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Detection of parathion and patulin by quartz-crystal microbalance functionalized by the photonics immobilization technique

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ABSTRACT

Oriented antibodies are tethered on the gold surface of a quartz crystal microbalance through the photonics immobilization technique so that limit of detection as low as 50 nM and 140 nM are achieved for parathion and patulin, respectively. To make these small analytes detectable by the microbalance, they have been weighed down through a "sandwich protocol" with a second antibody. The specificity against the parathion has been tested by checking the immunosensor response to a mixture of compounds similar to parathion, whereas the specificity against the patulin has been tested with a real sample from apple puree. In both cases, the results are more than satisfactory suggesting interesting outlook for the proposed device.

1. Introduction

The effective detection of small molecular weights analytes is of paramount importance in a wide range of scientific topics like investigating the molecular recognition phenomena and sensing of toxic molecules (Cooper and Singleton, 2007; Geschwindner et al., 2012; Jones et al., 2013; Vashist and Vashist, 2011). In particular, in the field of environmental monitoring it would be of great importance the availability of cost-effective and sensitive tools allowing the detection of low soluble and harmful compounds like steroids, herbicides, pesticides, toxins and combustion products like polycyclic aromatic hydrocarbon (PAH). As case studies to test our approach, we focused on parathion (IUPAC name O,O-diethyl O-4-nitrophenil phosphorothioate, MW=297 Da) and patulin (IUPAC name 4-hydroxy-4,6-dihydrofuro[3,2-c]pyran-2-one, MW=154 Da), which share a relatively low molecular weight and high interest for environment and health safety. Parathion is an organophosphate pesticide widely used to enhance agricultural production, but for its toxicity (Milles and Salt, 1950) it is now forbidden within the European Union which sets the limits of pesticide residues in food between 50 and 100 μg/kg (Commission Regulation (EC) no. 839/2008). Patulin is an example of mycotoxin which is most likely to be found in crops as a result of fungal infection. Both molecules are highly resistant to degradation and the patulin high toxicity for human and animal health has been recently pointed out in a review by Puel et al. (2010). Patulin level in food is strictly regulated in European countries (Commission Regulation (EC) no. 1881/2006) which set a maximum level of 50 μg/kg for fruit juices and derived products, 25 μg/kg for solid apple products and 10 μg/kg for baby foods. Both parathion and patulin are usually quantified by exploiting expensive, time consuming and relatively complex techniques like high-performance-liquid-chromatography (HPLC) and/or mass spectrometry [see (Blasco et al., 2004; Carabias Martinez et al., 1992; Kwakman et al., 1992) for parathion and (Berthiller et al., 2014; Pereira et al., 2014) for patulin]. Thus, the lack of any commercial and standard immunochemical methods underpins the research for biosensor based detection allowing *in situ* and real-time analysis for environmental monitoring and food quality control.

Amperometric devices are used for parathion in view of their feature to provide cheap, rapid and effective analysis of aqueous samples if the molecules to be detected are electroactive. Zen et al. (1999) developed a sensitive technique for the detection of parathion using a Nafion-coated glassycarbon electrode thus reaching a limit of detection (LOD) of 50 nM. Other sensing strategies are based on electrodes functionalized using enzymes like organophosphorus hydrolase. Exploiting this principle Mulchandani et al. (2001) were able to detect methyl-parathion and paraoxon with a LOD of 20 nM. Even if this kind of devices

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offers several advantages for water analysis, electrochemical detection can be easily influenced by other oxidizable molecules eventually present in a real sample.

Electrochemical (Vidal et al., 2013), optical (Pereira et al., 2014) and piezoelectric (Pohanka et al., 2007; Prieto-Simón and Campàs, 2009), sensors and biosensors for the detection of mycotoxins are reported in literature, but quite few results are reported for patulin detection. A fluorescence assay was proposed by De Champdoré et al. (2007) with a LOD of 10 μg/L (less than 0.1 μM), but no test on a real sample was carried out. Damián Chanique et al. (2013) have developed a detection method based on the electrochemical reduction of patulin using glassy carbon electrodes. With this strategy they reached a LOD of 300 nM quantifying patulin in commercial apple juices. Starodub and Slishek (2012) proposed a nano-porosus silicon based immunosensor for measuring the level of patulin and T2 mycotoxin in real samples reaching a sensitivity of about 10 ng/mL for both pollutants. More recently, Pennacchio et al. (2014) proposed a competitive surface plasmon resonance (SPR) based bioassay with an estimated LOD of 0.1 nM, but it is worth noticing that the accuracy of SPR measurements can be influenced by interfering effects like temperature and sample composition fluctuation which produce a change in the refractive index not related to the analyte binding.

In view of their robustness, flexibility and cost-effectiveness, quartz crystal microbalance (QCM) technology has achieved an important role in fields like sensing, material science, environmental monitoring and protein studying (Vashist and Vashist, 2011). It is possible use QCM devices for small molecule detection exploiting several principles and configurations (Cooper and Singleton, 2007). All these advantages led to a wide range of publications involving QCM based detection of both pesticides and mycotoxins. For instance, Bi and Yang (2009) used molecular imprinted monolayers (MIMs) self-assembled onto the QCM gold electrode to effectively detect imidacloprid and thiacloprid pesticides in celery juice. They used an extremely sensitive QCM device getting a LOD of 1 μM. Concerning mycotoxin detection an indirect competitive immunological strategy has been adopted by Jin et al. (2009) for the quantification of aflatoxin B1. They significantly improved the sensitivity of the QCM based biosensor coupling the indirect competitive immunoassay with biocatalyzed deposition amplification using enzyme labeled secondary antibodies. Horseradish peroxidase was used to catalyze the oxidation of 4-chloro-1-naphthol to form an insoluble product which deposits onto the QCM electrode thus resulting in a huge increase in the sensor response. This procedure requires several time consuming incubation steps and allows to reach a LOD of about 32 pM.

Surface functionalization is one the main issue in biosensor development, in fact, recent publications show the strong interest in the research of innovative immobilization and functionalization strategies which provide better sensitivity and lower LOD (Jung et al., 2008; Nicu and Leïchlé, 2008). In particular, protein orientation is of paramount importance for immobilized antibodies which have to well expose their sensitive parts, the so called antigen binding sites, to effectively capture the antigens. Trilling et al. (2013) have recently investigated the relationship between analyte characteristics and capture molecule anchoring showing that the uniform orientation of the recognition elements provides a huge systematic improvement in sensitivity for weak interactions. They observed that the smaller the molecule, the lower the epitope number per analyte and, hence, the more important is the orientation of the sensitive biomolecule. By an appropriate antibody surface functionalization, Funari et al. (2013) were able to use a simple transducer like quartz-crystal microbalance (QCM) to detect a concentration of about 200 nM of parathion. This result was achieved by adopting the photonic immobilization technique (PIT) (Della Ventura et al., 2011), so that a gold surface fully covered by oriented antibodies was realized, but also by "weighing down" the molecule through the complexion of parathion with bovine serum albumin (BSA). Since not all the molecules are able to complex with BSA, in this paper we propose a more general approach leading to higher sensitivity and specificity. Essentially, parathion and patulin are "weighed down" by the same antibodies used for the detection onto QCM, mimicking the so called sandwich configuration widely used in the ELISA assays. To this end, the pollutant sample is mixed with an antibody solution before the latter is conveyed to the QCM and LODs of approximately 50 nM and 140 nM are achieved for parathion and patulin, respectively.

2. Materials and methods

2.1. Chemicals

Parathion (45607) and patulin (P1639) were purchased from Sigma-Aldrich. Anti-parathion (ABIN113883) and anti-patulin (AS11-1699) polyclonal antibodies were purchased as rabbit sera from antibodies-online.com and Agrisera respectively. The type G immunoglobulins were purified using the Protein A Antibody Purification Kit (PURE1A) from Sigma-Aldrich. 5,5′-dithiobis-(2-nitrobenzoic acid) also known as Ellman's reagent (D8130), bovine serum albumin (A2153) and the compounds used for the specificity tests, bisphenol A (239658), p-nonylphenol (46018), dichlorvos (45441), diazinon (45428) and paraoxon (36186), were from Sigma-Aldrich. The pollutant samples were prepared using PBS 1 × buffer solution in the fume hood. Helix water, sulfuric acid 98% and hydrogen peroxide 40% were used for the cleaning procedure of the QCM gold surfaces.

2.2. Patulin extraction from real sample

For the specificity test, we used real samples of patulin extracted from apple puree obtained from apple processing plant. To this end a commercial kit (Polyintell Affinimip® SPE cartridges) was used. The extraction was performed as follows: 10 g of apple puree were treated with 150 μL of a pectinase enzyme solution followed by 10 mL water and mixed. Solution was left at room temperature overnight, or for 2 h at 40 °C, centrifuged at 4500*g* for 5 min and then filtered with a 0.2 μM filter. This solution is used as the loading solution. SPE Cartridge was conditioned with 2 mL of acetonitrile (ACN), then with 1 mL of deionised water. 5 mL of the loading solution was put in the cartridge, which was subsequently washed with 4 mL of deionized water containing 1% of acetic acid. Water was forced down into the cartridge. The cartridge was treated with 1 mL of $CHCl_3$ and patulin was eluted with 2 mL of ACN containing 1% acetic acid. The SPE procedure lasted approximately 30 min. The elution fraction was then evaporated and dissolved in water containing 0.1% acetic acid. This fraction was submitted to a Perkin Elmer HPLC with UV detector to determine the patulin concentration. The same sample was used in QCM validation analysis.

2.3. UV laser source

The immunoglobulin samples were irradiated using the UV laser pulses provided by a custom femtosecond PHAROS laser system with high tunable pulse repetition rate coupled with a harmonic generator stage (HIRO) which allows the conversion to 515 nm, 343 nm and 258 nm wavelengths of the IR fundamental radiation. Both PHAROS and HIRO were from Light Conversion Ltd.

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