An efficient direct competitive nano-ELISA for residual BSA determination in vaccines

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Abstract A simple, fast, and highly sensitive direct competitive enzyme-linked immunosorbent assay (ELISA) based on bovine serum albumin (BSA) antigen labeled amine-terminated silicon dioxide (SiO2–NH–BSA) nanoparticles was developed to determine residual BSA in vaccines. As nano-ELISA using nanomaterials with a very high surface-to-volume ratio has emerged as a promising strategy, SiO2–NH–BSA nanoparticles were prepared in this study by the coupling of BSA to SiO2 nanoparticles modified with amidogen, followed by the quantification of BSA via a direct competitive binding of BSA-antigen-labeled SiO2 nanoparticles to anti-BSA antibody conjugated with horseradish peroxidase. The validation study showed that the linear range of this method was from 1 to 90 ng/mL (r = 0.998) and the limit of detection was 0.67 ng/mL. The intra-assay and interassay coefficients of variation were less than 10% at three concentrations (10, 40, and 70 ng/mL), and the recovery was 92.4%, indicating good specificity. As a proof of principle, this new method was applied in the analysis of residual BSA in five different vaccines. Bland–Altman plots revealed that there was no significant difference in the accuracy and precision between our new method and the most commonly used sandwich ELISA. From the results taken together, the new method developed in this study is more sensitive and facile with lower cost and thus demonstrated potential to be applied in the quality control of biological products.

Keywords Bovine serum albumin · Vaccines · SiO2 nanoparticles · Direct competitive nano-ELISA

Introduction

In the last two centuries, vaccines have been considered the safest and most effective tools for the prevention of contagious diseases [1]. Bovine serum is a main ingredient in culture medium used in cell-based vaccine production [2]. Since the presence of bovine serum can result in the development of an allergic reaction associated with serious adverse events after vaccination, the residual amounts of bovine serum in vaccines need to be determined [3]. Bovine serum albumin (BSA) is a major and characteristic component of bovine serum. Thus, the residual content of BSA is usually used as an important
Historically, a variety of analytical techniques have been reported for BSA detection, such as resonance light scattering [5, 6], fluorimetric determination [7, 8], sensor determination [9, 10], and spectrophotometry methods [11]. These spectral methods intrinsically have low specificity for BSA and a relatively high background resulting from other unknown proteins in both modified live and killed vaccines. Thus, it is great practical significance to accelerate analytical method improvement. Recently, enzyme-linked immunosorbent assay (ELISA) has been applied for BSA determination with high specificity and sensitivity based on the specific reaction of an antibody and an antigen [12, 13]. The most commonly used immunoassay for BSA detection is sandwich ELISA, which is also designated as the official method in the Pharmacopoeia of the People’s Republic of China [14] for residual BSA detection in vaccines [14]. However, these conventional sandwich ELISA methods usually require an expensive secondary antibody and have a narrow detection range. They also involve complicated and time-consuming procedures to block the unbound sites and repeated plate incubation and washing steps, which may take several days [15–17]. Therefore, it is still challenging but necessary to develop a method with improved simplicity, selectivity, and sensitivity for the detection of residual BSA in vaccines.

Use of nanomaterials as carriers for signal amplification of antibody recognition is an emerging and promising direction in enzymatic immunoassays [18, 19]. Compared with the traditional ELISA performed in 96-well (or 384-well) polystyrene plates, nano-ELISA using nanoparticles as the stationary phase has shown greatly increased efficiency because of the strong absorption ability and high surface-to-volume ratio of nanoparticles [20, 21]. Furthermore, the miniaturized nanoscale material lends itself to a small diffusion distance compared with traditional plate wells, and thereby promotes shorter reagent diffusion times. As an important nanomaterial with a wide range of application, SiO2 nanoparticles are characterized by easy preparation, large surfaces areas, high chemical stability, and good biocompatibility [22–25]. SiO2 nanoparticles have been used as carriers for drug delivery and release in the pharmaceutical field [26, 27]. DNA detection with horseradish peroxidase (HRP) labeling [28], 2,4-dichlorophenoxycetic acid detection with antibody conjugation [29], and analysis of herbal medicine with modified human serum albumin [30].

Herein we report the preparation of immobilized BSA antigen on SiO2 nanoparticles modified with amidogen through the carbodiimide method. The subsequent quantification of BSA in vaccines was achieved via a direct competitive ELISA using this BSA antigen conjugated with SiO2 nanoparticles, which competed to bind to the anti-BSA antibody conjugated with HRP. Compared with the classic sandwich ELISA, this method is independent of a secondary antibody, and thus simplified the process, decreased the cost, and improved the detection range. Furthermore, the introduction of the SiO2 nanoparticles with large surface areas made possible a substantial interaction between the antigen and the antibody, which led to significantly improved performance with shortened assay time and enhanced reproducibility. Validation of this new method revealed a good linear range, limit of detection, intra-assay and interassay coefficients of variation, specificity, and recovery as required for quantitative analysis. As a proof-of-principle study, this new method was examined in the analysis of residual BSA in five different vaccines. Statistical analysis by Bland–Altman plots showed that there was no significant difference in the accuracy and precision of the new method and the most commonly used sandwich ELISA. Therefore, the new direct competitive ELISA method using SiO2 nanoparticles developed in the present study demonstrated potential to be applied in the quality control of biological products.

Experimental

Apparatus and reagents

BSA, HRP, Dulbecco’s modified Eagle’s medium (DMEM), 3,3’5,5’-tetramethylbenzidine (TMB), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, N-hydroxysuccinimide, and 3-aminopropyltrimethoxysilane were purchased from Sigma-Aldrich (USA). Anti-BSA antibody, horse serum, pig serum, rabbit serum, dog serum, chicken serum, and mouse serum were purchased from Beijing Beiseebio Co. (Beijing, China). Tetraethyl orthosilicate, ammonia solution, ethanol, sodium periodate, hydrogen peroxide, and sodium borohydride were purchased from Sinopharm Chemical Reagent Co. (China). Five vaccines were purchased from Boehringer Ingelheim (USA). The other solvents and chemicals were analytical reagent grade.

The UV absorbance of samples was measured with a UV-155s spectrophotometer (Shanghai INESA Scientific Instrument Co.). The morphologies and sizes of the SiO2 nanoparticles were determined with a transmission electron microscope (Tecnai G20, FEI, USA). The thermogravimetric analysis (TGA) curves of SiO2 nanoparticles were obtained by the heating of powdered samples from room temperature to 800 °C under a nitrogen atmosphere with use of a TGA Q500 V20.13 Build 39 thermoanalysis system (TA Instruments, USA).

Preparation of SiO2–NH–BSA

First, amine-terminated SiO2 (SiO2–NH2) nanoparticles were synthesized by a two-step method according to the typical
Stöber process [31]. Briefly, in the first step, 9 mL tetraethyl orthosilicate and 27 mL ethanol were added to a 500-mL flask. Thereafter, 170 mL of 85% ethanol solution containing 0.15 mM ammonia solution was introduced in the flask under vigorous stirring and the mixture was reacted for 4 h at room temperature. In the second step, 10 mL 3-aminopropyltrimethoxysilane was added directly to the aforementioned mixture and the resulting mixture was allowed to react for 16 h at room temperature. The mixture was then centrifuged at 1760 g for 15 min and washed three times with anhydrous ethanol and deionized water respectively. Finally, the products were dried at 50 °C for further use.

Second, the SiO$_2$–NH$_2$–BSA nanoparticles were prepared by the carbodiimide cross-linking method [32] with use of the synthesized SiO$_2$–NH$_2$ nanoparticles. In brief, 1 g BSA was dissolved in 100 mL phosphate-buffered saline (PBS; 10 mM, pH 7.4) to generate a 10 mg/mL BSA solution. Then the milky liquid was added dropwise to the BSA solution with constant stirring. Finally, 40 mL of a 50 mg/mL 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide solution and 25 mg/mL N-hydroxysuccinimide solution was added to the aforementioned mixture under gentle stirring, and the resulting mixture was allowed to react for 12 h. The SiO$_2$–NH$_2$–BSA nanoparticles were obtained after centrifugation at 1760 g for 20 min and were washed three times with deionized water. The products were dried at 35 °C and stored at 4 °C for further use. The procedure for preparation of SiO$_2$–NH$_2$–BSA nanoparticles is illustrated in Fig. 1.

**Preparation of HRP-labeled anti-BSA antibody**

The HRP-labeled anti-BSA antibody was prepared by the sodium periodate method as previously described [33]. Briefly, 1 mL of 5 mg HRP in deionized water was mixed with 0.16 mL of 0.1 M sodium periodate solution and the mixture was incubated in the dark at room temperature for 20 min. Thereafter, the mixture was dialyzed against sodium acetate buffer (1 mM, pH 4.4) for 16 h at 4 °C and carbonate buffer (10 mM, pH 9.5, 0.586 g NaHCO$_3$ and 0.32 g Na$_2$CO$_3$ dissolved in 1000 mL water). Then, the resulting mixture was centrifuged at 1760 g for 5 min, and the supernatant was measured at 450 nm. The procedure for preparation of SiO$_2$–NH$_2$–BSA nanoparticles was illustrated in Fig. 1.

In this case, 2 mg anti-BSA antibody was dialyzed against the carbonate buffer (10 mM, pH 9.5) for 2 h at 4 °C. Then the dialyzed anti-BSA antibody was incubated with the aforementioned HRP at room temperature for 2 h. Afterward, 0.08 mL of fresh sodium borohydride (4 mg/mL) was added dropwise to the aforementioned mixture and the resulting mixture was allowed to react for 2 h at 4 °C. The resulting product was dialyzed against PBS (10 mM, pH 7.4) for 12 h at 4 °C. Finally, the HRP-labeled anti-BSA antibody was purified by a saturated ammonium sulfate method [34] to remove the extra HRP and then stored at 4 °C for further use.

**Direct competitive enzyme immunoassay**

The analytical method for BSA detection was based on a direct competitive enzyme immunoassay. Firstly, 50 mg SiO$_2$–NH–BSA nanoparticles was dispersed in 1.5 mL of PBS with sonication for 15 min. Subsequently, 0.25 mL BSA solution and 0.25 mL HRP-labeled anti-BSA antibody solution were added to the aforementioned milky liquid under gentle vortexing and allowed to react for 30 min. Secondly, the product was centrifuged at 1760 g for 5 min and washed three times with deionized water. Thirdly, the precipitate was dispersed in 1 mL TMB substrate solution (0.5 mL of 2 mg/mL TMB solution and 42 μL of 0.75% hydrogen peroxide dissolved in 9.5 mL of substrate buffer; 0.1 M citrate-phosphate buffer, pH 5.0) and the resulting mixture was incubated for 15 min at room temperature. Finally, the reaction was terminated with 0.5 mL of 2 M H$_2$SO$_4$ for 5 min, and the mixture was centrifuged at 1760 g for 5 min. The absorbance of the supernatant was measured at 450 nm. The procedure for the direct competitive enzyme immunoassay is illustrated in Fig. 2.

**Method optimization**

In this reported analytical method, it was important to optimize the ratio of the SiO$_2$–NH–BSA nanoparticles to the HRP-labeled anti-BSA antibodies given that the nanoparticles will bind to all the antibodies when the system does not contain any BSA antigen. Briefly, 10, 20, 30, 40, 50, 60, and 70 mg of SiO$_2$–NH–BSA nanoparticles in triplicate were dispersed in 1.75 mL PBS, and the resulting mixture was allowed to react at 4 °C for 20 min. Finally, the HRP-labeled anti-BSA antibody was purified by a saturated ammonium sulfate method [34] to remove the extra HRP and then stored at 4 °C for further use.

**Method validation**

After optimization of the ratio of SiO$_2$–NH–BSA nanoparticles to HRP-labeled anti-BSA antibodies to be used, the linear
range, limit of detection, intra-assay and interassay coefficients of variation, specificity, and recovery were determined. The BSA standard solution for establishing the standard curve was prepared by addition of 3 mg BSA to 3 mL DMEM. Subsequently, the solution was diluted to the necessary concentration (1, 5, 10, 30, 50, or 90 ng/mL). The BSA-free DMEM served as the standard blank solution (0 ng/mL). According to the aforementioned direct competitive enzyme immunooassay procedures, the calibration curve for BSA was obtained by detection of each point in triplicate. The limit of detection was defined as the average value minus three times the standard deviation. The intra-assay variation was determined with BSA standard solution at three concentrations (10, 40, and 70 ng/mL) with the reported analytical method in nonuplicate. The interassay variation was determined at the three concentrations on 7 days under the same conditions. The specificity of the reported analytical method was analyzed with sera from horse, pig, rabbit, dog, chicken, and mouse. These six sera were diluted to a total protein concentration of 5 mg/mL, subsequently spiked with BSA at 30 ng/mL or not spiked with BSA, and then detected by the immunoassay method described earlier. The recovery was determined by a certain amount of BSA (80%, 100%, and 120% of the BSA content in the vaccines) added to the vaccines.

Data processing and statistical analysis

To evaluate the dispersion of the results obtained with the present method and a conventional sandwich ELISA, Bland–Altman plots were analyzed with PRISM 5.0 [35, 36]. The mean concentrations were presented together with the relative differences between concentrations. The percentage difference was plotted as a Bland–Altman graph.

Results and discussion

Characterization

The synthesized SiO$_2$–NH–BSA nanoparticles were characterized by transmission electron microscopy and TGA. A transmission electron microscope image of SiO$_2$–NH–BSA nanoparticles with a diameter less than 100 nm is shown in Fig. 3. TGA, which can determine the content of BSA-labeled silica nanoparticles, was used to confirm the characterization of the SiO$_2$–NH–BSA nanoparticles. The weight loss curves of the SiO$_2$–NH$_2$ and SiO$_2$–NH–BSA nanoparticles are shown in Fig. 4. Clearly, for both curves the first half is similar, which was ascribable to the removal of water. The variation of the latter half of the curves was different because of the decomposition of organic functional groups on the surface of nanoparticles, centrifugal separation, and catalytic color development by the substrate solution for quantitative measurement. Ab antibody, Ag antigen, TMB 3,3′,5,5′-tetramethylbenzidine.

Fig. 2 The three steps of the direct competitive enzyme immunoassay procedure: competitive binding with horseradish peroxidase (HRP)-labeled anti-BSA antibody between residual BSA and SiO$_2$–BSA nanoparticles, centrifugal separation, and catalytic color development by the substrate solution for quantitative measurement. Ab antibody, Ag antigen, TMB 3,3′,5,5′-tetramethylbenzidine

Fig. 3 Transmission electron microscope image of SiO$_2$–NH–BSA nanoparticles

Fig. 4 Transmission electron microscope image of SiO$_2$–NH–BSA nanoparticles
the silica nanoparticles. The weight loss of SiO$_2$–NH–BSA nanoparticles was greater than that of SiO$_2$–NH$_2$ nanoparticles, and thus indicated that the silica nanoparticles were successfully labeled with BSA. With use of the Bradford method according to the Pharmacopoeia of the People’s Republic of China, it was calculated that about 9% (w/w) of the BSA was immobilized on the surface of the SiO$_2$ (see “Determination of the BSA bound to SiO$_2$” in the electronic supplementary material).

Optimization of the quantity of SiO$_2$–NH–BSA nanoparticles

Optimization of the ratio of the SiO$_2$–NH–BSA nanoparticles to the HRP-labeled anti-BSA antibodies not only eliminates the unwanted binding of the nanoparticles to the antibodies in the absence of BSA antigen in the system, but also contributes to the detection sensitivity and saves resources. The absorbance of the 1:100 diluted HRP-labeled anti-BSA antibody at 280 and 403 nm was detected, and was 0.054 and 0.025 absorbance units, respectively. Therefore, the molar ratio of HRP and IgG was 1.51, and the dilution of HRP-labeled anti-BSA antibody was 1:20,000. As shown in Fig. 5, when the quantity of SiO$_2$–NH–BSA nanoparticles was greater than 45 mg, the absorbance of the reaction system at 450 nm remained unchanged. Therefore, the optimal quantity of SiO$_2$–NH–BSA nanoparticles to be used was 50 mg.

Analytical figures of merit

Through the assaying of standard solutions at concentrations ranging from 1 to 90 ng/mL, a linear calibration curve was established as $y = -0.012x + 1.659$, with good correlation ($r = 0.998$). The limit of detection was determined to be 0.67 ng/mL. The precision of this direct competitive enzyme immunoassay was determined by the intra-assay and interassay coefficients of variation. The intra-assay precision was calculated by detection of three different concentrations (10, 40, and 70 ng/mL) in nonuplicate, and their coefficients of variation were 8.6%, 4.9%, and 7.7% respectively. The interassay precision was calculated by detection of the three different concentrations on different days, and their coefficients of variation were 8.3%, 4.9%, and 7.8% respectively. Thus, the intra-assay and interassay coefficients of variation were both less than 10%, which indicated that the reported analytical method exhibited high reproducibility. The specificity was determined by addition of a certain amount of BSA to six different sera. The recoveries were below 120% and above 80%, which was interpreted as there being no cross-reactivity. The six sera from horse, pig, rabbit, dog, chicken, and mouse were analyzed, and the recoveries ranged from 101.9% to 117.8%. These results indicated good specificity without cross-reactivity. To determine the recovery, a certain amount of BSA (6.5, 8.1, or 9.7 ng/mL) was added to the vaccine. The recoveries ranged from 89.7% to 95.7%, with the mean recovery being 92.4%. As shown in Table 1, the analytical figures of merit were compared with those of several other quantitative methods reported for BSA determination. The details of the analytical figures of merit can be seen in Tables S1, S2, S3, and S4. Thus, the results indicated that the reported analytical method could be applied to detect BSA in vaccines, and has the potential for the detection of biological samples.

Residual BSA determination in five different vaccines

The residual BSA content in five different vaccines was measured in quintuplicate by the present method. To determine the
reliability of this method, a conventional 96-well sandwich ELISA was also used to determine the residual BSA in these five vaccines.

The results of this comparative study are shown in Table S5 and also presented in Fig. 6. The best agreement of the results obtained from BSA concentration evaluation was observed in measurements of five different samples. The difference between the two measurements was plotted on the y-axis, and the average of the two measurements was plotted on the x-axis. The Bland–Altman plot demonstrated that the present method was subject to negative bias (1.39% in sample 1, 2.53% in sample 2, 3.77% in sample 3, 3.15% in sample 4, and 2.71% in sample 5) compared with the conventional sandwich ELISA method.

**Table 1** Comparison of analytical methods reported for determination of bovine serum albumin

| Method                      | Material                          | Detection range | Equipment demand | Detection cost | Specificity | Reference |
|-----------------------------|-----------------------------------|-----------------|------------------|----------------|-------------|-----------|
| Direct competitive nano-ELISA | Polyclonal primary antibody       | 1–90 ng/mL      | Low              | Medium         | High        | This work |
| Sandwich ELISA              | Polyclonal primary and secondary antibody | 0–64 ng/mL     | Low              | High           | High        | [12]      |
| Sandwich ELISA              | Monoclonal primary and secondary antibody | 0.5–40 ng/mL   | Low              | Extremely high | High        | [13]      |
| Capillary Western technology| Polyclonal antibodies             | 5.2–420 ng/mL   | High             | High           | High        | [4]       |
| Rayleigh light scattering   | Triangular silver nanoplates      | 200–24,900 ng/mL| Medium           | Low            | No          | [5]       |
| Fluorescence detection      | Fluorescence-turn-on bioprobe     | 300–50,000 ng/mL| Medium           | Low            | No          | [7]       |
| Luminescence biosensor      | Ruthenium (II) complex            | Not mentioned   | Medium           | Low            | No          | [10]      |

ELISA enzyme-linked immunosorbent assay

Fig. 6 Comparison of the present method and conventional sandwich enzyme-linked immunosorbent assay (ELISA) in five different vaccines presented as the mean value (bottom) and the Bland–Altman plot with differences presented as a percentage (top). The Bland–Altman plots demonstrate that the present method was subject to negative bias (1.39% in sample 1, 2.53% in sample 2, 3.77% in sample 3, 3.15% in sample 4, and 2.71% in sample 5) compared with the conventional sandwich ELISA method.
Conclusion

In this study, a simple, fast, and highly sensitive direct competitive enzyme immunoassay based on SiO$_2$–NH–BSA nanoparticles was developed for the quantitative determination of residual BSA in vaccines. This is the first work to immobilize BSA antigen on the SiO$_2$ nanoparticles. These SiO$_2$–NH–BSA nanoparticles were subsequently used for direct competitive binding to HRP-labeled anti-BSA antibody, which forms the basis of the ELISA developed in the present study. This new assay was validated and found to be suitable for the quantification of residual BSA in vaccines, with a wide linear range, a low limit of detection, good specificity, high recovery, and consistent reproducibility. Furthermore, this new method was verified to be statistically comparable to the classic sandwich ELISA in terms of accuracy and precision but was significantly more facile.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval This article does not contain any studies with human or animal subjects performed by any of the authors.

Informed consent Informed consent is not applicable because of the nature of this study.

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