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New advances in electrochemical biosensors for the detection of toxins: Nanomaterials, magnetic beads and microfluidics systems. A review

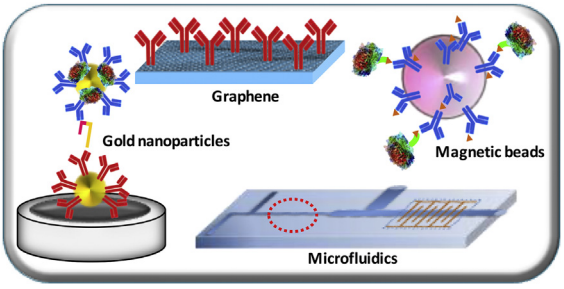
Laia Reverté [a], Beatriz Prieto-Simón [b], Mònica Campàs [a, *]

[a] IRTA, Carretera Poble Nou km. 5.5, 43540 Sant Carles de la Ràpita, Tarragona, Spain
[b] ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, Future Industries Institute, University of South Australia, SA 5095, Australia

HIGHLIGHTS

- Nanomaterials improve the performance of electrochemical biosensors.
- Carbon nanomaterials can act as electrocatalysts or label supports in biosensors.
- Metal nanomaterials can act as nanostructured supports or labels in biosensors.
- Magnetic beads are exploited as immobilisation supports and/or label carriers.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of nanotechnology in bioanalytical devices has special advantages in the detection of toxins of interest in food safety and environmental applications. The low levels to be detected and the small size of toxins justify the increasing number of publications dealing with electrochemical biosensors, due to their high sensitivity and design versatility. The incorporation of nanomaterials in their development has been exploited to further increase their sensitivity, providing simple and fast devices, with multiplexed capabilities. This paper gives an overview of the electrochemical biosensors that have incorporated carbon and metal nanomaterials in their configurations for the detection of toxins. Biosensing systems based on magnetic beads or integrated into microfluidics systems have also been considered because of their contribution to the development of compact analytical devices. The roles of these materials, the methods used for their incorporation in the biosensor configurations as well as the advantages they provide to the analyses are summarised.

1. Introduction

Over the past decade the number of publications describing the incorporation of nanomaterials in biosensors has exponentially increased. Several reviews on nanomaterial-based biosensors have recently been published with focus on certain types of target analytes, detection techniques and applications [1–12]. The analysis of the published literature reveals the well-established use of nanotechnology in biomedical applications, and the increasing interest of its application in the agro-food sector and the environmental field.

* Corresponding author.
E-mail address: monica.campas@irta.cat (M. Campàs).

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This paper aims to critically review research work that exploits nanotechnology for the development of biosensors. Due to the highly productive publication records in the field, the scope of the review is limited to electrochemical biosensors for the detection of toxins of interest in agro-food and environmental applications. Biosensing systems based on the use of magnetic beads and/or microfluidics are also considered due to their key contribution towards the development of compact analytical devices. Tables 1–3 summarise the electrochemical biosensors for mycotoxins, aquatic toxins and other analytes, respectively, reviewed according to the biorecognition element, electrochemical technique, performance parameters and analysed matrix.

2. Incorporation of nanomaterials in biosensors

The International Organisation for Standardisation [13] defines the term "nanomaterial" as a material with an external dimension, or an internal or surface structure in the nanoscale (1–100 nm). Nanomaterials have unique optical and electrical characteristics that make particularly interesting their incorporation in biosensor configurations by providing advantages, such as high sensitivity, low limits of detection (LODs) and reduced matrix effects. The features of certain nanomaterials, such as their biocompatibility, conductivity, catalytic activity or stability, show them an attractive choice for certain purposes: they are widely used as label supports and signal enhancers, as they increase the electroactive surface area, and might favour the electron transfer and amplify the electrochemical signals. Carbon and metal nanomaterials have often been incorporated in electrochemical biosensor configurations. Next, these works are reviewed and classified according to the purpose of their use in the biosensor configuration as well as the method used for the nanomaterial incorporation.

2.1. Carbon nanomaterials

The unique physical and chemical properties of carbon nanomaterials, such as ultra lightweight, high mechanical strength, excellent electrical and thermal conductivity, highly ordered structure and high surface area, are responsible for the increasing interest in their incorporation in biosensor configurations. Carbon nanotubes (CNTs), carbon nanospheres (CNSs), carbon nanohorns (CNHs), graphene oxide (GO) and graphene nanoribbons (GNRs) have been immobilised onto electrodes, sometimes the carbon nanomaterial being modified with biomolecules prior or after the immobilisation step, to amplify the electrochemical signal and decrease the limit of detection in biosensing systems. Another role of carbon nanomaterials in biosensors is to act as supports for the label in competition and sandwich assay formats, also enhancing the electrochemical response.

2.1.1. Carbon nanomaterials-modified electrodes

The modification of electrodes with carbon nanomaterials provides advantages such as larger surface areas that can lead to higher number of immobilised bioreceptors and enhanced electrochemical signals, which as a consequence result in higher sensitivities and lower limits of detection. Several techniques, alone or in combination, have been used for the immobilisation of carbon nanomaterials: adsorption, entrapment into polymers, covalent binding, electrochemical techniques and chemical vapour deposition. Below, examples of carbon nanomaterials-modified electrodes are described and organised according to the immobilisation method.

2.1.1.1. Adsorption. Due to its simplicity, adsorption has been widely used as immobilisation technique in biosensor development. Adsorption of multi-walled CNTs (MWCNTs) onto a gold electrode was used by Yao et al. [14] to develop an electrochemical biosensor for the detection of the mycotoxin sterigmatocystin (ST), a precursor of aflatoxin B_1 (AFB_1). The enzyme sensor prepared by covalent binding of aflatoxin–detoxifizyme (ADTZ), the enzyme responsible for the detoxification of AFB_1 and ST, to activated MWCNTs showed wider working range (0.04–1.33 μg mL^{-1}) and lower LOD (42 ng mL^{-1}) than those obtained by an equivalent sensor based on the adsorption of ADTZ on adsorbed MWCNTs [15]. The improved performance of the former relies on the active centre protection undertaken during the immobilisation procedure: ADTZ was saturated with its substrate ST to protect the active centre from being chemically modified and thus to maintain its activity. Similarly, Li and coworkers [16] developed an enzyme sensor for AFB_1 detection that incorporates MWCNTs adsorbed onto the electrode surface. The enzyme aflatoxin oxidase (AFO), embedded in a silica sol–gel, was covalently bound to the activated MWCNTs previously adsorbed. The sol–gel matrix provides the biocompatibility required to retain enzyme activity and prevents enzyme leakage. The biosensor exhibited a linear range from 1 to 225 ng mL^{-1} of AFB_1 with an LOD of 0.5 ng mL^{-1}. Upon CNTs adsorption, different approaches have been suggested to incorporate both bioreceptors and electron transfer mediators onto the already modified surface, aiming to improve stability and electron transfer. As an example, Fang et al. [17] reported the immobilisation of MWCNTs on glassy carbon electrodes, followed by the subsequent incorporation of Prussian Blue (PB), chitosan and glutaraldehyde. Chitosan provides the proper environment for the immobilisation of anti-*Clostridium difficile* toxin B antibody and avoids leakage of MWCNTs and PB, thus increasing the sensor stability. After toxin binding, graphene oxide (GO) conjugated with horse-radish peroxidase (HRP) and secondary HRP-labelled antibody was subsequently incubated as part of a multienzyme amplification step. The system showed a linear working range from 3 pg mL^{-1} to 320 ng mL^{-1} with an LOD of 0.7 pg mL^{-1}. This low LOD was attributed to the high amount of immobilised antibody, the excellent electrical conductivity of MWCNTs and improved electron transfer mediated by PB, and the signal amplification provided by the use of GO as enzyme/antibody carrier.

A particular study is that reported by Wang et al. [18] on a CNT-modified paper electrode for the detection of microcystin-LR (MC-LR). Paper impregnated with single-walled CNTs (SWCNTs) and anti-MC-LR antibody was shown as a simple and low-cost method of biosensor design. The change in conductivity of the paper electrode in the presence of MC-LR allowed the detection of MC-LR at an LOD of 0.6 ng mL^{-1} and a linear range up to 10 ng mL^{-1}.

Apart from CNTs, other carbon nanomaterials have also been adsorbed on electrodes. Srivastava et al. [19] developed an impedimetric immunosensor for the detection of AFB_1 incorporating GO. Anti-AFB_1 antibodies were conjugated to GO previously adsorbed on gold electrodes. The large available surface area and numerous carboxyl groups on the GO sheets provided a high loading of anti-AFB_1 antibodies, resulting in a wide linear detection range (0.5–5 ng mL^{-1}) and a low LOD (0.23 ng mL^{-1}). Zhang et al. [20] described the development of an immunosensor for the detection of MC-LR based on a competitive immunoassay, where MC-LR was covalently conjugated to carboxyl groups on the single-walled carbon nanohorns (SWCNHs) previously adsorbed on a glassy carbon electrode. The biosensor exhibited a wide linear response, 0.05–20 ng mL^{-1}, an LOD of 30 pg mL^{-1} and, in the analysis of polluted water, good agreement with the values obtained using high performance liquid chromatography (HPLC) as reference analytical method.

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Table 1
Electrochemical biosensors based on nanomaterials and/or magnetic beads for the detection of mycotoxins.

Analyte	Biorecognition element	Nano/magnetic material/flow	Detection technique	Linear range	LOD	Applicability	Ref.
Sterigmatocystin (ST)	Aflatoxin–Detoxifizyme (ADTZ)	MWCNTs	Differential Pulse Voltammetry (DPV)	0.04–1.33 μg mL^{-1}	42 ng mL^{-1}	–	[14]
ST	(ADTZ)	MWCNTs	DPV	0.08–0.66 μg mL^{-1}	0.08 μg mL^{-1}	–	[15]
Aflatoxin B_1 (AFB_1)	Aflatoxin Oxidase (AFO)	MWCNTs	Amperometry	1–225 ng mL^{-1}	0.5 ng mL^{-1}	–	[16]
AFB_1	Anti-AFB_1 MAb	GO	Electrochemical Impedance Spectroscopy (EIS)	0.5–5 ng mL^{-1}	0.23 ng mL^{-1}	–	[19]
MC-LR	Anti-MC-LR PAb	SWCNHs	DPV	0.05–20 ng mL-1	30 pg mL^{-1}	Lake water	[20]
ST	AFO	SWCNTs	Amperometry	0.01–1.48 μg mL^{-1}	3 ng mL^{-1}	–	[21]
Ochratoxin A (OTA)	IgG	MWCNTs	DPV	25–600 pg mL^{-1}	25 pg mL^{-1}	–	[22]
AFB_1	Anti-AFB_1MAb	GO	EIS	10 fg mL^{-1}–10 pg mL^{-1}	10 fg mL^{-1}	Corn	[27]
AFB_1	Anti-FAB_1 MAb	MWCNTs	EIS	0.1–10 ng mL^{-1}	0.03 ng mL^{-1}	Olive oil	[28]
Citrinin (CIT)	Horseradish Peroxidase (HRP)	MWCNTs	Amperometry	–	63 pg mL^{-1}	Rice	[30]
AFB_1	Anti-AFB_1 MAb	GO	Cyclic Voltammetry (CV)	0.125–1.5 ng mL^{-1}	0.15 ng mL^{-1}	–	[32]
OTA	Anti-OTA PAb	Au NPs	DPV	0.3–8.5 ng mL^{-1}	0.20 ng mL^{-1}	Wheat	[38]
OTA	Anti-OTA MAb	Au NPs	DPV	0.15–9.9 ng mL^{-1}	0.10 ng mL^{-1}	Wheat	[39]
OTA	Anti-OTA aptamer	IrO_2NPs	EIS	4 pg mL^{-1}–40 ng mL^{-1}	5.65 pg mL^{-1}	White wine	[40]
OTA	IgG	CeO_2 NPs	DPV	2.5–60 pg mL^{-1}	2.5 pg mL^{-1}	–	[41,42]
OTA	IgG	ZnO NPs	EIS	2.5–4.2 pg mL^{-1}	2.5 pg mL^{-1}	–	[43]
OTA	Anti-OTA MAb	Au NPs	DPV	0.01–100 ng mL^{-1}	8.2 pg mL^{-1}	Corn	[46]
AFB_1	Anti-AFB_1 MAb	Au NPs	Conductometry	0.5–10 ng mL^{-1}	0.1 ng mL^{-1}	Serum	[47]
AFB_1	Anti-AFB_1 MAb	Ni NPs	CV	0.05–1 ng mL^{-1}	0.32 ng mL^{-1}	–	[50]
AFB_1	Anti-AFB_1 MAb	Sm_2O_3 NRs	CV	10–700 pg mL^{-1}	58 pg mL^{-1}	–	[51]
Aflatoxin M_1 (AFM_1)	Anti-AFM_1 PAb	Au NPs	EIS	15–1000 pg mL^{-1}	12 pg mL^{-1}	–	[56]
OTA	Anti-OTA aptamer	Au NPs	CV	0.1–20 ng mL^{-1}	30 pg mL^{-1}	Red wine	[57]
OTA	Anti-OTA aptamer	MBs CdS QDs	Square Wave Stripping Voltammetry (SWSV)	0.5 pg mL^{-1}– 10 ng mL^{-1}	0.2 pg mL^{-1}	Red wine	[60]
AFB_1	Anti-AFB_1 MAb	GO Au NPs	CV	0.1–12 ng mL^{-1}	0.1 ng mL^{-1}	–	[63]
Deoxynivalenol (DON)	Anti-DON Mab	GO Au NPs	EIS	6–30 ng mL^{-1}	0.3 ng mL^{-1}	Corn, wheat & roasted coffee	[64]
AFM_1	Anti-AFM_1 MAb	MBs	Chronoamperometry (CA)	0.01–0.25 ng mL^{-1}	10 pg mL^{-1}	Milk	[68]
OTA	Anti-OTA MAb	MBs	DPV	0.26–8.87 ng mL^{-1}	0.12 ng mL^{-1}	Red wine	[70]
Zearalenone (ZEA)	Anti-ZEA MAb	MBs	Amperometry	–	11 pg mL^{-1}	Corn, powdered baby food & cereal milkshakes	[73]
ZEA	Anti-ZEA MAb	MBs	DPV	–	7 pg mL^{-1}	Corn, powdered baby food & cereal milkshakes	[72]
OTA	Anti-OTA aptamer	MBs	DPV	0.78–8.74 ng mL^{-1}	70 pg mL^{-1}	Wheat	[76]
OTA	Anti-OTA aptamer	MBs	DPV	0.11–15 ng mL^{-1}	0.11 ng mL^{-1}	Wine	[77]
ZEA	Anti-ZEA MAb	MBs Microfluidics	Amperometry	–	0.83 ng mL^{-1}	Corn, powdered baby food & cereal milkshakes	[79]
ZEA	Anti-ZEA MAb	MBs Microfluidics	Amperometry	–	0.4 ng mL^{-1}	Corn, powdered baby food & cereal milkshakes	[80]
ZEA	Anti-ZEA MAb	MBs Microfluidics	CV	20–500 pg mL^{-1}	20 pg mL^{-1}	Feedstuffs	[81]
DON	Anti-DON Fab fragment	MBs Multiplexed	CA	0.1–4.5 μg mL^{-1}	63 ng mL^{-1}	Breakfast cereals & baby food	[83]
ZEA	Anti-ZEA MAb	MWCNTs Flow system	Amperometry	–	0.77 ng mL^{-1}	Corn silage	[85]
CIT	Anti-CIT MAb	Microfluidics	Amperometry	0.5–50 ng mL^{-1}	0.1 ng mL^{-1}	Rice	[86]
DON	Anti-DON Mab	Microfluidics	Amperometry	6.25–250 ng mL^{-1}	6.25 ng mL^{-1}	Wheat	[87]

Table 2
Electrochemical biosensors based on nanomaterials and/or magnetic beads for the detection of aquatic toxins.

Analyte	Biorecognition element	Nano/magnetic material/flow	Detection technique	Linear range	LOD	Applicability	Ref.
Microcystin-LR (MC-LR)	Anti-MC-LR PAb	SWCNTs	Amperometry	$\leq$10 ng mL^{-1}	0.6 ng mL^{-1}	Lake water	[18]
Microcystins (MCs)	Anti-MC-LR MIP	MWCNTs	Potentiometry	0.74–1.2 ng mL^{-1}	0.66 ng mL^{-1}	Artesian well water	[24]
Okadaic acid (OA)	Anti-OA MAb	Graphene	Square Wave Voltammetry (SWV)	$\leq$5 ng mL^{-1}	19 pg mL^{-1}	Mussels	[31]
MC-LR	Anti-MC-LR MAb	MWCNTs	EIS	0.05–20 ng mL^{-1}	40 pg mL^{-1}	–	[33]
MC-LR	Anti-MC-LR MAb	SWCNTs	DPV	0.01–7.0 ng mL^{-1}	2.31 pg mL^{-1}	Reservoir water	[35]
Brevetoxin B (BTX-2)	Anti-BTX-2 MAb	GNRs MBs	SWV	1 pg mL^{-1}–10 ng mL^{-1}	1 pg mL^{-1}	Mussels, razor clams & cockles	[36]
MC-LR	Anti-MC-LR MAb	GO CNSs	DPV	0.05–15 ng mL^{-1}	16 pg mL^{-1}	Reservoir, tap & river water	[37]
BTX-2	Anti-BTX-2 MAb	Au NPs	DPV	0.03–8 ng mL^{-1}	10 pg mL^{-1}	Mussels, razor clams & cockles	[44]
MC-LR	Anti-MC-LR MAb	Ag NPs	Capacitance	10 fg mL^{-1}–1 ng mL^{-1}	7 fg mL^{-1}	Drinking, tap & raw water	[45]
MC-LR	Anti-MC-LR MAb	Au NPs	DPV	0.05–15 ng mL^{-1}	20 pg mL^{-1}	Crude algae	[48]
MC-LR	Anti-MC-LR MAb	Au NPs	Capacitance	0.1 pg mL^{-1}–0.1 ng mL^{-1}	10 fg mL^{-1}	*Microcystis aeruginosa* culture	[49]
MC-LR	Anti-MC-LR MIP	TiO2 NTs	Photoelectrochemistry (PEC)	0.5–100 ng mL^{-1}	0.1 ng mL^{-1}	–	[55]
MC-LR	Anti-MC-LR MAb	CdSe QDs	SWSV	0.227–50 ng mL^{-1}	99 pg mL^{-1}	*Microcystis aeruginosa* cultures	[59]
MC-LR	Anti-MC-LR Ab	GO PtRu alloy	Amperometry	0.01–28 ng mL^{-1}	9.63 pg mL^{-1}	Water	[66]
MC-LR	Anti-MC-LR Ab	MBs	DPV	0.01–200 μg mL^{-1}	4 ng mL^{-1}	–	[67]
MC-LR	Anti-MC-LR MAb	MBs	CA	0.4–20 ng mL^{-1}	0.4 ng mL^{-1}	*Microcystis aeruginosa* culture & natural bloom	[69]
OA	Anti-OA MAb	MBs	DPV	–	0.38 ng mL^{-1}	Mussels	[74]
OA	Anti-OA MAb	MBs	DPV	0.78–500 ng mL^{-1}	0.5 ng mL^{-1}	Mussels	[75]
OA	Anti-OA MAb	MBs Flow system	Amperometry	0.19–25 ng mL^{-1}	0.15 ng mL^{-1}	Mussels	[78]
BTX-2 Dinophysistoxin-1 (DTX-1)	Anti-BTX-2 MAb Anti-DTX-1 MAb	MBs Cd NCs Cu NCs Multiplexed Flow system	Square Wave Anodic Stripping Voltammetry (SWASV)	5 pg mL^{-1}–5 ng mL^{-1}	1.8 pg mL^{-1} (BTX-2) 2.2 pg mL^{-1} (DTX-1)	Mussels, razor clams & cockles	[84]

Table 3
Electrochemical biosensors based on nanomaterials and/or magnetic beads for the detection of other analytes.

Analyte	Biorecognition element	Nano/magnetic material/flow	Detection technique	Linear range	LOD	Applicability	Ref.
Clostridium difficile Toxin B (Tcd B)	Anti-Tcd B MAb	MWCNTs GO	DPV	3 pg mL^{-1}– 320 ng mL^{-1}	0.7 pg mL^{-1}	Human stool	[17]
Cholera Toxin (CT)	Anti-CT MAb	MWCNTs	Adsorptive Square Wave Stripping Voltammetry (AdSWSV)	10 fg mL^{-1}– 100 ng mL^{-1}	1 fg mL^{-1}	Tap water & domestic waste water	[23]
Anthrax Protective Antigen (Anthrax PA)	Anti-Anthrax PA peptide	MWCNTs	SWV	–	33 pg mL^{-1}	Blood plasma	[25]
Ricin	Anti-ricin PAbs	MWCNTs	Amperometry	0.625–25 ng mL^{-1}	0.56 ng mL^{-1}	–	[29]
Staphylococcal Enterotoxin B (SEB)	Anti-SEB PAb	Au NPs MWCNTs	DPV	0.05–15 ng mL^{-1}	10 pg mL^{-1}	Watermelon juice, apple juice, soy milk & pork food	[34]
Anthrax PA	Anti-Anthrax PA MAb	Au NPs	CV	8.1 ng mL^{-1}– 81 μg mL^{-1}	0.17 fg mL^{-1}	Human serum	[52]
SEB	Anti-SEB Mab	Nanoporous alumina	EIS	–	10 pg mL^{-1}	–	[53]
Ricin	Anti-ricin PAb	Nanoporous alumina	EIS	–	0.5 pg mL^{-1}	Milk, vegetable soup & tomato juice	[54]
Botulinum Neurotoxin Type A (BoNT)	Anti-BoNT MAb	Au NPs	CV	4–35 pg mL^{-1}	1 pg mL^{-1}	Full & skim milk	[58]
Tcd B	Anti-Tcd B Ab	MWCNTs Au NPs	DPV	1–80 ng mL^{-1}	0.3 ng mL^{-1}	–	[61]
SEB	Anti-SEB PAb	MWCNTs Au NPs	Conductometry	0.5–83.5 ng mL^{-1}	0.5 ng mL^{-1}	Milk	[62]
Anthrax PA	Anti-Anthrax PA aptamer	GO	Field-effect transistor (FET)	1 fg mL^{-1}–10 pg mL^{-1}	1 fg mL^{-1}	–	[65]
Anthrax PA	Anti-Anthrax PA aptamer	GO Au NPs	FET	–	0.1 fg mL^{-1}	–	[65]
Staphylococcal protein A (prot A)	Anti-prot A PAb	MBs	Amperometry	17 fg mL^{-1}– 2.6 pg mL^{-1}	3.9 fg mL^{-1}	Raw milk	[71]
Cholera Toxin B (CTB)	Anti-CTB PAb	MBs	Amperometry	–	1 ng mL^{-1}	–	[82]
BoNT	Anti-BoNT aptamer	Microfluidics	Amperometry	1 ng mL^{-1}–1 μg mL^{-1}	1 ng mL^{-1}	–	[88]

2.1.1.2. Entrapment and/or covalent binding. Carbon nanomaterials have also been immobilised onto the electrode surface via covalent binding and/or entrapment into polymers. Chen and co-workers [21] developed an enzyme sensor for ST by co-entrapment of AFO and SWCNTs in a chitosan film adsorbed on a gold electrode. The nanocomposite matrix provided a favourable environment for the enzyme, which preserves the enzyme activity and promotes the direct electron transfer between the enzyme active site and the electrode surface. As a result, an LOD of 3 ng mL^{-1} was attained. Also based on carbon nanomaterials entrapment into a chitosan film, Kaushik et al. [22] developed an immunosensor for the detection of ochratoxin A (OTA): chitosan-MWCNTs nanobiocomposites were adsorbed on indium tin oxide (ITO) electrodes for the subsequent co-immobilisation via adsorption of immunoglobulin G (IgG) and bovine serum albumin (BSA). The high antibody loading together with increased electroactive surface area enhanced the electron transport, providing a linear range between 25 and 600 pg mL^{-1} and an LOD of 25 pg mL^{-1}. MWCNTs have also been embedded in Nafion for the ultrasensitive detection of cholera toxin (CT) [23]. Anti-CT antibodies were entrapped in a poly(3,4-ethylenedioxythiophene) (PEDOT) film electropolymerised onto the MWCNT-modified Nafion layer, providing high conductivity and a large number of recognition sites. Voltammetric measurements using the sandwich immunosensor, which exploits liposomic magnification, provided a linear range of 10 fg mL^{-1} −100 ng mL^{-1} and an LOD of 1 fg mL^{-1}. This low value was certainly achieved because of the cooperative functions of Nafion, MWCNTs, PEDOT and the liposomes used as labels.

A particular case is that in which CNTs were exploited as supports for the surface imprinting of plastic antibodies, also known as molecularly imprinted polymers (MIPs), for the detection of MCs [24]. The molecularly imprinted MWCNTs were integrated as ionophores into a polyvinylchloride (PVC) selective membrane on a carbon electrode for the development of a potentiometric sensor. Results showed similar behaviour for all tested MCs (MC-LR, MC-YR and MC-RR), with an LOD below 1 ng mL^{-1}, and non-significant interfering effects from sulphate, iron and ammonium ions, chloroform and tetrachloroethylene.

Conductive polymers have also been used to covalently bind carbon nanomaterials on the electrode surface. Such is the case of the immunosensor for the detection of anthrax protective antigen described by Huan et al. [25]. Firstly, aniline monomers were electropolymerised onto the surface of a glassy carbon electrode to form a polyaniline (PANI) film. Afterwards, activated MWCNTs were covalently bound to PANI. Remaining carboxylic groups of MWCNTs were then conjugated to a specific peptide used as a biorecognition molecule. An LOD of 33 pg mL^{-1} was attained, which was 13-fold lower in comparison to that of a sensor in which a self-assembled monolayer was used for the immobilisation of the same peptide [26]. The use of a PANI layer not only allowed the nanomaterial immobilisation, but also provided good electrochemical conductivity. Similarly, Wang et al. [27] entrapped reduced GO (rGO) during the electrochemical co-polymerisation of pyrrole (Py) and pyrrolepropylic acid (PPa). Anti-AFB_1 antibodies were covalently bound to the carboxyl groups of the PPa. The authors reported a working range of 10 fg mL^{-1}–10 pg mL^{-1} and a very low LOD of 10 fg mL^{-1}. The improved electroactivity provided by PPy, and the electric conductivity, rigidity and high stability rendered by rGO contributed to the excellent performance of the biosensor.

Although not being a polymer, the use of ionic liquids for nanomaterial entrapment deserves to be mentioned in this section. Yu et al. [28] developed an impedance-based immunosensor for AFB_1, where a nanobiocomposite film based on a mixture of MWCNTs and ionic liquids was used for the entrapment of anti-AFB_1 antibodies on glassy carbon electrodes. The enhanced electron

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transfer provided by the presence of MWCNTs and the ideal environment for the antibody created by the 3D-structured composite film, allowed the detection of AFB_1 between 0.1 and 10 ng mL^{-1}, with an LOD of 0.03 ng mL^{-1}. The applicability of the developed immunosensor was demonstrated by the analysis of AFB_1 in spiked olive oil extracts.

Inclusion of carbon nanomaterials into carbon pastes to fabricate screen-printed electrodes is of special interest. Suresh et al. [29] mixed MWCNTs with paraffin oil and graphite powder to develop a sandwich-type immunosensor for the detection of ricin. The presence of MWCNTs widened the linear working range obtained with an equivalent biosensor without MWCNTs (0.625–25 ng mL^{-1} and 2.5–25 ng mL^{-1}, respectively). In another example, Zachetti et al. [30] mixed MWCNTs with mineral oil, HRP and ferrocene, for the development of an amperometric biosensor for the quantification of citrinin (CIT). The LOD was 63 pg mL^{-1} and good recoveries were obtained in the analysis of rice samples. This type of immobilisation provides good stability, preventing possible leakage of biosensor components. The success of the incorporation of carbon nanomaterials in carbon pastes, led to the commercialisation of screen-printed electrodes already modified with carbon nanomaterials. Commercial graphene-modified carbon screen-printed electrodes have been used in the development of an electrochemical immunosensor for okadaic acid (OA) [31]. The authors electrografted a carboxyphenyl monolayer on the printed electrodes for the subsequent covalent immobilisation of anti-OA antibodies. OA and OA-ovalbumin (OA-OVA) in solution compete for their binding to the immobilised antibody. A decrease of the reduction current of $[Fe(CN)_6]^{4-/3-}$ was observed for the lowest OA concentrations, caused by the blocking effects of bound OA-BSA which hindered the electron transfer. The immunosensor exhibited good sensitivity and an LOD of 19 pg mL^{-1}, attributed to the electrochemical properties of graphene and the stability of the carboxyphenyl layer. Additionally, the application of the immunosensor to the quantification of OA in certified mussel samples showed no significant matrix interferences.

2.1.1.3. Electrochemical methods. Carbon nanomaterials have also been immobilised on electrodes using electrochemical techniques. The simplest electrochemical approach is based on electrophoretic deposition (EPD), which allows the deposition of positively charged material on the anode. Following this approach, Srivastava et al. [32] immobilised rGO modified with Mg^{2+} ions on ITO electrodes (Fig. 1). Then, they covalently bound anti-AFB_1 antibodies to the carboxyl groups on the rGO. The presence of $AFB_1$was directly related to the increase in the anodic peak observed by cyclic voltammetry, which was suggested to be the result of the improved electron transfer induced by the toxin. The LOD was as low as 0.15 ng mL^{-1}, probably because of both the large area of the GO sheets, which provided high loading of antibodies, and their excellent electrical conductivity.

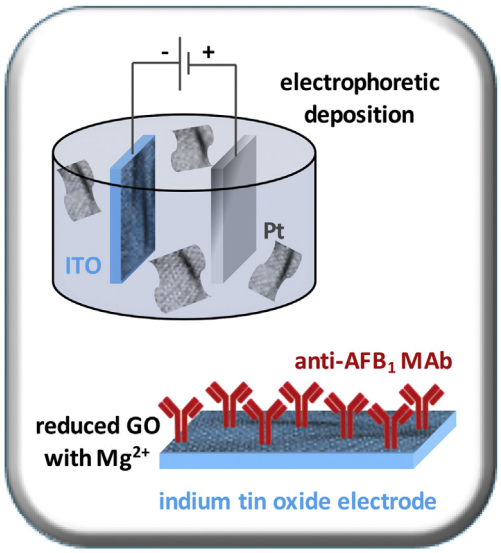

Fig. 1. Example of biosensing strategy based on rGO as antibody immobilisation support.

2.1.1.4. Chemical vapour deposition. Vertically aligned MWCNTs have been grown on patterned Si substrates via water-assisted chemical vapour deposition [33]. These MWCNTs were functionalised and modified with MC-LR, providing an electrode array used in a competitive-type immunoassay. This immunosensor was able to detect this cyanobacterial toxin in a concentration range from 0.05 to 20 ng mL^{-1}.

2.1.2. Carbon nanomaterials as label supports

As previously mentioned, carbon nanomaterials can also play a role as label supports when incorporated in biosensors. In this case, apart from being conjugated to the label, they have also been modified with the detection antibody or the antigen, depending on the assay format. Such mixed conjugates have been incorporated in sandwich and competition configurations providing advantages such as higher number of labels and electrochemical signal amplification. Next, a few examples of the different configurations are described.

Tang et al. [34] doped MWCNTs with nanosilica and HRP, and used them as labels of the anti-staphylococcal enterotoxin B (SEB) antibodies in a sandwich-type immunosensor. Thionine was electropolymerised on graphite electrodes to mediate the HRP-catalysed reaction, and Au nanoparticles (NPs) were added to the system to enhance the electrochemical reduction of H_2O_2. Combining the catalytic properties of CNTs and the bioelectrocatalytic signal amplification, they obtained a dynamic range of 0.05–15 ng $mL^{-1}$and an LOD of 10 pg mL^{-1}. Results from the analysis of spiked watermelon and apple juices, soy milk and pork food showed excellent correlation with the values obtained using a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Competition-type immunoassays and sensors have also been developed using carbon nanomaterials as bioreceptor/label immobilisation supports. Tian et al. [35] conjugated peroxidase-mimicking DNAzyme and MC-LR to SWCNTs. This multi-labelled MC-LR competed with free MC-LR in solution for their binding to capture antibodies immobilised on SWCNTs previously adsorbed on the electrode. The electrocatalytic current decreased linearly as the MC-LR amount increased from 0.01 to 7.0 ng mL^{-1}, and the LOD was 2.31 pg mL^{-1}. This low LOD was attained because the SWCNTs allowed loading multiple label enzymes and also promoted the electron transfer between the electrolyte and the electrode. Application of the immunosensor to the MC-LR determination in spiked reservoir water provided good recoveries and agreement with LC-MS/MS results. Tang et al. [36] developed another competition-type immunoassay, using magnetic beads (MBs), for the detection of brevetoxin B (BTX-2). In this case, graphene nanoribbons (GNRs) were used as label (guanine) and antigen (BSA-BTX-2) carriers. Free BTX-2 and labelled BSA-BTX-2 competed in solution for their binding to anti-BTX-2 antibodies immobilised on MBs. Once the immunocomplexes were formed, they were magnetically captured and transferred to an electrochemical cell containing the redox catalyst $Ru(bpy)_3^{2+}$, which mediates the oxidation of guanine. Oxidation current decreased with the increase of BTX-2 in the sample, providing a linear range from

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1 pg mL^{-1} to 10 ng mL^{-1} and an LOD of 1 pg mL^{-1}. Carbon nanospheres (CNSs) have also been integrated into biosensor configurations as enzyme/antibody carriers for signal amplification. In the work described by Zhao et al. [37], anti-MC-LR antibodies conjugated to HRP-labelled CNSs competed for their binding to MC-LR free in solution and MC-LR covalently attached to GO/chitosan, previously immobilised on glassy carbon electrodes by adsorption. The immunosensor exhibited a wide linear range (0.05–15 ng mL^{-1}) and an LOD of 16 pg mL^{-1}, well below the World Health Organisation (WHO) provisional guideline limit of 1 μg L^{-1}.

2.2. Metal nanomaterials and semiconductor quantum dots

Metal nanoparticles (NPs) and nanorods (NRs) have been incorporated in electrochemical biosensor configurations with two main purposes: the fabrication of nanostructured supports and their use as signal enhancers. They possess high electrocatalytic activity, stability and biocompatibility, and they can be easily functionalised. They favour electron transfer and, as a result, higher sensitivities and lower LODs are attained by metal nanomaterial-modified electrochemical biosensors.

2.2.1. Metal nanomaterials-modified electrodes

As previously shown for carbon nanomaterials, metal nanomaterials immobilisation has also been pursued by several strategies addressed to incorporate them on the electrode surface: adsorption, entrapment into polymers, self-assembly, and electrochemical methods. Examples are described below.

2.2.1.1. Adsorption. As shown before, adsorption is the simplest approach. Castillo's group developed competitive indirect [38] and direct [39] immunosensors for the detection of OTA. To compete with OTA in solution for the binding to anti-OTA antibodies, Au NP-modified OTA-BSA conjugates were adsorbed on screen-printed carbon electrodes. Au NPs provided a higher number of effective immobilised antigen sites and enhanced the charge-transfer kinetics of the voltammetric measurements. As a result, the immunosensors with NPs showed lower LODs compared to those without NPs: 0.20 ng mL^{-1} *vs.* 0.86 ng mL^{-1}, and 0.10 ng mL^{-1} *vs.* 0.30 ng mL^{-1}, for the indirect and direct approaches, respectively. The immunosensors were successfully applied to the analysis of OTA-spiked and certified wheat samples.

Metal NPs have also been adsorbed onto modified electrode surfaces. Such is the case of the label-free impedimetric aptasensor for OTA, developed by Rivas et al. [40], which incorporates IrO_2 NPs onto the electrode. Herein, IrO_2 NPs were adsorbed on the thionine film electrodeposited on a screen-printed carbon electrode. An amino-terminated DNA aptamer, specific for OTA, was electrostatically immobilised on the IrO_2 NPs. The impedimetric biosensor allowed the determination of OTA in the range of 4 pg mL^{-1} to 40 ng mL^{-1} and with an LOD of 5.65 pg mL^{-1} (5.65 ng kg^{-1} in wine).

2.2.1.2. Entrapment. In some cases, metal NPs are embedded into polymers and the resulting nanobiocomposites are then adsorbed on electrodes. Some examples are the immunosensors for OTA developed by Kaushik et al. [41,42]. Nanocomposites, prepared by dispersion of CeO_2 NPs in sol–gel [41] or chitosan [42], were adsorbed on ITO electrodes. Afterwards, rabbit IgG and BSA were co-immobilised on the nanocomposites via electrostatic interactions. The NPs increased the number of antigen sites and the electroactive surface area, enhancing the electron transport. As a result, an LOD of 2.5 pg mL^{-1} was achieved. The authors followed the same approach but using ZnO NPs instead of CeO_2 NPs, obtaining similar results for OTA detection [43].

Metal NPs have also been encapsulated into dendrimers: the large surface area of dendrimers has been combined with the good conductivity of metal NPs to create 3D metal-dendrimer nanocomposites to be used as nanostructured supports. Tang et al. [44] used this approach to develop an electrochemical immunosensor for the direct detection of BTX-2. Au NPs were synthesised and simultaneously encapsulated into amino-terminated dendrimers, which afterwards were adsorbed on ester-modified gold electrodes. BSA-BTX-2 conjugate was then immobilised on the nanostructured surface. HRP-labelled anti-BTX-2 antibody competed with free and immobilised BTX-2. The immunosensor response was measured using *o*-phenylendiamine and H_2O_2. As expected, the 3D-network provided the highest sensitivity, wider linear range and lower LOD, the latter being 0.01 ng mL^{-1}, compared to 0.5 or 0.1 ng mL^{-1} for immunosensors without Au NPs or dendrimers, respectively.

2.2.1.3. Self-assembly. Taking advantage of the high affinity of thiol groups for the surfaces of some metals, self-assembly has been exploited to immobilise NPs on thiol-modified electrodes. Loyprasert et al. [45] immobilised Ag NPs on thiourea-modified electrodes. Anti-MC-LR antibodies were adsorbed on the Ag NP-modified surface. Label-free capacitance measurements provided linearity between 10 fg mL^{-1} and 1 ng mL^{-1} and an LOD of 7 fg mL^{-1} (80 fg mL^{-1} using the same approach without NPs). The immunosensor was used to analyse spiked drinking and raw water, showing a good correlation with the results obtained by HPLC coupled to photodiode array detection (HPLC-DAD). The low LOD allowed the dilution of samples to be analysed, reducing interference effects from the matrix. A similar approach was used by Liu et al. [46] for the development of an immunosensor for OTA. In this case, 1,6-hexanedithiol was assembled on gold electrodes and Au NPs were assembled on it. OTA-OVA conjugate was adsorbed on the Au NPs and a competitive indirect assay was performed. The working range was linear between 10 pg mL^{-1} and 100 ng mL^{-1} with an LOD of 8.2 pg mL^{-1}. Moreover, matrix effects were negligible and good recoveries were obtained for the analysis of corn samples.

Self-assembly of metal NPs on amine groups has also been reported. Such are the cases of the immunosensors developed by Liu et al. [47] for AFB_1, and Tong et al. [48] and Lebogang et al. [49] for MC-LR. In these works, Au NPs were self-assembled on amino-modified electrodes, where the specific antibodies were later adsorbed. Liu et al. [47] also immobilised HRP on the Au NPs, which enabled the direct electrochemical measurement of HRP. The mycotoxin presence induced local changes of conductivity, resulting in a linear range from 0.5 to 10 ng mL^{-1} with an LOD of 0.1 ng mL^{-1}. Tong et al. [48] performed hydroquinone-mediated differential pulse voltammetry measurements, obtaining an LOD of 20 pg mL^{-1} for MC-LR. Finally, Lebogang et al. [49] measured capacitance changes in the presence of MC-LR, without the need of redox mediators or enzymes. This was possible thanks to the high surface area provided by the NPs that enabled the immobilisation of more antibodies than on an unmodified electrode. They obtained an LOD of 10 fg mL^{-1} and good correlation with ELISA results.

2.2.1.4. Electrochemical methods. Electrophoretical deposition (EPD) has been used to immobilise Ni NPs on ITO electrodes [50]. Anti-AFB_1 antibodies were covalently bound to the Ni NP-modified electrodes. The electrochemical biosensor revealed linearity between 0.05 and 1 ng mL^{-1} and an LOD of 0.32 ng mL^{-1}. EPD has also been used to immobilise Sm_2O_3 NRs on ITO surfaces [51]. The films were then modified with anti-AFB_1 antibodies. Cyclic voltammetry and impedance spectroscopy studies showed that the NRs increased the active area, enhanced the electron transport and improved the electrocatalytic behaviour. This was reflected in the

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broad linear range (10–700 pg mL^{-1}) and the low LOD (58 pg mL^{-1}) achieved. In some works, NPs are synthesised *in situ* by electroplating, process that also implies their immobilisation on electrodes. Electroplating of gold salts onto bare gold electrodes has been used to fabricate nanostructured gold electrodes. These electrodes were modified with a peptide specific for the detection of anthrax protective antigen [52]. In this case, the combination of the nanostructuration of the electrode and the immobilisation of the peptide through a DNA monolayer to favour electron transfer provided an LOD of 170 pg mL^{-1}.

Nanoporous anodised aluminium oxide has also been used as electrode substrate for the development of electrochemical immunosensors for SEB [53] and ricin [54]. Nanoporous alumina was nano-patterned via a three-step process: annealing, electropolishing and anodisation. Upon silanisation with 3-aminopropyltriethoxysilane, glutaraldehyde was used as a linker to immobilise the corresponding antibody. Impedance spectroscopy measurements allowed the detection of SEB and ricin at concentrations as low as 10 pg mL^{-1} [53] and 0.5 pg mL^{-1} [54], respectively.

In a different approach, highly ordered and vertically aligned TiO_2 NTs were used as support to synthesise MIPs for MC-LR [55]. The PPy-based MIP for MC-LR was used to develop a photoelectrochemical sensor, which attained a linear range from 0.5 to 100 ng mL^{-1} and an LOD of 0.1 ng mL^{-1}. Although MIPs usually suffer from lower affinities than antibodies, the sensor exhibited appropriate sensitivity probably because of the high specific surface area and the photoelectric activity of TiO_2.

2.2.2. Metal nanomaterials as label supports and/or signal enhancers

As previously mentioned, metal NPs have also been conjugated to the bioreceptor to perform as label supports and/or signal enhancers. Vig et al. [56] conjugated Au NPs to anti-AFM_1 antibodies to compete for their binding between free AFM_1 in solution and immobilised BSA-AFM_1 in an impedimetric direct competitive immunosensor. Silver was then electrodeposited using the catalytic effect of the immobilised Au NPs. Impedance measurements resulted in a wide working range (15–1000 pg mL^{-1}) and a low LOD (12 pg mL^{-1}) due to the silver enhancement.

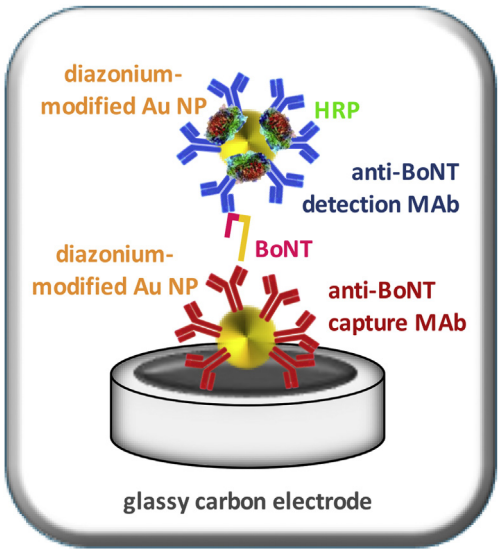

Fig. 2. Example of biosensing strategy based on Au NPs as antibody immobilisation support and label carriers.

Metal NPs have also been used to enhance the transduction signals arising from redox labels. Kuang et al. [57] developed a signal-off aptamer-based sensor for the detection of OTA using two oligonucleotides complementary to the DNA aptamer. The first complementary oligonucleotide was used to immobilise the aptamer on the electrode through hybridisation. The second complementary oligonucleotide, an Au NP-functionalised DNA, was hybridised to the aptamer to amplify the redox signal from the Methylene Blue intercalator, used as electrochemical probe. OTA binding forces the release of both the aptamer and Au NP-functionalised DNA and thus decreases the amount of MB to be detected. The effective sensing range was from 0.1 to 20 ng mL^{-1} with an LOD of 30 pg mL^{-1}. The method was used to analyse red wine samples showing good correlation with ELISA results.

The sandwich immunosensor developed by Liu and co-workers [58] for the detection of botulinum neurotoxin type A (BoNT) harnesses the use of NPs to modify the electrode surface and to perform as label supports and signal enhancers. On the one hand, the authors electrografted diazonium salt-functionalised Au NPs on glassy carbon electrodes; capture anti-BoNT antibodies were then covalently linked to the immobilised Au NPs (Fig. 2). On the other hand, Au NPs were used to co-immobilise detection antibody and HRP and to amplify the signal. The biosensor exhibited a working range of 4–35 pg mL^{-1} with an LOD of 1 pg mL^{-1}. The analysis of milk samples showed good recoveries without sample dilution, indicating negligible interference effects.

A particular case of NPs are quantum dots (QDs), which are semiconductor nanocrystals with unique intrinsic properties. Although QDs have been usually exploited as optical (fluorescent) labels, their redox or electrocatalytic behaviour has favoured their application as electrochemical labels. An example is the immunosensor for the detection of MC-LR developed by Yu et al. [59]. CdSe QDs, with a CdSe core encapsulated in the shell of ZnS and a polymer, were conjugated to anti-MC-LR antibodies and used in a competitive direct immunoassay. The immunocomplexes formed on the electrode surface were then dissolved to release cadmium ions measured by square wave stripping voltammetry. A dynamic range of 0.227–50 ng mL^{-1} and an LOD of 99 pg mL^{-1} were obtained. The biosensor was successfully applied to the analysis of *Microcystis aeruginosa* cultures. Another example of the use of QDs as electrochemical labels is the aptamer-based sensor for the detection of OTA developed by Tong et al. [60]. MBs were modified with a capture DNA that was subsequently hybridised to an OTA aptamer and a padlock DNA. OTA-aptamer binding induced aptamer release and padlock DNA-capture DNA hybridisation. Next, capture DNA was isothermally amplified via linear rolling circle amplification reaction, generating a long DNA sequence. CdS QD-labelled DNA probes were then added, which hybridised with the long sequence, and the electrochemical measurement was performed. The biosensor detected OTA down to 0.2 pg mL^{-1} with a dynamic range of more than 4 orders of magnitude. In the analysis of red wines, good correlation with ELISA results was obtained. The exploitation of QDs and nanoclusters (NCs) in biosensors for multiplexed detection is shown in detail in section 3.3.

2.3. Combination of carbon and metal nanomaterials

There are a few biosensors combining both carbon and metal nanomaterials with synergistic or unrelated purposes. In the work developed by Xu and co-workers [61], MWCNTs and Au NPs were immobilised on a glassy carbon electrode with a common purpose. A silica/thionine nanocomposite was electrodeposited on the electrode previously modified with adsorbed MWCNTs. Au NPs were then linked to thionine by electrostatic adsorption and covalent binding. After immobilisation of anti-*C. difficile* toxin B

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antibodies on the nanocomposite, the assay was performed. This label-free biosensor was able to detect *C. difficile* toxin B from 1 to 80 ng mL^{-1} with an LOD of 0.3 ng mL^{-1}. Chen [62] also combined MWCNTs and Au NPs to modify interdigitated microelectrodes. A MWCNT-chitosan composite, adsorbed on the microelectrodes, was modified with Au NPs which performed as immobilisation support for anti-SEB antibodies conjugated to HRP. The presence of the analyte in the sample induced local conductivity variations, providing a linear range between 0.5 and 83.5 ng mL^{-1} and an LOD of 0.5 ng mL^{-1}. More sophisticated is the combination of GO and Au NPs proposed by Srivastava et al. [63] and Sunday et al. [64], where the NPs were synthesised *in situ* simultaneously to the reduction or functionalisation of GO, respectively. Srivastava et al. [63] immobilised then anti-AFB_1 antibodies on the Au NP-rGO nanocomposite to develop a label-free immunosensor able to detect AFB_1 between 0.1 and 12 ng mL^{-1} with an LOD of 0.1 ng mL^{-1}. Sunday et al. [64] developed a label-free immunosensor for deoxynivalenol (DON) based on the modification of the electrode surface with a Nafion membrane containing Au NP-modified graphene. Anti-DON antibodies were immobilised on the functionalised graphene. The immunosensor was able to detect the mycotoxin in the range of 6–30 ng mL^{-1} with an LOD of 0.3 ng mL^{-1}. Results for the analysis of certified corn, wheat and roasted coffee samples using the immunosensor were in agreement with those obtained by an ELISA kit.

The next two examples describe the use of carbon and metal nanomaterials for different purposes. The first work is a field-effect transistor (FET) aptamer-based sensor developed for the detection of anthrax protective antigen [65]. Comparison between a GO-based direct assay and a GO/Au NP-based sandwich assay showed slightly better results for the latter. Although the direct assay, based on capture aptamer-modified GO nanosheets immobilised on the electrode, already provided an LOD of 1 fg mL^{-1}, the sandwich assay, also incorporating detection aptamer-conjugated Au NPs, resulted in one order of magnitude lower LOD (0.1 fg mL^{-1}). Similarly, Wei et al. [66] developed an immunosensor for MC-LR that combines capture antibody-modified GO sheets adsorbed on a glassy carbon electrode and mesoporous PtRu alloy-labelled detection antibody. The alloy allowed the H_2O_2 electrochemical detection, providing a MC-LR detection range from 0.01 to 28 ng mL^{-1} and an LOD of 9.6 pg mL^{-1}. The biosensor provided appropriate quantification of the cyanotoxin in polluted water.

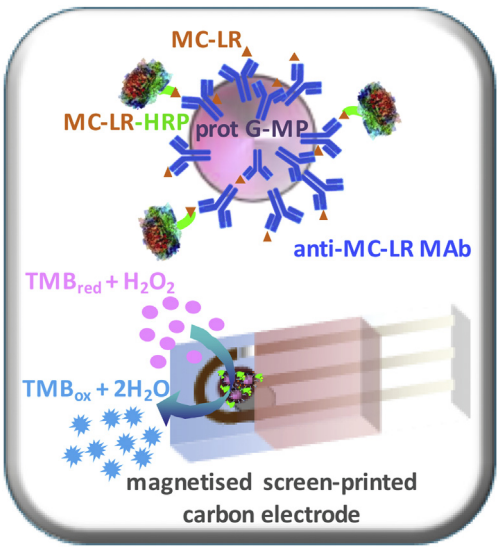

Fig. 3. Example of biosensing strategy based on magnetic particles as antibody supports.

3. Use of magnetic beads as supports and/or carriers

Magnetic beads (MBs) have been recently exploited as supports and/or carriers. MBs have particular characteristics that make them useful in biosensor configurations: they are biocompatible, they have large surface area, they can be easily functionalised, and their location and transport can be controlled by a magnetic field.

3.1. Magnetic beads in batch-mode biosensors

Sandwich-type configurations incorporating MBs have been reported. As an example, Ge et al. [67] co-immobilised anti-MC antibody and HRP on silica-coated ferroferric oxide (Fe_3O_4) NPs, and used them as carriers. Once the sandwich immunoreaction was completed, electrochemical measurements were recorded according to the oxidation of the thionine mediator, which was catalysed by both the HRP and Fe_3O_4 NPs (peroxidase mimetics). This immunosensor provided a linear working range from 0.01 to 200 μg mL^{-1} and an LOD of 4 ng mL^{-1}. In this case, the magnetic properties of the particles were not exploited for their immobilisation on electrodes, but for both the washing steps in the conjugate synthesis and the electrochemical signal enhancement.

The use of MBs as antibody immobilisation support is described in several magneto-controlled competitive immunoassays and immunosensors. In the simplest approach, the immunocomplexes are first formed and later captured on magnetised electrodes for the electrochemical measurement. In the work described by Paniel et al. [68], free and HRP-labelled AFM_1 competed for an anti-AFM_1 antibody conjugated to MBs (via protein G affinity) in solution. The immunocomplexes were then captured on screen-printed carbon electrodes by external magnets, and the 1-methoxy-phenazine-methosulfate (MPMS)-mediated electrochemical detection of the HRP enzyme used as label was performed. The LOD attained was 10 pg mL^{-1}. A similar approach was followed by Reverté et al. [69], who immobilised anti-MC-LR antibodies on protein G-modified MBs for the subsequent competition between free MC-LR and MC-LR-HRP tracer (Fig. 3). In this case, the HRP label was mediated by 3,3′,5,5′-tetramethylbenzidine (TMB) in solution, resulting in an LOD of 0.4 ng mL^{-1}. Vidal et al. [70] developed an immunosensor for the detection of OTA, also based on the competition between free and HRP-labelled toxin for their binding to biotinylated anti-OTA antibodies immobilised on streptavidin-coated MBs. Hydroquinone in solution was used as electrochemical mediator and an LOD of 0.12 ng mL^{-1} was obtained. With the aim of simplifying the experimental procedure, immobilised redox mediators have been used. Esteban-Fernández de Ávila et al. [71] developed a direct competitive magnetoimmunosensor for the detection of staphylococcal protein A (prot A). In this case, HRP-labelled immunocomplexes were captured on tetrathiafulvalene (TTF)-modified screen-printed gold electrodes, providing an LOD as low as 3.9 fg mL^{-1}. As mentioned in section 2.1.2, epoxy-modified MBs have also been used as anti-BTX-2 antibody immobilisation support, the antigen and the guanidine label being linked to GNRs [36]. A step further is the work described by Hervás and co-workers [72] in which both the competition between Zearalenone (ZEA) and ZEA-HRP for their binding to MAb-coated MBs and the electrochemical measurement were performed on the magnetised screen-printed electrodes. This format allowed lowering the LOD from 11 to 7 pg mL^{-1}, compared to the assay with separated competition and measurement steps [73]. Antigens have also been conjugated to MBs. As an example, Hayat et al. [74] conjugated biotinylated OA to streptavidin-modified MBs for their magnetic immobilisation on electrodes.

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The indirect competitive immunosensor, with detection and ALP-labelled secondary antibodies and 1-naphthyl phosphate as enzyme substrate, provided an LOD of 0.38 ng mL^{-1}.

A special configuration has been proposed by Hayat et al. [75], who developed a non-competitive label-free immunosensor for the detection of OA, conjugating anti-OA antibodies to protein G-MBs. The label-free detection was possible because of the hindered charge transfer from the redox couple $[Fe(CN)_6]^{4-/3-}$ in solution in the presence of OA/anti-OA antibody complex on the electrode. This immunosensor attained an LOD of 0.5 ng mL^{-1}.

MBs have also been used in aptamer-based configurations. As when conjugating MBs to antibodies, some authors performed the competition step in solution to form the immunocomplexes that are later captured on the electrode surface, by using external magnets, for the electrochemical measurement. Bonel et al. [76] conjugated streptavidin-coated MBs to a biotinylated OTA aptamer, which competed for its binding to free OTA and OTA-HRP tracer. Immunocomplexes where finally captured on magnetic screen-printed carbon electrodes and the hydroquinone-mediated HRP electrochemical detection provided an LOD of 70 pg mL^{-1}. A more sophisticated approach is that described by Barthelmebs and co-workers [77], in which indirect and direct competitive strategies for the detection of OTA were compared, both being entirely performed on screen-printed electrodes. In the indirect approach, biotinylated OTA was immobilised on streptavidin-coated MBs and compete with free OTA for their binding to HRP-labelled aptamer. MPMS redox mediator was added in solution. In the direct approach, OTA aptamer was covalently linked to the MBs via carbodiimide reaction and, after immobilisation on the electrode, free OTA and OTA-ALP competed for the aptamer binding. 1-naphthyl phosphate was used as enzyme substrate for the electrochemical measurement. The direct competitive approach lowered the LOD from 1.1 to 0.11 ng mL^{-1}. OTA was successfully analysed in wheat and wine samples using the immunosensors developed by Bonel et al. [76] and Barthelmebs et al. [77], respectively.

3.2. Magnetic beads in flow systems and microfluidics devices

With the aim to provide compact analysis devices for environmental surveillance and food control as well as other applications, MBs combined with immunoassays/sensors have been integrated in flow systems and microfluidics devices, providing advantages such as short analysis times and low sample and reagents volumes. Some of the immunosensors developed in the section 3.1 have been incorporated in flow systems. As an example, Dominguez et al. [78] injected MB-OA conjugates in a flow system and captured them on the electrode surface. Then, the competition took place and the electrochemical measurement was performed, both on the magnetised electrode. The magnetic field was enough to keep the MBs on the electrode, even despite the continuous flow. The authors achieved an LOD of 0.15 ng mL^{-1}, lower than in batch-mode (0.38 ng mL^{-1}) [74], probably due to the signal enhancement caused by the short diffusion distance in the continuous/stopped flow system.

The first step in the development of miniaturised compact systems was taken by Hervás et al. [79], who electrokinetically injected the enzymatic product from the MB-Ab-ZEA-HRP immunocomplexes into a microfluidics platform where the amperometric detection took place, providing an LOD of 0.83 ng mL^{-1}. In the following work [80], the whole system was integrated into the microfluidics chip, configuration that provided a lower LOD (0.4 ng mL^{-1}). A similar microfluidics immunosensor for ZEA but using 4-*tert*-butylcatechol as a redox mediator has also been reported [81]. Similarly, Bunyakul et al. [82] used MBs as immobilisation support for anti-cholera toxin subunit B (CTB) antibodies to develop a microfluidics immunosensor (Fig. 4). For the detection, they synthesised a tracer consisting of CTB receptor ganglioside GM_1 linked to liposomes, which encapsulated $[Fe(CN)_6]^{4-/3-}$and were used as signal amplifiers. Reagents were first mixed and then injected into the magneto-controlled microfluidics device. Once immunocomplexes were formed and captured by the magnet, *n*-octyl-β-D-glucopyranoside detergent was added to lyse the liposomes and release the $[Fe(CN)_6]^{4-/3-}$ on an interdigitated ultramicroelectrode array (IDUA), providing an LOD of 1 ng mL^{-1}.

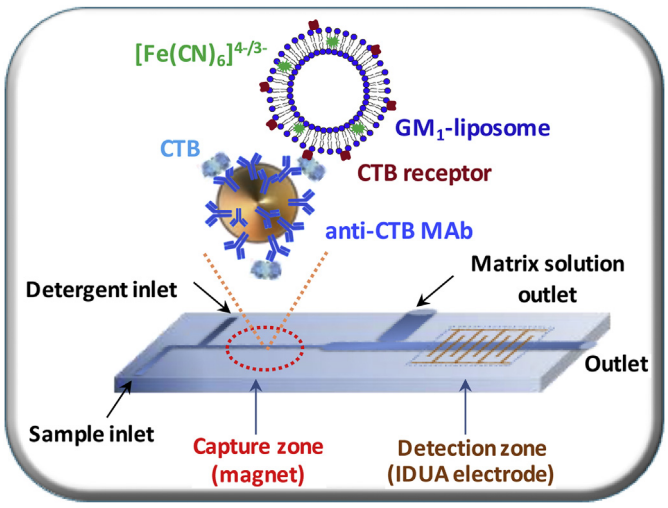

Fig. 4. Example of biosensing strategy based on magnetic particles as antibody supports in a microfluidics device.

3.3. Magnetic beads in multiplexed systems

Moving towards multiplexed electrochemical detection for simultaneous analysis of samples, Romanazzo et al. [83] developed an immunoassay for DON on a strip with 8 magnetised screen-printed carbon electrodes. Biotinylated anti-DON antibodies were used in the competition between free DON and human serum albumin (HSA)-DON immobilised on tosyl-modified MBs. Then, avidin and biotinylated HRP were added. Once immunocomplexes were formed, TMB in solution mediated the HRP reduction, and the electrochemical measurements provided an LOD of 63 ng mL^{-1}. The simultaneous analysis on the strip allowed processing a high number of samples in short time. Regarding multiplexed detection with discrimination of different analytes, a direct competitive immunoassay for the simultaneous detection of BTX-2 and dinophysistoxin-1 (DTX-1) has been developed [84]. Antibodies against BTX-2 and DTX-1 were co-immobilised on MBs and distinguishable metal nanoclusters (NCs) (cadmium and copper) were employed as both antigen-BSA conjugate carriers and labels. Both competition step and electrochemical measurements were performed in a flow-through detection cell with a sequential injection and using external magnets. The stripping voltammetry of the dissolved labels provided a wide linear range for both analytes (0.005–5 ng mL^{-1}), and LODs of 1.8 pg mL^{-1} and 2.2 pg mL^{-1} for BTX-2 and DTX-1, respectively. Additionally, the analysis of spiked samples provided toxin contents in good agreement with those obtained by ELISA.

4. Other flow systems and microfluidics devices

The integration of electrochemical immunoassays and

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immunosensors in flow and microfluidics systems has also been achieved without the use of MBs. Panini et al. [85] developed an immunosensor for the determination of ZEA using a continuous-flow/stopped-flow system. Anti-ZEA antibodies were immobilised on a rotating disk, where the competition between ZEA and the tracer ZEA-HRP took place. HRP catalysed the oxidation of the redox mediator 4-*tert*-butyl-catechol to 4-*tert*-butyl-*o*-benzoquinone, which was subsequently detected on a MWCNT-modified gold electrode, placed in front of the disk. This strategy provided a fast (15 min) and sensitive detection of ZEA, with an LOD of 0.8 ng mL^{-1}, and was successfully applied to the analysis of corn silage samples. The same group developed a microfluidics system for the detection of CIT [86]. CIT-OVA conjugates were immobilised on a cysteamine-modified gold disk electrode and competed with free CIT for antibody binding. Indirect detection using IgG-HRP and catechol was performed on an adjacent electrode. The system attained an LOD of 0.1 ng mL^{-1} with a total assay time of 45 min. Olcer et al. [87] have recently developed an immunosensing microfluidics platform for the detection of DON. Anti-DON antibodies were directly immobilised on a Prot A-modified working electrode to obtain a proper antibody orientation. Upon competition between free DON and DON-HRP, electrochemical measurements were performed using TMB as redox mediator, providing an LOD of 6.25 ng mL^{-1}. DON was successfully detected in wheat samples with an analysis time of 15 min. Finally, aptamers have also been included in microfluidics systems, in this case for the detection of BoNT [88]. The device had a zigzag-shaped channel to mix the reagents and a serpentine channel over the electrode to pull liquids through the fluidics network. Streptavidin-labelled dendrimer NPs located on the electrode were used to immobilise the biotin-labelled anti-BoNT aptamer, which was also labelled with fluorescein. In the presence of the neurotoxin, the aptamer conformation allowed the interaction between the fluorescein and an HRP-labelled anti-fluorescein IgG, providing the corresponding electrochemical signal in the presence of TMB. The system detected 1 ng mL^{-1} of BoNT in less than 15 min, with a high degree of autonomy and without the requirement of external pumps.

5. Conclusions

Over the last few years, researchers working on biosensors have widened their interest towards new fields of application. The environmental and agro-food sectors need to be modernised in terms of routine analysis methods. The recent advances in electrochemical biosensors, incorporating nanotechnological concepts, and their integration into compact analysis devices, may contribute to guarantee food safety, to facilitate environmental surveillance and to protect human and animal health.

When reviewing the biosensors with nanomaterial-based supports, all works claim advantages in terms of larger electroactive surface area, promoted electron transfer, enhanced electrochemical signals and lower LODs. Proper evaluation of the improvement achieved with these novel biosensors is not always possible, since comparison of equivalent systems is often difficult to perform. To a certain extent, the integration of nanomaterials could only add sophistication to the configuration. To guarantee the benefits from their incorporation, other parameters such as biosensor lifetime should also be considered. The global evaluation of the system will help to conclude if the sophistication is worthwhile. Nevertheless, a general statement is accepted attributing significantly better performance to biosensors based on bionanotechnological approaches. What is more, the advantages of one type of nanomaterial over another are also difficult to clear up, although one can figure out that the composition, structure and dimension may play significant roles. As an example, graphene is gaining ground to carbon nanotubes due to the better adhesion provided by its flat two-dimensional configuration [89] and the larger availability of active sites [90].

The role of nanomaterials in electrochemical biosensors can be focused on nanostructuring of electrode supports and/or on performing as labels, signal enhancers and label supports. The method used for the immobilisation of the nanomaterial on the electrode certainly plays an important role in the biosensor performance, as it also does the function of the nanomaterial in the electrochemical transduction system (e.g. label, electrocatalysts and/or label carrier).

Adsorption is a simple technique to incorporate carbon nanomaterials in biosensors, but sometimes suffers from leakage and always from random immobilisation. Although biorecognition molecules can be attached to nanomaterials by covalent bonds that favour the stability and allow oriented immobilisation, the initial nanomaterial adsorption process compromises the biosensor performance. When combined with polymers, adsorbed bionanocomposite matrixes may increase the immobilisation stability but diffusion barriers are created, which increase the LODs. The use of carbon nanomaterial pastes, although still limiting the diffusion processes, increases the stability and additionally allows the simultaneous immobilisation of biorecognition molecules and redox mediators. Electrochemical techniques allow addressed immobilisations and are compatible with the subsequent detection methods. Depending on the electrochemical technique (e.g. electrophoretic deposition, electropolymerisation and electrografting), bonds of different nature and different immobilisation conformations are created. Metal nanomaterials have also been immobilised on electrodes for subsequent biomolecule immobilisation. Again, adsorption and entrapment into polymers, as well as electrochemical techniques, have been proposed, but self-assembly on thiolated surfaces certainly is the preferred choice because of the easy and effective immobilisation process.

Regarding the incorporation of nanomaterials in electrochemical transduction schemes, metal nanomaterials are more commonly found. Metals can directly act as electroactive labels, and they can also enhance electrochemical signals arising from redox probes because of their electrocatalytic activity. Another advantage of metal nanomaterials is the possibility to use them in multiplexed array systems. Although to a minor extent, carbon nanomaterials have also been incorporated to improve the electrochemical transduction. In this case, their most common role is to act as support for the simultaneous immobilisation of the biorecognition element (antigen or antibody/aptamer, depending on the assay format) and the label. The electrocatalytic behaviour of carbon nanomaterials together with the high number of immobilised labels result in electrochemical signal amplification and lower LODs.

As the described works demonstrate (a considerable number of them having been developed for MC-LR and OTA), nanomaterials provide advantages in terms of lower LODs and wider working ranges, rendering widely applicable biosensors. However, to really foster the use of biosensors in the agro-food and environmental sectors, compact analysis devices, sensitive, robust, reliable and easy to handle even by non-trained people, are desired. The integration of electrochemical biosensors into microfluidics is a challenge that is being achieved, but the implementation of such devices in daily life still requires more effort. Nevertheless, the progress in this area is so fast that, provided the scientific community focuses not only on the development of the bioanalytical systems but also on their validation, it will not take a long time to implement them in routine analysis.

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Laia Reverté Laia studied the degree in Biotechnology and acquired the master in Immunology. She is currently a PhD student at IRTA in collaboration with the department of Biotechnology of Universitat Autònoma de Barcelona. Her thesis focuses on the development of cytotoxicity assays and biosensors for the detection of emerging marine toxins. She has published 1 review and 2 articles, 1 more article being in progress.

Dr. Beatriz Prieto-Simón is a research fellow at the University of South Australia leading a research line on nanostructured electrochemical biosensors. She has made significant contribution to the fields of biosensors, biomaterials, nano- and biotechnology, and the crossing over into areas such as environmental control and food safety. Currently her research is focused on combining the advantages of novel nanostructured materials, site-specific immobilisation of bioreceptors, and new electrochemical techniques, to provide platforms for sensitive and selective multiplexed detection. She is leading an ARC project focused on developing electrochemical sensors as early alert screening tools for water quality assessment. She is currently co-supervising 4 PhD students. http://www.unisanet.unisa.edu.au/staff/homepage.asp?Name=Beatriz.PrietoSimon

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Dr. Mònica Campàs is researcher at IRTA, where she leads a research line on biosensors for the detection of toxins. Her research interests lie in the development of colorimetric assays and electrochemical biosensors using new biorecognition elements, immobilisation techniques and transduction strategies. Presently, her work focuses on the development and applicability of systems for the detection of emerging marine toxins and the use of diatoms as natural nanostructured supports in biosensors. She has published 45 articles and 8 book chapters. She has been the principal investigator of 8 projects. She has directed 1 PhD thesis, 3 more being in progress. http://www.irta.cat/ca-es/Persones/Pagines/4599.aspx

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