RESEARCH

Heterologous immunoassay strategy for enhancing detection sensitivity of banned dye rhodamine B in fraudulent food

Jin Wang[1†], Xing Shen[1†], Peng Zhong[1], Zhaodong Li[1], Qiushi Tang[1], Xinan Huang[2], Anatoly V. Zherdev[3], Boris B. Dzantiev[3], Sergei A. Eremin[3,4], Zhili Xiao[1], Hongtao Lei[1,5*] and Xiangmei Li[1*]

Abstract

Background: The high quality of antibody (Ab) is critical for an immunoassay; usually, an Ab with low affinity is often regarded as a "bad" one in the immunoassay development. How to use a "bad" Ab to develop a highly sensitive immunoassay is still a huge challenge.

Methods: In this study, a heterologous immunoassay strategy was designed to enhance the sensitivity for the detection of banned dye, rhodamine B (RB), in fraudulent food. The RB Ab could not recognize RB by pairing with homologous coating antigen (Ag). However, the Ab showed unexpected high specificity and sensitivity recognition after being replaced by heterologous coating Ag. Indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed based on the heterologous strategy.

Results: The detection limit of icELISA for chilli powder, Chinese prickly ash, hot-pot seasoning, and chilli sauce was 0.002 μg/kg, and the recoveries of the four samples ranged from 76.0 to 102.0%, with the coefficient of variation between 3.9 and 18.8%. Parallel experiment for 20 market samples with high-performance liquid chromatography (HPLC) was performed on to confirm the performance of the practical application of the developed icELISA, and the

---

*Correspondence: hongtao@scau.edu.cn; lixiangmei12@163.com
†Jin Wang and Xing Shen have equally contributed to this work
[1] Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou 510642, China
Full list of author information is available at the end of the article

Wang *et al. Chem. Biol. Technol. Agric.*   (2021) 8:17

Page 2 of 10

results of the two methods had good correlation. Molecular modeling inferred that the carboxyl group of hapten and its exposure level played an important role in the hapten-Ab recognition.

Conclusions: The proposed icELISA can be used for the surveillance screening of RB in a range of seasoning foods,
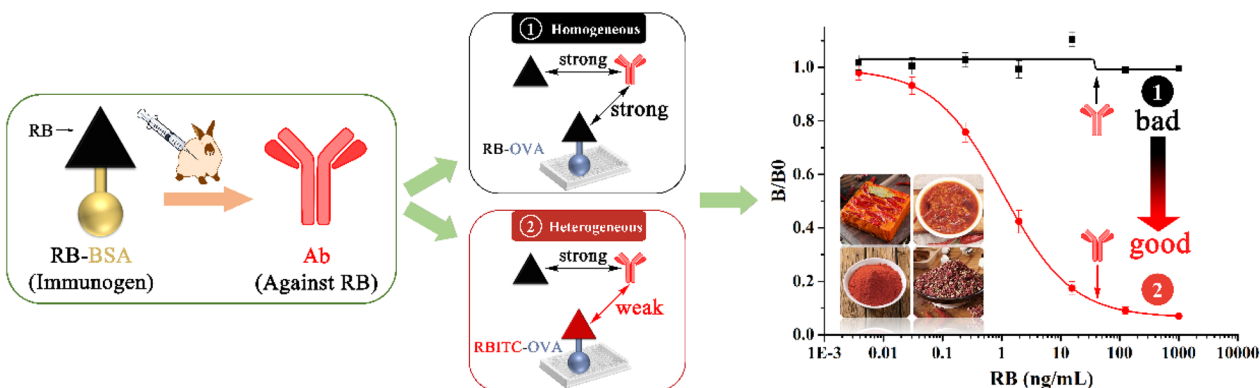
and the heterologous strategy is an effective approach to enhance the sensitivity in an immunoassay.

Keywords: Rhodamine B, ELISA, Immunoassay, Antibody, Hapten, Heterologous

Background

Rhodamine B (RB) (Fig. 1), a synthetic dye with fluorescent properties, is commonly used in textile, pharmaceutical industries, and bioanalytical chemistry [1]. However, there were some reports that RB was illegally used in food to enhance and/or maintain the appearance of food products, especially in chilli powder, chilli pepper, sauce, etc. [2–4], which was a typical food fraud [5]. The carcinogenicity, reproductive and developmental toxicity, neurotoxicity, and chronic toxicity of RB toward humans and animals have been experimentally proved [6, 7]. The European Food Safety Authority classified RB as a potentially genotoxic and carcinogenic illegal dye [2], and the USA also set RB as an illegal colorant in the "Colors in Food Regulations" [1]; moreover, RB was listed in "List of non-edible substances and illegally food additives in food" in China [8].

The usual analytical method for the banned dye is high-performance liquid chromatography (HPLC) coupled with different detectors, such as mass spectrometer [9], fluorescence detector (FLD) [2], etc. Other instrumental methods such as capillary electrophoresis [10], spectro-fluorimetrum [11], and spectrophotometrum [12, 13] have also been reported. Generally, these instrumental methods are accurate and sensitive. However, they are laborious, time-consuming, expensive, and need trained personnel, limiting the productivity of testing large batches of samples.

Immunoassay based on the specific interaction between an antibody (Ab) and corresponding hapten can meet the analytical requirement with high sensitivity, effective cost, and specificity, and plays an important role outside of the laboratory [14, 15]. It is well known that high sensitivity of Ab is critical for an immunoassay development. Usually, an Ab with a low sensitivity that
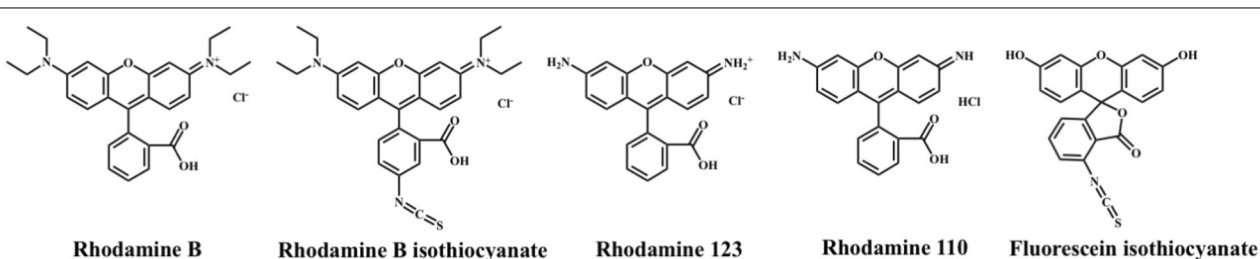

Fig. 1 Structure of RB and some analogues

cannot meet detection requirements will be regarded as a "bad" one and have to be abandoned, and thus, the researchers will be forced to generate another "good" one with a high sensitivity [16], which is time-consuming and labor-intensive. Are there any other approaches to enhance the sensitivity of the "bad" Ab? Instead of just abandon it. On one hand, a phage display Ab library is constructed and used to screen high-affinity single-chain Ab fragment (scFv). Compared with the parent monoclonal Abs (mAbs), the affinity of the obtained scFv can be greatly improved [17]. On the other hand, signal amplifying materials or novel signal sensor elements are often used to improve the sensitivity [18–22]. However, all these strategies will still take much time and effort, some of which cannot be easily performed. Therefore, an easy-handling strategy to enhance the sensitivity is still of high interest.

In this study, a heterologous strategy was designed to develop a high-sensitivity indirect competitive enzyme-linked immunosorbent assay (icELISA) by the "bad" Ab for the detection of illegally added RB in Chinese seasoning foods. The "bad" Ab could not recognize RB by pairing with homologous coating Ag. However, the Ab showed unexpected high specificity and sensitivity recognition after being replaced by heterologous coating Ag. Molecular modeling inferred that the carboxyl group of heterologous coating Ag and its exposure level played an important role in the Ag-Ab recognition. Therefore, this study used a heterologous strategy to convert possible "bad" Abs into "good" Abs. This method is simpler, faster, and more efficient than re-preparing ideal Abs, and it can also avoid misdiagnosis and waste of Abs.

Methods
Materials and reagents
RB (99.0%) was obtained from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). RBITC, Rhodamine 110 (R110), Rhodamine 123 (R123), and fluorescein isothiocyanate (FITC) were all purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO), BSA, and OVA were purchased from Shanghai Boao Biotechnology Co., Ltd. (Shanghai, China). HRP-conjugated goat anti-rabbit IgG was obtained from Boster Biotech Co., Ltd. (Wuhan, China). 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), tetramethylbenzidine (TMB), and complete and incomplete Freund's adjuvants were purchased from Sigma (St. Louis, MO, USA). Methanol, acetone, and tween-20 were obtained from Tianjin Damao Chemical Reagent Co., Ltd. (Tianjin, China). Alumina-N (1 g/6 mL) was purchased from Shanghai ANPEL Scientific Instrument Co. Ltd. (Shanghai, China).

The microtiter plates were purchased from the JET BIO-FIL Co. (Guangzhou, China). The New Zealand rabbits, weighing 1.5–2.0 kg, provided by Guangdong Medical Laboratory Animal Center.

Instruments
Ultraviolet spectra were recorded on a UV-2550 spectrophotometer (Shimadzu, Japan). Absorbance was read with a Multiskan MK3 microplate reader (Thermo Scientific, Hudson, USA). HPLC–FLD analysis was carried out on the LC-20 AT HPLC system (Shimadzu, Japan).

Preparation of hapten–protein conjugates
Immunogen 1
The EDC method was used to synthesize RB–BSA conjugate [23]. Briefly, 15 mg of BSA was added to 2 mL of phosphate buffer (PBS, pH 7.4, 10 mM) containing RB (10 mg), followed by the addition of EDC (20 mg) under continuous stirring for 30 min in a dark chamber. The reaction mixture was dialyzed against PBS (pH 7.4,10 mM) at 4 °C for 3 days, and then, the immunogen 1 was obtained. UV spectrometry was used to confirm the conjugate.

Coating antigen (Ag) 1
RB–OVA conjugate was synthesized by the method described above, but BSA was replaced by OVA.

Immunogen 2
RBITC–BSA conjugate was prepared by direct reaction of RBITC and BSA [24]. Briefly, 10 mg of RBITC was dissolved in 300 μL of DMSO, which was added to 2 mL of carbonate buffer (CB, pH 9.6, 50 mM) containing BSA (15 mg). The mixture reacted for 1 h under continuous stirring and then dialyzed against PBS (pH 7.4, 10 mM) at 4 °C for 3 days, and then, the immunogen 2 was obtained. UV spectrometry was used to confirm the conjugate.

Coating Ag 2
RBITC–OVA conjugate was synthesized using the same method above, just use OVA instead of BSA.

Other coating Ags
The preparation method of R110-OVA and R123-OVA was the same as that of RB-OVA, while FITC-OVA was the same as RBITC-OVA.

Generation of pAbs
The generation of pAbs was similar to the previous work [25]. Briefly, two kinds of immunogens, RB–BSA and RBITC-BSA, were injected to the New Zealand rabbits, respectively. Each rabbit was injected mixture of 0.5 mg immunogen and equal volume of Freund's complete

Wang *et al. Chem. Biol. Technol. Agric.*   (2021) 8:17

Page 4 of 10

adjuvant. Freund's incomplete adjuvant was used for the boost immunity every 28 days. After the third immunization, the serum titer and inhibition began to be monitored. The serum titer and inhibition effect were not changed after the fifth immunization. Therefore, the blood was collected, centrifuged, aliquoted, and stored at − 20 °C for further use. The negative serum was obtained from the rabbit without immunization. The Abs immunized by RB–BSA and RBITC-BSA were labeled as Ab1 and Ab2, respectively.

Establishment of icELISA

Briefly, 96-well polystyrene microtiter plates were coated (100 μL per well) with two coating Ags (RB-OVA, RBITC-OVA) in CB (pH 9.6, 50 mM), respectively, and then incubated at 37 °C for 16 h. After washing twice with PBST (pH 7.4, 10 mM, containing 0.05% tween-20), then blocked with 5% skimmed milk (120 μL/well) for 3 h at 37 °C, the plates were dried at 37 °C for 1 h. Then, 50 μL of RB standard solution (the concentrations were 0, 0.004, 0.03, 0.24, 1.95, 15.63, 125, and 1000 ng/mL, respectively) and Ab (19.5 ng/mL) were added to each well successively, and the reaction was carried out in a 37 °C water bath for 40 min. After washing five times, goat anti-rabbit IgG-HRP (200 ng/mL, 100 μL/well) was then added to each well. After another 30 min of incubation at 37 °C, the plates were washed five times again. Finally, TMB solution (400 μL of 0.6% TMB-DMSO and 100 μL of 1% H_2O_2 diluted with 25 mL of citrate–acetate buffer, pH 5.5) was added with 100 μL/well and then incubated for 10 min. The reaction was quenched by addition of 50 μL of 2 M H_2SO_4/well, and the absorbance was recorded at 450 nm.

Data analysis

Competitive curves were obtained by plotting absorbance against the logarithm of analytic concentration. The sigmoid curves were fit using OriginPro 9.1 software (OriginLab Corp., Northampton, MA, USA) according to the following formula:

$$Y = \frac{A - D}{\left[1 + (x/C)^B\right]} + D,$$

where A is the asymptotic maximum (maximum absorbance in absence of analyte, Amax), B corresponds to the curve slope at the inflection point, C is the concentration of RB resulting in 50% inhibition of binding to Ab (IC_{50}), and D is the asymptotic minimum (background signal).

The limit of detection (LOD) was defined as the standard concentrations that inhibited 10% of RB standard binding to the Ab, and the detectable concentration range

was defined as the standard concentration that inhibited 20 ~ 80% of RB standard binding to the Ab. The A_{max}/IC_{50} ratio was used to estimate the effect of a certain factor on the icELISA performance, in which a higher ratio indicated a higher sensitivity. Cross reactivity (CR) was calculated as follows [26]:

$$CR\% = \frac{IC_{50(RB)}}{IC_{50(\text{cross reactant})}} \times 100.$$

Molecular modeling

A domain (chain A 1–80) of BSA from the crystal structure was chosen as the carrier protein model (PDB ID 4F5S), and the three-dimensional model of RB–BSA and RBITC–BSA conjugate was performed on SYBYL-X 2.1.1 software package (Tripos, Inc., USA). The energy minimization of RB–BSA and RBITC–BSA model was also performed on SYBYL-X 2.1.1 using staged minimization method: the ligands, the side chains, and the biopolymer without C-Alpha were minimized in sequence. The distance between haptens and BSA was measured by SYBYL-X 2.1.1 program package (Tripos, Inc., USA).

Sample preparation

Chilli powder, Chinese prickly ash, hot-pot seasoning, and chilli sauce were bought from the local supermarkets. 1 g of each sample was added into a glass vial, followed by the addition of 10 mL n-hexane/acetone (80/20, v/v). The samples were vigorously vortexed for 30 s and then roller mixed for 10 min at room temperature. The upper phase was collected after centrifugation at 5000 g for 5 min. The bottom residue was extracted again following the above-mentioned procedure. The supernatants were combined and subjected to SPE cleanup. Alumina-N (1 g/6 mL) cartridge was used for purification. The cartridge was preconditioned with 5 mL of methanol followed by 5 mL of N-hexane:acetone (8:2, v/v) prior to the addition of the extracted supernatant. After the extract was drained through the cartridge, the cartridge was washed with 4 mL of N-hexane. The SPE cartridge was dried for at least 1 min. RB was eluted from the cartridge with 5 mL of methanol. The eluent was diluted 10 × with PBST for icELISA analysis, and directly used for HPLC–FLD analysis.

HPLC confirmation

HPLC–FLD method was developed on C_{18} column at an excitation wavelength of 550 nm and an emission wavelength of 580 nm. The mobile phase consisted of (A) methanol and (B) water: 0.2% formic acid (75:25, v/v). The HPLC calibration curve for RB was constructed in

the range of 0, 0.5, 1, 5, 10, 25, 50, and 100 ng/mL at the retention time of 4.7 min.

Analysis of market samples

Twenty natural samples (chilli powder: No. 1–5; hot-pot seasoning: No. 6–10; Chinese prickly ash: No. 11–15; chilli sauce: No. 16–20) were extracted by the above method and detected by both icELISA and HPLC–FLD.

Results and discussion

Preparation of immunogen and coating Ags

RB was covalently coupled with carrier protein to obtain immunogen 1 (RB–BSA) and coating Ag 1 (RB-OVA) by EDC method (Fig. 2a). Meanwhile, because RBITC has an additional isothiocyanate group compared with RB, it is used as hapten 2 (Fig. 2b), which provides more opportunities to obtain ideal RB Ab. UV spectra of RB, RBITC, BSA, OVA, RB–BSA, RBITC–BSA, RB–OVA, and RBITC–OVA are shown in Fig. 2c–d. BSA and OVA have the maximum absorption peak at 280 nm, and RB has a maximum absorption peak at 553 nm, while the characteristic absorption peaks of the conjugate RB–BSA and RB–OVA were at 565 nm, which differed significantly from the spectrogram of RB, and BSA/OVA, indicated that the coupling

of RB–BSA/OVA was successful [25]. Similar analysis was carried out for RBITC–BSA/OVA, and therefore, the coupling of RBITC–BSA/OVA was confirmed to be successful.

Comparison of assay design

In the present study, two Abs and two coating Ags were used for the development of the homologous or heterologous icELISA design to achieve the best sensitivity.

Ab1 showed relatively high titer combining with coating Ag1, but no significant inhibition was observed even at 1 μg/mL of the RB standard (Additional file 1: Fig. S1). To further investigate the performance of Ab1, several structural analogues of RB, including RBITC, R110, R123, and FITC (Fig. 1), were used to couple with OVA as coating Ags. No binding ability was found at all for Ab1 with R110–OVA, R123–OVA, and FITC–OVA. However, Ab1 exhibited high titer and 88.5% inhibition rate (Additional file 1: Fig. S1) when combined with RBITC–OVA, instead of the torpidity in sensitivity based on the homologous combination with RB–OVA. This may be due to the structural differences between R110, R123, and FITC compared with RB, although all compounds contain the same unit in their molecular structure, while RBITC only has an additional isothiocyanate group. Therefore, we inferred that the conjugation site and coupling group of isothiocyanate may retain the main geometric and
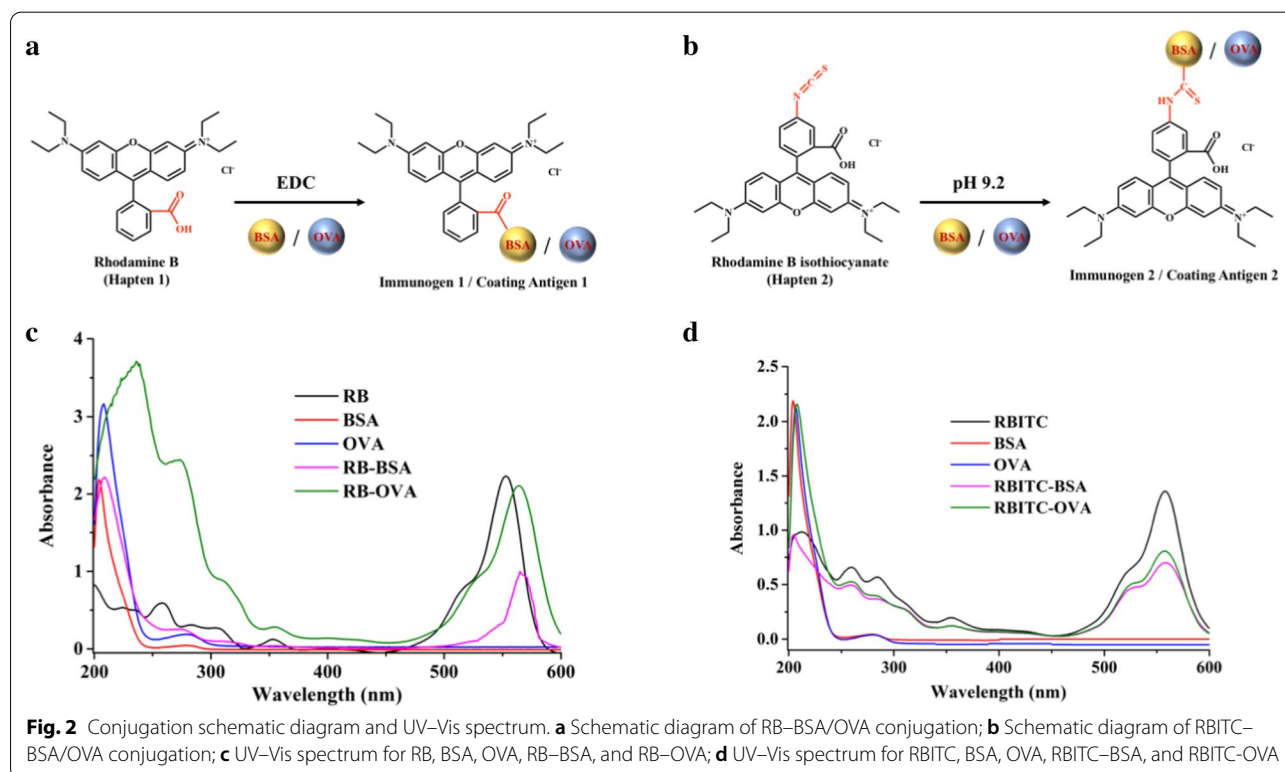

Fig. 2 Conjugation schematic diagram and UV–Vis spectrum. **a** Schematic diagram of RB–BSA/OVA conjugation; **b** Schematic diagram of RBITC–BSA/OVA conjugation; **c** UV–Vis spectrum for RB, BSA, OVA, RB–BSA, and RB–OVA; **d** UV–Vis spectrum for RBITC, BSA, OVA, RBITC–BSA, and RBITC-OVA

Wang *et al. Chem. Biol. Technol. Agric.*　(2021) 8:17

Page 6 of 10

Table 1 Comparison of homologous and heterologous ELISA

Ab	Coating Ag	Method	A_{max}	IC$_{50}$ (ng/mL)	LOD (ng/mL)	Detectable range (ng/mL)
Ab1	RB–OVA	icELISA	1.01	[a]/	/	/
Ab1	RBITC–OVA	icELISA	1.09	1.0	0.051	0.15~6.72
Ab2	RB–OVA	icELISA	1.03	*0.09*	*0.002*	0.008~1.15
Ab2	RBITC–OVA	icELISA	0.97	0.82	0.11	0.23~2.91
Ref Ab [27]	RBITC–OVA	[b]dcELISA	/	0.4	<10	0.10~1.80
Ref Ab [4]	RB–OVA	icELISA	/	0.94	0.1	0.10~20.00

[a] /, unavailable; [b]dcELISA, direct competitive ELISA

italic values indicate the IC$_{50}$ and LOD values achieved by the best Ag-Ab pairing
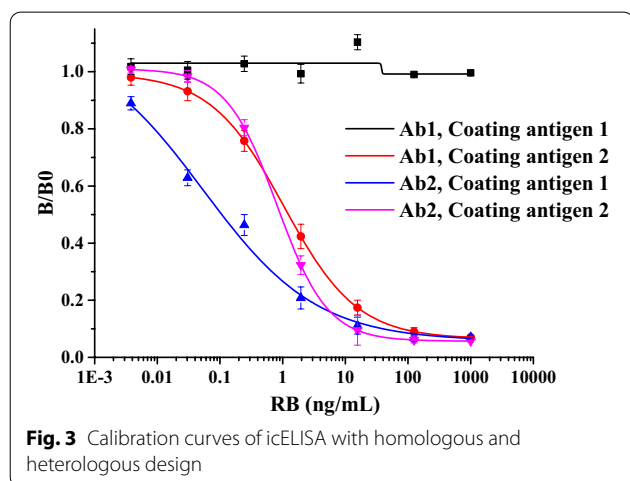

Fig. 3 Calibration curves of icELISA with homologous and heterologous design

Table 2 The IC$_{50}$ and CR values of anti-RB Ab determined by icELISA

Analytes	IC$_{50}$ (μg/L)	CR%
RB	0.09	100%
RBITC	0.13	69%
R110	96	<0.1%
R123	108	<0.1%
FITC	618	<0.1%
Amaranth	>1000	<0.1%
Malachite green	>1000	<0.1%
Erythrosine	>1000	<0.1%
Quinoline yellow	>1000	<0.1%
Indigo	>1000	<0.1%
Sunset yellow	>1000	<0.1%
Tartrazine	>1000	<0.1%
Chrysoidine	>1000	<0.1%
Brilliant blue	>1000	<0.1%
Sudan I	>1000	<0.1%
Sudan II	>1000	<0.1%
Carmine	>1000	<0.1%
Brilliant green	>1000	<0.1%
Crystal violet	>1000	<0.1%

electronic property of the analyte, which is better than R110, R123, and FITC.

Although the homologous combination of Ab1 and RB–OVA did not show noticeable detection capability for RB, the heterologous combination of Ab1 and RBITC–OVA exhibited excellent sensitivity with IC$_{50}$ of 1.0 ng/mL. Similarly, the homologous combination of
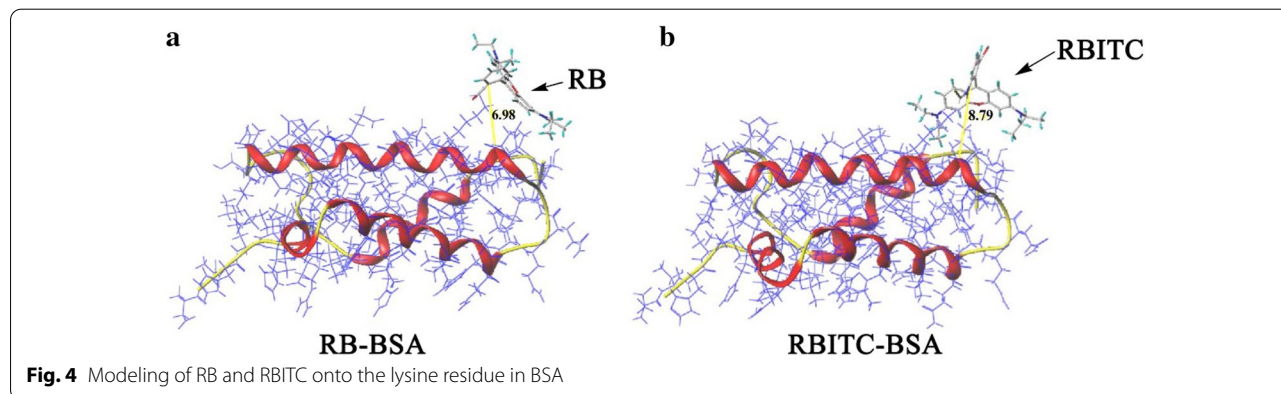

Fig. 4 Modeling of RB and RBITC onto the lysine residue in BSA

Wang *et al. Chem. Biol. Technol. Agric.*   (2021) 8:17

Page 7 of 10

Table 3 Recovery of the icELISA and HPLC–FLD for the determination of RB in spiked Samples ($n=4$)

Samples	Spiked level (µg/kg)	icELISA			HPLC–FLD		
		Mean ± SD (µg/kg)	Recovery (%)	CV (%)	Mean ± SD (µg/kg)	Recovery (%)	CV (%)
Chilli powder	0.5	0.5 ± 0.02	102.0	3.9	0.47 ± 0.02	94.0	4.3
	1.0	0.8 ± 0.1	82.0	12.5	0.9 ± 0.1	90.0	11.1
	5.0	4.8 ± 0.8	96.0	16.7	4.7 ± 0.5	94.0	10.6
Chinese prickly ash	0.5	0.47 ± 0.04	94.0	8.5	0.49 ± 0.03	98.0	6.1
	1.0	0.95 ± 0.5	95.0	5.3	0.8 ± 0.09	80.0	11.3
	5.0	3.8 ± 0.7	76.0	18.0	4.3 ± 0.2	86.0	4.7
Hot-pot seasoning	0.5	0.46 ± 0.04	92.0	8.7	0.46 ± 0.04	92.0	8.7
	1.0	0.81 ± 0.1	81.0	12.3	0.85 ± 0.1	85.0	11.8
	5.0	4.8 ± 0.9	96.0	18.8	4.3 ± 0.3	86.0	7.0
Chilli sauce	0.5	0.48 ± 0.02	96.0	4.2	0.5 ± 0.03	100.0	6.0
	1.0	0.88 ± 0.05	88.0	5.7	0.8 ± 0.06	80.0	7.5
	5.0	4.5 ± 0.3	90.0	6.7	5.1 ± 0.1	102	2.0

Table 4 Analysis of RB in blind samples by icELISA and HPLC–FLD

Samples		icELISA (µg/kg)	HPLC–FLD (µg/kg)	Samples		icELISA (µg/kg)	HPLC–FLD (µg/kg)
Chilli powder	1	ND	ND	Chinese prickly ash	11	ND	ND
	2	ND	ND		12	ND	ND
	3	ND	ND		13	ND	ND
	4	ND	ND		14	ND	ND
	5	ND	ND		15	ND	ND
Hot-pot seasoning	6	ND	ND	Chilli sauce	16	ND	ND
	7	ND	ND		17	ND	ND
	8	ND	ND		18	ND	ND
	9	ND	ND		19	1.2 ± 0.2	1.1 ± 0.1
	10	ND	ND		20	4.3 ± 0.2	4.0 ± 0.1

ND not detected

Ab2 and RBITC–OVA showed IC_{50} of 0.82 ng/mL, while the heterologous combination of Ab2 and RB–OVA exhibited IC_{50} of 0.09 ng/mL. Therefore, the sensitivity of heterogeneous strategy is much higher than that of homologous strategy. This result may be caused by the difference between the two coupling sites. We speculate that RBITC is conjugated at the isothiocyanate site, while RB is conjugated at the carboxyl site (Fig. 2). The LOD of the best heterologous combination of Ab2 and RB–OVA was 0.002 ng/mL, and the detection range ranged from 0.008 to 1.15 ng/mL (Table 1, Fig. 3), which is the highest immunoassay sensitivity reported for RB so far [4, 27].

Comparing the Ab used by Song and Ab1, the former demonstrated a good sensitivity (IC_{50}, 0.94 ng/mL) based on the homologous strategy [4]. However, the same design failed in our study and no inhibitory effect was observed. However, after switching to a heterologous coating Ag, the sensitivity (IC_{50}, 1.0 ng/mL) was consistent with Song's report. This phenomenon indicates that a "bad" Ab in one experimental design may be good in another. The heterologous coating Ag reduces the ability of the Ab to recognize it, and improves the ability of the analyte to compete with the Ab, thereby increasing the sensitivity of the analytical method.

Molecular modeling

To better understand the resultant high sensitivity from the heterogeneity strategy, molecular modeling was applied to model the conformational changes of BSA after coupling with RB and RBITC (Fig. 4). Crystal structure of BSA (PDB ID: 4F5S) was used for modeling. To show the conformation of RB or RBITC clearly, a domain of

Wang *et al. Chem. Biol. Technol. Agric.*   (2021) 8:17

Page 8 of 10

BSA containing three α-helical structures (chain A 1–80) was chosen as BSA model, and the Lys41 was chosen as the coupling group of proteins with the hapten. After energy stage minimization, the conformations of the RBITC–BSA exhibited clearly that the carboxyl group of RBITC–BSA was exposed outside. However, the carboxyl group of RB–BSA was occupied. On the other hand, the influence of the negative charge was still noticeable [28], which clearly showed that exposure of immunogen carboxyl group was the key factor to produce high-quality Ab. Moreover, the distance between α-helical and RBITC (8.79 Å) was longer than that of α-helical and RB (6.98 Å), which indicated that the characteristic structure was more exposed, and thus, it was easier to produce Abs with high specificity and sensitivity [29].

Specificity
The CRs of RB Ab with other structural analogues and food colorants are shown in Table 2. The results showed that the CRs of RB Ab to RBITC were 69%, while the CRs to other structural analogs and food colorings, such as R110, R123, etc., were less than 0.1%. This is because the characteristic structure of RBITC is similar to that of RB, so the Ab has a strong ability to recognize it.

Recovery
Chilli powder, Chinese prickly ash, hot-pot seasoning, and chilli sauce were confirmed to be free of RB, spiked with RB at three concentrations (0.5, 1.0, and 5.0 μg/kg). HPLC–FLD method was performed in parallel with the icELISA method for confirmation (Table 3). Initially, we directly extracted RB from each sample, the recovery rates of RB in chilli powder and chilli sauce ranged from 82.0 to 102.0%, and 88.0–96.0%, respectively. However, poor recoveries (< 40%) were obtained from Chinese prickly ash and hot-pot seasoning, which may be due to the complex matrix interference, such as high oil content and the presence of different types of pigments, which will cause greater background interference in the detection. Therefore, SPE purification was tried to overcome this technical difficulty, and the results proved that SPE purification can effectively eliminate matrix interference, and the recovery rates of Chinese prickly ash and hot-pot seasoning were increased to 76.0–95.0% and 81.0–96.0%, respectively. The recovery rates of the confirmation method had a good correlation with that of the icELISA, and the correlation coefficient was greater than 0.99, indicating that the established icELISA was accurate.

Analysis of market samples
Four kinds of samples, including chilli powder, Chinese prickly ash, hot-pot seasoning, and chilli sauce, were bought from the local supermarkets and farmer's markets, which were analyzed by both the developed icELISA and HPLC–FLD. As shown in Table 4, RB was not detected in the chilli powder, Chinese prickly ash, and hot-pot seasoning. However, RB was detected in two chili sauce samples, with a detection rate of 10%, indicating that the illegal addition of RB is still serious and needs to strengthen supervision and management. The icELISA method which we have established is simple, fast, and accurate, and can provide practical technical support to the regulatory authorities.

Conclusion
In this study, two RB Abs and several coating Ags were prepared, and the Ag–Ab pairing results showed that the sensitivity of the heterologous strategy was much better than that of the homologous strategy. Meanwhile, molecular modeling showed that the carboxyl group and its exposure to the organism were the key factors for producing Abs with high specificity and sensitivity in the hapten design. Therefore, this study used a heterologous strategy to convert possible "bad" Abs into "good" Abs, and a sensitive icELISA method was established on this basis, with a LOD of 0.002 μg/kg for chilli powder, Chinese prickly ash, hot-pot seasoning, and chilli sauce. The recoveries of the four samples ranged from 76.0 to 102.0%. Parallel experiment for 20 market samples with HPLC was carried out to confirm the performance of the practical application of the developed icELISA. The results of the two methods had good correlation, indicating that the proposed icELISA is accurate and reliable, and can be used for the surveillance screening of RB in a range of seasoning foods.

Supplementary Information

Additional file 1.

Abbreviations
Ab: Antibody; BSA: Bovine serum albumin; DMSO: Dimethyl sulfoxide; EDC: 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride; FITC: Fluorescein isothiocyanate; FLD: Fluorescence detection; icELISA: Indirect competitive enzyme-linked immunosorbent assay; IC$_{50}$: Half inhibitory concentration; HPLC: High-performance liquid chromatography; LOD: Limit of detection; mAb: Monoclonal antibody; pAbs: Polyclonal antibodies; OVA: Ovalbumin; RB: Rhodamine B; RBITC: Rhodamine B isothiocyanate; R110: Rhodamine 110; R123: Rhodamine 123; scFv: Single-chain antibody fragment; TMB: Tetramethylbenzidine.

Wang *et al. Chem. Biol. Technol. Agric.*   (2021) 8:17

Page 9 of 10

Authors' contributions
JW, XS, PZ, ZL, and QT synthesized all haptens and prepared Abs; AVZ, BBD, and SAE analyzed experimental data; XH, and ZX in charge of molecular modeling; JW and XL wrote this manuscript; HL and XL designed the experiments, provided experimental guidance. All authors read and approved the final manuscript.

Availability of data and materials
All data generated and analyzed during this study are included in this manuscript.

Ethics approval and consent to participate
The experimental rabbits used in this experiment come from Guangdong Medical Laboratory Animal Center, which have experimental animal production licenses. The type, quantity, and grouping conform to the 3R principle. All animals in this experiment were conducted in a licensed laboratory, which complies with the welfare principle. It is ethical for the animals to be euthanized after the experiment. The original and translated versions of "Ethical Approval of Animal Experiment" were given in Additional file 1.

Consent for publication
All authors have approved to submit this work to Chemical and Biological Technologies in Agriculture. They declare that there is no conflict of interest in relation to the submission of the article.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Guangdong Provincial Key Laboratory of Food Quality and Safety, South China Agricultural University, Guangzhou 510642, China. [2] Tropical Medicine Institute & South China Chinese Medicine Collaborative Innovation Center, Guangzhou University of Chinese Medicine, Guangzhou 510405, China. [3] A.N. Bach Institute of Biochemistry, Federal Research Centre Fundamentals of Biotechnology, Russian Academy of Sciences, Leninsky prospect 33, Moscow 119071, Russia. [4] Department of Chemical Enzymology, Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119998, Russia. [5] Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510642, China.

References
1. Alesso M, Bondioli G, Talío MC, Luconi MO, Fernández LP. Micelles mediated separation fluorimetric methodology for Rhodamine B determination in condiments, snacks and candies. Food Chem. 2012;134(1):513–7.
2. Qi P, Lin Z, Li J, Wang C, Meng W, Hong H, Zhang X. Development of a rapid, simple and sensitive HPLC–FLD method for determination of rhodamine B in chili-containing products. Food Chem. 2014;164:98–103.
3. Chen D, Huang YQ, He XM, Shi ZG, Feng YQ. Coupling carbon nanotube film microextraction with desorption corona beam ionization for rapid analysis of Sudan dyes (I–IV) and Rhodamine B in chilli oil. Analyst. 2015;140(5):1731–8.
4. Song S, Lin F, Liu L, Kuang H, Wang L, Xu C. Immunoaffinity removal and immunoassay for rhodamine B in chilli powder. Int J Food Sci Technol. 2010;45(12):2589–95.
5. Spink J, Moyer DC. Defining the public health threat of food fraud. J Food Sci. 2011;76(9):R157–63.
6. Jain R, Mathur M, Sikarwar S, Mittal A. Removal of the hazardous dye rhodamine B through photocatalytic and adsorption treatments. J Environ Manage. 2007;85(4):956–64.
7. Hood RD, Jones CL, Ranganathan S. Comparative developmental toxicity of cationic and neutral rhodamines in mice. Teratology. 1989;40(2):143–50.
8. List of non-edible substances and food additives that may be illegally added in food (first batch). 2008. http://www.gov.cn/gzdt/2008-12/15/content_1178408.htm.
9. Botek P, PouStka J, Hajslova J. Determination of banned dyes in spices by liquid chromatography-mass spectrometry. Czech J Food Sci. 2007;25(1):17–24.
10. Desiderio C, Marra C, Fanali S. Quantitative analysis of synthetic dyes in lipstick by micellar electrokinetic capillary chromatography. Electrophoresis. 1998;19(8–9):1478–83.
11. Wang CC, Masi AN, Fernández L. On-line micellar-enhanced spectrofluorimetric determination of rhodamine dye in cosmetics. Talanta. 2008;75(1):135–40.
12. Pourreza N, Rastegarzadeh S, Larki A. Micelle-mediated cloud point extraction and spectrophotometric determination of rhodamine B using Triton X-100. Talanta. 2008;77(2):733–6.
13. Biparva P, Ranjbari E, Hadjmohammadi MR. Application of dispersive liquid–liquid microextraction and spectrophotometric detection to the rapid determination of rhodamine 6G in industrial effluents. Anal Chim Acta. 2010;674(2):206–10.
14. Chen X, He J, Tan G, Liang J, Hou Y, Wang M, Wang B. Development of an enzyme-linked immunosorbent assay and a dipstick assay for the rapid analysis of trans-resveratrol in grape berries. Food Chem. 2019;291:132–8.
15. Guan T, He J, Liu D, Liang Z, Shu B, Chen Y, Liu Y, Shen X, Li X, Sun Y. Open surface droplet microfluidic magnetosensor for microcystin-LR monitoring in reservoirs. Anal Chem. 2020;92(4):3409–16.
16. Wirtz R, Zavala F, Charoenvit Y, Campbell G, Burkot T, Schneider I, Esser K, Beaudoin RL, Andre R. Comparative testing of monoclonal antibodies against *Plasmodium falciparum* sporozoites for ELISA development. Bull World Health Organ. 1987;65(1):39.
17. Hu ZQ, Li HP, Wu P, Li YB, Zhou ZQ, Zhang JB, Liu JL, Liao YC. An affinity improved single-chain antibody from phage display of a library derived from monoclonal antibodies detects fumonisins by immunoassay. Anal Chim Acta. 2015;867:74–82.
18. Li D, Ying Y, Wu J, Niessner R, Knopp D. Comparison of monomeric and polymeric horseradish peroxidase as labels in competitive ELISA for small molecule detection. Microchim Acta. 2013;180(7–8):711–7.
19. Wen J, Chen J, Zhuang L, Zhou S. SDR-ELISA: Ultrasensitive and high-throughput nucleic acid detection based on antibody-like DNA nanostructure. Biosens Bioelectron. 2017;90:481–6.
20. Wen W, Yan X, Zhu C, Du D, Lin Y. Recent advances in electrochemical immunosensors. Anal Chem. 2017;89(1):138–56.
21. Zhou Y, Huang X, Zhang W, Ji Y, Chen R, Xiong Y. Multi-branched gold nanoflower-embedded iron porphyrin for colorimetric immunosensor. Biosens Bioelectron. 2018;102:9–16.
22. Yu F, Vdovenko MM, Wang J, Sakharov IY. Comparison of enzyme-linked immunosorbent assays with chemiluminescent and colorimetric detection for the determination of ochratoxin A in food. J Agric Food Chem. 2011;59(3):809–13.
23. Ye L, Wu X, Xu L, Zheng Q, Kuang H. Preparation of an anti-thiamethoxam monoclonal antibody for development of an indirect competitive enzyme-linked immunosorbent assay and a colloidal gold immunoassay. Food Agric Immunol. 2018;29(1):1173–83.
24. Shechter Y, Schlessinger J, Jacobs S, Chang KJ, Cuatrecasas P. Fluorescent labeling of hormone receptors in viable cells: preparation and properties of highly fluorescent derivatives of epidermal growth factor and insulin. PNAS. 1978;75(5):2135–9.
25. Zhang Y, Wang L, Shen X, Wei X, Huang X, Liu Y, Sun X, Wang Z, Sun Y, Xu Z. Broad-specificity immunoassay for simultaneous detection of ochratoxins A, B, and C in millet and maize. J Agric Food Chem. 2017;65(23):4830–8.
26. Abad A, Moreno MJ, Montoya A. A monoclonal immunoassay for carbofuran and its application to the analysis of fruit juices. Anal Chim Acta. 1997;347(1–2):103–10.

Wang *et al. Chem. Biol. Technol. Agric.*   (2021) 8:17

Page 10 of 10

27. Oplatowska M, Elliott CT. Development and validation of rapid disequilibrium enzyme-linked immunosorbent assays for the detection of Methyl Yellow and Rhodamine B dyes in foods. Analyst. 2011;136(11):2403–10.
28. Zeck A, Weller MG, Niessner R. Characterization of a monoclonal TNT-antibody by measurement of the cross-reactivities of nitroaromatic compounds. Fresen J Anal Chem. 1999;364(1–2):113–20.
29. Estévez M-C, Galve R, Sánchez-Baeza F, Marco M-P. Direct competitive enzyme-linked immunosorbent assay for the determination of the highly polar short-chain sulfophenyl carboxylates. Anal Chem. 2005;77(16):5283–93.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.