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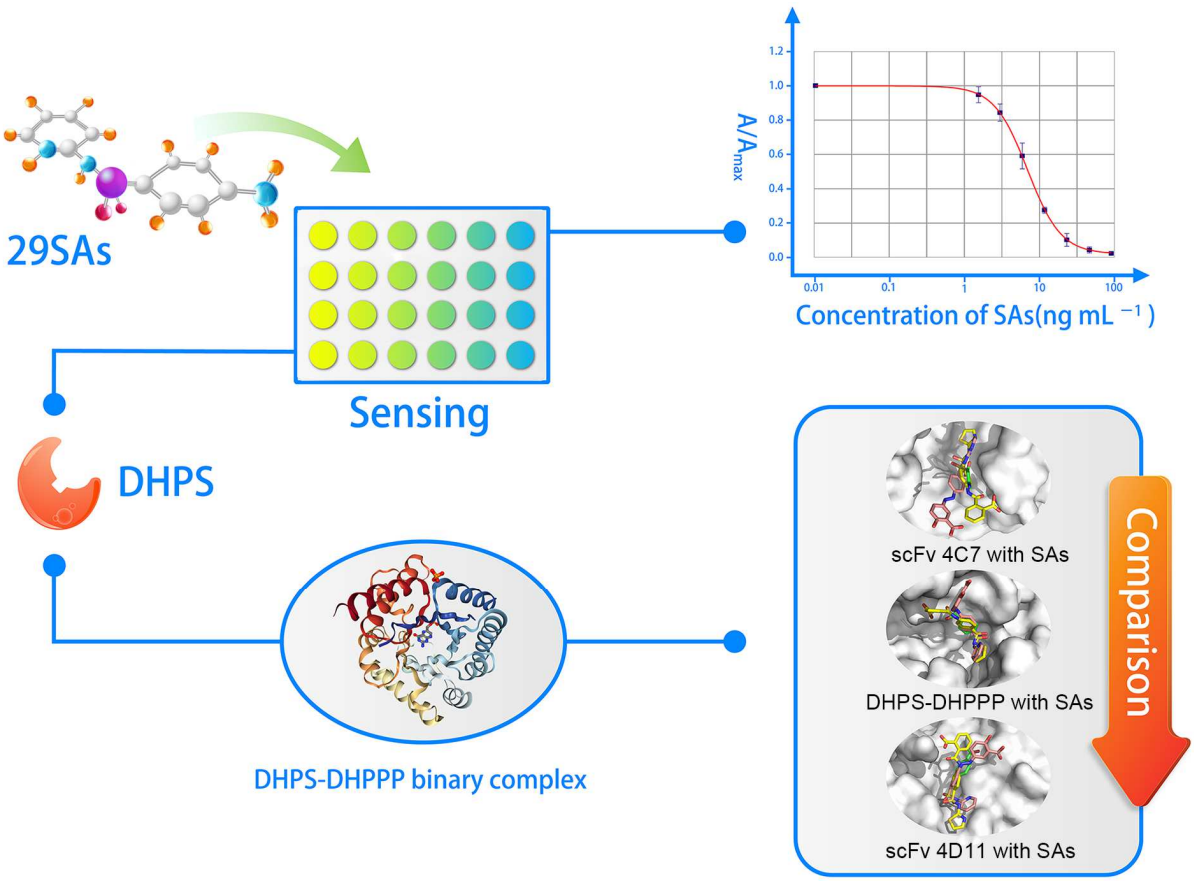

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Dihydropteroate synthase based sensor for screening multi-sulfonamides residue and its comparison with broad-specific antibody based immunoassay by molecular modeling analysis

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ABSTRACT

A biosensor that could simultaneously detect multi-analyte as many as in a single test is more favored when facing lots of samples for screening purpose. In such a biosensor, the recognition element with broad specificity and highly affinity is a key reagent for detection spectrum. Here we report a dihydropteroate synthase (DHPS) based biosensor for detecting 29 sulfonamides (SAs) in milk. The biosensor was established in a competitive format where HRP-labeled tracer and free SAs competitively binding to the limited amount of DHPS-DHPPP (6-hydroxymethyl-7,8-dihydropterin diphosphate) binary complex. Compared to other biosensors based on antibodies with the same objective, the current sensor employing DHPS provided not only highly sensitivity but also preponderant uniform affinities for all individual sulfonamide, which has never been achieved before in any immunosensor. However, the difference of recognition mechanism between DHPS and antibody remains unknown. To address this question, we deconstructed the variable region of two monoclonal antibodies produced by our group and then compared the intermolecular forces and key contacting amino acid residues of both DHPS and antibodies to several typical SAs with help of molecular modeling analysis. Results showed the orientation of the ligands in the binding site played a significant role during the recognition with proteins. The optimized assay provided a limit of detection (LOD) of 1.15-14.91 ng mL^{-1} and dynamic range was ranging from 1.54-126.85 ng mL^{-1} for 29 SAs. The developed biosensor finally was validated for five SAs in spiked milk with recovery values ranging from 72.7-129.3% and coefficients of variation less than 16.0%. The study showed that the biosensor based on DHPS make it a suitable screening method for the simultaneous determination of total SAs residues in food matrices.

KEYWORDS

Sulfonamides, Dihydropteroate synthase, Biosensor Molecular, Modeling analysis

1. Introduction

Sulfonamides (SAs) are a group of synthetic and broad-spectrum antimicrobial agents that are widely used in human medicine as well as in veterinary practice for the treatment of infectious diseases and as growth-promoting feed additives [1]. In the European Union, 11% of total sold veterinary antimicrobial agents from 25 member states are SAs on 2011, while, the consumption of SAs in animal husbandry was estimated to be around 6721.8 tons in year 2013 in China, accounting for 8.6% of total consumption [2, 3]. A summary of national veterinary drugs residue screening program of China from 2012-2016 showed that sulfonamide residues are 11.5% of total positive animal-origin food samples. The term sulfonamide is a generic name for derivatives of sulfanilamide, which differ from each other by the substituents in the $-SO_2NH-$ (N_1) or the aromatic amino group (N_4) (**Fig. S1**). Nowadays, 16 SAs were approved for veterinary practices by the Ministry of Agriculture and Rural Affairs of China, and at least nine and six SAs are permitted to be used in veterinary medicine in the Netherland and the Czech Republic, respectively [4, 5]. The presence of SAs in food of animal-origin could cause hypersensitive allergic reactions and drug-resistance problems to human beings, and some individual sulfonamide also have been proved providing potential carcinogenic character [6]. To protect consumers from the risk related to sulfonamide residues, China, USA and European Union have been established the maximum residue limits for such drugs in food [7-9].

For surveillance of the residues of SAs in food samples, rapid, sensitive and selective methods are required. Biosensors, such as immunosensors traditionally based on antibodies, are gaining in confidence owing to their simplicity, high sensitivity, and cost-effectiveness, which make them particularly useful in screening purpose [10-14]. During the past decades,

numerous immunosensors for SAs have been reported in the literature [4-6, 15-19]. However, all those immunosensor always suffer from non-uniform affinity, i.e., each of which cannot recognize all type of SAs below 100 ng mL^{-1}. Apparently, for efficient surveillance purposes, a method would be more qualified if which able to detect as many as SAs instead of each individual sulfonamide in a single test. But, the heterogeneity of the R groups of sulfonamide molecules (**Fig. S1**) invariably limits the possibilities to raise antibodies with broad specificity. Thus, the inherent heterogeneity of SAs combined with highly discrimination character of antibody to slightly change of target molecule leading to produce real antibodies with quite uniform affinities to SAs theoretically is impossible. To overcome the limitation of antibody, the development of alternatives to antibodies for all SAs with not only highly affinity but also broad specificity is an active and tremendously important area of research.

As SAs are designed to inhibit dihydropteroate synthase (DHPS) within bacteria, the use of DHPS for detection purposes seemed a promising approach [20]. We have already prepared DHPS from R6 strain of *S. pneumoniae* and firstly developed a microplate assay for multi-SAs detection [21]. In the assay, 9 SAs and *p*-aminobenzoic acid (PABA) could be detected at concentrations less than 100 ng mL^{-1} and 28 SAs with IC_{50} values ranging from 426-50,000 ng mL^{-1}, which could not meet the requirements of regulatory agencies [7]. We then used DHPS-DHPPP binary complex (DHPPP is 6-hydroxymethyl-7,8-dihydropterin diphosphate, which acts as the first substrate for DHPS) instead of DHPS alone to develop a fluorescence polarization assay (FPA), demonstrating quite uniform affinities for all SAs tested [22]. However, the overall sensitivity of the FPA is usually lower and may not be acceptable when analysis needs to be run at trace levels or sample extract needs to be highly diluted to remove matrix effect. Moreover, limited approaches are available to enhance the

FPA sensitivity in large part because it lacks a signal amplification step during the detection procedure [23]. In this study, we prepared a new DHPS* protein from ATCC25922 strain of *E. coli* (To distinguish the DHPS from *S. pneumoniae*, we named the DHPS protein from *E. coli* as DHPS*). Both DHPS and DHPS* were used to develop a direct competitive ELISA-type biosensor for the detection of multi-SAs after selecting an appropriate HRP-labeled sulfonamide derivative and extensively optimizing the assay conditions. To interpret the uniform affinity of the DHPS-based biosensor which exhibits obvious superior performance than immunoassay, we then prepared the single chain variable fragments (scFv) derived from monoclonal antibody (MAb) 4D11 [6]. The three-dimensional structures of scFv 4C7 (modelled previously) and scFv 4D11 were constructed by homology modeling and then docked with several typical SAs [24]. The driving forces and contacting amino acids that binding SAs with antibodies and DHPS protein were compared and discussed. The information about the differences between antibody and DHPS recognition may give insight into design and selection more effective haptens of SAs, production and evolution of antibody to a desired affinity and specificity. The biosensor based on DHPS derived from R6 strain of *S. pneumoniae* was finally applied to determine milk samples spiked with SAs. The results demonstrate the developed biosensor is a suitable method to be used as a fast, simple, sensitive screening tool for multi-SAs residue detection.

2. Experimental

The details on the materials, apparatus and protocols for preparing DHPS* from the strain of ATCC25922 of *E. coli*, synthesizing hapten-HRP conjugates, producing scFv 4D11 against SAs are provided in the ***Supporting Information***.

2.1. Development of biosensor for multi-SAs

Briefly, a fixed amount of DHPS diluted in coating buffer (100 μL/well) was immobilized on the surface of microplate overnight at 4°C. After washing, the microplate was blocked with blocking buffer (150 μL/well) and then the solution was discarded followed by three times washing. For each well, 50 μL of standard solution (or sample diluted by assay buffer), 50 μL of hapten-HRP dissolved in assay buffer containing DHPPP (1/1500 dilution) were added to each well and incubated for 30 min at 37°C. After the final three times washing, 100 μL of the TMB and H_2O_2 solution (fresh prepared) was added and incubated for 15 min at room temperature. The enzymatic reaction was stopped by adding 2 M H_2SO_4 (50 μL/well) and the optical density of each well was measured at 450 nm. A four-parameter logistic equation was used to fit the biosensor data by OriginPro 8.0 (OriginLab Corp., Northampton, MA, USA) as follows:

$$Y = (A - D) / [1 + (X/C)^{B}] + D \quad (1)$$

Where A=response at high asymptote, D=response at low asymptote, *X*=calibration concentration, C=concentration corresponding to 50% specific binding (IC_{50}), and B=slope. The cross-reactivity (CR) of each sulfonamide was calculated based on the IC_{50} values according to the following equation:

$$\text{Cross-reactivity (CR)} = IC_{50}\text{ (sulfamethazine, SMZ)} / IC_{50}\text{ (analog)} \times 100\ \% \quad (2)$$

2.2. Optimization of assay condition for biosensor

In the optimization procedure, we used SMZ as reference analyte to select the appropriate conditions. To evaluate the effect of hapten structures on the sensitivity of biosensor, seven HRP conjugates incorporating different haptens were tested. A set of experimental parameters, including the concentration of DHPS and appropriate hapten-HRP dilution, coating condition, buffer composition, assay temperature, and assay time for the

reaction was studied to improve the performances of biosensor. The detailed optimization procedure was provided in the ***Supporting Information***.

2.3. Molecular modeling and docking

The crystal structure of DHPS from *S. pneumoniae* were obtained from RCSB Protein Data Bank with PDB ID 2VEG. The three-dimensional structure of scFv 4C7 was modelled in our published report [24]. The process of antibody homology modeling of scFv 4D11 and ligand-protein/ligand-antibody docking was done according to our previous work and described in detail in ***Supporting Information*** [25].

2.4. Analysis of samples

The optimized biosensor was applied to whole milk (fat content 3.6%) and skimmed milk (no fat). All those negative samples were provided by the National Veterinary Drug Safety Evaluation Center (Beijing, China) and kept frozen until use. Before recovery study, the effect of milk matrix on the performance of biosensor was firstly evaluated by comparing the standard curve of SMZ obtained in assay buffer with those in diluted milk. For recovery studies, whole milk samples were fortified with five analytes, SMZ, sulfadimethoxine (SDM), sulfaquinoxaline (SQX), sulfamonomethoxine (SMM), and sulfamethoxazole (SMX) at 30, 50, and 60 μg L^{-1} respectively, and skimmed milk samples were spiked with these five analytes at 18, 30, and 36 μg L^{-1} respectively. Then 5-fold and 3-fold dilution with assay buffer was used to avoid matrix interferences for whole milk and skimmed milk, respectively. The average absorbance values (A) determined at each concentration by the optimized biosensor were interpolated with a standard curve prepared in assay buffer with each data point being an average of three replicates.

3. Results and discussion

*3.1. Preparation of DHPS**

The *folP* gene encoding DHPS* protein was successfully obtained by PCR amplification using genomic DNA extracted from the ATCC25922 strain of *E. coli* (**Fig. S2**) and then cloned into cloning vector. As shown in **Fig. S3**, seven clones randomly picked up were confirmed by PCR with the expected size of about 910 bp for *folP* gene. Then plasmid DNA isolated from one of these clones was tested via restriction analysis with fragments in size of about 5717 bp and 910 bp for pEASYTM-T1 and *folP* respectively by *Nde*I and *Xho*I digest (**Fig. S4**). Next, the *folP* gene was transformed into expression plasmid pET-28b, which were confirmed by PCR amplification (**Fig. S5**), restriction analysis (**Fig. S6**) with the expected size shown in the gel and sequence analysis. Finally, DHPS* protein was expressed in *E. coli* BL21 (DE3) and purified by Ni^{2+} chelation affinity chromatography and ion exchange. The data from SDS□PAGE and western□blot analysis are in good consistency with the expected size of 30 kD for DHPS* in **Fig. S7**. Both DHPS protein were used to develop biosensor for SAs. **Fig. S8 (A)** revealed that the identity and similarity between amino sequence originated from *S. pneumoniae* and *E. coli* were 34.3% and 49.5%, respectively. However, as depicted in **Fig. S8 (B)**, the three-dimensional structures of DHPS from *S. pneumoniae* and DHPS* from *E. coli* were more conservative with RMSD of 1.048 Å than primary sequences. Moreover, through the crystal structures of *E. coli* DHPS* (**Fig. S8B**) complexed with DHP and SN (PDB ID 1AJ0), key residues Arg63 and Ser219 which hydrogen bonded with SN were aligned with Arg58 and Ser235 in *S. pneumoniae* DHPS, respectively.

3.2. Development and optimization of biosensor

The DHPS proteins were immobilized on the surface of microplate and then the mixture of sulfonamide at different concentrations, hapten-HRP conjugates and DHPPP at a fixed concentration were added (**Scheme 1**). The DHPPP firstly binds to DHPS and formed as DHPS-DHPPP complex. Then, sulfonamide and hapten-HRP competitively combine for the limited binding site on the DHPS. With more sulfonamide, a lower absorbance signal (A) value is obtained.

As can be observed in **Fig. 1A**, both DHPS-DHPPP complexes could bind with seven hapten-HRP, which demonstrated that these all haptens (**Fig. S9**) have successfully linked to HRP. Unlike noncompetitive biosensor, where an excess of reagents is used to increase detectability, limiting the concentrations of reagents is required in competitive biosensor to obtain the best sensitivities [26]. Accordingly, the concentration of DHPS and CS-HRP was firstly optimized by chessboard experiment (**Table S1**) and the results showed that the optimal DHPS concentration and CS-HRP dilution were at 6 μg mL^{-1} and 1/250 dilution respectively at which the A_{max} (the absorbance value at zero analyte concentration) of biosensor was around 1.5 and A_{100}/A_{max} value (A_{100}, the absorbance value at 100 ng mL^{-1} of SMZ as model analyte) was comparatively low.

The concentrations of Tris-HCl and $MgCl_2$ in the coating buffer could influence the binding of DHPS-DHPPP complex with SAs. As shown in **Fig. 1B**, the A_{max} increased and then decreased when the concentrations of $MgCl_2$ varied from 0 to 100 mM with fixed concentration of Tris-HCl. It is reasonable to believe that Mg^{2+} is a cofactor of DHPS, and high concentration of $MgCl_2$ has a detrimental effect on the interaction of DHPS and the SAs, which may due to ionic forces [27, 28]. With the consideration of the A_{max}, the concentrations

of 20 mM Tris-HCl and 30 mM $MgCl_2$ were chosen for further optimization. The coating temperatures and time were then optimized and the results were summarized in **Table S2**. As shown, the IC_{50} values of SMZ were 9.83 and 9.56 ng mL^{-1} at 4°C for 16 h and 37°C for 2 h, respectively. Although the A_{max} was higher at 4°C for 16 h, the A_{min} higher as 0.5 was not acceptable. Therefore, coating at 37°C for 2 h was selected as the optimum coating temperature and time. The composition of blocking buffer is critical to assay sensitivity when an enzyme was used as recognition element [29]. Two kind of blocking buffers (i.e., 2% skim milk and 5% fetal calf serum) and buffer without blockers were evaluated for their effectiveness in blocking non-occupied sites of coated microplates. The results in **Table S2** showed that the blocking buffer with 2% skim milk could significantly block the plate and resulted in the lowest IC_{50} value of 7.35 ng mL^{-1} for SMZ.

The assay buffer provides the reaction micro-environment for the competitively binding DHPS between SAs and CS-HRP, thus, the composition of assay buffer obviously has direct and vital influence on the performance of biosensor. The effect of the concentration of Tris-HCl and $MgCl_2$ is shown in **Fig. 1C** and **Fig. 1D** respectively. Similar with the coating buffer, the influence of Tris-HCl and $MgCl_2$ in assay buffer followed similar tendencies. Of all Tris-HCl concentrations evaluated in the range from 5-100 mM, the lowest A/A_{max} was obtained at 20 mM, and the lowest A/A_{max} was obtained when the concentration of $MgCl_2$ was 5 mM (A represents the absorbance value at 50 ng mL^{-1} of SMZ as model analyte). The same optimization procedure for DHPS* and SAs was conducted and the optimal conditions were summarized in **Table S3-S4**. In a biosensor for screening purpose, short assay time is one of key advantages over instrumental methods, on the contrary, enough time is required for highly sensitivity of biosensor caused from the completion of effective competition among reagents.

Thus, the assay time for the developed biosensor should be compromise between sensitivity and speed. We varied assay time from 10 to 60 min at 37°C and assessed the effect over the A_{max} and IC_{50} of the biosensor. The variation of the IC_{50} and the A_{max} exhibited was shown in **Fig. 1E**, showing no observed differences for IC_{50} (8.3 and 7.6 ng mL^{-1} for DHPS; 18.4 and 15.3 ng mL^{-1} for DHPS*) of the biosensor when incubating time was 30 min and 60 min. Thus, the assay time in 30 min was acceptable both for speed and sensitivity.

3.3. Sensitivity and cross-reactivity of the biosensors

Under these optimized conditions, the sensitivity and specificity of both biosensors based on DHPS and DHPS* were calculated by using 29 structurally similar SAs and PABA. **Fig. 1F** (based on DHPS) and **Fig. 1G** (based on DHPS*) show the typical standard curves of two biosensors for five SAs (SMZ, SDM, SQX, SMM, and SMX), which are officially required to be monitored by the Ministry of Agriculture and Rural Affairs of China. The all IC_{50} values were used to calculate the CRs and summarized in **Table S5**. All Data show that in the assay based on DHPS, the IC_{50} values for the 29 SAs (besides PABA) varied from 2.95 to 56.22 ng mL^{-1} and in the assay based on DHPS*, the IC_{50} values for 29 SAs varied from 6.16 to 182.27 ng mL^{-1}. And in the assay based on DHPS, most of the IC_{50} values were below 10 ng mL^{-1}, and the lowest value of 2.53 ng mL^{-1} was achieved by PABA. As it can be observed in **Table S5**, all SAs tested were highly recognized in this biosensor, with IC_{50} values far below the maximum residue limits. In addition, other frequently used antibiotics, e.g., trimethoprim (TMP), chloramphenicol (CM), kanamycin (KAN) and ampicillin (AMP), did not interfere in this assay. To the best of our knowledge, the best immunoassays based on antibodies for SAs, such as MAb 4D11, 4C7 (**Fig. 2**) and 4E5 could not detect all SAs with IC_{50} values below 100 ng mL^{-1} [6, 18]. Furthermore, this biosensor based on DHPS exhibited uniform CR values

ranged from 16.06% to 306.1% (besides PABA, **Fig. 3**), whereas the immunoassays based on MAb 4D11, 4C7, 4E5 or any reported other immunoassays showed very large varied CR values among SAs [6, 18, 30]. The results demonstrated that the prepared DHPS was highly broad-specific and sensitive. Though there was lower sensitive for 5 SAs monitored in China when compared to MAb 4D11 based assay, it is reasonable to conclude that, compared with antibody-based assay, DHPS-based biosensor is more appropriate for multi-SAs detection. As shown in **Fig. S10**, compared with our previous developed FPA, this DHPS-based biosensor obtained higher sensitivity [22].

3.4. Molecular basis for recognition difference between antibodies and DHPS

In this study, three ligands PABA, phthalylsulfathiazole (PST), and sulfasalazine (SPH) were docked into DHPS-DHPPP complex, scFv 4C7 and scFv 4D11 respectively. As shown in **Fig. 4A**, despite different substitutes on the N_1 and N_4 position of common structure of SAs, three ligands with almost the same orientation were bound in the binding pocket of DHPS. But for scFv 4C7 and 4D11 depicted in **Fig. 4E** and **4I**, these three ligands revealled distinctly different orientations when combined into the cavity of antibodies. Probably due to the difference of ligand orientations, diverse affinities of proteins against these ligands were generated. As illustrated in **Fig. 2**, **Fig. 3** and **Table S5**, DHPS can recognize PABA, PST, and SPH simultaneously with IC_{50} values ranged from 2.53-56.22 ng mL^{-1}. But antibodies showed different performance, for example, in contrast with negligible recognition between SPH and scFv 4C7, scFv 4D11 has low recognition ability towards SPH. The residues in scFv 4C7, mainly Gly165 and Gly234 formed hydrogen bonds between ligands and antibody. And for scFv 4D11, residues Arg76 operated as one of the key amino acids to capture ligands into the binding site. More importantly, due to one more hydrogen bond between SPH and scFv 4D11

rather than scFv 4C7, higher affinity was obtained by scFv 4D11.

3.5. Analysis of samples

One of the most common challenges of immunoassays for food analysis is matrix interference, which could reduce the sensitivity and reliability of the competitive immunoassay and cause false positives by lowering the color development [31]. In the present study, interferences are quantified by comparing a standard curve obtained in assay buffer with a calibration curve generated in the sample matrix. If the two curves are superimposed, the effect of the matrix is not significant, and then, the samples can be analyzed according to the calibration curve prepared in the assay buffer [16]. To study the possible matrix effect in this study, whole milk and skimmed milk were chosen as test samples. As reported previously, dilution was the most common screening method used to reduce such matrix effects [32]. However, dilution of milk did not eliminate matrix effects in our study. Thus, skimmed milk was added to assay buffer for correcting the matrix interferences. Standard curves produced in whole milk and skimmed milk after 5-fold dilution and 3-fold dilution with the assay buffer (containing 2.5% skimmed milk) respectively were superimposed, indicating that the matrix interferences could be corrected by 2.5% skimmed milk (**Fig. 1H**).

To further evaluate the robust of developed DHPS-based bissensor for food sample analysis, we determined the accuracy (% recovery) and precision (% CV) values with five SAs spiked in the whole milk and skimmed milk, respectively. The results are summarized in **Table 1**. Using the developed method, recoveries for SMZ, SDM, SQX, SMM, and SMX were between 72.7% and 129.3%. In addition, the precision between analyses remained below 16.0%. The results above indicating that this biosensor potentially can be used in

practical applications.

4. Conclusion

A simple, rapid and highly sensitive biosensor based on DHPS-DHPPP binary complex for the detection of class-SAs was developed. The sensitivity and selectivity of the developed biosensor were significantly improved by optimizing some physical and chemical conditions, which showed IC_{50} values for all 29 SAs tested in assay buffer below 100 ng mL^{-1}. Moreover, other compounds like TMP, CM, AMP and KAN do not interfere in this assay. This method could be utilized in the detection of sulfonamide residues in whole milk and skimmed milk. Dilution was an effective and simple mean to reduce the matrix effect, and furthermore, satisfactory recoveries with low CVs were observed in the analysis of spiked samples indicating that the final developed biosensor is a good screening method for class SAs detection. Moreover, the structural information obtained from this report, mainly ligand orientation and hydrogen bonding could provide potentially useful guideline for general hapten design and antibody evolution for SAs. Since interference will occur, this DHPS-based biosensor was not applicable for use in other complicated samples, especially containing a high content of PABA or pterin-related compounds.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at

References

[1] S. Wang, H. Zhang, L. Wang, Z. Duan, I. Kennedy, Analysis of sulphonamide residues in edible animal products: A review, Food Addit. Contam. 23 (2006) 362–384.

[2] K. Grave, J. Torren-Edo, A. Muller, C. Greko, G. Moulin, D. Mackay, Variations in the sales and sales patterns of veterinary antimicrobial agents in 25 European countries, J Antimicrob. Chemoth. 69 (2014) 2284–2291.

[3] Q. Zhang, G. Ying, C. Pan, Y. Liu, J. Zhao, Comprehensive evaluation of antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance, Environ. Sci. Technol. 49 (2015) 6772–6782.

[4] M. Franek, I. Diblikova, I. Cernoch, M. Vass, K. Hruska, Broad-specificity immunoassays for sulfonamide detection: Immunochemical strategy for generic antibodies and competitors, Anal. Chem. 78 (2006) 1559–1567.

[5] P. Cliquet, E. Cox, W. Haasnoot, E. Schacht, B.M. Goddeeris, Generation of group-specific antibodies against sulfonamides, J. Agric. Food Chem. 51 (2003) 5835–5842.

[6] Z. Wang, R.C. Beier, Y. Sheng, S. Zhang, W. Jiang, Z. Wang, J. Wang, J. Shen, Monoclonal antibodies with group specificity toward sulfonamides: Selection of hapten and antibody selectivity, Anal. Bioanal. Chem. 405 (2013) 4027–4037.

[7] PRC Ministry of Agriculture Bulletin No 235, Maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, (2002).

[8] Food and Drug Administration, New animal drug for use in animal feed; Code of Federal Regulations, FDA, Washington, DC, (1988) Title 21, Part 558.

[9] Commission regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off. J. Eur. Union, (2010) L15, p. 63.

[10] N. Pastor-Navarro, Á. Maquieira, R. Puchades, Review on immunoanalytical determination of tetracycline and sulfonamide residues in edible products, Anal. Bioanal. Chem. 395 (2009) 907–920.

[11] J. Isanga, B.N. Tochi, D. Mukunzi, Y. Chen, L. Liu, H. Kuang, C. Xu, Development of a specific monoclonal antibody assay and a rapid testing strip for the detection of apramycin residues in food samples, Food Agric. Immunol. 28 (2016) 49–66.

[12] J. Isanga, D. Mukunzi, Y. Chen, S. Suryoprabowo, L. Liu, H. Kuang, C. Xu, Development of a monoclonal antibody assay and a lateral flow strip test for the detection of paromomycin residues in food matrices, Food Agric. Immunol. 28 (2017) 355–373.

[13] J. Isanga, D. Mukunzi, Y. Chen, S. Suryoprabowo, L. Liu, H. Kuang, Development of a monoclonal antibody assay and immunochromatographic test strip for the detection of amikacin residues in milk and eggs, Food Agric. Immunol. 28 (2017) 668–684.

[14] Y. Chen, L. Liu, Z. Xie, J. Zhu, S. Song, H. Kuang, Gold immunochromatographic assay for trimethoprim in milk and honey samples based on a heterogenous monoclonal antibody, Food Agric. Immunol. 28 (2017) 1046–1057.

[15] W. Haasnoot, G. Cazemier, J.D. Pre, A. Kemmers-Voncken, M. Bienenmann-Ploum, R. Verheijen, Sulphonamide antibodies: From specific polyclonals to generic monoclonals, Food Agric. Immunol. 12 (2000) 15–30.

[16] H. Zhang, L. Wang, Y. Zhang, G. Fang, W. Zheng, S. Wang, Development of an enzyme-linked immunosorbent assay for seven sulfonamide residues and investigation of matrix effects from different food samples, J. Agric. Food Chem. 55 (2007) 2079–2084.

[17] X. Liang, H. Ni, R. Beier, Y. Dong, J. Li, X. Luo, S. Zhang, J. Shen, Z. Wang, Highly broad-specific and sensitive enzyme-linked immunosorbent assay for screening sulfonamides: Assay optimization and application to milk samples, Food Anal. Method 7 (2014) 1992–2002.

[18] Q. Zhou, D. Peng, Y. Wang, Y. Pan, D. Wan, X. Zhang, Z. Yuan, A novel hapten and monoclonal-based enzyme-linked immunosorbent assay for sulfonamides in edible animal tissues, Food Chem. 154 (2014) 52–62.

[19] Y. Chen, L. Liu, L. Xu, S. Song, H. Kuang, G. Cui, C. Xu, Gold immunochromatographic sensor for the rapid detection of twenty-six sulfonamides in foods, Nano Res. 10 (2017) 2833–2844.

[20] C. Levy, D. Minnis, J.P. Derrick, Dihydropteroate synthase from *Streptococcus pneumoniae*: Structure, ligand recognition and mechanism of sulfonamide resistance, Biochem J. 412 (2008) 379–388.

[21] X. Liang, Z. Wang, C. Wang, K. Wen, T. Mi, J. Zhang, S. Zhang, A proof-of-concept receptor-based assay for sulfonamides, Anal. Biochem. 438 (2013) 110–116.

[22] Z. Wang, X. Liang, K. Wen, S. Zhang, C. Li, J. Shen, A highly sensitive and class-specific fluorescence polarisation assay for sulphonamides based on dihydropteroate synthase, Biosens. Bioelectron. 70 (2015) 1–4.

[23] D.S. Smith, S.A. Eremin, Fluorescence polarization immunoassays and related methods for simple, high-throughput screening of small molecules, Anal. Bioanal. Chem. 391 (2008) 1499–1507.

[24] Y. Li, K. Wen, X. Yu, C. Li, Z. Wang, Construction of a single chain variable fragment antibody against sulfonamides and its interaction with sulfathiazole, Chin. J. Anal. Chem. 43 (2015) 366–370.

[25] K. Wen, G. Nölke, S. Schillberg, Z. Wang, S. Zhang, C. Wu, H. Jiang, H. Meng, J. Shen, Improved fluoroquinolone detection in ELISA through engineering of a broad-specific single-chain variable fragment binding simultaneously to 20 fluoroquinolones, Anal. Bioanal. Chem. 403 (2012) 2771–2783.

[26] A. Abad, A. Montoya, Development of an enzyme-linked immunosorbent assay to carbaryl. 2. Assay optimization and application to the analysis of water samples, J. Agric. Food Chem. 45 (1997) 1495–1501.

[27] M.-K. Yun, Y. Wu, Z. Li, Y. Zhao, M.B. Waddell, A.M. Ferreira, R.E. Lee, D. Bashford, S.W. White, Catalysis and sulfa drug resistance in dihydropteroate synthase, Science 335 (2012) 1110–1114.

[28] K.E. Hevener, M.-K. Yun, J. Qi, I.D. Kerr, K. Babaoglu, J.G. Hurdle, K. Balakrishna, S.W. White, R.E. Lee, Structural studies of pterin-based inhibitors of dihydropteroate synthase, J. Med. Chem. 53 (2010) 166–177.

[29] J. Zhang, Z. Wang, K. Wen, X. Liang, J. Shen, Penicillin-binding protein 3 of *Streptococcus pneumoniae* and its application in screening of β-lactams in milk, Anal. Biochem. 442 (2013) 158–165.

[30] Y. Chen, L. Guo, L. Liu, S. Song, H. Kuang, C. Xu, Ultrasensitive immunochromatographic strip for fast screening of 27 sulfonamides in honey and pork liver samples based on a monoclonal antibody, J. Agric. Food Chem. 65 (2017) 8248–8255.

[31] J. Sun, B. Liu, Y. Zhang, S. Wang, Development of an enzyme-linked immunosorbent assay for metolcarb residue analysis and investigation of matrix effects from different agricultural products, Anal. Bioanal. Chem. 394 (2009) 2223–2230.

[32] Z. Wang, S. Zhang, I.S. Nesterenko, S.A. Eremin, J. Shen, Monoclonal antibody-based fluorescence polarization immunoassay for sulfamethoxypyridazine and sulfachloropyridazine, J. Agric. Food Chem. 55 (2007) 6871–6878.

Figure captions

Scheme 1. The schematic illustration of the developed biosensor in this study.

Fig. 1. (**A**) Recognition between DHPS-DHPPP complex (and DHPS*-DHPPP complex) and hapten-HRP conjugates. (**B**) Optimization of concentrations for Tris-HCl and $MgCl_2$ in coating buffer for DHPS. (**C**) Optimization of concentrations for Tris-HCl in reaction buffer for DHPS and DHPS*, respectively. (**D**) Optimization of concentrations for $MgCl_2$ in reaction buffer for DHPS and DHPS*, respectively. A_{max} represents the absorbance value at zero analyte concentration; A represents the absorbance value at 50 ng mL^{-1} of SMZ as model analyte. (**E**) Optimization of reaction time for DHPS and DHPS*, respectively. The IC_{50} values were detected by using SMZ as model analyte. (**F**) Calibration curves for SMM, SQX, SMX, SDM and SMZ in the assay based on DHPS. (**G**) Calibration curves for SMM, SQX, SMX, SDM and SMZ in the assay based on DHPS*. Here, A represents the absorbance value measured by using SMZ as model analyte. (**H**) Standard curves of SMZ in whole milk and skimmed milk after appropriate dilution based on DHPS.

Fig. 2. The IC_{50} values of biosensor assay based on DHPS and immunoassays based on MAb 4D11 and 4C7 against SAs.

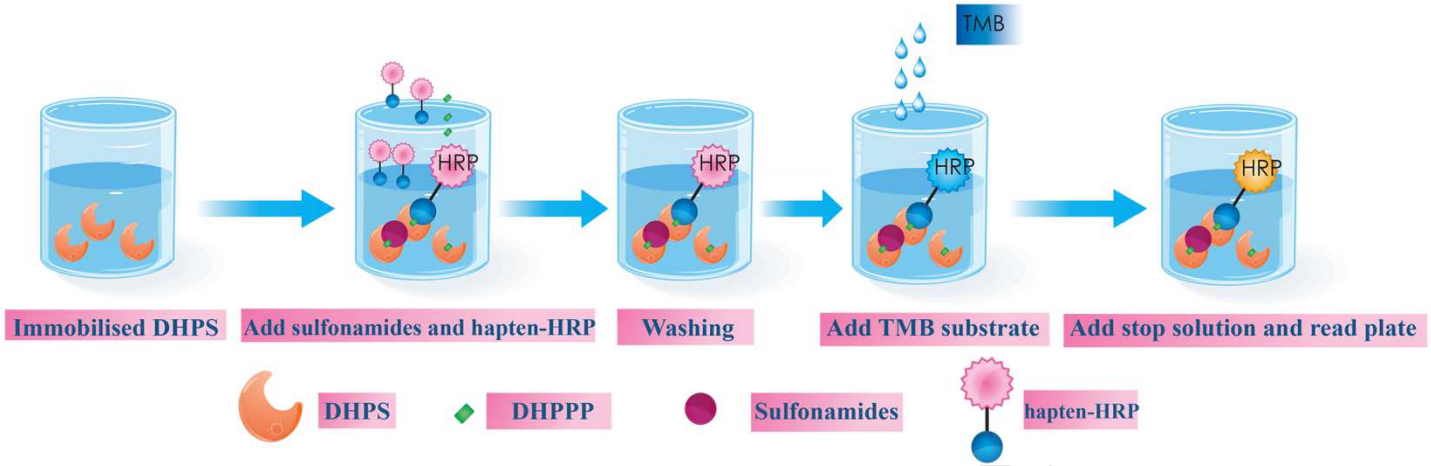
Fig. 3. The cross-reactivity based on DHPS and immunoassays based on MAb 4D11 and 4C7 against SAs. In DHPS and MAb 4D11 based biosensor, cross-reactivity was calculated referred to SMZ; In MAb 4C7 based assay, cross-reactivity was calculated referred to STZ.

Fig. 4. (**A**) Structure of *S. pneumoniae* DHPS in complex with first substrate DHPPP and PABA (green), PST (yellow) and SPH (pink). (**B**) The interaction between PABA (green) and DHPS-DHPPP complex. (**C**) The interaction between PST (green) and DHPS-DHPPP complex. (**D**) The interaction between SPH (green) and DHPS-DHPPP complex. (**E**) Modelled structure of scFv 4C7 and PABA (green), PST (yellow) and SPH (pink). (**F**) The

interaction between PABA (green) and scFv 4C7. (**G**) The interaction between PST (green) and scFv 4C7. (**H**) The interaction between SPH (green) and scFv 4C7. (**I**) Modelled structure of scFv 4D11 and PABA (green), PST (yellow) and SPH (pink). (**J**) The interaction between PABA (green) and scFv 4D11. (**K**) The interaction between PST (green) and scFv 4D11. (**L**) The interaction between SPH (green) and scFv 4D11.

Table 1. Recovery studies from whole milk and skimmed milk with five SAs at three levels by DHPS-based biosensor.

	Added ($ng\ mL^{-1}$)	**SMZ**		**SDM**		**SQX**		**SMM**		**SMX**	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Whole milk	30	96.1	2.7	89.9	10.5	83.3	6.4	98.1	2.8	104.2	7.9
	50	108.8	6.1	86.0	2.0	104.0	8.7	88.9	1.6	91.2	1.1
	60	86.1	15.8	100.3	9.8	92.7	8.4	83.6	5.5	86.6	3.2
Skimmed milk	18	72.7	3.0	129.3	5.3	97.2	7.0	83.4	7.1	109.6	10.5
	30	101.9	9.9	117.5	3.8	107.8	5.7	89.8	8.8	121.7	7.1
	36	94.9	16.0	111.7	3.0	104.3	1.6	109.5	12.4	122.5	3.2



Scheme 1

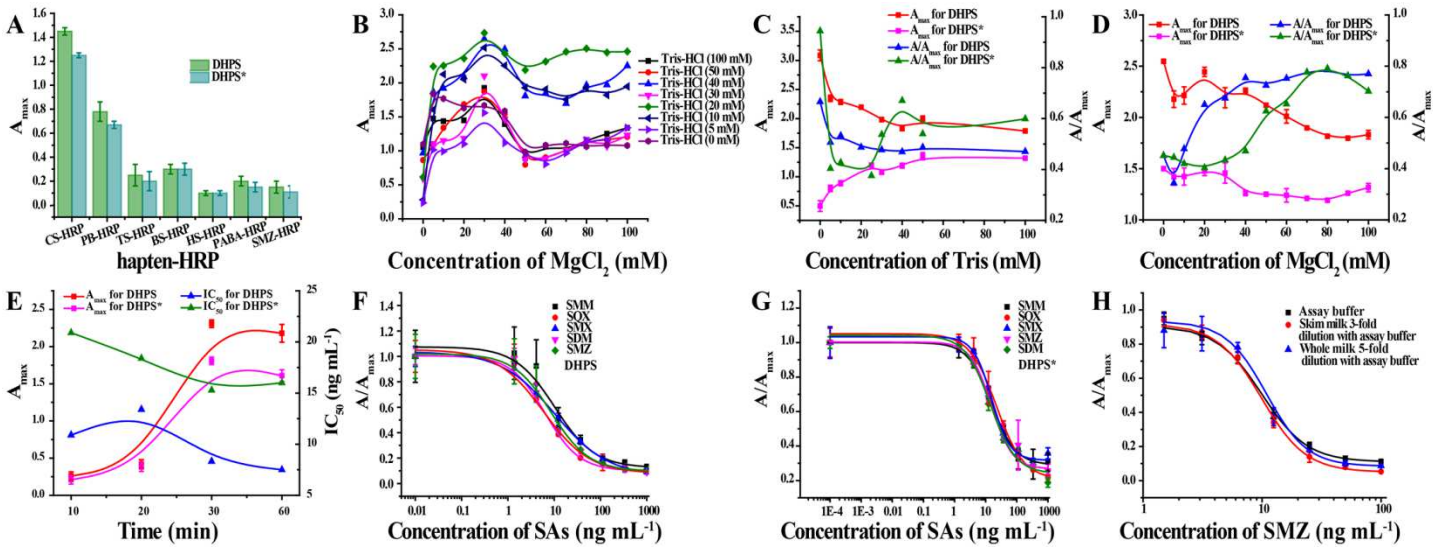


Fig. 1

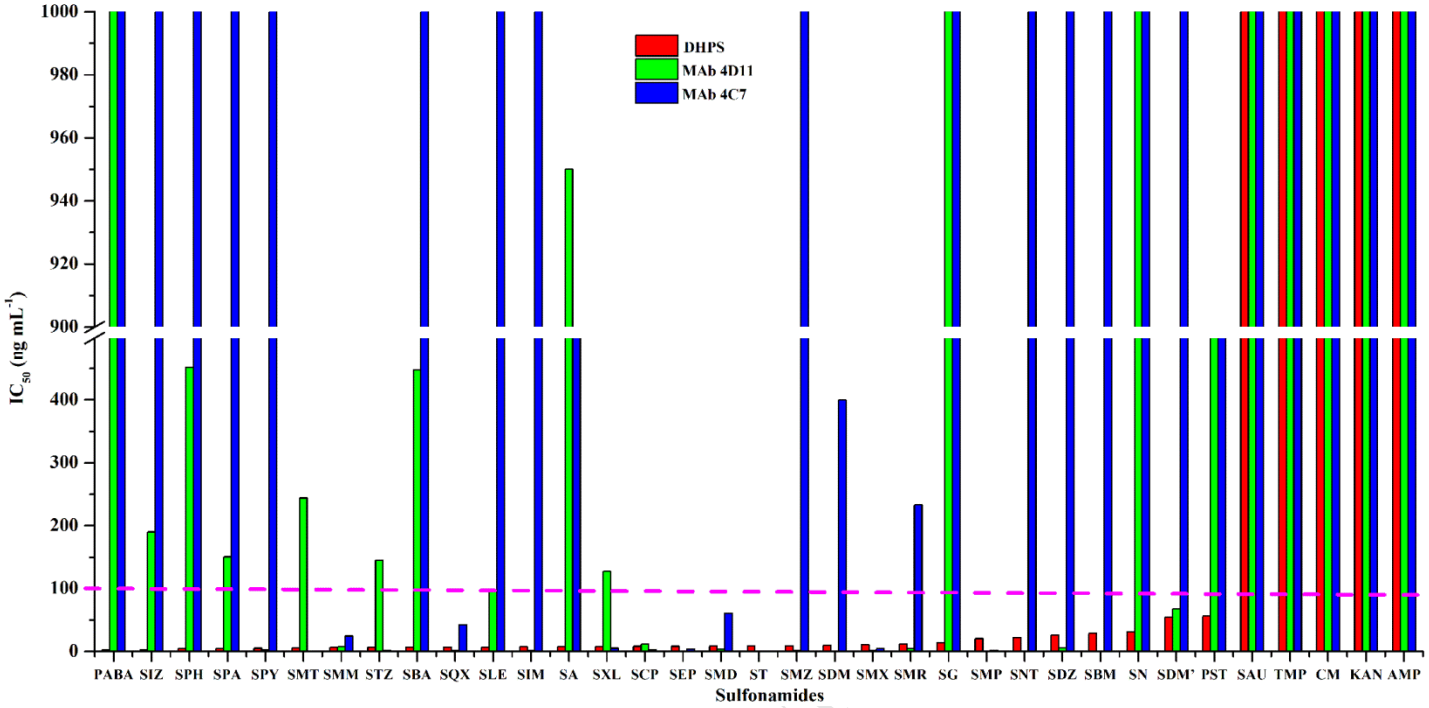


Fig. 2

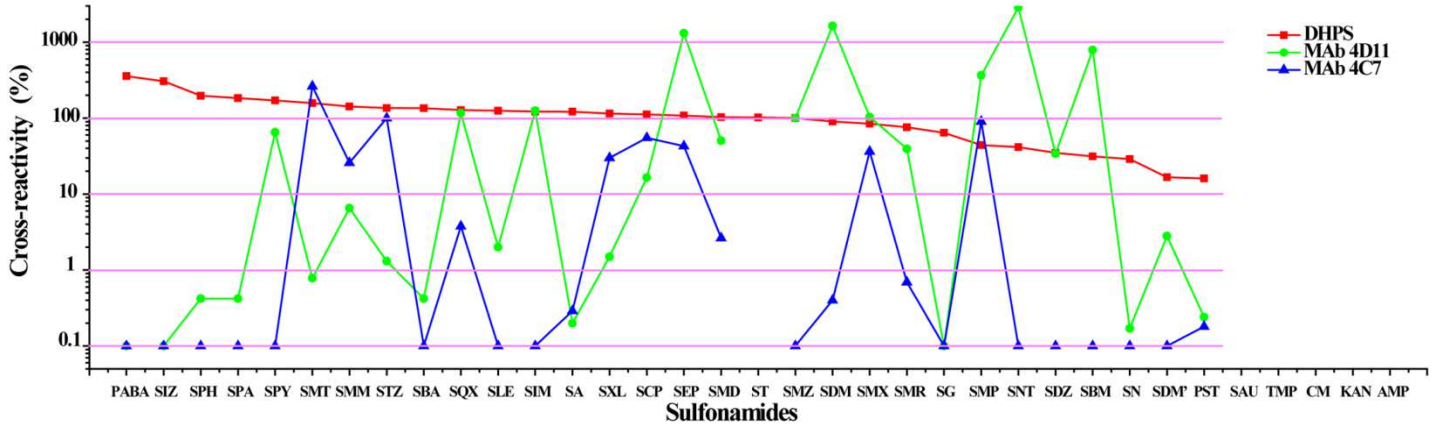


Fig. 3

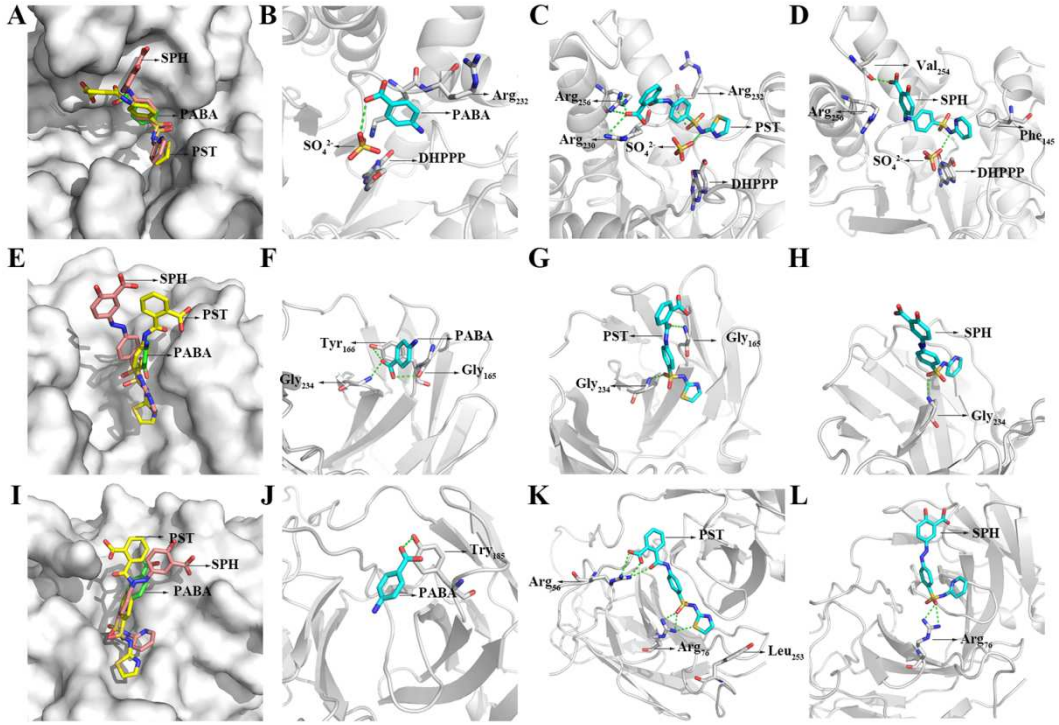

A
SPH
PABA
PST
B
Arg232
PABA
SO4 2-
DHPPP
C
Arg256
Arg232
PST
Arg230
SO4 2-
DHPPP
D
Val254
SPH
Arg256
Phe145
SO4 2-
DHPPP
E
SPH
PST
PABA
F
Tyr166
PABA
Gly234
Gly165
G
PST
Gly165
Gly234
H
SPH
Gly234
I
PST
SPH
PABA
J
Try185
PABA
K
PST
Arg256
Arg76
Leu253
L
SPH
Arg76

Fig. 4

HIGHLIGHTS

- A simple, rapid and highly sensitive biosensor based on DHPS-DHPPP binary complex for the detection of class-SAs was developed.
- The sensitivity and selectivity of the developed biosensor were significantly improved by optimizing some physical and chemical conditions.
- The biosensor could detect at least 29 SAs below the MRLs.
- The DHPS-DHPPP binary complex was used to detect SAs in milk showing high accuracy and precision.
- The difference of recognition mechanism between DHPS and antibody was determined by molecular modeling analysis.