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Bioimprinting for multiplex luminescent detection of deoxynivalenol and zearalenone

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ABSTRACT

A sensitive tool for simultaneous quantitative determination of two analytes in a single spot with the use of a bioimprinted protein is presented for the first time. BSA is chosen as a scaffold for generation of binding sites specific towards two compounds. A multiplex immunosorbent assay for screening of two cereal-born mycotoxins, deoxynivalenol and zearalenone, in wheat and maize is realized with the use of fluorescent silica coated quantum dots as labels. Water-soluble fluorescent nanostructures consist of core/shell Cd-QDs enrobed in silica shells to ensure their solubility. The mycotoxins are simultaneously detected by scanning the assay outcome at two different wavelengths, since two QD@SiO_2 labelled conjugates fluorescent in different parts of the spectrum. The assay is combined with a rapid extraction technique. The limits of detection for the simultaneous determination were 100 and 700 μg kg^{-1} in both matrices for zearalenone and deoxynivalenol, respectively. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to confirm the obtained results.

1. Introduction

Molecular imprinting is a promising approach to create tailor-made binding sites complementary to template molecules in shape, size and functional groups in (bio)polymer scaffolds [1]. Molecularly imprinted polymers (MIPs) have already been widely used both in solid phase extraction [2,3] and as receptors in different types of immunoassay [4–6]. MIPs' benefits include their robustness, high mechanical and thermal stability in harsh conditions (broad pH and t° ranges) compared to antibodies. Imprinted sites are initially created via interaction between monomers and a template molecule [7]. The same procedure could also be applied for generation of specific sites in biomolecules (bioimprinting). Bioimprinting addresses an issue of re-using conventional polymer particles. The production of imprinted proteins (IPrs) is cheaper, faster, and more environmental friendly than conventional MIP production. Nowadays only a few articles were devoted to bioimprinting, mainly to bioimprinting of enzymes [8–10] for selective recognition and catalysis. To the best of our knowledge only one manuscript described the biomimicking of a low molecular weight compound using a protein as a carrier, aflatoxin B1 for subsequent use of this imprinted protein in the electrochemical sensor [11]. This technique proposed a sensitive detection in a micro- nanomolar concentration range that indicates the great potential of bioimprinting.

As the recent trends in assay development are related to multiplex detection and real-time monitoring, all possibilities to decrease time and cost of an analysis receive a high priority. This is illustrated by a constantly increasing amount of publications describing simultaneous detection of multiple analytes. Together with chromatography, cost-effective and user-friendly immunochemical techniques, such as ELISA (Bio-Plex®, Qiagen®, PathScan®), multiplex bead array assays (Luminex xMAP, BD Biosciences[12,13]), fluorescent polarization immunoassay [14], electrochemical biosensors [15], lateral flow dipsticks [16,17], membrane-based flow-through [18], and gel-based [19,20] rapid tests were developed and commercialized. These multiplex immunoassays were based on placing immunoreagents, specific towards different targets, on separate spots (test-zones, tubes, wells, beads etc.) within one test system. Such multiplex principle was demanded because the signals provided by two enzymes cannot be separated; a separation of signals provided by two organic dyes requires statistical data processing due to their asymmetrical unsharpened emission peaks which are broadened by red-tails [21].

Introduction of quantum dots (QDs) as labels for immunoassay

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allowed multiplex detection in one single spot due to the unique size-dependent optical properties. QDs are monodisperse crystalline clusters with physical dimensions smaller than the bulk-exciton Bohr radius that had a definite effect on their photoluminescent characteristics [22–25]. Since QDs emission is size-dependent, they can be tailored in a controlled way by adjusting the synthesis conditions to assure a fluorescence emission matching any wavelength of the visible region. Their fluorescence is intense and highly stable against photobleaching. QDs have high quantum yield, broad absorption and narrow, symmetric emission spectra and long excited-state decay lifetimes [26,27]. This broad absorption bandwidth, related to the presence of multiple electronic states at higher energy levels, allows simultaneous excitation of multicolor QDs by using a single light source, and therefore their use in a single-spot multiplexing without any mathematical or statistical processing of obtained results. This is why using of multicolored QDs in lateral-flow is very beautiful and user-friendly [28–31], but not so interesting as multiplex electrochemiluminescence immunoassay [32,33] and multiplex immunosorbent systems based on QDs [34–36] where detection of two or three analytes actually happens in one spot by scanning of its outcome at different wavelengths. In addition, QDs are currently of considerable interest, not only because of their unique properties but also because of their dimensional similarities with biological macromolecules (e.g. proteins).

This manuscript is the first manuscript to describe imprinting of a protein by two different templates and the only paper devoted to use of imprinted proteins in multiplex immunoassay. Two foodborne toxins, deoxynivalenol (DON) and zearalenone (ZEN) were chosen as a model system. The choice was based on (i) their possible co-occurrence in cereals [37,38]; (ii) different structures of the targets (Fig. S1) that facilitate bioimprinting and decrease any possible cross-influence of the targets on their separate specific detection; (iii) their quite high established maximum permitted limits in unprocessed cereals, 100 μg kg^{-1} and 350 μg kg^{-1} for ZEN, for wheat and maize, respectively, and 1750 μg kg^{-1} for DON both in wheat and maize [39]. This study aimed at a quantitative immunochemical screening of two mycotoxins in maize and wheat using the engineered receptor for simplification of the traditional multiplex analysis procedure. To make it happen a combination of QDs-based multiplexing with imprinted proteins (IPr) as recognition element was used.

2. Materials and methods

2.1. Materials

Green and red light emitting Cd-based QDs were synthesized by barely modified protocols that can be found in literature [40,41]. The synthesis of DON-OVA was performed according to [42], whereas the synthesis of ZEN-OVA was presented in [43]. A list of the used reagents is presented in SI. Microtiter plates (96 flat-bottom wells, opaque black with high binding capacity) were purchased from Nunc A/S (Roskilde, Denmark). Size distribution of the particles was measured by dynamic light scattering (DLS) using the Zetasizer Nano ZS (Malvern, England). All measurements were carried out at 25 °C. Bright-field transmission electron microscopy (TEM) images were taken using a Cs-corrected JEOL 2200 FS microscope (Peabody, MA, USA) operating at 200 kV. UV–vis absorption spectra of QDs were recorded by a Shimadzu 1800 spectrophotometer. For fluorescence measuring an Infinite Tecan Plate Reader (Tecan, Switzerland) was used by changing of emission wavelength depending on the QDs fluorescence peak position: 547 and 632 nm for green and red QD@SiO2, respectively. Bright-field transmission electron microscopy (TEM) images were taken using a Cs-corrected JEOL 2200 FS microscope. Emission spectra were recorded on a Perkin Elmer LS55 fluorescence spectrometer. Naturally contaminated wheat and maize samples (different varieties) were collected from different fields in Flanders, Belgium. A procedure for sample preparation and LC-MS/MS technique are described in details in SI.

2.2. Bioimprinting

Bioimprinting was performed in accordance with the technique of Liu et al. [9] Briefly, 1 mg of BSA was dissolved in 1 mL of citrate buffer (pH~4) and stirred for 1 min. After that pH of the protein solution was adjusted to 3.0 using 0.1 M HCl, and the solution was stirred at room temperature for 10 min. A mixture of ZEN and DON (60 μg of each in methanol) was added to the protein solution, and the mixture was stirred for 10 additional min, after that pH of the system was adjusted to 8 using 0.1 M NaOH. One hundred μL of 1% glutaraldehyde was added, and the mixture was stirred at 4 °C for 30 min, then the mixture was statically incubated at the same temperature overnight. Thereafter the obtained IPr was purified by dialysis against 10 mM of PBS for 48 h. The non-imprinted protein (nIPr) was prepared according to the same protocol, but excluding the template molecules (the mixture of ZEN and DON). After washing the IPr and nIPr were stored at 4 °C.

2.3. Screening of binding potential of IPrs

IPr was immobilized on a microwell plate, and ZEN and DON in different ratios were added to each well. The plate was gently shaken for 3 h on a horizontal shaker, and then the mycotoxins solutions were taken from each well for determination of the concentration of free analytes by LC-MS/MS. The amount of the mycotoxins bound to the modified BSA was calculated by subtracting the amount of free analytes from the initial amount added to the mixture.

2.4. Labelling of mycotoxins-protein conjugates with silica coated quantum dots (QD@SiO2)

The ratio QD@SiO_2 /myxotoxin-protein conjugate equal to 1/3 was used. Green-and red-emitted QD@SiO_2 solution (~4.4×10^{-4} μmol in PBS) was dropwise added to the conjugate solution (~1.3×10^{-3} μmol) under constant stirring, and incubated for 24 h at RT under stirring. Coupled and uncoupled QD@SiO_2 were separated using NHS-activated magnetic beads, then the conjugates were washed using dialysis (2 days, 4 °C, against PBS). To control the binding efficiency gel-electrophoresis was used. The QD@SiO2–labelled conjugates and free QD@SiO2 nanoparticles were loaded onto a 1.5% agarose gel (in TBE buffer) and run under 150 V for 1 h, then imaged under UV light (λex = 365 nm). Free QD@SiO2 and the labelled conjugates (DON-QD@SiO2 and ZEN-QD@SiO2) moved through agarose gel at different rates, based on each particle's size and charge carried. The labelled conjugates were kept at 4 °C within 6 months.

2.5. IPr-based fluorescent labelled immunosorbent assay (IPr-FLISA)

A 96-well opaque black microtiter plate was coated with 100 μL/well of the obtained IPr (diluted in 0.05 M sodium carbonate buffer, pH 9.6) overnight at 4 °C. The plates were covered with an adhesive plate sealing film to prevent evaporation. The plates were then washed three times with PBS-Tween 20 (PBST, 0.05%, v/v) and later blocked with PBS containing casein (0.75%, w/v, 300 μL/well) for 1 h at 37 °C. After blocking each plate was washed twice with PBS-Tween 20 (0.05%, v/v). Standard solutions (diluted in PBS) or the diluted sample extract (50 μL/well) were added simultaneously with the mixture of DON-QD@SiO_2 and ZEN-QD@SiO_2 (25 μL of each in PBST), and incubated at RT for 1 h by agitation on a horizontal shaker. After the incubation the plates were washed four times with PBST. The content of each well was re-dissolved in 100 μL of PBS and fluorescence was measured at two different wavelength to determine each target: the maximum emission peak of ZEN-QD@SiO_2 was at around 547 nm, whilst DON-QD@SiO_2 emitted with the maximum at around 632 nm. All measurements were made in triplicate. The sigmoidal standard curves were plotted in the semi-logarithmic scale: absolute or relative fluorescence intensity against the logarithm of the analyte concentration. Each standard curve