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Disposable label – free immunosensor on screen – printed electrodes for the detection of Aflatoxin B1 in food

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***Abstract* – An impedimetric label – free immunosensor on disposable screen-printed carbon electrode (SPCE) for Aflatoxin B1 detection has been developed in this work. SPCE surface was modified with gold nanoparticles and the monoclonal anti-AFB1 was immobilized on the working electrode through a cysteamine layer. After each coating step, the modified surface was characterized by Cyclic Voltammetry and Electrochemical Impedance Spectroscopy. The charge transfer resistance (R_{ct}) was chosen as the best parameter that describes the reproducible change in electrical properties of the electrode surface at different AFB1 concentrations and it was used to investigate the analytical parameters of the developed immunosensor. Under optimized conditions, the immunosensor showed a linear relationship between 0.01 and 4 ng/mL with a low detection limit of 0.01 ng/mL, making it suitable to detect AFB1 amount in many common food products. Lastly, the immunosensor was used to measure AFB1 in nuts samples with good results.**

I. INTRODUCTION

Health hazards from food products can be caused by infectious agents and toxic compounds: living microorganisms ingested with food can cause infectious diseases, while toxic substances lead to acute poisoning or have a long□term negative impact on the health of consumers [1]. Among toxic compounds, mycotoxins are a large group of secondary metabolites produced by certain fungi, with toxic effects against vertebrates and other organisms; they affect a broad range of agricultural products including cereals, cereal based foods, dried fruits, wine, milk, coffee beans, cocoa bakery or meat products, which are the basis of the economies of many developing countries [2].

In particular, Aflatoxins are toxic compounds which are produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* growing on a variety of food products and are known to be carcinogenic, mutagenic, teratogenic and immunosuppressive. Among them, aflatoxin B_1 (AFB_1), which is the most abundant and carcinogenic, was classified as a carcinogenic substance of group 1 by the International Agency for Research on Cancer (IARC).

In the European Union, the acceptable limits established by Commission Regulation (EC) N° 1881/2006 fro AFB_1 in various foodstuffs are ranged from 8 µg/kg for groundnuts to 0.1 µg/kg for dietary foods intended specifically for infants.

Several methods for aflatoxin B1 determination have been developed and reported in the literature. Conventional analytical methods for mycotoxin detection include thin-layer chromatography (TLC), high-performance liquid cromatography (HPLC) coupled with ultraviolet (UV), diode array (DAD), fluorescence (FD) or mass spectrometry (MS) detectors, gas chromatography (GC) coupled with electron capture (ECD), flame ionization (FID) or MS detectors and enzyme-linked immunoassay (ELISA). Though these methods are well known for their accurate and precise detection of mycotoxin in food or feed samples, they require skilled operators, extensive sample pretreatment, equipment and may lack accuracy at low analyte concentration [3, 4, 5]. Therefore, there is an increasing demand for rapid, sensitive and specific assay technique for the routine analysis of foods and beverages.

Electrochemical biosensors may be a good choice due to their fast, simple, and low-cost detection capabilities for biological binding events. In particular, among biosensors, the electrochemical immunosensors are currently widely investigated for toxin and mycotoxin detection due to their specific immuno-interaction, sensitive recognition and simple fabrication [6]: to transform the toxin interaction to an analytical signal, a variety of electrochemical techniques have been used, such as amperometric, potentiometric, conductimetric, impedimetric.

Among these, the application of Electrochemical Impedance Spectroscopy (EIS), as a transduction technology, enables the label-free detection and quantification of the immune complex and, thus, for the development of biosensors for food hazards [7]. EIS is a powerful informative and non-destructive technique due to the small voltage excitation used during detection, which can be used to study the electrical properties of the sensing device interface and tracing the reactions occurring on it.

An important aspect that affects the fabrication of an immunosensor is the choose of the immobilization technique of the sensing molecule on a proper transducer, in order to improve sensitivity, specificity and analyte – binding capacity. As regards the transduction surface, in recent years, the application of disposable screen – printed carbon electrodes (SPCEs), characterized by low – cost fabrication and mass production, have attracted an increasing interest for the development of immunosensor [8, 9, 10].

In real – time monitoring, signal amplification for label – free detection of small molecules such as AFB1 is a real challenge; nanomaterials, such as carbon nanotubes, metal nanoparticles, nanowires, nanocomposite and nanostructurated materials are playing an increasing role in the design of sensing and biosensing systems with interest for applications in food analysis [11]. Exploiting the advantages of gold nanoparticles (AuNPs), which have been extensively used as matrices for the immobilization of macromolecules, such as proteins, enzymes and antibodies, in addition to providing a microenvironment similar to what obtained under physiological conditions [12], it is possible to design a new electrochemical sensor by the modification of a working electrode surface.

Moreover, AuNPs have attracted considerable attention in electroanalysis because of their excellent physical and chemical properties, such as high surface to volume ratio, good electrical properties, strong adsorption ability and good surface properties. As highlighted in our previous work [8], the electrodeposition of metallic nanoparticles on an electrode surface is the best process deposition from solution; it is easy, fast and generates a stable surface. In addition, electrochemical deposition allows Au(III) to be addressed only on a polarised working electrode as Au(0) not on auxiliary or reference electrodes. Compared to gold electrodes, the electrodeposition of metallic nanoparticles on SPEs is more advantageous because of the unique properties offered by metallic nanoparticles [13].

On the basis of all previous considerations, the aim of this work is the development of an impedimetric label-free immunosensor for the detection of AFB1 realized on an AuNP-modified SPCE. Each step of electrode modification was characterized through EIS and CV, as well as the analytical performances of the immunosensors.

Finally the developed immunosensor, under optimized operative conditions, was used to quantify AFB1 in spiked nuts samples.

II. MATERIALS AND METHODS

A. *Chemicals*

Glutaraldehyde solution ($C_5H_8O_2$, 50 wt % in H_2O), cysteamine (C_2H_7NS, <98%), gold (III) chloride hydrate ($HAuCl_4$, 99.9%), sulfuric Acid (H_2SO_4, 99.9%), ethanolamine ($NH_2CH_2CH_2OH$), >99.5%) potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, 99%) were purchased from Sigma-Aldrich (Milan, Italy). Potassium ferrocyanide ($[Fe(CN)_6]^{4-}$) was obtained from Carlo Erba reagent (Milan, Italy). Anti-Aflatoxin B1 antibody (anti-AFB1) (1 µg/mL) was purchased from Microtech and Aflatoxin B1 were obtained from LKT Laboratories (Saint Paul, USA). NaH_2PO_4, Na_2HPO_4, NaCl and KCl used in the preparation of phosphate – buffered saline (PBS 0.1 M KCl, pH 7.4) were also obtained from Sigma Aldrich (Milano, Italy).

B. Apparatus

The electrochemical measurements were carried out with a compuer – controlled Autolab PGSTAT 204 Potentiostat (Methrom), equipped with an impedance module (FRA32M) and the experimental date were analysed with Nova software (Metrohm). Screen-printed carbon electrodes (SPCEs) based on a three-electrode layout (working/auxiliary/reference) were produced according to Albanese et al. 2012 [14]. The diameter of the working electrode was 2.8 mm.

C. *Immunosensor manufacturing*

Before any modification, SPCEs were cleaned through an electrochemical treatment at 1.7 V vs. Ag/AgCl as a reference electrode for 360 s in PBS. The electrochemical AuNPs deposition was carried out according to our previous study [8] using a solution of 1 M $HAuCl_4$ in 0.5 M H_2SO_4 under a constant potential of -0.4 V vs. Ag/AgCl for 300 s. After that, cysteamine 20 mM was dropped onto the AuNPs modified working electrode and a constant potential of 1.2 V vs. Ag/AgCl fro 20 min was applied. After the electrode was rinsed with water to remove physicallty – adsorbed cysteamine, 100 ⌈L of glutaraldehyde solution 5% (v/v) were dropped onto the modified electrode for 30 min and then, again, rinsed with water.

Different concentrations of anti – AFB1 solution (0.5 ⌈g/mL, 1 ⌈g/mL, 2 ⌈g/mL) were dropped on the modified electrode for 30 min at room temperature. After the immobilization step, ethanolamine 1 M (pH 8.5) for 15 min was used to block unreacted active sites.

D. *Experimental Measurement*

Electrochemical Impedance Spectroscopy (EIS) and Cyclic Voltammetry (CV) were used to characterized each step of the electrode modification. For the impedance measurements, a sinusoidal AC potential (10 mV) in the frequency range from 0.1 to 10^4 Hz was superimposed to 0.00 mV DC potential. The impedance spectra were plotted in the form of Nyquist plots, where the complex impedance is displayed as the sum of real (Z') and imaginary (Z'') components. All measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-/3-}$, in PBS pH 7.5 as background electrolyte at room temperature. The voltammetric measurements were performed from -0.6 to 0.6 V vs Ag/AgCl with a scan rate of 0.05 mV/s; the redox couple used for the CV was the same used for EIS measurements. For the AFB1 analysis, 50 ⌈L of AFB1 at different concentrations in PBS were dropped onto the working area of the immunosensor and incubated for 30 min. Before the impedance measurements, the immunosensor was rinsed thoroughly with copious amounts of PBS.

E. *Preparation of nuts sample for AFB1 measurement*

For nuts sample analysis, two grams of crushed homogenate were added in 10 mL of 70% methanol solution, oscillated for 5 min and centrifuged at 4000 rpm/min for 10 min at room temperature. 0.5 mL of supernatant were added to 0.5 mL of deionized water and mixed fully. Before the extraction procedure, samples were spiked with known concentrations of AFB1 in order to obtain 0.1 ppb, 0.5 ppb, 1 ppb, 2 ppb.

III. RESULTS

A. *Surface modification of the electrode and immobilization process*

The surface modification of the Au electrodes for the preparation of AFB1 immunosensor was monitored using EIS and CV.

As expected, the working electrode modified with AuNPs exhibited an increase of the anodic and cathodic peaks (Fig. 1); this behavior suggests a slight improvement in the electrocatalytic properties of the electrode produced by the addition of the AuNPs, which facilitated the electron-transfer process.

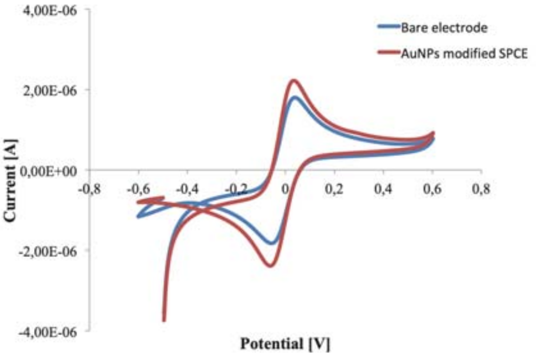

Fig.1. Cyclic Voltammograms of bare and AuNPs-modified SPCE recorded in 1 mM $[Fe(CN)_6]^{3-/4-}$ in PBS pH 7.5

Impedimetric spectra for the bare SPCE and AuNPs-modified SPCE are shown in fig. 2.

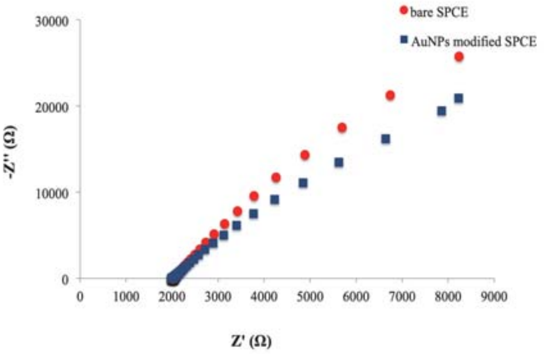

Fig.2. Nyquist plots in impedance measurements of bare and AuNP-modified SPCEs

The linear spectra, observed before and after the electrodepositon of AuNPs, is caused by a faster mass-transfer limited process due to electron flow from the electron surface in the bulk solution. This behavior indicates a purely capacitive response of the electrode properties [15].

The cysteamine layer, as well as the consecutive immobilization of monoclonal anti-AFB1 and ethanolamine, caused a decrease of the electron transfer rate with a lowering of both anodic and cathodic peaks due to the hindering effect of the layers [8] (Fig. 3).

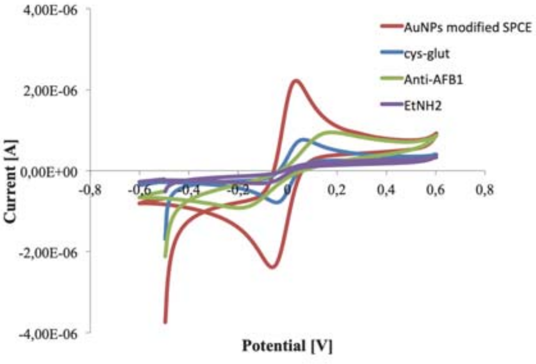

Fig.3. Cyclic Voltammograms in 1 mM $[Fe(CN)_6]^{3-/4-}$ in PBS pH 7.5 during the fabrication of immunosensors

Nyquist plots related to each preparation step showed a change of both real (Z') and imaginary (Z'') component of the total impedance (Z) of the system (Fig. 4).

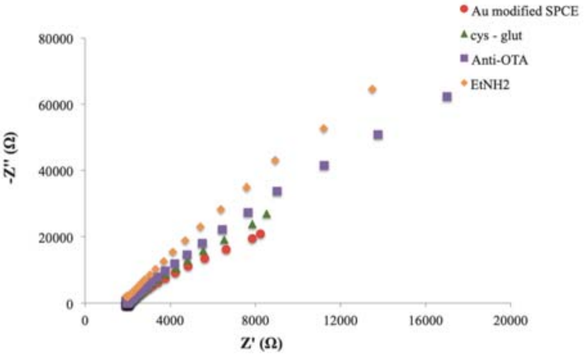

Fig.4. Nyquist plots in impedance measurements during the fabrication of the immunosensor

In particular, the increase of Z components is due to a change in the electron transfer resistance caused by the blocking layer coating on the surface of the Au modified electrode, which became thicker with the progress of functionalization of the electrode surface. A further increase of the impedance was observed when the anti-AFB1 was immobilized.

B. Optimization of anti-AFB1 concentration

Because the performances of an immunosensor depends strongly on the immunoreaction between the antigen and the antibody, the optimization of the amount of anti-AFB1, that affect the capability of the antibody to detect the target in the range of interest, is a crucial step in the immunosensor construction. For this purpose, immunosensors were developed with three different amounts of monoclonal anti-AFB1 (0.5 μg/mL, 1 μg/mL, 2 μg/mL) and after incubated with different concentrations of AFB1. In all cases, a decrease of the impedance in Nyquist plots was observed by increasing AFB1 amount.

All Nyquist plots were fitted by the Randle's equivalent circuit (fig. 5), who showed that the Electron Transfer Resistance (R_{ct}) was the parameter most influenced by the increasing of AFB1 amount. This parameter was used to characterize the immunosensors fabricated.

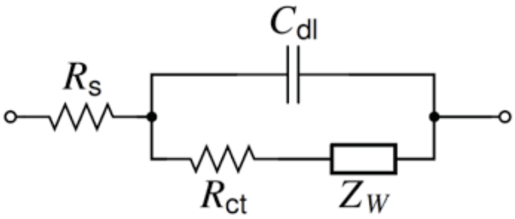

Fig.5. Randle's circuit for impedimetric data fitting

The calibration curves for all immunosensors were obtained by plotting the value of AFB1 concentration versus $\Delta R_{ct}\%$. ΔR_{ct} %was calculated by the following equation:

$$\Delta R_{ct}\% = \frac{R_{ct(antiAFB1)-R_{ct}(AFB1)}}{R_{ct(antiAFB1)}} * 100 \tag{1}$$

where $R_{ct(antiAFB1)}$ is the value of electron transfer resistance when anti-AFB1 is immobilized on the electron surface and $R_{ct(AFB1)}$ is the value if electron transfer resistance after the bind between anti-AFB1 and AFB1. The calibration curves of the AFB1 immunosensors with 0.5 μg/mL, 1 μg/mL and 2 μg/mL were shown in Fig. 6, while in Table 1 were summarized the principal analytical parameters of the three developed immunosensors

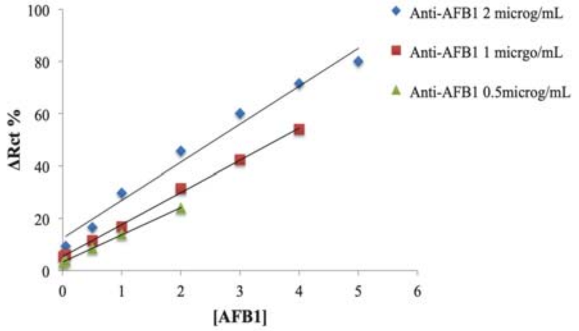

Fig.6. Calibration curves of AFB1 immunosensors at 0.5 μg/mL, 1 μg/mL and 2 μg/mL anti-AFB1

Table 1. Comparison among analytical parameters of developed immunosensors.

Anti-AFB1 amount [μg /mL]	Sensitivity [ΔR_{ct}%/(ng/mL)]	Linear Range [ng/mL]	LOD [ng/mL]
0.5	10.44	0.01 - 2	0.01
1	12,27	0.01 - 4	0.01
2	14.53	0.05 - 5	0.05

The immobilization of the highest anti – AFB1 amount leads to highest sensitivity even if best result for LOD were registered with 0.5 - 1 anti – AFB.

As reported in our previous study [16], a higher antibodies amount allow obtaining higher sensitivity due to the higher antigen-binding capacity but a lower antibody amount allow detecting lower AFB1 amount, allowing estimating smaller variations of impedance.

The reproducibility, calculated on five different immunsensors, showed a good relative standard deviation (RSD) for all immunosensors: 4.02% for 0.5 μg /mL anti-AFB1, 4.89% for 1 μg /mL anti-AFB1 and 5.12% for 2 μg /mL anti-AFB1.

Because the immunosensor developed with 1 μg /mL anti – AFB1 showed the lowest LOD, an acceptable linear range and a good sensitivity, it is chosen as the best one for the analysis in food products.

Taking into consideration the dilution factors used for the AFB1 detection in food matrices that can range from 5 to 20, the developed immunosensor shows a detection limit of 0.05 μg /kg and 0.2 μg /kg respectively, highlighting its potential as device for fast Aflatoxin B1 measurement in infant and adult food products.

C. *Measurement of AFB1 in nuts sample*

The immunosensor developed with 1 μg /mL antibody was used to analyze nuts samples spiked with known concentrations of AFB1. The results were reported in Table 2.

Table 2. AFB1 results nuts samples obtained by immunosensor.

Spiked concentration [ppb]	Found concentration [ppb]	Recovery [%]
0.1	0.11	90
0.5	0.49	98
1	1.05	95
2	1.99±0.03	99,5

The results highlight the immunosensor capability as device for fast Aflatoxin B1 measurement in many food products

IV. CONCLUSIONS

A label-free immunosensor for AFB1 detection based on EIS on a modified SPCE is reported in this work.

The modification of carbon electrode surface with AuNPs led to an improvement of the electrochemical parameters of the electrode.

The immobilization technique was optimal and the anti-AFB1 concentration of 1 μg/mL was the best, in order to obtain a device with good analytical performances, making the developed immunosensor suitable to detect AFB1 amount in many common food products.

Finally, under optimized conditions, the developed immunosensors was used to analyze nuts sample spiked with known concentrations of AFB1, obtaining good results.

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