Bulgarian Chemical Communications, Volume 48, Number 4 (pp. 689 – 693) 2016

Quartz crystal microbalance-based unlabeled immunosensor for the determination of aflatoxin B1

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Received April 19, 2016; Revised May 22, 2016

The aim of this work is to describe our first results obtained by a quartz crystal microbalance (QCM)-based unlabeled immunosensor for quantification of afllatoxins. Aflatoxins are natural mycotoxins that enter the food chain by contamination of crops and nuts, potentially posing carcinogenic risks to animal and human health. On choosing aflatoxin B1, immuno-QCM approaches were examined including sandwich assays with monoclonal antibodies. The immuno-QCM assay leads to the sensitive detection and quantification of aflatoxin B1 down to 0.01 ng/ml. The approach discussed here is used as a model system that could easily be adapted for aflatoxin detection in a variety of food or animal feed samples using a simple methanol/water solution as an extraction solvent.

Keywords: immune-quartz crystal microbalance, immunosensor, aflatoxin B1.

INTRODUCTION

Aflatoxins are natural mycotoxins that enter the food chain by contamination of crops and nuts, potentially posing carcinogenic risks to animal and human health. In fact, the European Committee Regulation (ECR) has established the maximum acceptable level of AFB1 in cereals, peanuts and dried fruits for direct human consumption: 4 ng/g for total aflatoxins (AFB1, AFG1, AFB2, AFG2) and 2 ng/g for AFB1 alone [1]. Aflatoxin B1 (AFB1) is the most common mycotoxin produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus* that grow on food crops during their production and storage. It exhibits carcinogenic, teratogenic, mutagenic and immunosuppressive properties and has been regarded as a human carcinogen by the International Agency for Research on Cancer [2].

Analytical methodology must allow the determination of aflatoxins at least below the specific regulatory levels [3]. The measurement of antibody or antigen concentrations based on biospecific recognition interactions such as immunoassay and biosensors, has been considered a major analytical method used in clinical diagnosis, environment, and biochemical studies and has generated much interest due to its cost-effectiveness, sensitivity and specificity [4].

Immunosensing is a very active research field. The inherent combination of the exquisite molecular recognition ability of antibodies and the philosophy of rapid, continuous, reversible and automatic analysis of chemical sensors utilized in immunosensors is very useful in many fields [5]. In this sense, batch immunoassays can solve analytical problems that require a high number of determinations, while immunosensing is the better choice when automation and rapid results are needed [6].

The general strategy for immunosensor construction is to place the biological material in close contact with the transducer in order to obtain high sensitivity and to minimise the time of measurement. In a sandwich assay, after interaction between immobilised antibodies (Ab) and free antigens (Ag), second antibodies (Ab*), directed toward a second binding site of the Ag are added; at this point, Ag is "sandwiched" between two antibodies (Ab and Ab*). Transducers, such as piezoelectric sensors (quartz crystal microbalance, QCM) allow label-free detection with a direct quantification of the immunocomplex (Ab–Ag). For QCM, only a few examples are based on the use of a direct assay (not to be confused with the direct competitive assay) where the simple binding between an antigen and an antibody is detected. In fact, for low-molecular weight analytes such as aflatoxins the sensitivity of QCM is usually not sufficient when a direct antibody/antigen

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interaction is adopted. For this reason competitive and sandwich formats (without any labeled molecules) are highly preferred [7]. Quartz crystal microbalance (QCM) has been extensively investigated as a transducer in chemical and biological sensing [8, 9]. A unique feature of QCM is that it can be designed as an immunosensor for directly detecting biomolecules [10, 11]. The QCM immunosensors measure the resonant frequency (F_0) using the standard oscillator technique, and the frequency change (ΔF) is usually explained by Sauerbrey equation, which states that the decrease in F_0 ($-\Delta F$) is linearly proportional to the increase in surface mass loading of QCM [12]. One of the biggest challenges for the development of QCM immunosensor devices for the antigen–antibody interaction is to understand the interaction process on the probe surface [13-15]. Thus, the surface properties of the immobilized antibody should be reproducible, stable, and selective to a corresponding antigen [16, 17].

The aim of this work is to describe the observation of a quartz crystal microbalance (QCM)-based unlabeled immunosensor for quantification of toxins suspended in methanol/water solution that can also serve as an extraction solvent (Figure 1). These are the first results of this kind of observation.

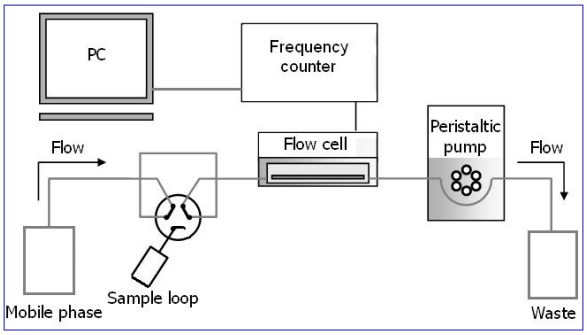

Fig. 1. Quartz crystal microbalance technology

MATERIALS AND METHODS

Aflatoxin B_1, the monoclonal antibodies against AFB_1 from rabbit (r-anti-AFB1) and the monoclonal antibodies against AFB_1 from mouse (m-anti-AFB1) were purchased from Sigma (St Louis, MO, USA). All chemicals and solvents used were of analytical grade and were used without further proceeding.

Aflatoxin B1 standards

Aflatoxin B1 analytical standard containing 5 μg AFB1 was solved in 1 ml of 100% methanol. A main stock of 100 ppb was prepared in 60:40 methanol/water solvent and used to prepare several standards by diluting the main stock in the same solvent. Considering our objective of quantifying low aflatoxin concentrations (less than 100 ppb), a narrow 2-fold dilution range of aflatoxin standards was prepared [18].

Obtaining of polymer layer of polyacrylonitrile-polyacrylamide over QCM-resonator

The particles of the polyacrylonitrile-polyacrylamide copolymer were obtained according to the method used in [19]. Then 0.100 g of the obtained copolymer was diluted in 1 mL dimethylformamide (DMF) and the solution was stirred for 15 min on a magnetic stirrer. After 1:1 dilution with anizol the prepared polymer solution was cast as a thin layer over the QCM- resonator using spin coating (Figure 2).

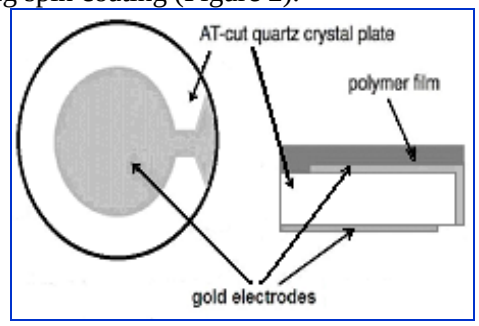

Fig. 2. Quartz crystal resonator with thin polymer layer

Method of oxidation of anti-aflatoxin B1 antibody

The working concentration of monoclonal m-anti-AFB1 was 0.67 mg/mL Oxidation of the carbohydrate moieties of the antibody with periodic acid (0.04 mmol/L in 0.05 mmol/L acetate buffer, pH 5.0) was performed according to Zabrosky and Ogletree. The unreacted periodic acid was removed with 0.025 mmol/l ethylene glycol. The oxidized antibody was dialyzed against 50 mmol/l phosphate buffer with pH 6.0 for 18-24 h [19].

Covalent immobilization of oxidized m-anti-AFB1 on polymer film over QCM-resonator

The immobilization of m-anti-AFB1 was performed in the following manner: 5 mL of oxidized dialytic solution of antibody was added to a resonator. The process was implemented for 24 hours at t = 4°C in dark.

QCM-based immunosensor for aflatoxin B1 using sandwich immunoassay

Saline was used as a basic solution for QCM (0.9% sodium chloride solution). Different dilutions of aflatoxin B1 prepared using 60:40 methanol/water solvent in separate 1 ml microcentrifuge tubes (100 μl of each standard) were added with 1 μg (5 μl) of r-anti-AFB1. Toxin

capture was performed by gently shaking the tubes at room temperature.

All solutions of AFB1 were previously incubated with r-anti-AFB1 to avoid unspecific adsorption onto the polymer layer. Including one of the AFB1 solutions in the system causes a decrease in the frequency of oscillation of the resonator due to the formation of a sandwich between the antibodies and AFB1 (Figure 3).

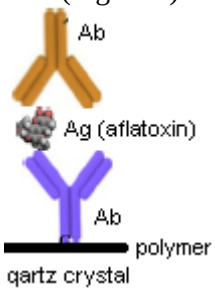

Fig. 3. QSM-immunosensor

Recovery of immunosensor

After 20 min treating with citrate-phosphate buffer (containing 4.7 g/l citric acid and 9.2 g/l dibasic sodium phosphate (Na_2HPO_4) anhydrous) at pH 5.0 the sandwich is destroyed and the sensor is regenerated.

Modified Lowry method

The protein content was measured using a modified Lowry method [20]. The principle behind the Lowry method for determining protein concentrations lies in the reactivity of the peptide nitrogens with the copper [II] ions under alkaline conditions, and the subsequent reduction of the Folin-Ciocalteu phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids.

First, reagent A: 2% Na_2CO_3 in 0,1N NaOH, reagent B: 1% $CuSO_4.5H_2O$ and reagent C: 2% sodium tartrate, were prepared. Reagent D was prepared by mixing reagents B and C at a ratio of 1:1. Reagent D was prepared just prior to use of reagents A and D at a ratio of 1:50.

After immobilization, the QCM-resonator was put into 1 ml of distilled water. Thereafter 5 ml of reagent D were added, and the solution was left for 160 min with occasional stirring. Then, 0.5 ml of 1N Folin-Ciocalteu reagent (phosphomolybdotungstate) were added. After 45 min, the absorbance was measured at 750 nm against a control that contained the same components, but without the immobilized QCM-resonator. The amount of protein was determined by the standard. As a reference a solution of m-anti-AFB1 was used.

RESULTS AND DISCUSSION

100 µl solution of 0,01 ng/ml AFB1 decreased the signal by 175Hz due to the accumulation of mass on the resonator. The increased amount of mass can be attributed to the formation of the sandwich assay or a non-specific binding of the components of the solution with the resonator (Figure 4a).

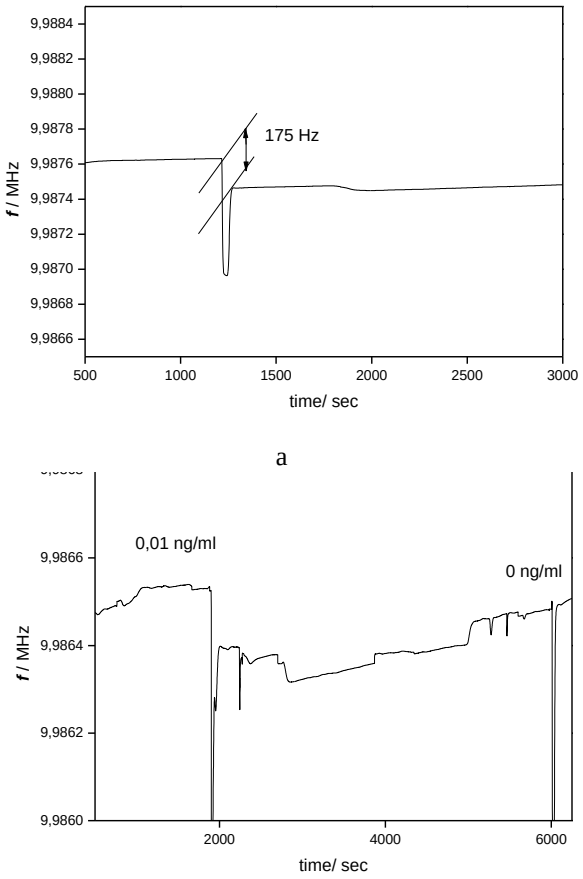

Fig. 4. Frequency dependence of the QCM-resonator: a) 100 µl 0,01 ng/ml AFB1; b) 100 µl r-anti-AFB1 without AFB1 after regeneration

The vibration frequency of the QCM-resonator is inversely proportional to the mass that has been deposited on it. Since the mass of AFB1 is very small, we increased it multifold by adding in advance r-anti-AFB1 to the primary solution containing the toxin. This method makes possible the determination of very small concentrations of AFB1.

After the regeneration of the sensor, the acid buffer was replaced with saline. The solution flow containing 100 µl of r-anti-AFB1 without AFB1 did not change the frequency of the resonator, which indicated that there was no non-specific binding (Figure 4b).

Table 1. Standard solutions for determination of protein according to the Lowry method

Solution	Control	1	2	3	4	5	6	7	8	9
1 mg/ml mouse IgG	-	0.1 ml	0.15 ml	0.2 ml	0.25 ml	0.3 ml	0.35 ml	0.4 ml	0.45 ml	0.5 ml
Dist. H_2O	1 ml	0.9 ml	0.85 ml	0.8 ml	0.75 ml	0.7 ml	0.65 ml	0.6 ml	0.55 ml	0.5 ml

The calibration curve was prepared by the Lowry method, using an m-anti-AFB1 solution at 1 mg/ml concentration as a standard protein solution. Out of the solution, the following solutions were prepared (Table 1):

To each tube 5 ml of reagent D were added and after stirring for 10 min at room temperature, to each tube 0,5 ml 1N solution of Folin-Ciocalteu were added. After 45 min, the solutions were tested by photometry at 750 nm against a control (Figure 5).

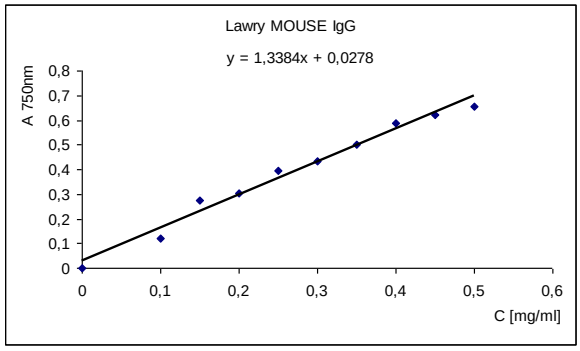

Fig. 5. Calibration Lawry curve

The quantity of immobilized antibodies on the QCM-resonator was 0.094 mg, entirely consisting of covalent immobilized immunoglobulin.

CONCLUSIONS

This method combined the sandwich competitive immunoassay with the amplification method of covalent immobilization onto the QCM-surface for AFB1 detection. The immuno-QCM assay leads to a sensitive detection and quantification of aflatoxin B1 from 100 down to 0.01 ng/ml. The approach discussed here is used as a model system that could easily be adapted for aflatoxin detection in a variety of food or animal feed samples using a simple methanol/water solution as an extraction solvent.

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ИМУНОСЕНЗОР ЗА ОПРЕДЕЛЯНЕ НА АФЛАТОКСИН B1

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Постъпила на 19 април 2016 г.; коригирана на 22 май 2016 г.

(Резюме)

Настоящото проучване представя нашите първи резултати, получени с имуносензор базиран на кварцова кристална микровезна (QCM) за количествено определяне на афлатоксини. Афлатоксините са природни микотоксини, които постъпват в храната от замърсени зърна и ядки. Те са причина за канцерогенни заболявания при човека и животните. Афлатоксин B1 е избран за изследване с имуно-QCM сандвич метод с моноклонални антитела. Анализът показва добра чувствителност на откриване и количествено определяне на афлатоксин B1 до 0.01 нг/мл. Подходът, обсъден тук, се използва като моделна система, която лесно може да се адаптира за откриване на афлатоксин в различни проби от храни и фуражи, използвайки разтвор метанол/ вода като екстракционен разтворител.