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Sensitive QD@SiO2-based immunoassay for triplex determination of cereal-borne mycotoxins

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ABSTRACT

A sensitive tool for simultaneous quantitative determination of three analytes in one single well of a microtiter plate is shown for the first time. The developed technique is based on use of colloidal quantum dot enrobed into a silica shell (QD@SiO2) derivatives as a highly responsive label. Silica-coated quantum dots were prepared and subsequently modified via the co-hydrolysis with tetraethylorthosilicate (TEOS) and various organosilane reagents. Different surface modification schemes were compared in terms of applicability of the obtained particles for the multiplex immunoassay, *e.g.* stability and simplicity of their conjugation with biomolecules. As model system a multiplex immunosorbent assay for screening of three mycotoxins (deoxynivalenol, zearalenone and aflatoxin B1) in cereal-based products was realized via a co-immobilization of three different specific antibodies (anti- deoxynivalenol, anti-zearalenone and anti-aflatoxin B1) in one single well of a microtiter plate. Mycotoxins were simultaneously determined by labelling their conjugates with QD@SiO_2 emitting in different parts of the visible spectrum. The limits of detection for the simultaneous determination were 6.1 and 5.3, 5.4 and 4.1, and 2.6 and 1.9 μg kg^{-1} for deoxynivalenol, zearalenone and aflatoxin B1 in maize and wheat, respectively. As confirmatory method, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used.

1. Introduction

Colloidal semiconductor nanocrystals, quantum dots (QDs), exhibit interesting optical properties, governed by the strong quantum confinement effect [1]. Namely, there optical properties are size-dependent. QDs are one of the most promising nanoprobes for chemical, biomedical and therapeutic labelling and imaging because they are characterized by a narrow, size-tunable, symmetric emission spectrum and quite high photochemical stability [2,3]. But for these kinds of applications some requirements have to be met, *i.e.* QDs should (a) be stable in aqueous solutions over a wide pH range (b) have a high quantum yield and photostability (c) have functional groups available for conjugation on their surface (d) not evince a high non-specific interaction with proteins/carriers/interferences [4–6]. QDs with high fluorescence quantum yield and narrow size distribution are synthesized at high temperature in organic solvents [7–10]. There are two main strategies employed for hydrophilization of QDs: ligand exchange [7,11–12], including the silanization of the particles [3,13] and encapsulation with amphiphilic polymers [14–16] or liposomes [17]. All mentioned techniques were repeatedly described and claimed to be effective. Currently, the use of QDs in immunoassay is starting to take great interest [18–20]. The main advantages of QDs over traditionally used enzymatic labels are their sensitivity caused by their high photoluminescence brightness, low sensitivity to environmental conditions, rapid and easy detection of analytical signal, low rate of false results [21]. Polymer- coated QDs have been widely applied in different variations of immunoassays, but they usually show a quite high propensity towards non-specific interaction with the carrier surface [22]. Variation of polymer composition [23] and QDs purification technique [14] allows to significantly decrease this interaction, but not completely eliminate it. Loading of water-insoluble QDs into liposomes could also reduce this undesirable interaction [17], but a huge drawback of liposomes is their sensitivity towards environmental influences

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and the consequent instability *in vivo* and during storage. Encapsulation of QDs into a silica shell is a promising approach towards hydrophilization because the silica surface is non-toxic, chemically inert, optically transparent and easy to functionalize [24] and the obtained derivatives can be further employed in immunoassay [25].

Nowadays the amount of publications covering easy-to-operate immunochemical tests for simultaneous determination of several analytes in various matrices is constantly rising. Different labels have been developed for application in immunochemical assays in order to reach the highest possible sensitivity. All published multiplex immunoassays were designed by placing immunoreagents, specific towards different targets, on separate spots within one test system. This multiplex approach was used because the signals provided by two enzymes cannot be separated. Separation of the signals provided by two organic dyes requires use of some statistical methods due to their asymmetrical unsharpened emission peaks which are broadened by a red-tail [26,27]. Our recent publications presented a double- analyte multiplex (DAM) where the immobilization of two antibodies specific to different analytes into the same well for simultaneous fluorescent detection of two analytes was performed by employing of QDs with different colors of emission as labels [27]. Replacement of the label for the QDs encapsulated into liposomes resulted in the significant increase of assay sensitivity [28].

This work presents a synthesis of QD@SiO2 particles together with their following application in the multiplex immunosorbent assay. To the best of our knowledge this is the first manuscript to describe the immobilization of three antibodies specific towards different analytes into the same well of microtiter plate for simultaneous fluorescent detection of three analytes, so called triple-analyte multiplex (TAM) and the first manuscript presenting an application of silica-coated QDs in multiplex immunoassay.

2. Experimental section

2.1. Reagents and materials

Hydrophobic Cd-based QDs were synthesized as described in [10]. Tetraethyl orthosilicate (TEOS), (3-Glycidyloxypropyl)-trimethoxysilane (GLYMO), 2-[methoxy(polyethyleneoxy) 6-9 propyl]trimethoxysilane (PEG- silane), (3-aminopropyl) triethoxysilane (APTES), 3-(trihydroxysilyl)propylmethylphosphonate monosodium salt (THPMP), polyethylene glycol dodecyl ether (Brij L4), 3-(2- pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), dithiothreitol (DTT), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), N,N′-dicyclohexylcarbodiimide (DCC), O- (carboxymethyl)hydroxylaminehemihydrochloride(CMO), Tween 20 (Tween; polyoxyethylenesorbitan monolaurate), bovine serum albumin (BSA), albumin from chicken egg. white (OVA), casein sodium salt from bovine milk, skim milk powder, phosphate buffered saline. tablets, carbonate bicarbonate buffered saline tablets, Tris-Borate-EDTA buffer 10x concentrate were purchased from Sigma-Aldrich (Bornem, Belgium). Agarose was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Aflatoxin B1 (AFB1), zearalenone (ZEN), deoxynivalenol (DON) were supplied by Fermentek (Jerusalem, Israel). Imject cBSA Immuno Modulator and protein concentrators (9 K, 20 mL) were purchased from Thermo Scientific (Rockford, USA). Lipoid S75 was purchased from Lipoid GmbH, (Ludwigshafen, Germany). All other chemicals and solvents were of analytical grade. Microtiter plates (96 flat-bottom wells with high binding capacity; black Maxisorp) were purchased from Nunc A/S (Roskilde, Denmark). Polyclonal rabbit anti-mouse immunoglobulins (2.1 g L^{-1}) were obtained from Dako Denmark A/S (Glostrup, Denmark). The anti-ZEN monoclonal antibody was obtained in our laboratory and characterized by a high ZEN (100%) and α-zearalenol (69%) recognition (cross-reactivities for α-zearalanol, zearalanone, β-zearalenol and β- zearalanol were 42%, 22%, < 1% and < 1%, respectively). Monoclonal anti-AFB1 antibody was obtained from Soft Flow Hungary Ltd (Pecs, Hungary) and it was characterized with 79% cross-reaction with aflatoxin M1, 33% with aflatoxin M2, 76% with AFB2, 55% with AFG1, 6% with AFG2 and none at all with AFB2a and AFG2a. Preparation of AFB1-cBSA was described in [29], the synthesis of DON-OVA was performed according to [14], whereas the synthesis of ZEN-OVA was presented in [30].

Size distribution of the particles was measured by dynamic light scattering method 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 operating at 200 kV. 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, 590 and 632 nm for green, orange and red QD@SiO2, respectively. Bright-field transmission electron microscopy (TEM) images were taken using a Cs-corrected JEOL 2200 FS microscope. UV–vis absorption spectra of QDs were measured by a Shimadzu 1800 spectrophotometer. Photoluminescence (PL) spectra were recorded on a Perkin Elmer LS55 fluorescence spectrometer.

2.2. Water-solubilization of QDs through silica coating

Hydrophobic QDs were encapsulated in silica shells (QD@SiO2) through a water-in-oil microemulsion process. For this, 1 nmol of QDs was mixed with 2 mL of hexane and 0.64 mL of Brij L4 and the suspension was stirred for 20 min One hundred μL of Milli-Q water and 20 μL of ammonia solution (25%) were added dropwise with followed 1 h stirring of the mixture to ensure stability and homogeneity of the microemulsion. After addition of TEOS (20 μL) the reaction was left under stirring for 48 h. At the last stage GLYMO was added in amount as 3% from TEOS, and the suspension was stirred extra 24 h for agening. The microemulsion was precipitated by following addition of a large volume of ethanol (1.5 mL) and the nanoparticles were collected by centrifugation (3000*g*, 10 min). The nanoparticles were purified by repeated centrifugation cycles in Milli-Q water (10,000*g*, 30 min, to remove the remained Brij), in hexane (1 time) and in ethanol (2 times). and finally re-dispersed in Milli-Q water. Finally, aqueous dispersions of the composite particles were obtained via sonication.

2.3. Labelling of mycotoxins-protein conjugates with QD@SiO2 for following use in FLISA

As QD@SiO2 particles contain epoxy groups on their surface the easiest reaction of a nucleophilic substitution was used to couple the nanoparticles with the mycotoxin-protein conjugates. The ratio QD@SiO2/monoclonal antibody equal to 1/3 was used for QDs-labelling. Green-, orange- and red-emitted QDs solution ($\sim 4.4 \times 10^{-4}$ μmol in PBS) was dropwise added to the mycotoxin-protein solution ($\sim 1.3 \times 10^{-3}$ μmol) under constant stirring. The mixture was gently stirred for 24 h at RT. The unreacted and nonspecific sites were blocked with a 10 mg mL^{-1} BSA–PBS solution. Characterization of the conjugations was done by a gel electrophoresis and DLS.

All conjugates were kept at 4 °C within 6 months. Efficient binding of QD@SiO2 nanoparticles to biomolecules was demonstrated by gel-electrophoresis. Free QD@SiO2 and the obtained conjugates (QD@SiO2–antibody or QD@SiO2–mycotoxin-protein) move through agarose gel at different rates, based on each particle's size and charge carried. The QD@SiO2–labelled conjugates