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Silica-coated liposomes loaded with quantum dots as labels for multiplex fluorescent immunoassay

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ABSTRACT

This manuscript describes synthesis and followed application of silica-coated liposomes loaded with quantum dots as a perspective label for immunoaasay. The hollow spherical structure of liposomes makes them an attractive package material for encapsulation of multiple water-insoluble quantum dots and amplifying the analytical signal. Silica coverage ensures the stability of the loaded liposomes against fusion and internal leakage during storage, transporting, application and also provides groups for bioconugation. For the first time these nanostructures were employed for the sensitive multiplex immunochemical determination of two analytes. As a model system mycotoxins zearalenone and aflatoxin B1 were detected in cereals. For simplification of multiassay results' evaluation the silanized liposomed loaded with QDs of different colors were used. The IC_{50} values for the simultaneous determination of zearalenone and aflatoxin B1 were 16.2 and 18 μg kg^{-1} for zearalenone and 2.2 and 2.6 μg kg^{-1} for aflatoxin B1 in wheat and maize, respectively. As confirmatory method, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used.

1. Introduction

Since 1965, when Bangham et al. have discovered and described liposomes structures [1], new perspectives for drug delivery [2], bioimaging [3–5], cosmetics and food applications [6], chemical analysis [7] were opened. Liposomes (lipid vesicles) are colloidal spherical composites of lipid bilayers self-assembled in aqueous media. The large surface area and big internal volume allow them to carry plenty of molecules or nanoparticles. Therefore, loaded liposomes are promising labels for chemical analysis able to improve its sensitivity due to their signal-amplification properties.

Loaded liposomes were successfully applied in different immunoassays, such as the microtiter-plate sorbent assay [8–11], flow-injection analysis [12–14], lateral flow on-site tests [15], chemiluminescent [16] and electrochemical [17] biosensors and also microarray [18]. Quantum dots (QDs) have been incorporated into liposomes and mostly used for labeling in biochemical and biomedical aims [19–22]. Recently the liposomes loaded with QDs (LQDs) were successfully applied by our group as label for the high sensitive immunoassay [23,24]. Nevertheless, liposomes could be sensitive towards external influences in experimental conditions and during the storage [25,26]. Semipermeability of liposomes membrane, so essential for medical and pharmaceutical aims, could do a bad turn in chemical analysis. The possible osmosis of QDs through the phospholipid bilayer would results in luminescence decrease, which is undesirable for immunolabel. Leakage of the phospholipid membrane can be halted through the coverage of liposomes with a polymer net [27,28] or silica cover [29,30]. Silica coverage prevents the aggregation of liposomes and the leakage of their content, increasing their stability and facilitating their desiccation and therefore their storage. Synthesis of liposome-silica derivatives is a widely used method to stabilize the lipid bilayer [31–34].

To the best of our knowledge this article is the first one to describe the application of silica-coated liposomes loaded with QDs (SLQDs) as a novel sensitive label for the multiplex immunoassay. The multiplex procedure was based on simultaneous determination of two analytes (mycotoxins zearalenone (ZEN) and aflatoxin B1 (AfB1)) in the single well of microtiter plate by co-immobilization of two specific antibodies there. This technique was the first time described by us using QDs-labeled conjugates [35]. Replacement of the label for the SLQDs resulted in the significant increase of assay sensitivity.

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2. Experimental section

2.1. Reagents and materials

Lipoid S75 was purchased from Lipoid GmbH, (Ludwigshafen, Germany). 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), (3-aminopropyl)triethoxysilane (APTES), aflatoxin B1 (AfB1), zearalenone (ZEN), dithiothreitol (DTT), O-(carboxymethyl) hydroxylamine hemihydrochloride (CMO), sodium fluoride, albumin from chicken egg white (OVA), casein sodium salt from bovine milk, skim milk powder, were purchased from Sigma-Aldrich (Bornem, Belgium). Imject cBSA Immuno Modulator and protein concentrators (9 K, 20 mL) were purchased from Thermo Scientific (Rockford, USA). Nuclepore track-etched membranes were purchased from Whatman (Belgium). Microtiter plates (96 flat-bottom wells with high binding capacity; black Maxisorp) were purchased from Nunc A/S (Roskilde, Denmark). All other chemicals and solvents were of analytical grade.

Polyclonal rabbit anti-mouse immunoglobulins (2.1 g L^{-1}) were obtained from Dako Denmark A/S (Glostrup, Denmark). The anti-ZEN monoclonal antibody (1 g L^{-1}) was 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) [36]. Monoclonal anti-AfB1 antibody (1.3 g L^{-1}) 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 [37]. CdSe/CdS/ZnS QDs with green and orange emission were prepared and stabilized by octadecylamine [38]. Preparation of AfB1-cBSA was described in [23], whereas the synthesis of ZEN-OVA was presented in [36].

Size distribution of the liposomes 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. An Infinite Tecan Plate Reader (Tecan, Switzerland) was used to measure fluorescence through variation of the emission wavelength, depending on the QDs fluorescence peak position: 540 and 594 nm for green and orange-emitting QDs, respectively.

2.2. Preparation of silica-coated liposomes loaded with QDs (SLQDs)

Preparation of QDs-loaded liposomes was done according to a protocol of thin-film evaporation described by us [23,24]. The phospholipid material (Lipoid S75, 134 μmol) and the water insoluble QDs (1.5 nmol) were dissolved in chloroform (2 mL) for their homogeneous mixing. Afterward chloroform was removed by rotary evaporation at 45 °C and ten mL of carbonate buffer were added to a dried lipid film left on the walls of a flask. The mixture was vigorously stirred in a water bath at 45 °C for 45 min. The lipid suspension was extruded through the polycarbonate membrane (the mean pore size 450, 200, and then 100 nm). The separation of LQDs from non-entrapped QDs and from excess phospholipids was realized by ultracentrifugation (300 000 g, 30 min, 4 °C) in a medium possessing an increasing sucrose density gradient (10–60%). LQDs were concentrated in a 30%-sucrose layer. LQDs were collected and, after removal of the sucrose residue with protein concentrator tubes, the pellet was re-dissolved in carbonate buffer (pH ~ 9.6) and used for further experiments. The obtained LQD solution was stored at 4 °C.

For silanization of QDs-loaded liposomes the 2.5-molar excess of APTES was directly added to the LQDs and the mixture was stirred during 48 h in the dark. For SLQDs drying sodium fluoride (4% molar excess with respect to the initial concentration silanization agent) was added and the mixture was stirred during 48 h at RT in the dark. Then the sample was dried at 40 °C for 24 h.

2.3. Syntheses of SLQDs-labeled antigens

APTES used for the silanization provides active amino groups on the liposomes' surface. For the synthesis of SLQDs-labeled conjugates a technique based on the modification of analyte-labeled proteins and SLQDs with SPDP and followed their coupling was applied. The analogue techniques were described for the production of ZEN-LQDs [24] and AfB1-LQDs [23]. SPDP (5 μmol) was dissolved in ethanol and dropwise added to the SLQDs solution (2 mL). The reaction mixture was stirred for 2 h at RT. The excess of SPDP was removed by the protein concentrator tube. In parallel, modification of analyte-protein was done. SPDP (6.5 μmol in ethanol) was dropwise added to the ZEN-OVA and AfB1-cBSA solutions (2 μmol) and the reaction mixture was stirred at RT for 30 min. The excess of SPDP was removed by the protein concentrator tube and dithiothreitol (4.2 μmol) was added to the SPDP-modified protein conjugate. The reaction mixture was stirred during 30 min at RT, and then excess of dithiothreitol was removed by the protein concentrator tube. A portion of the modified SLQDs (1.5 mL) was added dropwise to the obtained thiolated ZEN-OVA and AfB1-cBSA. Reaction was continued under constant stirring overnight at RT. The modified liposomes were separated from excess protein by gel-filtration using Sephadex G-75. The prepared conjugates were kept at 4 °C.

2.4. Fluorescent labeled immunoassay (FLISA)

The 96-well opaque black microtiter plates were coated with rabbit anti-mouse IgG antibody (100 μL/well; 5 μg/mL in 0.05 M sodium carbonate buffer, pH 9.6) for 2 h at 37 °C. Then the plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST) and blocked for 1 h at 37 °C with PBS containing 2% casein (w/v). Further, the plates were washed two times with PBST. The mixture of anti-ZEN and anti-AfB1 antibodies (50 μL of anti-ZEN antibody in dilutions of 1/2500 and 50 μL of anti-AfB1 antibody in dilution of 1/45000) was added and the plates were incubated for 2 h at 37 °C. The plates were washed three times with PBST. Fifty μL/well of standard mycotoxin solutions (in the range of 0.001–1000 ng mL^{-1} in PBS) or diluted sample extract were added simultaneously with the mixture of ZEN-SLQDs and AfB1-SLQDs (25 μL of each reagent, dilutions of 1/45 and 1/65 for ZEN-SLQDs and AfB1-SLQDs, respectively). After 1 h-incubation the plates were washed with PBST. The content of each well was re-dissolved in 100 μL of PBS and luminescence was measured using an Infinite Tecan Plate Reader (Tecan, Switzerland).

The standard FLISA sigmoidal calibration curve was plotted on a semilogarithmic scale: absolute or relative luminescence intensity against the logarithm of the analyte concentration. This relation is described by the Rodbard function:

$$y = (A - D)/[1 + (x/C)^b] + D$$

where A is maximum luminescence intensity value, D is minimum luminescence intensity value, C is IC_{50} concentration of analyte, b is slope of the curve in the IC_{50} plot. The limit of detection (LOD) was defined as the concentration that caused the analytical signal to decrease more than three times the signal-to-noise ratio (based on the results of 20 measurements).

2.5. Sample preparation

For the preparation of wheat and maize samples, a portion of ground cereal (10 g) was extracted with 40 mL of methanol/water