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SHORT COMMUNICATION

Comparison of monomeric and polymeric horseradish peroxidase as labels in competitive ELISA for small molecule detection

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Abstract We have developed a simple and sensitive competitive enzyme-linked immunosorbent assay (ELISA) to determine aflatoxin B1 (as a model small analyte) and using streptavidin-polymeric horseradish peroxidase complex (SApolyHRP) as a label for signal amplification. The performance of the assay was evaluated by comparing it with the classical indirect competitive ELISA using HRP labeled anti-mouse IgG as the tracer antibody. The results indicate that the SApolyHRP-based competitive ELISA exhibits a typically 2.4-fold steeper slope of the linear working range of the calibration curve compared to the monomeric HRP based classical ELISA, i.e., the sensitivity was increased. The SApolyHRP conjugate causes a typically 19-fold stronger signal generation in comparison to the traditional HRP labeled anti-mouse IgG at the same concentration (25 ng mL^{-1}). Moreover, the SApolyHRP-based assay has a much wider linear range and a 3.8-fold better signal-to-noise ratio. Considering its simplicity, sensitivity and ease of operation, this competitive ELISA is considered to be a promising tool for small molecule immunodetection.

Keywords ELISA · Signal amplification · PolyHRP · Small molecule

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Introduction

Due to the attractive advantages like high sensitivity, speed, ease to operate, general applicability, high throughput, safety, and low cost, immunoassays, e.g., the enzyme-linked immunosorbent assay (ELISA), have been widely used for the detection of a great number of antigens. This trend has been greatly encouraged by the availability of automated devices and convenient, reliable commercial kits.

Despite fascination superior to the technology of last generation, ELISA has always been in evolution to deal with the increasing test standards and demands [1]. Like other analytical methods, increasing sensitivity is the never-stopping goal of analysts using immunoassays. One possibility is to employ signal amplification, e.g., by increasing the specific activity of enzyme conjugates. Classical coupling techniques for enzyme labeling (typically horseradish peroxidase, HRP) of antibodies or antigens preparation mainly includes the glutaraldehyde [2] and periodate methods [3]. These methods permit a maximum ratio of 2–3 HRP molecules labeled to one antibody molecule due to their inherent limitation of coupling efficiency or influence on immunological activity [4]. Therefore, alternative approaches are investigated steadily. There exist only few data on the use of engineered polymeric HRP (polyHRP) as label. Already in the nineties, Vasilov and Tsitsikov reported on the synthesis of streptavidin-polyHRP complexes (SApolyHRP) [5]. They can contain up to 400 enzyme molecules. The technique is used by providers of commercial SApolyHRP conjugates, generally. For example, Damen et al. used this type of conjugate for sensitive detection of anti-HIV antibodies [6]. Dhawan reported on the synthesis of a 20 amino acid peptide containing 20 lys

residues and its conjugation to activated anti-human IgG [7]. The increased number of primary amines was then coupled with activated HRP. A 15-fold signal amplification of the ELISA was obtained due to higher number of enzyme molecules attached per IgG molecule. In another approach, Marquette et al. prepared macromolecular complexes, composed of dextran bearing both biotin and amine residues, followed by grafting of activated HRP onto the primary amine functions [8]. With a peroxidase/dextran molar ratio of 24 ten times increase of the detection limit of anti-HIV antibodies was obtained. Recently, Charbgoo et al. described synthesis of a streptavidin-dextran-polyHRP complex [9]. A sevenfold increase in signals from ELISA for tissue plasminogen activator was demonstrated in comparison to a commercially available standard streptavidin-HRP complex.

To the best of our knowledge, no data on signal amplification using polyHRP in competitive ELISA of small molecules exist. Herein, we report an ultrasensitive and simple colorimetric competitive ELISA for the detection of the mycotoxin aflatoxin B_1 (AFB_1) using SApolyHRP as label for signal amplification (Fig. 1b). AFB_1 was chosen as the model for a small analyte considering also the importance of its analysis on food safety, being one of the most toxic and carcinogenic contaminants ubiquitous in the human food supply [10]. The maximum residue level of AFB_1 in foodstuff in the European Union (EU) is 2 μg kg^{-1} according to Commission Regulation (EU) No. 165/2010. To systematically evaluate the performance of the developed SApolyHRP based competitive ELISA using biotinylated anti-AFB_1 (BioAb) and SApolyHRP, the BioAb-SApolyHRP format was compared with the traditional indirect competitive ELISA using naked mouse anti-AFB_1 as detection antibody (DetecAb) and the HRP labeled anti-mouse IgG as tracer antibody (TracAb). This classical DetecAb-TracAb format for AFB_1 detection was schematically shown in Fig. 1a.

Experimental

Materials

The affinity-isolated and lyophilized mouse monoclonal antibody against AFB_1 (anti-AFB_1, clone 1F2, 0.5 mg/vial) was from our group [11]. AFB_1, AFB_1-BSA, NHS-LC-biotin, 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Streptavidin-polyHRP40 conjugate (SApolyHRP) was obtained from Senova GmbH (www.senova.de). HRP labeled anti-mouse IgG produced in horse (HRP-anti-mouse IgG) was purchased from Vector Laboratory (www.vectorlabs.com). Aflatoxin-free oat flakes (baby food) used as sample matrix was purchased from local supermarket in Munich. High-binding 96-well polystyrene microplates were purchased from Greiner Bio-One (www.greinerbioone.com). Slide-A-Lyzer MINI Dialysis Units (10–100 μL capacity, Cat.-No. 69576) were obtained from Pierce (www.piercenet.com). Buffers and solutions were prepared with the ultrapure water produced by Milli-RO 5 Plus and Milli-Q185 Plus (www.millipore.com). The phosphate buffered saline (PBS) consisted of 0.01 M phosphate buffer solution and 0.137 M NaCl (pH 7.4). The TMB substrate solution for color development, washing buffer and AFB_1 stock solution were prepared as described [11]. Other reagents, unless otherwise stated, were purchased from Sigma-Aldrich or Merck (www.merck.de). The microplates were washed automatically with a 96-channel plate washer (ELx405 Select) and the optical density (O.D.) was measured with a microplate reader (Synergy HT) both from Bio-Tek (www.biotek.com).

Biotinylation of anti-AFB_1 antibody

Anti-AFB_1 antibody was biotinylated through amine coupling using NHS-LC-biotin at a nominal 10:1 molar ratio of biotin to anti-AFB_1 [12]. Briefly, fresh NHS-LC-biotin solution (6 μL, 1 mg mL^{-1} in DMSO) was quickly added to anti-AFB_1 stock solution (100 μL, 2 mg mL^{-1} in PBS). This reaction mixture was then incubated at 37 °C for 1 h with shaking. To eliminate unreacted biotin, the resulting solution was dialyzed at 4 °C against 1 L of PBS buffer with three changes for 24 h. The obtained biotinylated anti-AFB_1 was stored at 4 °C until use.

Preparation of sample matrix

To simulate the analysis of a real sample, aflatoxin-free oat flakes extract was used to dilute and prepare the standard solutions. Briefly, 100 mL methanol/water (80:20, v/v) was added to a 250 mL laboratory bottle holding 25 g oat flakes plus 5 g sodium chloride. Then, the bottle was immersed in a small basin full of crushed ice to prevent evaporation of liquid caused by excessive heat during the subsequent homogenization of oat flakes using T25 Ultra-Turrax® disperser (IKA, www.ika.com). After homogenization for 5 min, the mixture was filtered through filter paper and the filtrate was collected in a brown laboratory bottle. This resulting extract was immediately stored at −20 °C before use. When in use, the extract was diluted with PBS (1:3, v/v) and later used as the diluent solution for preparation of AFB_1 standards.

Immunoassay procedures

PolyHRP-based competitive ELISA for AFB_1 detection using biotinylated anti-AFB_1 plus SApolyHRP (BioAb-SApolyHRP format)

The high-binding 96-well microplate was coated with AFB_1-BSA in PBS (100 ng mL^{-1}, 100 μL/well) overnight

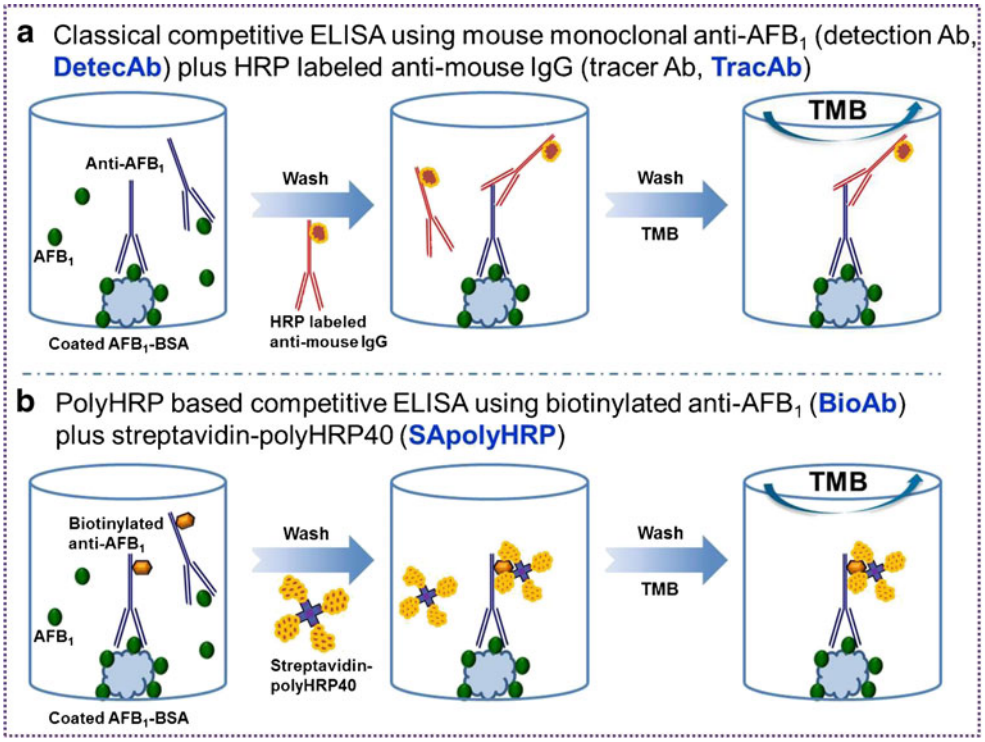

Fig. 1 Schematic (not in scale) of **a** the classical indirect competitive ELISA using mouse monoclonal anti-AFB_1 as detection antibody and HRP labeled anti-mouse IgG as the tracer antibody (DetecAb-TracAb format), and **b** the polyHRP-based competitive ELISA using biotinylated anti-AFB_1 and streptavidin-polyHRP40 (BioAb-SApolyHRP format)

at 4 °C. After washing, the plate was blocked with 1 % casein/PBS (300 μL/well) for 1 h and subsequently washed. Then, biotinylated anti-AFB_1 (5 ng mL^{-1}, 100 μL/well) in PBS and serial concentrations of AFB_1 standard (0, 1, 5, 25, 50, 75, 100 and 1,000 pg mL^{-1}, 100 μL/well) in diluted oat flakes extract were added to the wells. The immunoreaction was allowed to proceed for 1 h. After washing, the plate was incubated with SApolyHRP (25 ng mL^{-1}, 100 μL/well) for 30 min. After another washing, TMB substrate (100 μL/well) was added and the plate was incubated for 15 min. Finally, the optical density (O.D.) was recorded at 450 nm after stopping the color development with 5 % sulfuric acid (100 μL/well). All incubations unless otherwise specified were performed at RT with shaking and each washing step involved three changes of washing buffer (300 μL/well).

Classical indirect competitive ELISA for AFB_1 detection using anti-AFB_1 and HRP labeled anti-mouse IgG (DetecAb-TracAb format)

With minor modifications, the ELISA was performed as described above for the BioAb-SApolyHRP format. The biotinylated anti-AFB1 and anti-mouse IgG were used at concentrations of 5 ng mL^{-1} and 200 ng mL^{-1}, respectively. Further, instead of 15 min, the substrate incubation was continued for 20 min.

Comparison of the BioAb-SApolyHRP and the DetecAb-TracAb competitive ELISAs

BioAb-SApolyHRP and DetecAb-TracAb competitive ELISAs were performed on three plates for each format. For better comparison, the reagents prepared in the same batch were used for both formats (e.g., buffers, AFB_1-BSA coating antigen solution, AFB_1 standards, TMB substrate) and their similar steps were operated almost in parallel, e.g., coating, blocking, the addition of AFB_1 and biotinylated or unconjugated anti-AFB_1, color development).

Data analysis

Standard curves were obtained by plotting the signal responses (O.D.) against the logarithm of analyte concentrations using Origin software (Origin 7.0). The 4-parameter logistic equation $y = A_2 + (A_1 - A_2)/[1 + (x/x_0)^p]$ was used for curve fitting in the whole concentration range, where A_1 is the maximum signal at no analyte, A_2 is the minimum signal at infinite concentration, p is the curve slope at the inflection point, and x_0 is the IC_{50} (analyte concentration causing a 50 % inhibition of the maximum response, a measure of immunoassay detectability). S/N ratio was calculated from maximum signal (A_1)/minimum signal (A_2) from the above equation [13]. In this paper, the actual background (%) was defined as the ratio of observed minimum signal (O.D.min)/maximum signal (O.D.max)×100.

Moreover, the (y vs $log(x)$) linear equation $y = b + k\,log(x)$ was used for curve fitting in the linear (working) range, where k is the slope.

Results and discussion

Sensitivity, precision, and accuracy, e.g., are terms, which are sometimes very loosely used and therefore, generate confusion. Obviously, results of enzyme immunoassay cannot be meaningfully interpreted nor can theoretical considerations be made if such important concepts are not clearly distinguished [14]. Defined by the dose–response curve, sensitivity corresponds to the change in response (dR) per unit amount of reactant (dC) and equals dR/dC (not necessarily constant). It is used in this sense throughout the manuscript and should be discriminated from the limit of detection (LOD), which refers to the calculated analyte concentration corresponding to signal response of the blank plus three times of its standard deviation (SD) [15]. Compared to other parameters like working range, the LOD seems less practical in realistic analysis. With regard to signal amplification in competitive immunoassay, the successful amplification should be reflected first on its improvement of assay sensitivity, as was illustrated by Ambrosi et al. [16] on studying signal enhancement in sandwich ELISA using gold nanoparticles.

Immunoassay optimization

For BioAb-SApolyHRP ELISA, the cost-effective NHS-LC-biotin was used to biotinylate the anti-AFB_1 since it can provide high biotinylation efficiency supported by earlier research data. Mock and Bogusiewicz [17] investigated six commonly used commercial biotinylation reagents against various groups of IgG, and found that the NHS-LC-biotin revealed the highest biotinylation efficiency (4.5 mole of biotin labeled per mole of IgG). The biotinylated anti-AFB_1 obtained in this way worked well. The optimization test showed that very low concentration of BioAb (5 ng mL^{-1}) and SApolyHRP (25 ng mL^{-1}) can lead to sufficient O.D. response with good performance achieved (data not shown). These parameters were thus used throughout this study. For the classical DetecAb-TracAb ELISA, the lower the anti-AFB_1 concentration used, as expected, the lower IC_{50} gained. When the low concentration of 5 ng mL^{-1} was used, the relatively high concentration of HRP-anti-mouse IgG (200 ng mL^{-1}) was needed to produce only adequate O.D. response (data not shown). Considering neglectable influence of biotinylation on immunological activity of antibody, the anti-AFB_1 concentration (5 ng mL^{-1}), the same as BioAb concentration aforementioned, and HRP labeled anti-mouse IgG (200 ng mL^{-1}) were used as the optimized parameters in DetecAb-TracAb format, to compare the signal amplification capability between the monomeric HRP and the polyHRP.

Comparison of analytical performance between the two competitive ELISA formats

Under optimized conditions, as shown in Fig. 2a, the dose-response curves of both BioAb-SApolyHRP and DetecAb-TracAb competitive ELISA formats exhibit the typical inverse sigmoidal pattern. The O.D. decreases with the increasing AFB_1 concentration. For the BioAb-SApolyHRP format, the logistic regression equation is:

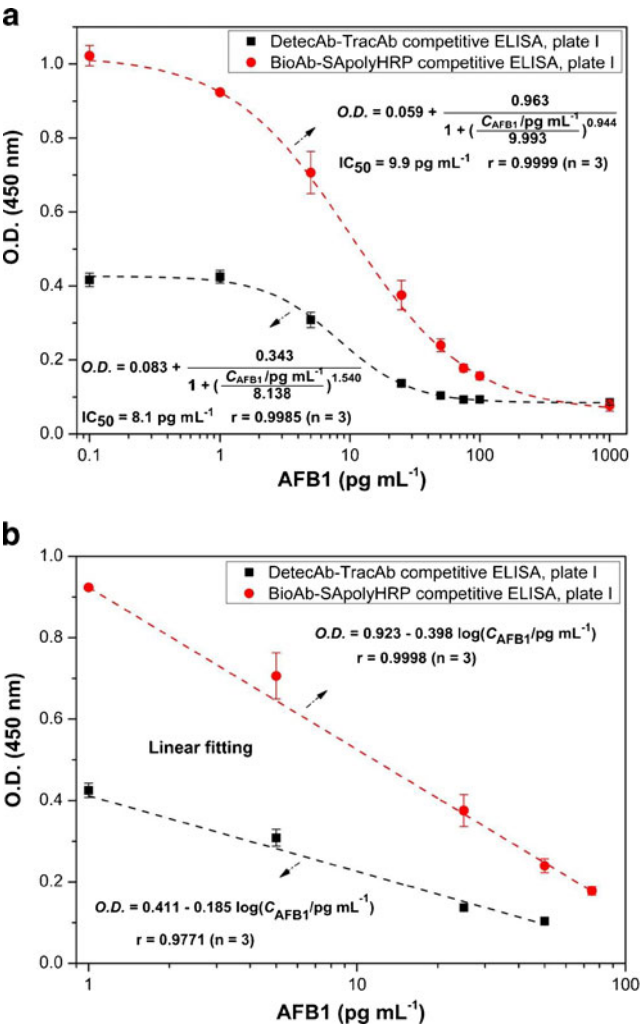

Fig. 2 Calibration curves of competitive ELISAs for detection of AFB_1 in oat flakes extract with polyHRP (●) and HRP (■) as labels (**a**) and the intervals used for linear fitting of the calibration curves (**b**). Other conditions, coating: AFB_1-BSA, 100 ng mL^{-1} in PBS (pH 7.4) overnight at 4 °C; blocking: 1 % casein/PBS, 1 h at RT; biotinylated anti-AFB_1 (●) or unconjugated anti-AFB_1 (■), 5 ng mL^{-1}, 1 h at RT; SApolyHRP (●, 25 ng mL^{-1}) or HRP labeled anti-mouse IgG (■, 200 ng mL^{-1}), 0.5 h at RT; color development, 15 min (●) or 20 min (■)

$$O.D. = 0.059 + \frac{0.963}{1 + \left(\frac{C_{AFB1}/\mathrm{pg\,mL^{-1}}}{9.993}\right)^{0.944}}$$

where C_{AFB1} is the AFB_1 concentration in diluted oat flakes extract. The coefficient of correlation was r= 0.9999 with the calculated IC_{50} of 9.9 pg mL^{-1}, LOD of 0.8 pg mL^{-1} and S/N ratio of 17.2. This BioAb-SApolyHRP competitive ELISA has a linear range defined as $IC_{20–80}$ of 2.3–43.3 pg mL^{-1}. Its linear regression equation (Fig. 2b) is $O.D. = 0.923 - 0.398\log\left(C_{AFB1}/\mathrm{pg\,mL^{-1}}\right)$ with coefficient of correlation r=0.9998, slope $k_{\text{BioAb-SApolyHRP}} = -0.398$ and sensitivity $dO.D./dC_{AFB1} = k_{\text{BioAb-SApolyHRP}}/(C_{AFB1}\ \ln 10)$. For the DetecAb-TracAb format, the logistic regression equation is:

$$O.D. = 0.083 + \frac{0.343}{1 + \left(\frac{C_{AFB1}/\mathrm{pg\,mL^{-1}}}{8.138}\right)^{1.540}}$$

The coefficient of correlation was r=0.9985 with the calculated IC_{50} of 8.1 pg mL^{-1}, LOD of 3.1 pg mL^{-1} and S/N ratio of 5.0. This DetecAb-TracAb competitive ELISA has a linear range defined as $IC_{20–80}$ of 3.3–20.0 pg mL^{-1}. Its linear regression equation is $O.D. = 0.411 - 0.185\log\left(C_{AFB1}/\mathrm{pg\,mL^{-1}}\right)$ with coefficient of correlation r=0.9771, slope $k_{\text{DetecAb-TracAb}} = -0.185$ and sensitivity $dO.D./dC_{AFB1} = k_{\text{DetecAb-TracAb}}/(C_{AFB1}\ \ln 10)$. Thus, the direct comparison of the two formats in terms of sensitivity can be revealed by the amplification factor, which equals the ratio of sensitivities, i.e., amplification factor=$k_{\text{BioAb-SApolyHRP}} : k_{\text{DetecAb-TracAb}}$ ((−0.398) : (−0.185)). This factor was calculated to be 2.1. From the parameters above, it can be concluded that the developed BioAb-SApolyHRP competitive ELISA using polyHRP as label leads to the performance improvement in terms of sensitivity, width of linear range, and S/N ratio, whereas assay time, IC_{50} and LOD values were comparable to the traditional DetecAb-TracAb format. As shown in Table 1, both assay formats can be used to determine AFB_1 according to EU rules in cereal samples, i.e., LOD values and working ranges are clearly below the set MRL of 2 μg kg^{-1}.

To further demonstrate the capability of the developed polyHRP based ELISA, the overall performance including precision and reproducibility of the two formats were evaluated on three plates for each format. The intraassay precision (an expression of well-to-well consistency) was assessed by assaying the AFB_1 samples of three replicates (n=3) on one plate. The intraassay variation coefficients (CVs, %) for BioAb-SApolyHRP \DetecAb-TracAb ELISAs were 2.7\4.3, 0.2\4.1, 8.1\6.8, 10.5\4.2, 7.1\4.6, 5.7\4.7, 6.8\8.9 and 3.6\8.8 at 0, 1, 5, 25, 50, 75, 100 and

Table 1 Comparison between the BioAb-SApolyHRP ELISA and the DetecAb-TracAb format based on the average of parameters obtained on three plates

Format	Logistic fitting					Linear fitting		Precision (CVs %)		Background (%)	S/N	Analysis time
	LOD (μg kg^{-1})	IC_{50} (μg kg^{-1})	r	Linear range (μg kg^{-1})		Slope (k)	r	Intraassay (n=3)	Interassay (n=3)	O.D.min/ O.D.max	Upper asymptote (A_1)/Lower asymptote (A_2)	
				IC_{20}	IC_{80}							
A: BioAb-SApolyHRP	0.077± 0.070	0.182± 0.019	0.9999± 0.0030	0.043± 0.005	0.776± 0.069	(−0.446)± 0.054	0.9908± 0.015	0.2–10.5	4.7–16.1	7.2±0.9	18.4±3.5	1 h 45 min
B: DetecAb-TracAb	0.048± 0.008	0.138± 0.006	0.9985± 0.0006	0.054± 0.005	0.344± 0.042	(−0.188)± 0.008	0.9771± 0.019	4.1–8.9	0.3–14.7	21.3±0.7	4.8±0.3	1 h 50 min
Ratio (A/B)	1.6±1.4	1.3±0.1	N/A	N/A	N/A	2.4±0.3	N/A	N/A	N/A	0.33±0.04	3.8±0.7	N/A

N/A not applicable

1,000 pg mL^{-1} of AFB_1, respectively. Likewise, the interassay precision (an expression of plate-to-plate consistency) was estimated by analyzing the AFB_1 samples on three plates (n=3) using the mean results of three replicates of each plate. The interassay CVs for BioAb-SApolyHRP ELISA and DetecAb-TracAb ELISA were 4.7–16.1 % and 0.3–14.7 %, respectively. Thus, the precision and reproducibility for both formats are similar and within the acceptable range. Moreover, the overall performance for each format, expressed as the average of the parameters from three plates, was summarized in Table 1. It could be found that, for detection of AFB_1 in oat flakes extract, the polyHRP based BioAb-SApolyHRP ELISA showed a 2.4±0.3 -fold signal amplification in terms of sensitivity, i.e., the slope of the calibration curve was increased significantly, compared to the monomeric HRP based traditional DetecAb-TracAb ELISA. Considering the concentrations of SApolyHRP (25 ng mL^{-1}) and HRP labeled anti-mouse IgG (200 ng mL^{-1}) used in the comparison, there can be estimated an about nineteen-fold signal amplification resulting from the polyHRP when the same concentration of enzyme conjugates were used in both formats. Almost no appreciable signal difference existed between the O.D.max (blank) and the O.D.min when 25 ng mL^{-1} HRP labeled anti-mouse IgG was used in the aforementioned DetecAb-TracAb ELISA (data not shown). Moreover, both formats have comparable IC_{50} and LOD values. However, the polyHRP based BioAb-SApolyHRP ELISA still demonstrated the performance improvement in regard to linear range (width about doubled) and S/N ratio (3.8±0.7 -fold increased) in comparison to the traditional DetecAb-TracAb ELISA. This indicated the polyHRP based BioAb-SApolyHRP ELISA to be a promising method for AFB_1 detection in practical samples. Also, the observed signal amplification may open up new possibilities for other small molecules detection.

Conclusion

In this work, an ultrasensitive competitive ELISA, based on polyHRP as label, was developed for the detection of AFB_1 in oat flakes extract. The optical detection signal was amplified by the polyHRP conjugate consisting of hundreds of HRP molecules pre-polymerized. This led to the very low concentration of biotinylated primary anti-AFB1 (5 ng mL^{-1}) required for adequate signal response and about 2.4-fold increase in sensitivity compared to the classical indirect competitive ELISA. The polyHRP conjugate showed a 19.2-fold ability of signal amplification in comparison to the traditional HRP-anti-mouse IgG at the same concentration. The developed polyHRP based competitive ELISA also revealed performance improvement in terms of working range, S/N ratio and analysis time. Considering its simplicity, high loading density of HRP signal molecules, safety and ease in operation and storage, the described competitive ELISA using polyHRP as label, was successfully demonstrated as a simple, cost-effective, highly sensitive ELISA for the small molecule detection in practical samples.

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