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Microfabricated biosensor for the simultaneous amperometric and luminescence detection and monitoring of Ochratoxin A

Scherrine A. Tria [a,d], David Lopez-Ferber [a,d], Catherine Gonzalez [a,d], Ingrid Bazin [a,d,*], Anthony Guiseppi-Elie [a,b,c,d,**]

[a] Ecole des mines d'Alès, LGEI, 6 avenue de Clavière, 30319 Alès Cedex, France
[b] Center for Bioelectronics, Biosensors and Biochips (C3B), The Dwight Look College of Engineering, Texas A&M University, College Station, TX 77843, USA
[c] Department of Biomedical Engineering, 5045 ETB, The Dwight Look College of Engineering, Texas A&M University, College Station, TX 77843, USA
[d] ABTECH Scientific, Inc., Biotechnology Research Park, 800 East Leigh Street, Richmond, VA 23219, USA

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ABSTRACT

The low molecular weight hapten, Ochratoxin A (OTA), is a natural carcinogenic mycotoxin produced by Aspergillus and Penicillium fungi and so it commonly appears in wines, other foods, and in the environment. An amperometric biosensor has been developed that uses the immobilized synthetic peptide, NFO4; which possesses a high binding affinity and thus provides for molecular recognition of OTA; simulating the mycotoxin-specific antibody. Biotransducers were produced from a microlithographically fabricated electrochemical cell-on-a-chip that uses the microdisc electrode array working electrode format augmented with microporous graphitized carbon (MGC) that was electrodeposited within a poly (aniline-*co*-meta-aminoaniline) electroconductive polymer layer. A redox mediator, iron-nickel hexacyanoferrate (Fe|NiHCF) was amperometrically deposited onto the MGC. The device was then dip-coated with monomer cocktail that yielded poly(HEMA-*co*-AEMA) foam that was prepared in-situ by UV crosslinking and by sequentially freezing followed by freeze drying of the chip to yield a 3-D support for the chelation of Zn^{2+} ions ($ZnCl_2$) and the subsequent immobilization of N-terminus his-tagged peptide, NFO4. To conduct the biosensors assay, HRP conjugated OTA was added to the free OTA solutions and together competitively incubated on the biospecific MDEA ECC 5037-Pt|MGC|HCF|Hydrogel-NFO4 biotransducer. The amperometric response to peroxide was determined after 5 min of enzymatic reaction following addition of standard substrate H_2O_2/luminol. Simultaneous analysis of light emission signals (λ_{max}=425 nm) allowed direct comparison of amperometric and luminescence performance. Using chitosan foam and a luminescence bioassay we obtained maximum inhibition at 10 μg L^{-1} and half inhibition occurred at 2.1 μg L^{-1}. Using poly(HEMA-*co*-AEMA) hydrogel and an amperometric bioassay (50 s) we obtained maximum inhibition at 10 μg L^{-1} and half inhibition occurred at 2.8 μg L^{-1}.

1. Introduction

Haptens are small, low molecular weight molecules that are generally not antigenic by themselves but can induce an immune response when conjugated to a carrier protein and made to elicit the formation of antibodies against the hapten-protein complex (Shan et al., 2002). It is therefore difficult to obtain antibodies against haptens and thus challenging to build immuno-biosensors for such ubiquitous small molecules. Moreover, these small molecules have been suspected to be implicated in some disease. Of particular interest is the example presented by the mycotoxin, Ochratoxin A (OTA). OTA is a coumarinic mycotoxin produced mainly by several *Aspergillus ochraceus* and *Penicillium verrucosum* and is known to be powerful in its nephrotoxic, immunotoxic, teratogenic and carcinogenic effects (O'Brien and Dietrich, 2005; Pfohl-Leszkowicz et al., 2002; Smith et al., 1995). OTA is also capable of mimicking natural hormones and has been shown to be an endocrine disruptor leading to an increasing risk of cancer. This mycotoxin is found in a variety of improperly stored foods and beverages such as cereals and wine (Blesa et al., 2004a; Varga and Kozakiewicz, 2006; Zimmerli and Dick, 1995). Wine is widely consumed worldwide and represents the major intake of OTA (Jørgensen, 2005), which has been largely reviewed by several

* Corresponding author at: Ecole des mines d'Alès, LGEI, 6 avenue de Clavière, 30319 Alès Cedex, France.
** Corresponding author at: Center for Bioelectronics, Biosensors and Biochips (C3B), The Dwight Look College of Engineering, Texas A&M University, College Station, TX 77843, USA.
E-mail addresses: scherrine.tria@mines-ales.fr (S.A. Tria), Catherine.Gonzalez@mines-ales.fr (C. Gonzalez), ingrid.bazin@mines-ales.fr (I. Bazin), guiseppi@TAMU.edu (A. Guiseppi-Elie).

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authors (Blesa et al., 2004b; Varga and Kozakiewicz, 2006). The International Agency for Research on Cancer (IARC) has classified OTA in Group 2B (possibly carcinogenic agent) and a level of 2 μg kg^{-1} of OTA in wines has been established by the Regulatory Commission of the European Community (European Commission Commission Regulation, 2005; European Commission Regulation, 2010).

Standardized methods for OTA determination in food matrices are already available. The commonly used technique for quality control relies on immunoaffinity column (IAC), followed by reversed-phase high pressure liquid chromatography (HPLC) using fluorescence detection (Visconti et al., 1999; Aresta et al., 2006). Analytical methods to detect mycotoxins include thin-layer chromatography (TLC), liquid chromatography (LC), high-performance liquid chromatography (HPLC) with fluorescence or diode array detector, gas chromatography tandem mass spectrometry (GC–MS) or with tandem electron capture detection (GC–ECD), immuno-affinity column chromatography and enzyme-linked immunosorbent assays (ELISAs) (WHO, 2002). These techniques have been successfully applied to single as well as multiple-analyte detection of mycotoxins such as aflatoxins, Ochratoxin A, fumonisin, and deoxynivalenol (DON) in cereals, coffee, beer and wine (Bhat et al., 2010).

In order to protect the health of consumers, producers and the regulatory bodies concerned are always looking for new, faster, more convenient and less expensive methods of detection and/or monitoring. During the last decade, various immuno-biosensors have been developed against OTA (Alarcón et al., 2006; Liu et al., 2009; Prieto-Simón et al., 2008; Radi et al., 2009a, 2009b). Enzyme-Linked Immunosorbent Assays (ELISAs) have been developed and deployed for OTA. However, these have generally shown poor correlation coefficient (0.74) when compared to standard HPLC methods (Buckle et al., 1985; Huybrechts and Tangni, 2010). In addition, the coefficient of variation (CV) representing intra-assay and inter-assay variability among ELISAs can be quite high, being 3–22% (Candlish et al., 1986; Meulenberg, 2012). Detection systems with antibodies show high affinity and selectivity but depending upon the matrix or experimental condition, several disadvantages appear. Among these disadvantages are thermal denaturation and solvent effects. To overcome these drawbacks, several alternatives to OTA antibodies are being pursued for development. Among these multiple strategies are molecular recognition that relies upon molecular imprinted polymers (MIPs) (Ali et al., 2010; Yu and Lai, 2010), DNA aptamers (Cruz-Aguado and Penner, 2008a, 2008b) and phage display libraries (Giraudi et al., 2007).

In previous work, a new bioanalytical format that allows the rapid capture, concentration and detection of OTA was introduced (Soleri et al., 2015). In this enzyme-linked, peptide-based immunosorbent assay ELISA format (Bazin et al., 2013), N-terminal histidine tagged NFO4 was immobilized onto three-dimensional porous chitosan foam that was pre-adsorbed with divalent M^{2+} ions to allow pre-concentration and simultaneous detection of the mycotoxin on the high surface area biopolymer. OTA-HRP conjugate was added to the OTA containing standards or samples and together competitively incubated on the biospecific chitosan foam. Chemiluminescence signals were generated by the addition of H_2O_2/luminol and recorded (λ_{max}=425 nm) following 5 min of enzymatic reaction.

In the present study, we develop and demonstrate a novel amperometric peptide-biosensor wherein the NFO4 synthetic peptide (VYMNRKYYKCCK-NH_2) was immobilized as the biorecognition element in a similarly constructed biorecognition layer. A microfabricated, dual responsive electrochemical-cell-on-a-chip that is based on the microdisc electrode array format for its working electrodes, the ECC MDEA 5037-Pt/Au, has been previously developed (Guiseppi-Elie et al., 2005b), packaged (Rahman et al., 2009c) and fully characterized (Justin et al., 2009; Rahman et al., 2009a) to serve as a biotransducer for the amperometric or voltammetric measurement of analytes such as glucose and lactate (Kotanen et al., 2014; Guiseppi-Elie, 2011). With critical microdisc dimensions of 25 μm, this device was engineered to work with polymer hydrogel layers that naturally attenuate the diffusivity of small molecules compared to buffer (Kotanen et al., 2013; Palmisano et al., 2000). The biotransducers, developed in conjunction with ABTECH Scientific, Inc. (Richmond, VA, USA) were first chemically modified by immobilization of an electroactive layer of poly(aniline-*co*-aminoaniline) that served to entrain particles of mesoporous graphitized carbon (MGC) during electrodeposition. Onto the PAn entrapped MGC was then electrochemically deposited a mediator layer of nickel hexacyanoferrate (NiHCFe) nanocrystals that served to mediate peroxide response. This layer was subsequently decorated with a biorecognition layer that comprised the histadine tagged OTA linked via divalent zinc cations that were bound to a poly(HEMA-*co*-AEMA) hydrogel. Amperometric biosensor bioassays were performed in response to incubation of OTA samples and standards with OTA-HRP conjugates in the presence of H_2O_2/luminol. Performing the bioassay in a luminomitor permitted recording of the optical signal (λ_{max}=425 nm) as well as the amperometric signal as a function of time of the enzymatic reaction. This allows the direct comparison of the bioanalytical performance of the amperometric and luminescence biosensor formats.

2. Experimental section

2.1. Chemicals and reagents

2.1.1. Preparation of electroconductive hydrogels

Poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA) (CAS No.: 27119-07-9 15 wt% in water' MW=2,000,000, 6–10% sulfonation, ρ=1.10 g/mL), benzene sulfonic acid (BSA) (CAS No.: 98-11-3, $C_6H_5SO_3H$' MW=158.18), aniline monomer (An) (≥99.5%' CAS No.: 62-53-3' $C_6H_5NH_2$' MW=93.13), *m*-phenylenediamine monomer (AAn), (aka: meta-aminoaniline' CAS No.: 108-45-2' C_6H_4-1,3-$(NH_2)_2$' MW=108.14), Mesoporous Graphatized Carbon (MGC) (CAS No.: 1333-86-4' 20% graphite' particle size <500 nm (DLS)' pore size=0.25 cm^3/g' average pore diameter=137 Å' surface area=50–100 m^2/g), poly(N-vinylpyrrolidone) (pNVP) $(C_6H_9NO)_n$ (CAS No.: 9003-39-8' MW~1,300,000), Dulbecco's phosphate buffered saline, benzophenone (reagent grade 99%), ferrocene monocarboxylic acid (FcCOOH), and all other common solvents (dimethyl formamide, isopropyl alcohol) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and used as received.

2.1.2. Preparation of the redox mediator layer

Potassium chloride ACS reagent grade (CAS No.: 7447-40-7' KCl' MW=74.55)' nickel (II) chloride (CAS No.: 7718-54-9' $NiCl_2$' MW=129.60)' potassium ferricyanide (CAS No.: 13746-66-2' $K_3Fe(III)(CN)_6$, MW=329.24)' hydrochloric acid (CAS No.: 7647-01-0' HCl' MW=36.46) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and used as received.

2.1.3. Preparation of hydrogels

2-hydroxyethyl methacrylate (HEMA), tetra(ethylene glycol) diacrylate (TEGDA, technical grade), oligo(ethylene glycol)(400) methacrylate (OEG(400)MA), *N*-[Tris(hydroxymethyl)methyl]acrylamide (HMMA, 93%), polyvinylpyrrolidone (pNVP, M_w ~1,300,000), amino ethyl methacrylate (AEMA, 98%), the photoinitiator, 2,2-Dimethoxy-2-phenylacetophenone (DMPA, 99+%),

and all other common solvents and buffers were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The HEMA, methacrylate and diacrylate reagents were first passed over an inhibitor removal column (Sigma-Aldrich) in order to remove the polymerization inhibitors hydroquinone and monomethyl ether hydroquinone.

All aqueous solutions were prepared in deionized water (DI-water) prepared by purifying reverse osmosis (RO) water through a Milli-Q® plus (Millipore Inc.) ultrapure water system. Phosphate buffered saline (PBS) was used at 0.1 M at pH=7.2 unless otherwise stated. HEPES buffer was prepared at 0.025 M and pH=7.35.

2.2. Device fabrication and cleaning

The Electrochemical Cell-on-a-Chip Microdisc Electrode Arrays (ECC MDEA 5037-Pt' ABTECH Scientific, Inc.), an Ag/AgCl (3 M KCl) reference electrode (RE803; ABTECH Scientific, Inc.) and a large area platinum mesh counter electrode comprised a three-electrode electrochemical cell. The ECC MDEA 5037-Pt is a microlithographically fabricated dual analyte electrochemical transducer intended for amperometric and voltammetric biosensor application and has been fully described. The microlithographic fabrication and geometric patterning of the three-electrode electrochemical biotransducers have been previously described (Guiseppi-Elie et al., 2005a) and assembly and packaging of the chip in a manner suitable for implantation into small vertebrate animals has similarly been described (Rahman et al., 2009b). Developed for simultaneous monitoring of interstitial glucose and lactate (Jongwon et al., 2006), the transducer possesses 37 recessed microdiscs arranged in a hexagonal array. Each disc has φ=50 μm for a total working electrode area, WEA=7.3×10^{-4} cm^2 (Guiseppi-Elie et al., 2005a). In summary, the electrochemical transducers (0.2 cm × 0.4 cm × 0.05 cm) were fabricated from electron beam vapor deposited platinum (100 nm) on an adhesion promoting titanium/tungsten (Ti/W) layer (10 nm) onto a 0.5 mm thick electronics grade borosilicate glass (Schott D263). The electrodes were laid out as two separate three-electrode electrochemical cells and the metallization passivated with 0.5 μm thick silicon nitride (Si_3N_4). The nitride layer was eventually patterned and fluoroplasma etched to reveal the array of multiple microdiscs of the working electrode, the large area counter electrode (CEA=7.3×10^{-3} cm^2) and the shared reference electrode (REA=7.3×10^{-5} cm^2) that were connected to the five bonding pads. The MDEA 5037-Pt transducer was first ultrasonicated for 3 min in each of DI-water, isopropyl alcohol (IPA), and DI-water. Next, the transducer was placed in a UV-ozone cleaner (Boekel Industries) under irradiated ozone generation for 10 min followed by 1 min of ultrasonication in IPA. This was followed by plasma surface modification of the transducer to generate hydroxyl groups on the silicon nitride surface (Harrick Plasma Cleaner). The transducer was then immersed in PBS buffer, made the working electrode of a three electrode electrochemical cell and was cathodically cleaned by sweeping the potential between 0 and −1.2 V *vs.* Ag/AgCl at 100 mV/s for 40 cycles.

2.3. Electrodeposition of mesoporous graphitized carbon (MGC)

The solvent and UV-ozone cleaned MDEA 5037-Pt was modified by amperometric electrodeposition of the high surface area mesoporous graphitized carbon (MGC). The electropolymerization solution was prepared by adding MGC to 50/50 v/v/ DMF/H_2O (2 mg/mL) followed by the addition of aniline (0.1 M) and *m*-aminoaniline (0.1 M), the addition of pNVP (2 mg/mL) and finally PAMPSA (2 mg/mL). Two drops of Tween-20 was added to the final 10 mL of prepared suspension and the mixture sonicated at room temperature (RT). The device was potentiostatically held at +800 mV *vs.* Ag/AgCl and subjected to timed immersion into the electrodeposition solution for 50 s. The resulting device was designated MDEA-5037-Pt|MGC-PAn.

2.4. Electrodeposition of redox mediator layer (RML)

The MDEA-5037-Pt|MGC-PAn was further modified by amperometric electrodeposition of a solid-state mediator multi-layer following a modified literature procedure attributable to Karyakin (Chen et al., 2012; Karyakin and Karyakina, 1999; Krylov and Lisdat, 2007; Sitnikova et al., 2011). The device was potentiostatically held at −400 mV *vs.* Ag/AgCl and subjected to sequential immersion with controlled timing into four separate aqueous solutions held in a 96-well plate: A (5 s)=50 mM KCl in (0.01 M) 10 mM HCl (serving as acidic solvent), B (50 s)=3.0 mM $K_3Fe(III)(CN)_6$+3.0 mM $FeCl_2$ prepared in A; C (50 s)=1.5 mM $K_3Fe(III)(CN)_6$+3.0 mM $NiCl_2$ prepared in A and finally, D (5 s)=PBS 7.2. This procedure was repeated five times without interruption, effectively creating a layer-by-layer, multi-layered structure of iron (II,III) hexacyanoferrate(II,III) (Prussian Blue) formed in B followed by a layer of nickel(II) hexacyanoferrate [Ni(II)HCFe] formed in C and conditioned in PBS (D) and conditioned in dilute HCl (A). Following mediator deposition, the resulting MDEA 5037-Pt|MGC-PAn|HCF was tested in pH 7.2 buffered aqueous H_2O_2 solutions also held in the 96-well plate before being used in bioassay development. Electrodeposition of MGC and RML as well as amperometric biosensor performance were all done using a customized multimode potentiostat (BioSTAT, ESA Biosciences, Inc.) with custom cabling to the MDEA 5037-Pt transducer as shown in Fig. 1A. Electrical Impedance Spectroscopy (EIS) was performed on IME 1025-Pt devices (Yang et al., 2011) in freshly prepared 1× PBS (40 mV p-t-p, 10^{-1}–10^6 Hz, RT) using a Solartron 1260 Frequency Response Analyzer equipped with ZView and ZPlot software.

2.5. Device coating with an amine-rich hydrogel layer

MDEA 5037-Pt|MGC-PAn|HCF devices were dip-coated in a hydrogel monomer cocktail of HEMA (79 mol%), TEGDA cross-linker (3 mol%), OEG(400)MA (5 mol%), HMMA (5 mol%), pNVP (2 mol%), AEMA (5 mol%), and DMPA photo-initiator (1 mol%). These monomer were prepared in 20% v/v solvent of 1:1 water: ethylene glycol, degassed by bubbling with nitrogen, and once coated onto the device immediately UV-cross-linked under flowing nitrogen. Devices so prepared, MDEA 5037-Pt|MGC-PAn|HCF|Gel, were incubated in 0.1× PBS overnight to achieve hydration equilibrium and to release any unreacted monomer. The amine-rich hydrogel served as the chelation sites for Zn2+ ions and eventually his-tagged immobilization of NFO4 peptide, VYMNR-KYYKCCK-NH_2, (Synthetized by Smartox, France).

2.6. Peptide-based competitive enzyme-linked immunosorbent assay

After washing with PBS, MDEA 5037-Pt|MGC-PAn|HCF|Gel devices were placed in zinc chloride solution (100 mg L^{-1}) overnight at 20 °C (Soleri et al. 2015). They were then incubated with the his-tagged synthetic peptide NFO4 in azide containing carbonate buffered (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.2 g· L^{-1} NaN_3, pH 9.6) and were incubated at 37 °C for 3 h. Non-specific binding sites of the peptide-coated device were blocked with casein solution at RT for 3 h before performing the biommobilization. Devices were immersed in OTA-HRP (horseradish peroxidase label) (Abcam, France) conjugate (1,000 mg L^{-1}) that was combined with PBS containing unlabeled OTA (Sigma-Aldrich, France) of various concentrations (Soleri et al., 2015). The reaction was allowed to incubate for 30 min at 4 °C. After washing away unbound OTA with PBS, the device was quickly placed in 40 μl of luminol-hydrogen peroxide (Pierce, France) that was already in the luminometer tube

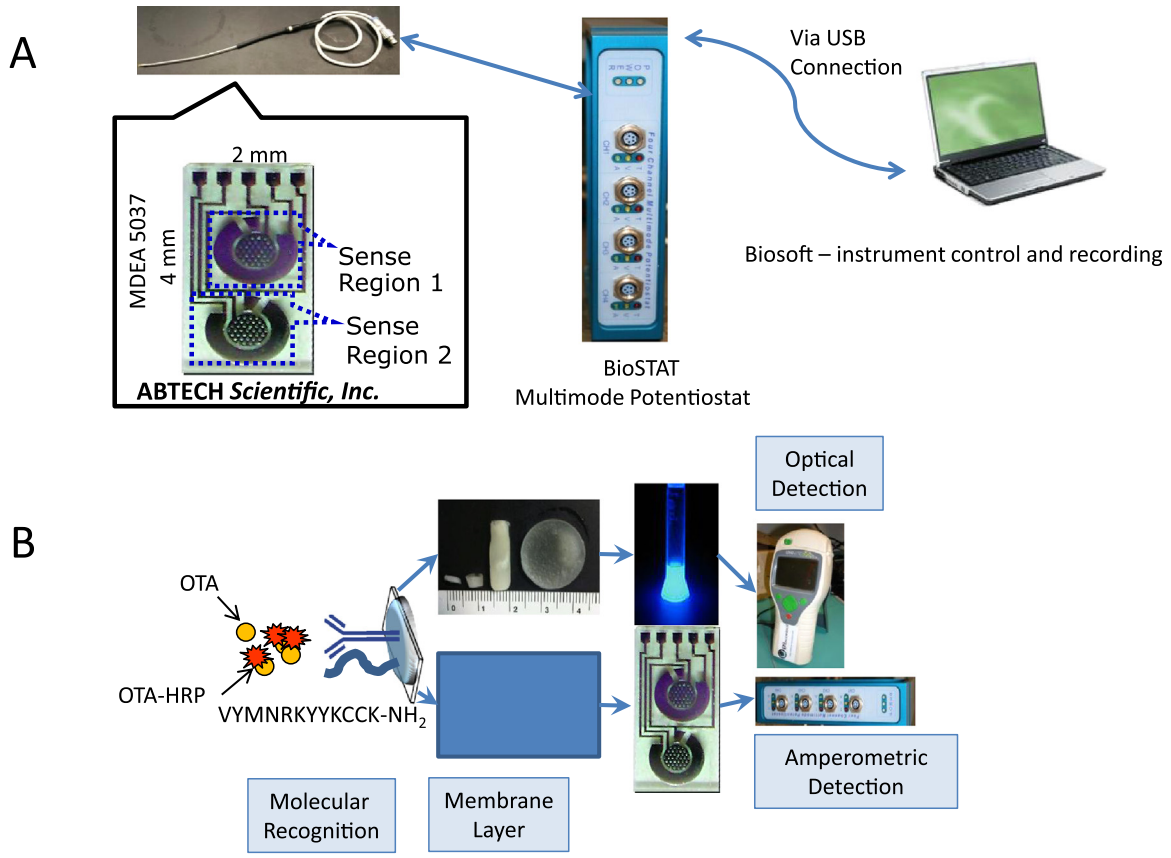

Fig. 1. A. Micrograph of the two channel electrochemical transducer, the MDEA 5037-Pt, and its interfacing to the four channel multimode potentiostat, the esa Biostat, wherein two channels are reconfigured to a single connector port. B. Schematic layout for the comparative evaluation of the two biosensor systems using the same molecular recognition elements. Above: Optical detection mode (luminescence) using a luminometer. Below: Amperometric detection mode using a multimode potentiostat.

at RT (3 M™ Clean-Trace™ NG Luminometer, UK). Luminescent data as well as amperometric data could be simultaneously collected. The results obtained were inversely proportional to the concentration of unlabeled OTA. During each test, nonspecific binding (negative control) was determined by using an incubation mixture (OTA-HRP) in which the peptide NFO4 was replaced by 100 µL of PBS buffer. Total analysis time per sample was ca. 40 min.

2.7. Antibody-based competitive enzyme-linked immunosorbent assay

After washing with PBS, MDEA 5037-Pt|MGC-PAn|HCF|Gel devices were placed in zinc chloride solution (100 mg L^{-1}) overnight at 20 °C (Soleri et al., 2015). They were then incubated in 6His-tagged-Protein G at 5 µg/ml for 3 h at 4 °C. Non-specific binding sites of the peptide-coated chip were blocked with casein solution at RT for 3 h before performing the immobilization of monoclonal antibody (AbCam, France) at the dilution 1/1000, for 3 h at 4 °C. Control samples remained in PBS until exposure to OTA-HRP (1000 mg L^{-1}). Devices were immersed in OTA-HRP (horseradish peroxidase label) conjugate (1000 mg L^{-1}) that was combined with PBS containing unlabeled OTA of various concentrations (Soleri et al., 2015). The reaction was left for 30 min at 4 °C. After washing away unbound OTA with PBS, the device was quickly placed in 40 µl of luminol-hydrogen peroxide (Pierce, France) that was already in the luminometer tube at RT.

3. Results and discussion

3.1. Hardware configuration and instrumentation set-up

The ESA Multimode Potentiostat was electrically reconfigured such that adjacent channels were merged to one of the four separate five-pin connectors. In this way, two channels (e.g. Ch1 and Ch 2) emerged from a single connector and similarly Ch 3 and Ch 4 emerged from another separate five-pin connector. The five pin configuration was made compatible with the five electrodes of the MDEA 5037 biotransducer chip via a compression fit, five-pin Omnetics quick-connect/disconnect connector that connected to the two working microdisc electrode array working electrodes, the two large area counter electrodes and a single shared reference electrode that reside on the chip. This instrumentation and device configuration allowed potentiostatic electrodeposition of MGC via aniline electropolymerization and hexacyanoferrate mediator multi-layer formation to be completed in a series of timed steps performed in sequence by immersion of the chip in the appropriate solution held in a 96-well plate at RT. In some instances, fabrication could be directly followed by amperometric characterization all done in one continuous operation within ~10 min. The experimental setup to allow a direct comparison of the chemiluminescence and amperometric responses corresponding to the two biosensor systems is schematically shown in Fig. 1B.

3.2. Peroxide response of biotransducer

It is customary to make high-solids suspension of MGC in highly volatile solvents and to deposit these by dropcasting onto micro- or macro-electrodes (Lu et al., 2014; Palani Barathi and

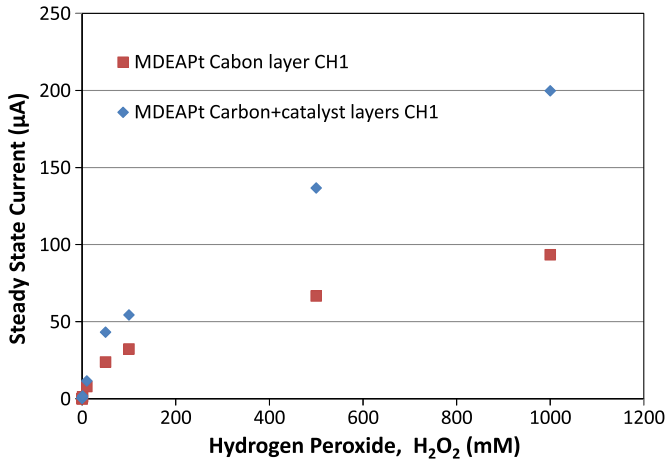

Fig. 2. Dose-response curve of steady state amperometric current (+650 mV) vs. concentration of hydrogen peroxide, H_2O_2 (mM), for a single device wherein the microdiscs of the WEA were: Ch1 was modified with the MGC layer and Ch1 was further modified by electrodepositon of the redox mediator layer.

Minnal, 2011; Thangaraj and Senthil Kumar, 2013). Here electropolymerization of aniline (Lin and Cui, 2006) and meta-aminoaniline to yield a copolymer was used as an additive microfabrication technique to simultaneously guide and immobilize the MGC to the microdisc electrodes (Du et al., 2010; Kotanen et al., 2014) of the MDEA as well as to introduce surface 1° amine functional groups for subsequent reaction including complexation with Zn^{2+} ions for the reversible immobilization of his-tag-peptides such as NFO4 and for attachment of the poly(HEMA-*co*-AEMA) hydrogel.

Subsequent to PAn-MGC electropolymerization, cathodic electrodeposition of five alternating layers of Prussian Blue: iron(II,III) hexacyanoferrate and nickel hexacyanoferrate according to the method of Karyakin (Sitnikova et al., 2011) produced a biotransducer with a redox mediator layer that was designed to respond to the changing levels of hydrogen peroxide that accompany HRP consumption of this reagent under bioassay conditions. The MDEA 5037-Pt|MGC-PAn|HCF was tested in pH 7.2 buffered aqueous H_2O_2 solutions also held in the 96-well plate before being used in bioassay development. Fig. 2 shows the dose-response curves of steady state amperometric current (+650 mV) *vs.* concentration of hydrogen peroxide (mM), for a single device wherein

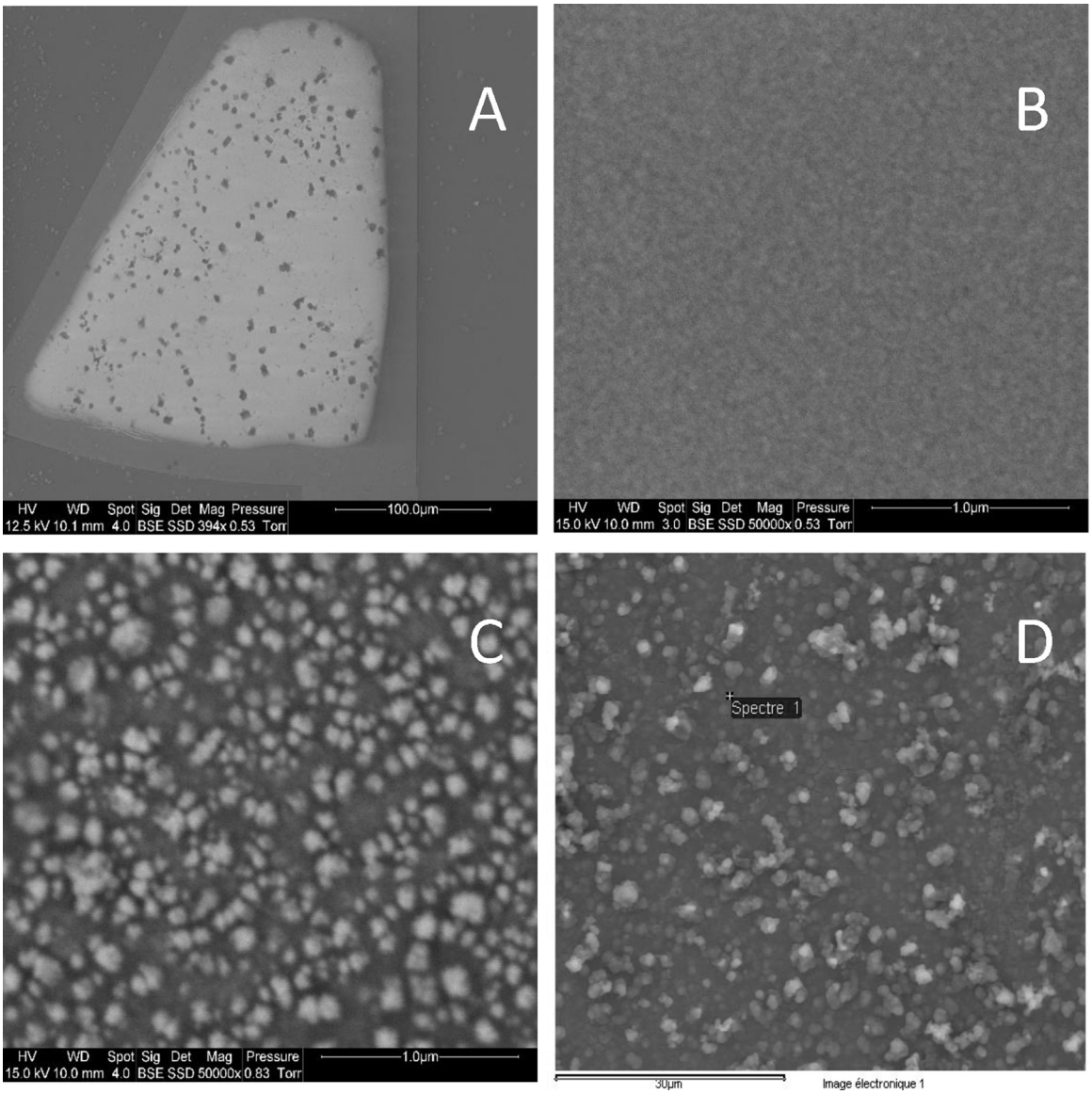

Fig. 3. SEM of (A) Silverized and chloridized reference electrode (100 µm), (B) The unmodified Pt microdisc electrode (1.0 µm), (C) Electropolymerized poly(aniline-*co*-meta-aminoaniline) with mesoporpous graphitized carbon (1.0 µm), and (D) the electrodeposited Fe|NiHCF multi-layer on Pt microdisc electrode (30.0 µm).

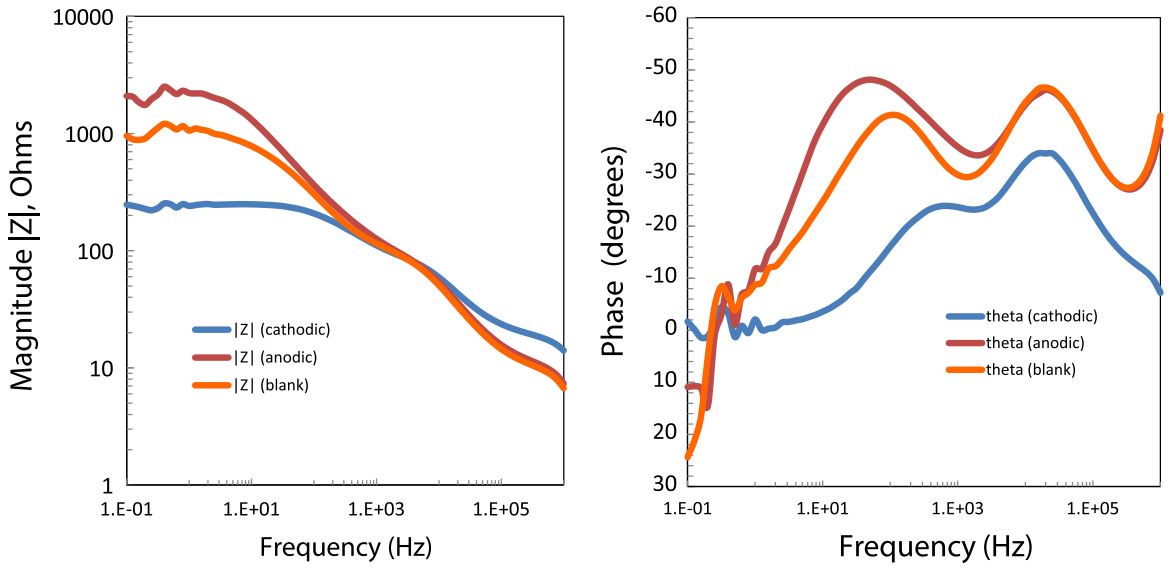

Fig. 4. Bode plots of the electrical impedance, magnitude (LHS) and phase angle (RHS), showing the blank IME device, the of electroconductive hydrogel electropolymerized onto the IME device and following multilayer mediator deposition. (40 mV p-t-p, 10^{-1}–10^{6} Hz, RT).

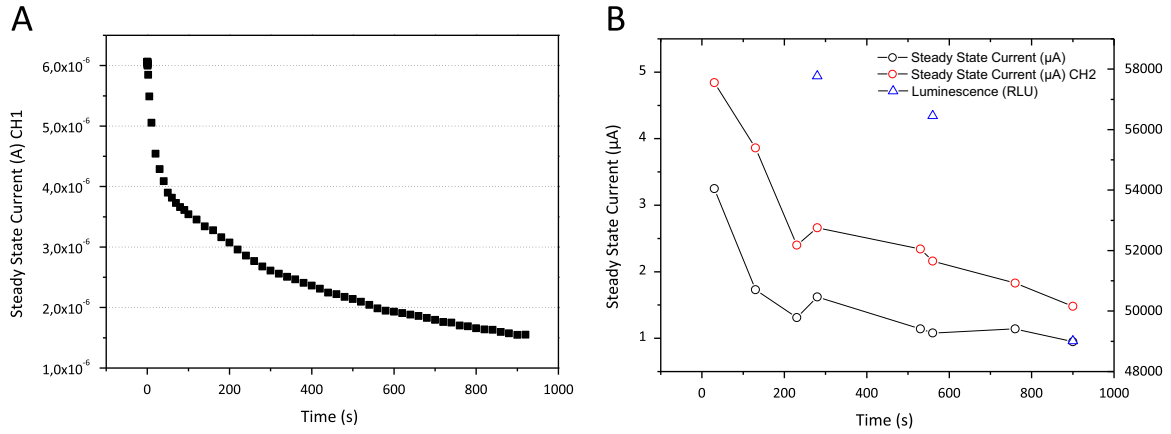

Fig. 5. A. Amperometric current response of Ch1 of an MDEA-PtIPAn-MGCINi-FeHCFIHG-NFO4 at 650 mV following incubation with OTA and OTA-HRP and following addition of 50/50 H_2O_2 and luminol solution. The current reflects decreases in peroxide concentration. B. Simultaneous measurement of the amperometric current response of Ch1 and Ch2 of an MDEA-PtIPAn-MGCINi-FeHCFIHG-NFO4 at 650 mV (LHS) and the luminesence (RHS) following incubation with OTA and OTA-HRP and following addition of 50/50 H_2O_2 and luminol solution.

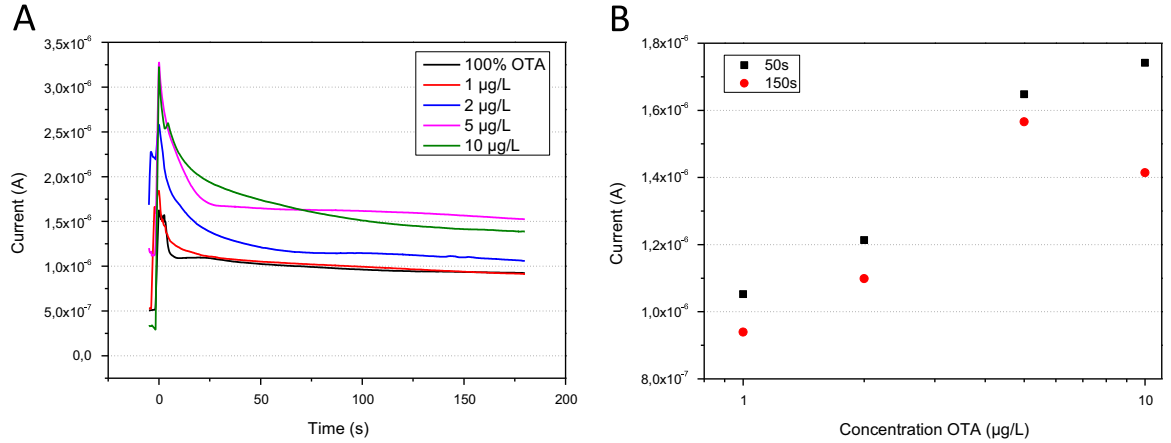

Fig. 6. Amperometric response of MDEA-PtIPAn-MGCINi-FeHCFIHG-NFO4 at 650 mV following competitive incubation with various concentrations of OTA (1–10 ug/L). A. Temporal amperometric current response at 650mV following competitive incubation with various concentrations of OTA and OTA-HRP and following addition of 50/50 H_2O_2 and luminol solution. B. Amperometric dose-response at 50 s and 150 s over the OTA concentration range 1–10 ug/L of the NFO4 peptide biochip.

the microdiscs of the WEA are: Ch1 was modified with the MGC layer and Ch2 was further modified by electrodepositon (−400 mV) of the redox mediator layer. The presence of the mediator layer clearly renders the device more sensitive to peroxide over this concentration range.

3.3. Electronmicroscopy of the biotransducer

Scanning electron microscographs (SEM) of the various steps in the realization of the amperometric transducer as shown in Fig. 3. Fig. 3A shows one of the silverized and chloridized reference electrodes of the chip. Fig. 3B is a micrograph of the pristine Pt surface (scale bar 1.0 μm) showing very little relief on that scale. Fig. 3C is a micrograph of the electropolymerized poly(aniline-*co*-meta-aminoaniline) containing entrapped mesoporpous graphitized carbon particles (scale bar 1.0 μm). MGC particles were found to be on the order of 70–100 nm in diameter. These particles are specified to have a pore volume of 0.25 cm^3/g, average pore diameter of ~137 Å (typical) and specific surface area 50–100 m^2/g. Fig. 3D is a micrograph of the electrodeposited Ni-FeHCF multilayer formed directly on platinum electrode (scale bar 30.0 μm). These particles are clearly angular, implying crystalline nature, and were ~35 nm. Energy dispersive X-ray analysis revealed the presence of Ni and Fe, although the ratio could not be specifically established.

3.4. Electrical impedance spectroscopy of biotransducer membranes

Shown in Fig. 4 is the electrical impedance spectra of MGC immobilized by anodic electropolymerization of poly(aniline-*co*-*m*-aminoaniline) (anodic) as well as following cathodic electrodeposition of the mixed HCF mediator layer (cathodic) onto the IME device (blank) as measured in pH 7.4 PBS at RT. The magnitude at low frequencies, approaching DC, informs the in-plane electrical conductivity of the membrane. The in-plane membrane magnitude is seen to increase, relative to PBS, following electropolymerization of the PAn-MGC composite layer. At pH 7.4 polyaniline is generally in the un-protonated form and is thus an insulator. What is noteworthy is that its overall conductivity is less than that of the PBS within which it is immersed. Upon cathodic electro-deposition of the Ni-FeHCF mediator layer, the impendence magnitude is seen to be less than that of free flowing PBS buffer. In general, cathodic conditions reduce PAn films and favor the pernigraldeine form. It is unclear if a redox relationship is established between the mediator and the p(An-*co*-AAn).

3.5. Biosensor-based Bioassay of OTA

A competitive ELISA based assay was realized on the functionalized microfabricated amperometric device with a concentration that was 10^{-6} M in the NFO4 peptide and a concentration that was 1000 μg $L^{-1}$of OTA-HRP. Both concentrations were selected based on previously published optimization studies (Soleri et al., 2015). After incubation of the device with OTA/OTA-HRP solution, devices were dipped into the luminol-hydrogen peroxide solution and the amperometric response was recorded continually by the potentiostat and periodically by the luminometer.

First, we test the efficiency of our new device by measuring the current response upon exposure to H_2O_2. As shown in Fig. 5A, the consumption of H_2O_2 by the OTA-HRP can be followed amperometrically as we observed a decrease in the amperometric current response from 6×10^{-6} A to approximately 1.5×10^{-6} A. These results are consistent with the consumption of H_2O_2 by HRP over time.

One advantage of current device configuration is simultaneous recording of the amperometric current response and the chemiluminescence. As shown in Fig. 5B, discrete current and luminescence measurements were recorded over a 15 minute period resulting in a decrease of the amperometric signal and an increase in the chemiluminescence signal over time. This figure also reflects the reproducibility of the two channels of the MDEA device. The noted difference is likely due to the difference in thickness of the hydrogel membrane layer that was deposited by dip coating of the discrete device in HEMA-AEMA cocktail. Dip coating creates a drip-edge (Kuznetsov and Xiong, 2002) which is thicker over Ch2 compared to Ch1 and which, as a form, supports more bioimmobilization' which can account for the larger signals on Ch2 compared to Ch1. The chemiluminescence signal, initially at background levels, rises rapidly to a maximum after 5 min and begins to decay thereafter.

Following demonstration that the device can be used in amperometric and luminescence mode, we then go on and test different concentrations of OTA (0 μg L^{-1}, 1 μg L^{-1}, 2 μg L^{-1}, 5 μg L^{-1}, 10 μg L^{-1}). Fig. 6A shows the temporal amperometric current response upon exposure to H_2O_2. We can observe a peak in the response corresponding to the time when the device is first dipped into the luminol-hydrogen peroxide solution followed by an approach to a steady state response. When we look at the transient response or rate (Fig. 6B), we can observe a linear dose response from 1.08 μA for 1 μg/L to 1.79 μA for 10 μg/L. While when you look at the results at the steady state current we observe an increase from 0.94 μA for 1 μg/L to 1.56 μA for 5 μg/L follow by a small decrease due to the hook effect at 10 μg/L with a response at 1.41 μA.

4. Conclusions

Microfabricated MDEA 5037-Pt dual channel chips can be physicochemically modified to support hydrogen peroxide discharge. The use of a Ni-FeHCF layer enhances the peroxide signal that is otherwise compromised when an electropolymerized polyaniline layer is first deposited to support hydrogel attachment. MDEA devices were found to be quite reproducible in their peroxide responses. However, bioassays implemented via bioimmobilization onto dip-coated poly(HEMA) foam compromised onboard channel reproducibility. Bioassays could be simultaneously amperometric and chemiluminescent. The amperometric implementation may be kinetic (rate) or steady state with the kinetic implementation offering a broader dynamic range. The use of amine rich biopolymers such as chitosan (previous works (Soleri et al., 2015)) or synthetic polymer (e.g. poly(HEMA-*co*-AEMA) hydrogel (current work) supports Zn^{2+} complexation and the use of his-tagged bioimmobilization to implement biosensor-based ELISA-type assays. The luminescence bioassay and the amperometric bioassay (50 s) are quite comparable in their dynamic range and detection limits.

References

Alarcón, S.H., Palleschi, G., Compagnone, D., Pascale, M., Visconti, A., Barna-Vetró, I., 2006. Monoclonal antibody based electrochemical immunosensor for the determination of ochratoxin A in wheat. Talanta 69, 1031–1037. http://dx.doi.org/10.1016/j.talanta.2005.12.024.

Ali, W.H., Derrien, D., Alix, F., Pérollier, C., Lépine, O., Bayoudh, S., Chapuis-Hugon, F., Pichon, V., 2010. Solid-phase extraction using molecularly imprinted polymers for selective extraction of a mycotoxin in cereals. J. Chromatogr. A 1217, 6668–6673. http://dx.doi.org/10.1016/j.chroma.2010.04.071.

Aresta, A., Vatinno, R., Palmisano, F., Zambonin, C.G., 2006. Determination of Ochratoxin A in wine at sub ng/mL levels by solid-phase microextraction coupled to liquid chromatography with fluorescence detection. J. Chromatogr. A 1115, 196–201. http://dx.doi.org/10.1016/j.chroma.2006.02.092.

Barathi, Palani, Kumar, A.S., Karthick, Minnal Ranjan Babu, 2011. Polynuclear nickel hexacyanoferrate/graphitized mesoporous carbon hybrid chemically modified

electrode for selective hydrazine detection. Int. J. Electrochem. 2011, 12 (Article ID 386023).
Bazin, I., Andreotti, N., Hassine, A.I.H., De Waard, M., Sabatier, J.M., Gonzalez, C., 2013. Peptide binding to ochratoxin A mycotoxin: a new approach in conception of biosensors. Biosens. Bioelectron. 40, 240–246. http://dx.doi.org/10.1016/j.bios.2012.07.031.
Bhat, R., Rai, R.V., Karim, A.A., 2010. Mycotoxins in food and feed: present status and future concerns. Compr. Rev. Food Sci. Food Saf. 9 (1), 57–81.
Blesa, J., Berrada, H., Soriano, J.M., Moltó, J.C., Mañes, J., 2004a. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. J. Chromatogr. A 1046, 127–131. http://dx.doi.org/10.1016/j.chroma.2004.06.086.
Blesa, J., Soriano, J.M., Moltó, J.C., Mañes, J., 2004b. Concentration of ochratoxin A in wines from supermarkets and stores of Valencian Community (Spain). J. Chromatogr. A 1054, 397–401. http://dx.doi.org/10.1016/j.chroma.2004.04.058.
Buckle, A.E., Coulter, J., Scudamore, K., 1985. Evaluation of an ELISA method for ochratoxin A analysis of cereals and compound feedstuffs. In: Proceedings of the 5th Meeting on Mycotoxins in Animal and Human Health. Edinburgh, Scotland, pp. 37–45.
Candlish, A.A.G., Stimson, W.H., Smith, J.E., 1986. A monoclonal antibody to ochratoxin A. Lett. Appl. Microbiol. 3 (1), 9–11.
Chen, X., Chen, Z., Tian, R., Yan, W., Yao, C., 2012. Glucose biosensor based on three dimensional ordered macroporous self-doped polyaniline/Prussian blue bicomponent film. Anal. Chim. Acta 723, 94–100.
Cruz-Aguado, J.A., Penner, G., 2008a. Fluorescence polarization based displacement assay for the determination of small molecules with aptamers. Anal. Chem. 80, 8853–8855. http://dx.doi.org/10.1021/ac8017058.
Cruz-Aguado, J.A., Penner, G., 2008b. Determination of ochratoxin A with a DNA aptamer. J. Agric. Food Chem. 56, 10456–10461. http://dx.doi.org/10.1021/jf801957h.
Du, D., Wang, M., Qin, Y., Lin, Y., 2010. One-step electrochemical deposition of Prussian Blue-multiwalled carbon nanotube nanocomposite thin-film: preparation, characterization and evaluation for H2O2 sensing. J. Mater. Chem. 20 (8), 1532–1537.
European Commission Regulation, 2005. European Commission Commission Regulation. No. 123/2005 Brussels.
European Commission Regulation, 2010. European Commission Commission Regulation, 2010. Off. J. Eur. Union 35, 7–8.
Giraudi, G., Anfossi, L., Baggiani, C., Giovannoli, C., Tozzi, C., 2007. Solid-phase extraction of ochratoxin A from wine based on a binding hexapeptide prepared by combinatorial synthesis. J. Chromatogr. A 1175, 174–180. http://dx.doi.org/10.1016/j.chroma.2007.10.057.
Guiseppi-Elie, A., Brahim, S., Slaughter, G., Ward, K., 2005a. Design of a subcutaneous implantable biochip for monitoring of glucose and lactate. IEEE Sens. J. 5 (3), 345–355.
Guiseppi-Elie, A., Brahim, S., Slaughter, G., Ward, K.R., 2005b. Design of a subcutaneous implantable biochip for monitoring of glucose and lactate. Sens. J. IEEE 5 (3), 345–355.
Guiseppi-Elie, A., 2011. An implantable biochip to influence patient outcomes following trauma-induced hemorrhage. Anal. Bioanal. Chem. 399 (1), 403–419.
Huybrechts, B., Tangni, E., 2010. Evaluation of Immunoassay Kits for Ochratoxin A Determination in Cereals. AFSCA-FAVV. CODA-CERVA, Belgium.
Jongwon, P., Chang-Soo, K., Minsu, C., 2006. Oxidase-coupled amperometric glucose and lactate sensors with integrated electrochemical actuation system. Instrum. Meas., IEEE Trans. 55 (4), 1348–1355.
Justin, G., Rahman, A.R.A., Guiseppi-Elie, A., 2009. Bioactive hydrogel layers on microdisk electrode arrays: cyclic voltammetry experiments and simulations. Electroanalysis 21 (10), 1125–1134.
Jørgensen, K., 2005. Occurrence of ochratoxin A in commodities and processed food–a review of EU occurrence data. Food Addit. Contam. 22 (1), 26–30. http://dx.doi.org/10.1080/02652030500344811.
Karyakin, A.A., Karyakina, E.E., 1999. Prussian Blue-based 'artificial peroxidase' as a transducer for hydrogen peroxide detection. Application to biosensors. Sens. Actuators B: Chem. 57 (1–3), 268–273.
Kotanen, C.N., Tlili, C., Guiseppi-Elie, A., 2013. Amperometric glucose biosensor based on electroconductive hydrogels. Talanta 103 (0), 228–235.
Kotanen, C., Karunwi, O., Guiseppi-Elie, A., 2014. Biofabrication using pyrrole electropolymerization for the immobilization of glucose oxidase and lactate oxidase on implanted microfabricated biotransducers. Bioengineering 1 (1), 85–110.
Krylov, A.V., Lisdat, F., 2007. Nickel hexacyanoferrate-based sensor electrode for the detection of nitric oxide at low potentials. Electroanalysis 19 (1), 23–29.
Kuznetsov, A.V., Xiong, M., 2002. Effect of evaporation on thin film deposition in dip coating process. Int. Commun. Heat Mass Transf. 29 (1), 35–44.
Lin, Y., Cui, X., 2006. Electrosynthesis, characterization, and application of novel hybrid materials based on carbon nanotube-polyaniline-nickel hexacyanoferrate nanocomposites. J. Mater. Chem. 16 (6), 585–592.
Liu, X.-P., Deng, Y.-J., Jin, X.-Y., Chen, L.-G., Jiang, J.-H., Shen, G.-L., Yu, R.-Q., 2009. Ultrasensitive electrochemical immunosensor for ochratoxin A using gold colloid-mediated hapten immobilization. Anal. Biochem. 389, 63–68. http://dx.doi.org/10.1016/j.ab.2009.03.019.
Lu, S., Lu, P., Li, C., Wang, C., Yu, J., 2014. Highly improved electrooxidation of captopril on copper hexacyanoferrate/ordered mesoporous carbon-modified glassy carbon electrode. Aust. J. Chem. 67 (6), 851–857.
Meulenberg, E.P., 2012. Immunochemical methods for ochratoxin A detection: a review. Toxins 4 (4), 244–266.
O'Brien, E., Dietrich, D.R., 2005. Ochratoxin A: the continuing enigma. Crit. Rev. Toxicol. 35 (1), 33–60.
Palmisano, F., Rizzi, R., Centonze, D., Zambonin, P.G., 2000. Simultaneous monitoring of glucose and lactate by an interference and cross-talk free dual electrode amperometric biosensor based on electropolymerized thin films. Biosens. Bioelectron. 15 (9–10), 531–539.
Pfohl-Leszkowicz, A., Bartsch, H., Azémar, B., Mohr, U., Esteve, J., Castegnaro, M., 2002. Mesna protects rats against nephrotoxicity but not carcinogenicity induced by ochratoxin a, implicating two separate pathways facta universitatis. Med. Biol. 9 (1), 57–63.
Prieto-Simón, B., Campàs, M., Marty, J.-L., Noguer, T., 2008. Novel highly-performing immunosensor-based strategy for ochratoxin A detection in wine samples. Biosens. Bioelectron. 23, 995–1002. http://dx.doi.org/10.1016/j.bios.2007.10.002.
Radi, A.-E., Muñoz-Berbel, X., Cortina-Puig, M., Marty, J.-L., 2009a. An electrochemical immunosensor for ochratoxin A based on immobilization of antibodies on diazonium-functionalized gold electrode. Electrochim. Acta 54, 2180–2184. http://dx.doi.org/10.1016/j.electacta.2008.10.013.
Radi, A.-E., Muñoz-Berbel, X., Lates, V., Marty, J.-L., 2009b. Label-free impedimetric immunosensor for sensitive detection of ochratoxin A. Biosens. Bioelectron. 24, 1888–1892. http://dx.doi.org/10.1016/j.bios.2008.09.021.
Rahman, A.R.A., Justin, G., Guiseppi-Elie, A., 2009a. Bioactive hydrogel layers on microdisk electrode arrays: impedimetric characterization and equivalent circuit modeling. Electroanalysis 21 (10), 1135–1144.
Rahman, A.R.A., Justin, G., Guiseppi-Elie, A., 2009b. Towards an implantable biochip for glucose and lactate monitoring using micro-disc electrode arrays (MDEAs). Biomed. Microdevices 11 (1), 75–85.
Rahman, A.R.A., Justin, G., Guiseppi-Wilson, A., Guiseppi-Elie, A., 2009c. Fabrication and packaging of a dual sensing electrochemical biotransducer for glucose and lactate useful in intramuscular physiologic status monitoring. Sens. J. IEEE 9 (12), 1856–1863.
Shan, G., Lipton, C., Gee, S.J., Hammock, B.D., 2002. Immunoassay, biosensors and other nonchromatographic methods. In: Lee, P.W. (Ed.), Handbook of Residue Analytical Methods for Agrochemicals. John Wiley & Sons, Ltd., Chichester, England.
Sitnikova, N.A., Borisova, A.V., Komkova, M.A., Karyakin, A.A., 2011. Superstable advanced hydrogen peroxide transducer based on transition metal hexacyanoferrates. Anal. Chem. 83 (6), 2359–2363.
Smith, J.E., Solomons, G., Lewis, C., Anderson, J.G., 1995. Role of mycotoxins in human and animal nutrition and health. Nat. Toxins 3 (4), 187–192, discussion 221.
Soleri, R., Demey, H., Tria, S.A., Guiseppi-Elie, A., Ibn Had Hassine, A., Gonzalez, C., Bazin, I., 2015. Peptide conjugated chitosan foam as a novel approach for capture-purification and rapid detection of hapten – Example of ochratoxin A. Biosens. Bioelectron. 67 (0), 634–641.
Thangaraj, R., Senthil Kumar, A., 2013. Simultaneous detection of guanine and adenine in DNA and meat samples using graphitized mesoporous carbon modified electrode. J. Solid State Electrochem. 17 (3), 583–590.
Varga, J., Kozakiewicz, Z., 2006. Ochratoxin A in grapes and grape-derived products. Trends Food Sci. Technol. 17, 72–81. http://dx.doi.org/10.1016/j.tifs.2005.10.007.
Visconti, A., Pascale, M., Centonze, G., 1999. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. J. Chromatogr. A 864, 89–101. http://dx.doi.org/10.1016/S0021-9673(99)00996-6.
WHO, 2002. Evaluation of Certain Mycotoxins in Food. Technical Report Series. WHO, Geneva, Switzerland, pp. 11–44.
Yang, L., Guiseppi-Wilson, A., Guiseppi-Elie, A., 2011. Design considerations in the use of interdigitated microsensor electrode arrays (IMEs) for impedimetric characterization of biomimetic hydrogels. Biomed. Microdevices 13 (2), 279–289.
Yu, J., Lai, E., 2010. Molecularly imprinted polymers for ochratoxin A extraction and analysis. Toxins (Basel), 1536–1553. http://dx.doi.org/10.3390/toxins2061536.
Zimmerli, B., Dick, R., 1995. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. J. Chromatogr. B Biomed. Sci. Appl. 666, 85–99. http://dx.doi.org/10.1016/0378-4347(94)00569-Q.