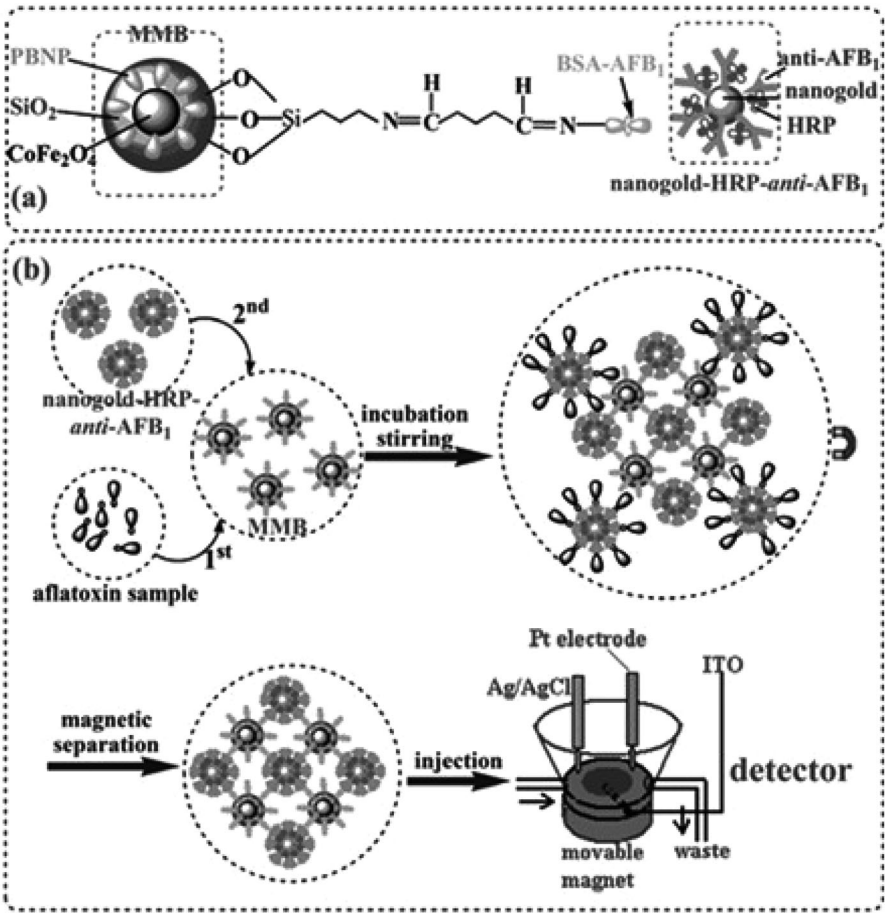

Fig. 3. (a) Schematic illustration of the multifunctional MBs and the AuNP-HRP-anti-AFB_1, and (b) measurement process involved in the competitive immunoassay methodology. Reprinted from [33] with permission. Copyright 2009 RSC.

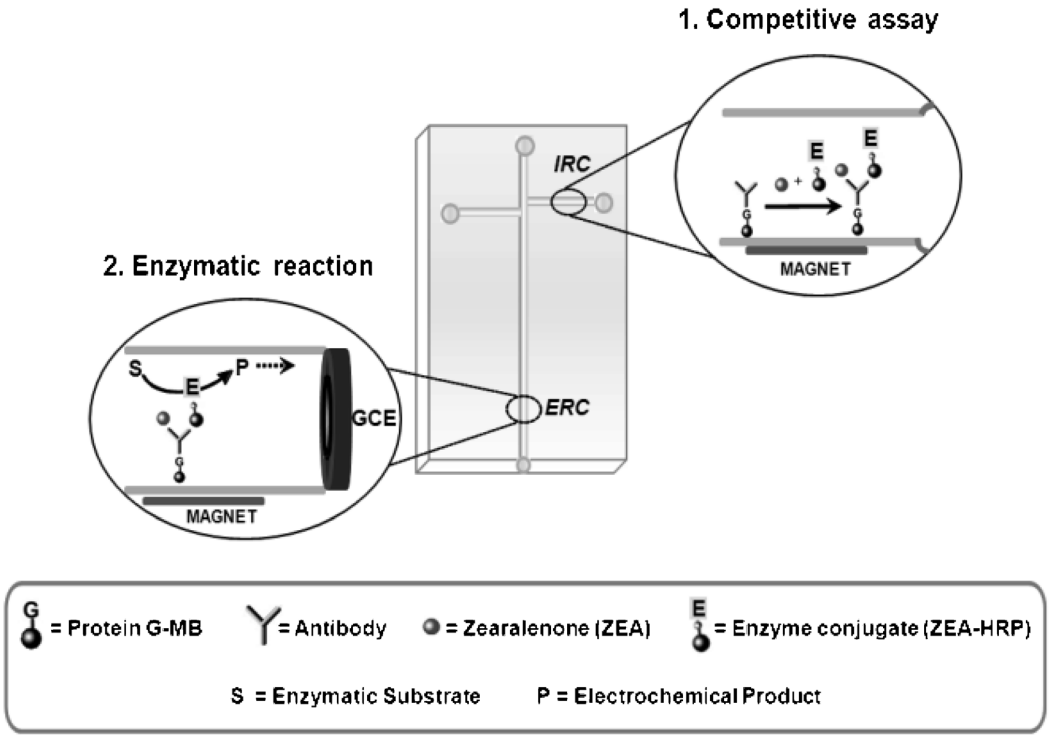

Fig. 4. Schematic display of the microfluidic layout and immunoassay principle used for the determination of ZEA. IRC, immunological reaction chamber; ERC, enzymatic reaction chamber. Adapted from [38] with permission. Copyright 2011 RSC.

safety of cereal-based food and feedstuffs [34]. This methodology was based on the use of a novel anti-DON Fab fragment and of immunomagnetic beads coupled with eight magnetized SPEs as electrochemical transducers. The experimental procedure consisted of coating MBs with the conjugated toxin and performing a competitive assay by adding the free toxin together with the Fab fragment, conjugated with a biotin group. In order to carry out the electrochemical detection, the immunological complex was linked to HRP via an avidin-biotin binding. Although the developed system resulted in sensitivity and selectivity characteristics similar to those offered by ELISA or SPR methodologies, the employed multielectrode strip could be applied to multianalyte detection. Also, this kind of transducer is easily miniaturized in a cost-effective and mass producible way thus opening a door to the development of a portable electrochemical instrument and miniaturized strip for lab-on-a-chip approach.

Another example is that described for the determination of okadaic acid (OA), a lipophilic marine biotoxin ingested by various species of shellfish, involving the use of super paramagnetic nanobeads [15]. Immobilized OA on streptavidin-coated MBs competed with free OA in solution for the anti-OA monoclonal antibody also in solution. A secondary antibody labelled with AP was used in the detection step which was carried out by DPV. The developed method was applied successfully to the determination of AO in mussels extracts demonstrating that the use of MBs reduced the analysis time and eliminated matrix effect and interferences.

Some electrochemical MBs-based immunosensing approaches exhibiting successful integration in microfluidic devices have been also reported. One of them, based on a sandwich configuration, was developed for the detection of cholera toxin subunit B (CTB) [35]. The sandwich format used comprised anti-CTB antibody and ferri/ferrocyanide encapsulating ganglioside GM_1 liposomes for signal amplification. Fluorescence and electrochemical detections were compared finding out that the electrochemical approach showed advantages in terms of flexibility and reliability of signal recording. A microfluidic device has been developed for a simplified detection of zearalenone (ZEA) on the basis of a competition scheme where this mycotoxin and an enzyme-labelled derivative compete for the binding sites of a specific antibody immobilized on protG-modified MBs.

Also, the possibility of using electrochemical detection coupled with a MBs-based immunoassay for the detection of ZEA in baby food was demonstrated [36,37]. Subsequently, the developed system was integrated in microfluidic chips following the scheme displayed in Figure 4, where the simple channel layout of a double-T microchip was used to perform sequentially the immunoreaction and the enzyme reaction by applying a program of electric fields [38]. Another system for the determination of ZEA in feedstuffs samples has been recently reported using a microbiochip microfluidic immunosensor coupled with flow injection (FIA) with Au/SPEs as the working electrode showing good sensitivity and accuracy, and minimizing the use of expensive reagents [39].

Table 4. Analytical characteristics for the detection of biological toxins detection using electrochemical magnetoimmunosensors.

Target	Label	Detection technique	Detection limit	Real sample	Ref.
AFB_1	HRP	CV	6 $pg\,mL^{-1}$	Paprika	[33]
DON	HRP	Chronoamp.	63 $ng\,mL^{-1}$	Cereals	[34]
OA	AP	DPV	0.38 $\mu g\,L^{-1}$	Mussels	[15]
CTB	AP	Coulometry	1.0 $ng\,mL^{-1}$	–	[35]
ZEA	HRP	Amp.	0.011 $\mu g\,L^{-1}$	Baby food	[36]
ZEA	HRP	DPV	0.007 $\mu g\,L^{-1}$	Baby food	[37]
ZEA	HRP	Amp.	0.4 $\mu g\,L^{-1}$	Infant foods	[38]
ZEA	HRP	Amp.	0.41 $\mu g\,kg^{-1}$	Feedstuffs	[39]

AFB_1: aflatoxin B_1; Amp.: amperometry; AP: alkaline phosphatase; CV: cyclic voltammetry; DON: deoxynivalenol; DPV: differential pulse voltammetry; HRP: horseradish peroxidase; OA: okadaic acid; ZEA: zearalenone.

Table 4 summarizes the main characteristics reported for the detection of biological toxins using electrochemical magnetoimmunosensors.

3 MBs-Based Electrochemical DNA Sensors

MBs have demonstrated to be versatile tools not only for the separation of nucleic acids but also as a platform for optimized DNA hybridization [7]. The hybridization event is detected at a different surface, the working electrode, the detection being based either on the intrinsic DNA electroactivity or on DNA labelling in order to amplify the measured signals. Moreover, MBs have been used to discriminate genetic alleles and thus identify the presence of single nucleotide polymorphisms (SNPs), in the study of mutational status or genotyping of an organism, drug discovery, and genetic disorders associated with point mutations, as well as in the detection of viruses, bacteria and food-borne pathogens [9].

LaGier et al. developed a label-free electrochemical method based on the use of DNA probe-coated MBs for the selective detection of *E. coli* 16S rRNA directly from lysed cells, by monitoring the guanine oxidation signal [40]. Using this approach, 10^7 *E. coli* cells could be detected in 4 h. A sensitive and selective genomagnetic assay for the electrochemical detection of *Salmonella* spp. was developed based on a double labelling polymerase chain reaction (PCR) strategy that generated an amplicon of the IS200 insertion sequence with double labelling: with a biotinylated capture primer to achieve immobilization on streptavidin-coated MBs, and with a digoxigenin signalling primer for electrochemical detection [41]. A PCR reactor for real-time electrochemical detection of *Salmonella* genome, in which the amplification and double-labelling were directly performed on the streptavidin-MBs by using magnetic primers, was also reported.

A highly sensitive assay for rapidly screening-out *Mycobacterium bovis*' (*M. bovis*) DNA in contaminated samples was also developed using electrochemical magnetogenosensing [42]. The assay consisted on specific amplification and double-tagging of the IS*6110* DNA fragment, highly related to *M. bovis*, followed by electrochemical detection of the amplified product. PCR amplification, carried out using a labelled set of primers, resulted in a double-tagged amplicon with both biotin and digoxigenin at each terminus. The double-tagged amplicon was detected using a magnetosensor combined with streptavidin-modified MBs (Figure 5). The assay allowed the detection of 10 fmol of PCR amplicon and showed promising features for the detection of *M. bovis* on dairy farms by screening for the presence of the bacterial DNA in milk samples.

Integration of streptavidin-coated paramagnetic microbeads modified with a biotinylated capture probe, with a commercially available microfluidic platform, resulted in a rapid and sensitive (0.2 nM detection limit) enzyme-linked electrochemical genosensor used for the determination of a PCR amplified fragment of the Cor a 1.04 gene (182 bp) of hazelnut [43]. A gravity-driven microfluidic-based electrochemical platform using micromagnetic beads as solid support for nucleic acid detection has been recently described and applied to the detection of specific DNA sequences of *Legionella pneumophila* with a detection limit of 0.33 nM [44]. The developed device combined a special chip containing eight polymer microchannels with a portable, computer-controlled, instrument where both hybridization and labelling events were performed on streptavidin-coated paramagnetic microparticles functionalized with a biotinylated capture probe.

MBs covered with biotin-labelled oligonucleotides have recently been used to isolate viral DNA sequences of human immunodeficiency virus (HIV) and influenza virus subtype H5N1 [45]. The viral nucleic acids were detected with carbon nanotubes (CNTs)-modified SPEs coupled with a micro-flow instrument. The described system could be useful for easy-to-use and rapid detection of viral nucleic acid.

The development of magnetoelectrochemical disposable DNA sensors for the detection of *E. coli*, based on the use of MBs and enzymatic signal amplification and the recognition of a characteristic region of the *lacZ* gene, was reported [46,47]. 326-bp amplicons were obtained by asymmetric PCR (aPCR) using genomic DNA or directly from cell cultures (daPCR) and hybridized on streptavidin-MBs modified with a specific biotinylated capture probe. After binding of a streptavidin-HRP polymer to the resultant biotinylated hybrids, the modified

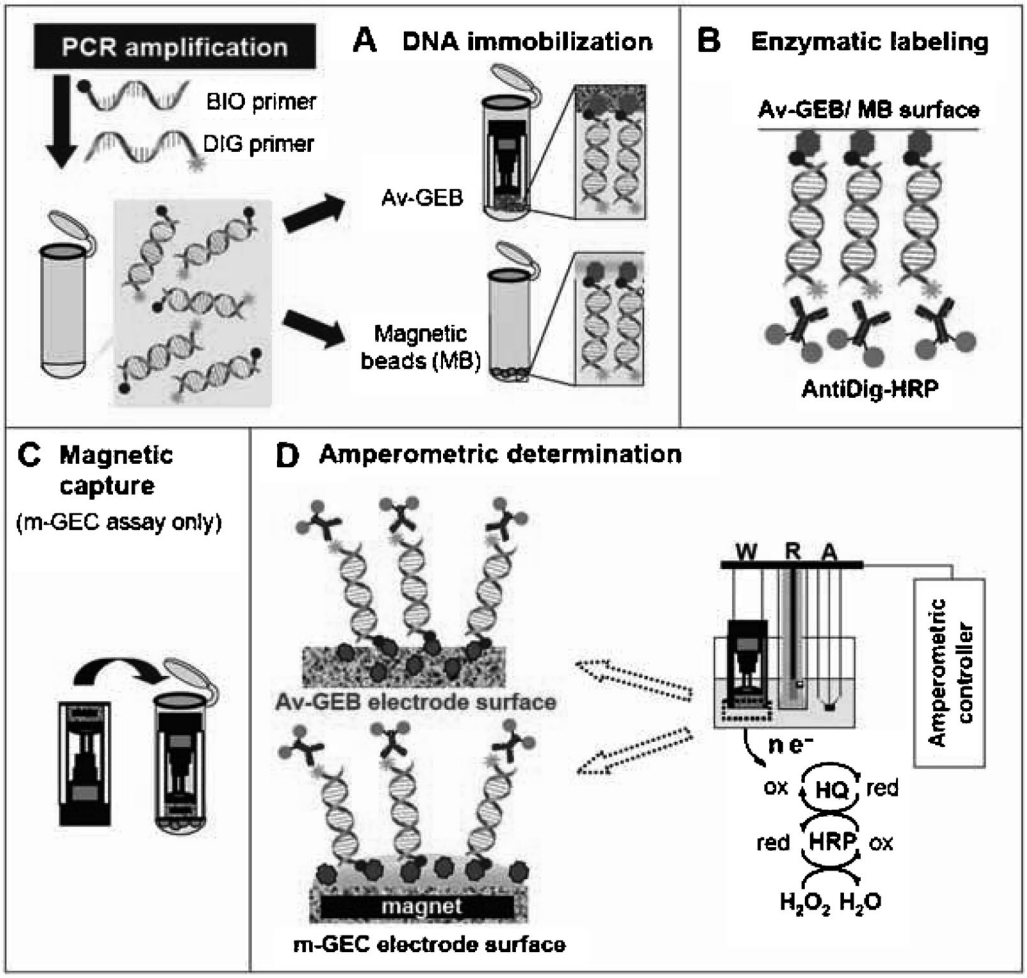

Fig. 5. Schematic display of the methodology employed for the detection of *M. bovis* using MBs-based electrochemical genosensors. Reprinted from [42] with permission. Copyright 2010 Spanish Society for Microbiology.

MBs were captured by a magnetic field on the surface of TTF-modified Au/SPEs. As low as 2.5 aM aPCR product could be detected with the developed methodology. A further approach involved the obtaining of the 326-bp amplicons directly from cell cultures (daPCR) achieving *E. coli* detection at a concentration level of 1 cfu /100 mL with no need for culture preconcentration steps. These results demonstrated the usefulness of the developed sensors as screening or alarm devices indicating the possible presence of coliforms, together with their applicability to drinking water quality assurance (no coliform bacteria per 100 mL of water). The same research group employed similar strategies in the development of DNA sensors for the detection of a characteristic 235-bp region of the gene coding for autolysin (LytA), a specific *pneumococcus* virulent factor [48]. The obtained results demonstrated the usefulness of the developed methodology for rapid, simple, specific, quantitative, and sensitive detection of aPCR amplicons with a detection limit of 1.1 nM. Moreover, amplified daPCR products could be prepared with as few as 2 cfu from *S. pneumoniae* cultures, which make these devices very promising for the rapid identification of these bioagents. Under the experimental conditions employed, the assay was capable of distinguishing between *S. pneumoniae* and other streptococci of the mitis group also known to contain a *lytA*-like gene.

An electrochemical competitive aptasensor for the detection of the mycotoxin ochratoxin A (OTA) has been described. The aptasensor was based on competition between the mycotoxin conjugated to the enzyme HRP and free OTA for streptavidin-MBs functionalized with a biotinylated aptamer [49]. This magnetic aptasensor showed a detection limit of 0.07 ng mL^{-1} and was accurately applied to extracts of certified and spiked wheat samples.

Table 5 summarizes the main analytical characteristics exhibited by electrochemical nucleic acid magnetosensors for the detection of bioterrorism and biohazard agents.

4 Magnetoimmuno-PCR Approaches

The combination of IMS, PCR, and electrochemical magnetogenosensing of the resulting amplicons allows the im-

Table 5. Analytical characteristics for the detection of bioterrorism and biohazard agents using electrochemical nucleic acid magnetosensors.

Target	Label	Detection technique	Detection limit	Real sample	Ref.
E. coli	G	DPV	10^7 cells	Cell culture	[40]
Salmonella	HRP	Amp.	2.8 fmol	–	[41]
M. bovis	HRP	Amp.	10 fmol	Milk	[42]
Halzenut	AP	DPV	0.2 nM	–	[43]
Legionella	AP	Chronoamp.	0.33 nM	–	[44]
HIV, influenza	DNA bases	SWV	–	Human serum	[45]
E. coli	HRP	Amp.	2.5 Am	Cell culture	[46]
E. coli	HRP	Amp.	0.01 cfu mL^{-1}	Cell culture	[47]
S. pneumoniae	HRP	Amp.	2 cfu	Urine, Blood	[48]
OTA	HRP	DPV	0.07 ng mL^{-1}	Wheat	[49]

Amp.: amperometry; AP: alkaline phosphatase; DPV: differential pulse voltammetry; G: guanine; HRP: horseradish peroxidase; HIV: human immunodefiency virus; OTA: ochratoxin A; *S. pneumoniae*: *Streptococcus penumoniae*; SWV: square wave voltammetry.

plementation of rapid, sensitive, and selective assays for the detection of bacterial agents.

Salmonella was detected using a strategy based on capturing and preconcentrating the bacteria from milk samples by IMS [50].The bacteria attached to the MBs were then lysed by heating treatment to release the genomic DNA. Amplification of the genetic material with a double-tagging set of primers was performed and the amplicons were detected by electrochemical measurement (Figure 6). Using this approach, a detection limit of 0.04 cfu mL^{-1} was obtained in skim milk samples preenriched for 6 h.

An electrochemical magnetoimmuno-PCR approach has been recently described for direct and highly sensitive detection of *S. pneumoniae* [51]. The amplified products obtained by daPCR from bacteria attached to specific

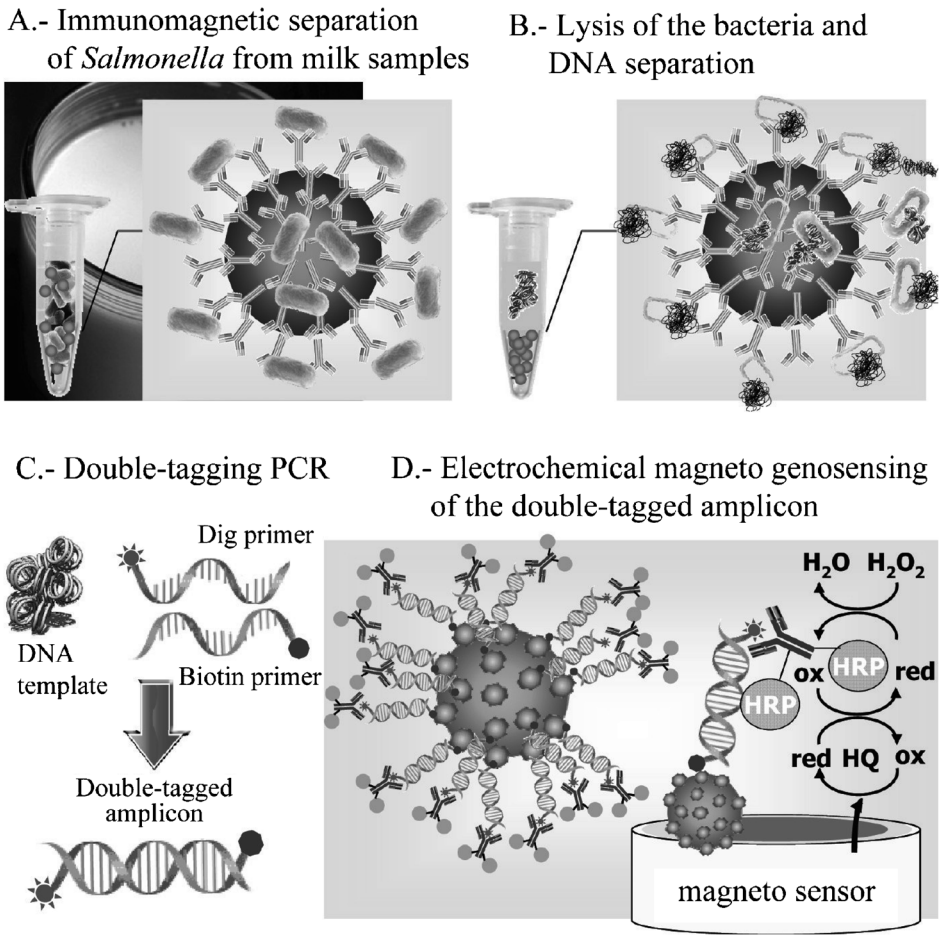

Fig. 6. Schematic display of the electrochemical magnetoimmuno-PCR approach for the detection *Salmonella*. Reprinted from [50] with permission. Copyright 2009 ACS.

capture antibody-modified MBs were detected similarly to that commented above using the electrochemical genosensor [48]. The developed methodology allowed an easy discrimination between only 100 cfu mL^{-1} *S. pneumoniae* cultures and high concentrations ($\sim 1.0 \times 10^8$ cfu mL^{-1}) of other streptococci, thus demonstrating the reliability of the assay for the specific detection of low pneumococcus concentrations.

5 Other Magnetosensor Approaches Employed in the Detection of Bioterrorism/Biohazard Agents

Some other approaches for the determination of bioterrorism/biohazard agents making use of other types of receptors and MBs have been described. A method for detecting wheat germ agglutininin (WGA) based on the use of glucosamine-modified MBs and daunomycin-labelled glucose as a probe was reported [52]. When labelled glucose was held in the binding site of WGA to the sugar, the peak current decreased. Given that the binding between WGA and labelled glucose depended on the glucosamine concentration of glucosamine-modified beads, the concentration was estimated from the change in the peak current. Other lectins not having the binding site to glucosamine or glucose did not produce any change of peak current. Therefore, this method would be powerful for evaluation of interaction between protein and sugar chain existing at cell surface. An ultrasensitive method to evaluate toxicity due to pesticides in a glass lab-on-a-chip by means of enzymatic inhibition of acetylcholinesterase immobilised on MBs and amperometric detection has been developed and applied to the detection of carbofuram down to the nanomolar (sub-ppb) level [53].

6 Conclusions

Micro-and nano-MBs play an important role in the development of electrochemical detection methodologies for biohazard and bioterrorism agents. MBs-based electrochemical immunosensors have been widely employed with this purpose, making possible to analyze environmental and food samples as long as antibodies against specific bioagents are available. There are not so many examples on magnetic genosensors, although interesting approaches have also been developed in this field, where high sensitivities are obtained through different amplification systems, such as PCR or enzymatic signal amplification. Sensitivity and specificity can be improved in a high extent using magnetoimmuno-PCR approaches, although very few examples have been described based on this innovative and combined new methodology. Future prospects should be focused on the extension of these magnetobioassays to develop highly demanded easy-to-use miniaturized and multiplexed bio-platforms for the automated detection of multiple biohazard and/or bioterrorism agents on one shot. In this regard, combination of MBs-based bioassays with microfluidic devices should facilitate implementing this kind of advanced bio-platforms for the direct decentralized detection and with a short time from sample acquisition to readout. Another important challenge for analytical chemists is to validate the developed devices with real environmental, food and clinical samples. Moreover, integration of the biosensing platforms in the clothing of risk people, such as soldiers, constitutes also an exciting new field of work involving multidisciplinary strategies and economical interests.

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