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Initial investigations for detection of aflatoxin B1 through nanostructured membranes

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Abstract

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Mycotoxins are toxic and cancerogenic compounds produced by certain types of fungi. Fungi that produce mycotoxins grow on feed and food products. Most mycotoxins are chemically stable and survive food processing. Their toxicity occurs at very low concentrations, and therefore, there is a need of sensitive and reliable methods for their detection. Along the used conventional methods, nanomaterials are becoming increasingly widespread materials for detection of different residue. They could be functionalized with various biomolecules, such as enzymes, antibodies, nucleic acids, making them suitable for detection of various substances, such as bacteria, food and feed toxins. Among mycotoxins, aflatoxins are the most significant mycotoxins for agriculture. They could induce various toxic processes in humans and animals. For this purpose, nanostructured polyethylene terephthalate (PET) membranes with pore size dimensions 170±4 nm and without being modified on pores surface were tested for aflatoxin B1 (AFB1) detection. The detection principle was based on two methods, namely by diffusion and cyclic voltammetry. The challenge was to see if the molecules of mycotoxin could enter in the nanopores and catch to the non-modified inner walls of the membrane. In cyclic voltammetry study when there is a presence of AFB1 inside the nanopore it induces blockage of the ionic current. Using a nanoporous membrane the mycotoxins could be detected at the nanogram level, indicating that such membranes are promising candidates for detection of very small quantities mycotoxins in aqueous solution. New experimental protocol allows obtaining information about the nanoporous membranes tested if they could play a role as detectors for mycotoxins.

Keywords: aflatoxin B1; cyclic voltammetry; diffusion; mycotoxins; nanodetectors; nanoporous membranes

Introduction

The growth of fungal species in various feeds and foods leads to the production of metabolic substances called mycotoxins. They are low molecular weight compounds produced by various genera such as *Aspergillus, Fusarium, Penicillium* spp. (Creppy, 2002).

As natural contaminants they occur in many feed matrices like wheat, barley, maize, beans, oilseeds and etc.

Guan et al. (2011) reported about 98% of the agricultural commodities, including corn, compound animal feeds, silage, cornmeal, puffed corn, wheat, bran, soybean, meal, rapeseed meal, cottonseed meal content various groups of mycotoxins.

The presence of mycotoxins in feed has severe implications on animal health even at very low levels, due to their mutagenic, teratogenic, carcinogenic, nephrotoxigenic, and immunosuppression effects (Fernández et al., 2017). The

most studied mycotoxins are aflatoxins due to their wide presence in food and feed materials (Marchese et al., 2018). This makes AFB1 a significant object of interest and the development of new analytical tools for its detection in food and feed matrices (Jallow et al., 2021).

Currently, chromatographic techniques are the most commonly used for mycotoxin analysis and provide adequate information on exposure levels.

According to Pascale and Visconti (2008), sensitive methods are needed to detect very low concentrations of mycotoxins.

Comparing with the traditional analytical methods used for mycotoxins analysis, the main advantages of nanobiosensors include the fast analysis time and rapid detection, high sensitivity, easy sample preparation, and low cost.

Nanomaterials such as gold, silver, metal oxides and quantum dots have been extensively employed for enhancing the detection capability of biosensors due to their remarkable optical, electronic, and thermal properties. This allow the increase of their sensitivity, stability, and selectivity towards mycotoxins (Santos et al., 2019).

Track-etched membranes are nanomaterials featuring nanochannels grafted with various polymers, which makes them promising for various applications (Barsbay and Güven, 2014). There are three main kinds of nanochannels, biological channels, synthetic solid state nanochannels, and hybrids of biological and synthetic nanochannels (Haque et al., 2013). The solid-state nanochannels fabricated in track-etched membranes have a great potential in biotechnological applications as biomolecule sensors (Ali et al., 2013), stimuli-responsive devices, molecular filters with high selectivity (Savariar et al., 2008), and nanofluidic diodes (Ali et al., 2009).

The development of biosensors for mycotoxins detection has risen sharply in the recent decades with a large number of different bio-sensing technologies application. In the mid-1980s, some research groups started electrochemical studies at electrodes coated with membranes comprising uniform cylindrical nanopores such as nanoporous anodic alumina membranes and track-etched polymer membranes. Mainly nanoporous alumina membranes have been deeply studied in electrochemical-based nanopore immunosensors (Toyos-Rodríguez et al., 2024).

Nanopore sensing relies on the monitoring of current fluctuations between two chambers filled with an electrolyte solution through a nanopores inside an insulating membrane (Xue et al., 2020). The passage of an analyte through the pores reduces the current recorded in a specific signature, associated to the size, charge or sequence of the target molecule (Toyos-Rodríguez et al., 2024).

The aim of the present study was to test, through the diffusion process and cyclic voltametry, the ability of nanostructured PET membranes to detect mycotoxins.

Materials and Methods

Instrument

Scanning Electron Microscope (SEM) measurements were performed on LYRA I XMU (TESCAN). After etching, the samples were sputter-coated with a thin layer of gold and inspected in order to determine the pore diameter.

In parallel the samples were studied using ATR-FTIR spectroscopy by directly fixing a small pirce of the membrane on top of the ATR accessory. The spectra were measured in the interval 4000–600 cm^{-1} by accumulating 100 scans at a resolution of 2 cm^{-1} on a FTIR Bruker Invenio R FT spectrometer with a diamond crystal ATR accessory (Pike Technology, Madison, WI, USA).

Chemical etching of irradiated polymer membranes

The nanoporous membranes used in this work were prepared by track-etched technology where the PET membranes (thickness 12 microns) were irradiated with swift heavy ions at GANIL accelerator (Caen, France) under normal beam incidence. The irradiated membranes were chemically etched in a way to create nanopores (channels) along the ion-tracks using alkaline solution. Basically, they were immersed in a 2N aqueous NaOH solution at 80°C for 4 min. They were then rinsed with deionized water and air-dried to evaporate residual water from the pore's channels.

As mycotoxin we used AFB1 in standard solution 25 µg/10 ml from Trylogy (USA).

Electrochemical investigations

Electrochemical measurements were performed inside an electrochemical cell on Potentiostat: Gamry 1000 by using as reference electrode: Ag wire and counter electrode: carbon rod. Ferrocene (98%) was obtained from Acros, $LiClO_4$ (99.9%) from Sigma Aldrich and acetonitrile (99.5%) from Honeywell. The mycotoxin was in solution in acetonitrile and to detect it in this medium ferrocene was selected as a redox probe. A solution of ferrocene at 10 mmol/L in 0.5 mol/L $LiClO_4$ in acetonitrile was used. A first series of experiment deal with the selection of a suitable working electrode for the electrochemical cell used. Platinum was the one that allows to clearly monitor the signal of ferrocene.

Results and Discussion

The obtained nanostructured PET membranes were tested if they could play a role as detectors of mycotoxins, through

the diffusion process. In Figure 1 are shown SEM image and FTIR spectrum of chemical etched PET membrane.

Basically, the peaks in FTIR spectra give an information about molecular bonding structure or functional groups existing in the material. To capture a mycotoxin molecule, maybe is necessary the presence of antibody or modification with an appropriate chemical agent. In our case, the nanoporous membranes were not chemically modified or coated with antibodies. The challenge was to see if it is possible for the mycotoxin molecule to attach to the inner walls of the nanopore through a diffusion process alone.

In first scenario, the nanostructured polymer membrane was immersed in AFB1 in standard solution with concentration 5 µg/ml. After overnight stay, the membrane was rinsed and examined by SEM and FTIR spectroscopy. In Figure 2 (cross-section SEM micrograph), no objects can be seen that has stuck to the inner walls of the nanopores. This is also confirmed by FTIR spectroscopy measurement (Figure 2), which shows that there are no changes in the band intensity or a new band which corresponds to aflatoxins structure appears.

This may be due to the fact that the mycotoxin molecules entering the nanopores are not trapped there and pass through. Therefore, they are not registered in the physicochemical studies. On the other hand, the membrane is probably not attractive. Because, without being immobilized with biorecognition objects, such as antibodies, it makes it difficult for mycotoxins to attach to the nanopores through the diffusion process. From these results, becomes clear that by diffusion process and without antibody immobilization or chemical structures modification the nanopores could not play a role as nanodetectors for mycotoxins.

On the other hand, it is interesting to mentioned that the same type nanoporous PET membrane but in other conditions was used as template for nano objects synthesis by diffusion process (Sertova et al., 2006). When expected the same type membranes to play a role as antigen trappers, obviously, the principle is not the same.

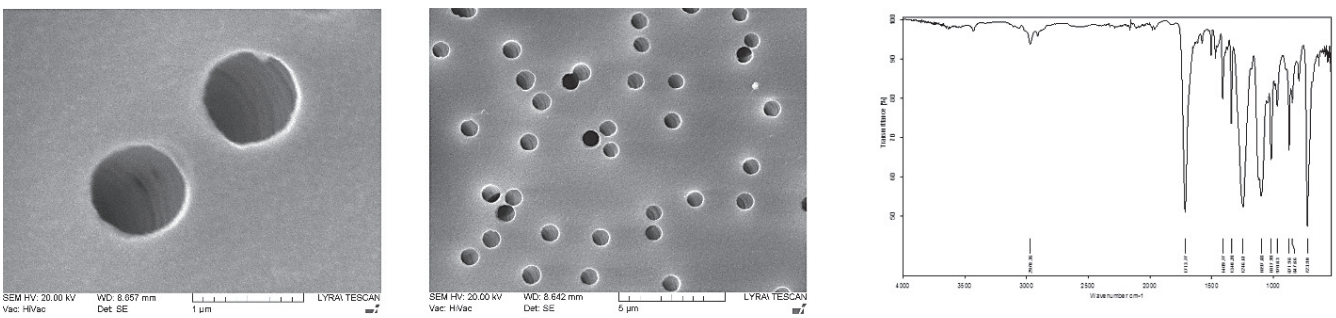

Fig. 1. SEM top-view and FTIR spectrum of the obtained single pore and PET nanoporous membrane

Source: Authors' own elaboration

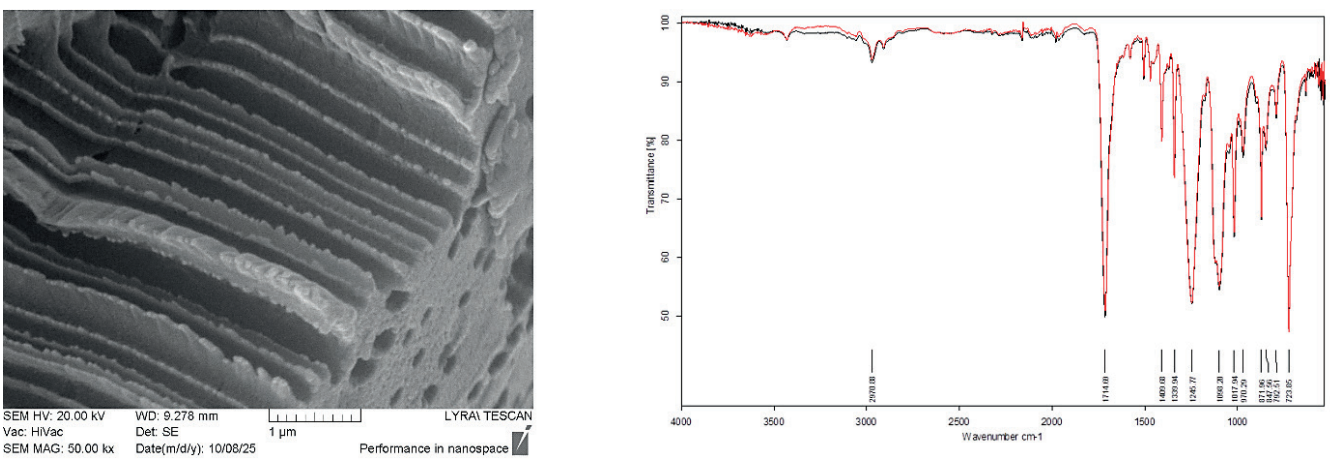

Fig. 2. Cross-sectional view SEM image and FTIR spectrum of non-modified PET membranes tested for AFB1 during diffusion

Source: Authors' own elaboration

Other type nanostructured polymeric membranes for detection of mycotoxins are synthesized in situ. They are used as for selective recognition of AFB1. Artificial binding sites were formed in the structure of the polymeric membranes by method of molecular imprinting (MIP). The fluorescent sensor system based on the optimized MIP membranes provided a possibility of AFB1 detection within the range 14 – 500 ng mL^{-1} (Sergeyeva et al., 2017).

Rovina et al. (2019) in their work developed third-generation enzymatic biosensor to quantify sterigmatocystin (STEH). It was based on a glassy carbon electrode modified with a composite of the soybean peroxidase enzyme (SPE) and chemically reduced graphene oxide. A third-generation enzymatic biosensor to quantify STEH in corn samples spiked with the mycotoxin. The biosensor was based on glassy carbon (GC) electrode, modified with a composite of SPE and chemically reduced graphene oxide (CRGO). The biosensor was also used to determine STEH in corn samples inoculated with *Aspergillus flavus*, which is an aflatoxins fungus producer. The biosensor showed a linear response in the concentration range from 6.9×10^{-9} to 5.0×10^{-7} mol L^{-1}. The limit of detection was 2.3×10^{-9} mol L^{-1}.

To see if the membrane could otherwise trap the mycotoxin molecules in aqueous solution, we moved on to a second scenario, and namely to test it through electrochemical investigations.

The idea was by cyclic voltammetry to determine whether there would be a decrease in the signal, which could be associated with the attachment of a mycotoxin molecules to the inner walls of the nanoporous membrane.

For this purpose, non-porous PET membrane was fixed on the surface of the working electrode no signal could be obtained after 24 hours as a consequence of the insulating effect of the film between electrode and electrolyte (Figure 3).

On the other hand, when a porous membrane was fixed on the surface of the working electrode, although no signal could be measured just after fixing the membrane, the ferrocene signal was detected after waiting overnight. This result could be interpretated as time needed to ferrocene in acetonitrile solution to penetrate inside the pore and reach the surface of the working electrode. Mycotoxin was added to the electrolyte in order to reach a concentration of 5 µg/mL. The signal was recorded regularly (Figure 4).

During the first 5 hours the signal slightly changes with no tendency (Figure 4). But between 5h20 and 12h00 a continuous decrease of both oxidation and reduction peak currents was monitored. This could be interpretated as the filling of the pores with mycotoxin reducing the accessibility of ferrocene to the platinum electrode surface area (Figure 5).

Probably specific interactions occur between the mycotoxin and carbonyl groups, which are formed during the etching of PET. Here we demonstrate a nanopore/nanochannel-based system capable of detecting low concentrations of mycotoxins without sample preparation.

If we assumed that the membrane pores are filled with a 5 µg/mL aflatoxin solution, the total amount of aflatoxin

Fig. 3. Photographs of lab electrochemical workstation and detection device built for measurement of AFB1, (Cergy Paris Université, LPPI)

Source: Authors' own elaboration

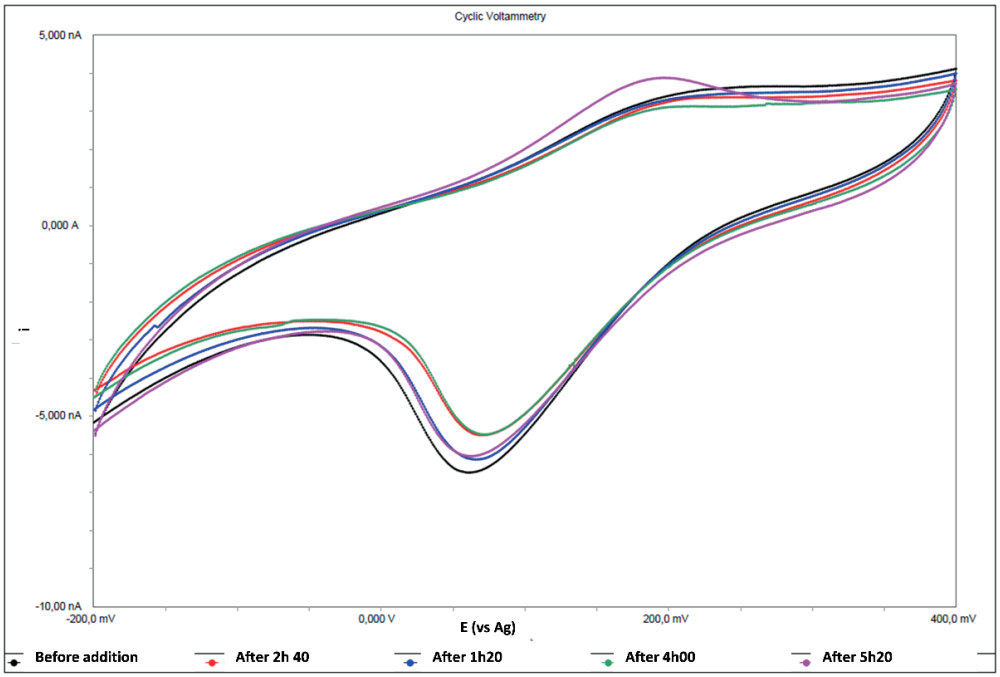

Fig. 4. Cyclic voltammograms of ferrocene solution (10 mM) in LiClO4 (0.5 M) in acetonitrile at 1 mV/s before and at different time during the first 5 hours of contact of the porous membrane with a solution of mycotoxin at 5 µg/mL

Source: Authors' own elaboration

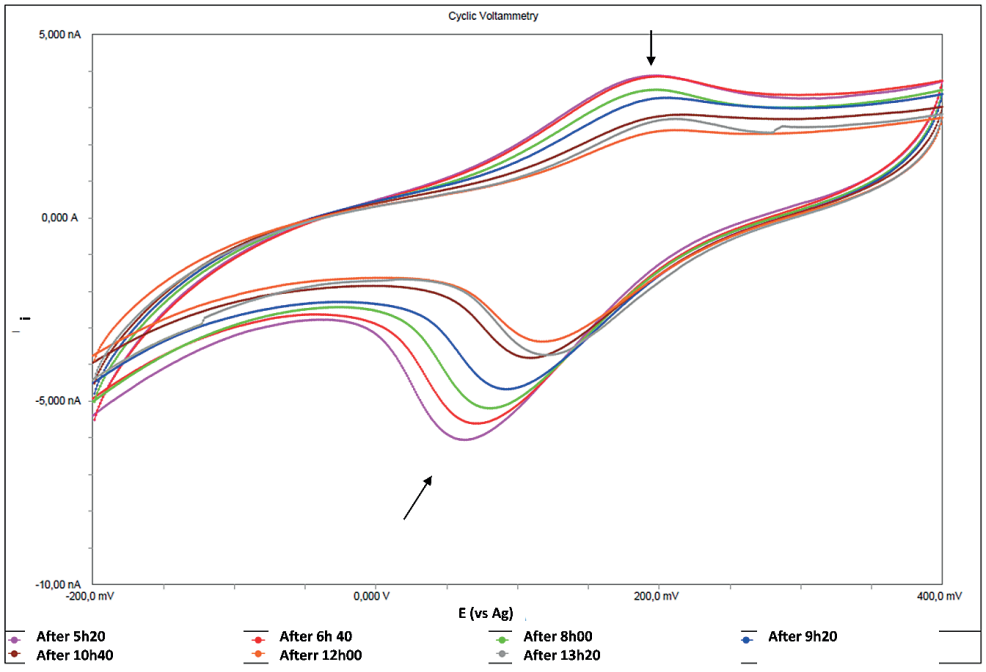

Fig. 5. Cyclic voltammograms of ferrocene solution (10 mM) in LiClO4 (0.5 M) in acetonitrile at 1 mV/s at different time between 5h20 and 13h20 of contact of the porous membrane with a solution of mycotoxin at 5 µg/mL

Source: Authors' own elaboration

contained in the nanoporous membrane could be calculated as follows:

$V_{pore} = \pi r^2 h$

$V_{pore} = \pi(85\times10^{-9})^2(12\times10^{-6}) = 2.72\times10^{-19}\ m^3$

Total pore volume is calculated as follows:

$V_{total} = 2.72x10^{-19}\times10^{9} = 2.72\times10^{-10} m^3$

$V_{total} = 2.72x10^{-10}\times10^{6} = 2.72\times10^{-4} mL$

Aflatoxin mass inside pores:

Mass = concentration x volume = 5000 ng/mL × 2.72×10^{-4} mL

Mass = 1.36 ng

The nanoporous membrane should contain 1.36 ng aflatoxin. This is expected to bring new benefits and it indicating that nanoporous PET membranes could be future tool to mycotoxin detection.

Similar to our research, Xue et al. (2020) observed current fluctuations between two chambers, filled with an electrolyte solution through a nanopore placed inside an insulating membrane. Passage of the analyte through the pore reduces the current registered in a specific signature related to the size, charge, or sequence of the target molecule.

On the other hand, Jiang et al. (2015) developed by linear sweep voltammetry a sensitive electrochemical molecularly-imprinted sensor for detection of AFB1, by electropolymerization of *p*-aminothiophenol-functionalized gold nanoparticles in the presence of AFB1 as a template molecule. The extraction of the template leads to the formation of cavities that are able to specifically recognize and bind AFB1 through π-π interactions between AFB1 molecules and aniline moities.

The presence of AFB1, ochratoxin A (OTA) or fumonisin B1 (FB1) inside the nanopore induces reversible blockage of the ionic current flowing through the nanopore, with distinct characteristics of blockage (Toyos-Rodríguez, 2024).

Silva et al. (2023) consider that other promising candidates for detection of mycotoxins in aqueous solution are unitary alphatoxin nanopore. In their study, they demonstrate that increasing the KCl concentration in solution causes an increase in the frequency of blockage of the ionic current induced by the presence of mycotoxins in aqueous alphatoxin nanopore. They understand that by changes in a blockage could be detected besides aflatoxins, OTA or FB1, where inside the nanopore they induced blockage of the ionic current which is unique to each of the three toxins.

Along the classical methods for mycotoxins detection, the sensors based on nanostructured materials are more hopeful because of less time consuming for analyses, rapid detection, and mobility.

Conclusions

Nowadays, improvements are still needed for more sensitive, accurate and rapid mycotoxin biosensors. In this connection our attention point to polymer nanoporous membranes as a new alternative for fast detectors of mycotoxins.

The results show that without any surface modification of the membrane mycotoxin's molecules can not catched in the inner walls of the nanopores through difussion process. This prompted us to change the methodology, where we demonstrate that by cyclic voltammetry is possible to detect the blockage of the ionic current induced by the presence of mycotoxins in the nanopores.

In addition, the ability to detect mycotoxins at very low level indicates that the nanoporous membranes could be promising tool for analysis of mycotoxins in aqueous solution. The obtained information will help us in our future investigations in manner to analyze very small quantities of mycotoxins and to determined limit detection.

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Conflicts of Interest

The authors declare no conflict of interest.

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