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Development of a fluorescence-linked immunosorbent assay for detection of avermectins using a fluorescent single-domain antibody†

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A fluorescent single-domain antibody (fluobody), consisting of a single-chain variable fragment antibody (scFv) and a green fluorescent protein extracted from aequorea coerulescens (AcGFP), was produced and used to develop a rapid and sensitive fluorescence-linked immunosorbent assay (FLISA) for the detection of avermectins (AVMs). The scFv gene was prepared by cloning V_H and V_L genes from a hybridoma cell line (2C11) and then fused to the C-terminus of AcGFP (fluobody) with a flexible peptide linker $(Gly_4Ser)_2$ between the two domains. After expression and purification, the functional fluobody was used to develop a one-step FLISA protocol for the determination of AVMs in milk samples. The 50% inhibition concentration (IC_{50}) value and the limit of detection (LOD) of the optimized immunoassay for abamectin (ABM) were 2.13 and 1.07 ng mL^{-1}, respectively. Cross-reactivity studies showed that the fluobody exhibited high affinity for the other four AVMs. The recoveries from the spiked milk samples ranged between 86.8 and 125.0%, with relative standard deviation lower than 10.2%. Moreover, the developed FLISA was applied to field samples, followed by confirmation with liquid chromatography-fluorescence detection (LC-FLD) analysis. The consistency of results between the immunoassay and instrumental techniques in detecting the presence of AVMs near the detection limit of the FLISA indicated that the newly developed method is suitable for rapid screening of AVM contamination in food samples prior to chromatographic analysis.

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1 Introduction

Avermectins (AVMs), produced by fermentation of the soil bacterium *Streptomyces avermitilis*, belong to the family of macrocyclic lactones. Five AVMs, namely abamectin (ABM), ivermectin (IVM), eprinomectin (EPR), doramectin (DOR) and emamectin (EMA), are widely used in agriculture and farm animals for the treatment of a broad spectrum of parasitic diseases.[1,2] Though their proper administration at the recommended doses does not pose a threat to human health, the extensive use of these compounds in food-producing animals can cause the accumulation of drug residues and intoxication in animals and humans.[3] In addition, AVMs are easily excreted in milk because of their high lipophilicity.[4] In order to ensure human food safety, the EU has set MRLs in milk samples, 40 ng mL^{-1} for moxidectin (MOX) and 20 ng mL^{-1} for EPR, whereas the use of ABM, DOR and IVM is prohibited in lactating species by the EU (European Commission 2010).

The most used AVM determination methods are based on high performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS).[5–9] However, these instrumental methods are time consuming and costly and sample preparation is demanding. Immunoassays have been confirmed as effective and economical screening methods by virtue of their high sample throughput, sensitivity and fast analysis. During the last two decades, several immunoassay methods have been developed to detect AVMs based on polyclonal and monoclonal antibodies (mAb).[10–13] However, these conventional antibodies are labelled with enzymes by means of chemical methods, which result in partial inactivation of the enzyme and the low stability of the reagents used for labelling, which in turn influence the specificity and sensitivity of immunoassays.[14,15] Recently, single-chain fragment variables (scFvs) fused with alkaline phosphatase (AP) and green fluorescent proteins (GFPs) have been reported to directly detect large molecular weight antigens such as pathogens, peptides, and microtubules.[16–18] The application of scFv–AP and scFv–GFP fusions in immunoassay methods for detecting small molecules has also been reported and the assays indicated an improvement in sensitivity.[19–22] Moreover, the use of this bifunctional

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antibody can avoid the chemical coupling of enzymes with antibodies and the use of a second antibody, and therefore shorten the analysis time.

For rapid and sensitive screening of AVMs, here a fast, simple, and sensitive indirect competitive fluorescence-linked immunosorbent assay (icFLISA) based on a bifunctional fluorescent single-domain antibody (fluobody) was developed. In our previous study, a hybridoma cell line (2C11) that secretes an mAb with broad specificity to a class of AVMs was produced.[10] Here we successfully produced a single-chain variable fragment (scFv) from 2C11 and then fused the scFv to the terminus of AcGFP (fluobody) with a flexible peptide linker $(Gly4Ser)_2$ between the two domains. As a previous study shows that scFv fused at the C-terminus (C-fluobody) is superior to that fused at the N-terminus of AcGFP (N-fluobody) as a probe for FLISA when expressed in *E. coli*,[22,23] the C-fluobody was used in the following research. After expression and purification, the functional fluobodies were applied to icFLISA for the detection of AVMs in milk samples. The results showed that the sensitivity of the developed FLISA was approximately 2 times that of the corresponding enzyme-linked immunosorbent assay (ELISA) based on mAb.

2 Materials and methods

2.1 Microbiology reagents and chemicals

ABM (purity 98.27%) and DOR (purity 94.33%) were obtained from Pfizer Co. (New York, NY, USA), IVM (purity 95.64%) and EMA benzoate (purity 98.26%) were sourced from CAU Newtech Development Co. (Beijing, China), and EPR (purity 96.85%) was a gift from Prof. Ming Wang (China Agricultural University, Beijing, China). Primers were synthesized by Shanghai Sangon Biotechnology (Shanghai, China). Restriction enzymes, Restriction enzymes *Sfi*I, T4 DNA ligase, dNTP and Pfu DNA polymerase were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). The hybridoma cell line 2C11, which secretes mAb against RAC, was produced in our laboratory previously. We used *Escherichia coli* strains *XL1-Blue* and *RV308*, which were stored in our laboratory. Kanamycin (>99% pure) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The oligonucleotide primers were synthesized using Invitrogen and used for polymerase chain reaction (PCR) cloning of the variable genes. The Aequorea coerulescens (AcGFP) gene sequence was from National Center for Biotechnology Information (NCBI). Clone vector pEASY-T3 was purchased from TransGen Biotechnology Co. (Beijing, China). The vector pET28b used for AcGFP–scFv expression was preserved in our laboratory. Restriction enzymes used in cloning and library construction were purchased from New England Biolabs (Beijing, P.R.C.). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was obtained from Promega (Madison, WI, USA). The anti-c-myc 9E10 antibody and the peroxidase-conjugated goat antimouse IgG (IgG-HRP) were obtained from Jackson Immuno Research Laboratories, Inc. (West Grove, PA, USA). The coating antigens of 4′-*O*-succinoyl-ABM–OVA were preserved in our laboratory. Tetramethylbenzidine (TMB) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ni-NTA agarose was purchased from GE Healthcare (Beijing, China). All other chemicals were standard commercial products of analytical reagent grade.

2.2 Apparatus

All aqueous solutions and buffers were prepared with water purified with a Milli-Q system (Millipore, Bedford, MA, USA). Black and white, high binding, 96-well polystyrene microplates for FLISA and ELISA were obtained from Costar (Cambridge, MA, USA). A SpectraMax M5 microplate reader from Molecular Devices (Downingtown, PA, USA) was used to measure the OD value and fluorescence intensity (FI) signal.

2.3 Buffers and standard solutions

The following buffers were used in the FLISA and ELISA: (1) coating buffer was 0.05 M carbonate buffer, pH 9.6; (2) blocking buffer consisted of 0.01 M phosphate-buffered saline (PBS), 0.5% BSA, 0.01% NaN_3, and 0.05% Tween 20, pH 7.4; (3) washing buffer was PBS with 0.05% Tween 20; (4) the substrate was 0.1% TMB and H_2O_2 in 0.05 M citrate buffer, pH 4.5; and (5) the stopping reagent was 2 M H_2SO_4. Working standard solutions of analytes in the range of 0.1 to 1000 ng mL^{-1} were prepared by dilution of stock solution with assay buffer.

2.4 Cloning of variable region genes and production of recombinant scFv antibodies

The total RNA was extracted from the 2C11 hybridoma cell line (about 1×10^7) using TRIzol reagent (Takara Biotechnology Co.). After reverse transcription from mRNA using a reverse transcription PCR kit (Promega Biotechnology Co.), the first cDNA was synthesized as a template, and the V_L and V_H genes were amplified by separate PCR primers.[24] The amplified products were purified using the agarose gel DNA purification kit and cloned into the pEASY-T3 vector for plasmid production. The extracted plasmid was used for sequencing. The V_L and V_H region sequences were aligned to immunoglobulin sequences in the international ImMunoGeneTics (IMGT) information system databases using the V-QUEST search tool.[25] After purification and quantification, overlapping of the collected V_H and V_L DNAs was then performed to obtain the scFv gene with P1–P4 primers (Table 1), resulting in the formation of the $(Gly_4Ser)_4$ linker from the overlapped gene portions residing between the V_H and V_L fragments.

2.5 Construction of AcGFP/scFv expression vector

The anti-AVMs-scFv PCR product and the synthesized AcGFP gene (from NCBI) were used as templates for constructing a fusion protein expression vector by means of splicing by overlapping extension PCR (SOE-PCR). Four primers (Table 1) for PCR were designed based on the scFv and AcGFP sequences to construct the AcGFP/scFv fusion protein with scFv at the C-terminus of AcGFP (C-fluobody). The P5 and P6 primers were used to amplify the AcGFP domains from the synthesized pET28b vector encoding AcGFP gene. The P1 and P7 primers

Table 1 Primers used for construction of fluobody[a]

Primer name	Sequence (5′ → 3′)
*P*1	CAAATTGTTCTCACCCAGTCT
P2	ggagccgccgccgccagaaccaccaccaccagaaccaccaccaccTTTCAGCTCCAGCTT
P3	ggcggcggcggctccggtggtggtggatccCAGATCCAGTTGGTGCAGTCT
P4	TGCAGAGACAGTGACCAGAGT
P5	GTGTCCAAAGGCGCTGAACTG
P6	GCTACCGCCACCGCCTTTGTACAGTTCATCCATACCGTGC
P7	GGCGGTGGCGGTAGCGGCGGTGGCGGTTCT CAAATTGTTCTCACCCAGTCT
P8	CTACAGCA GGCCCAGCCGGCC GTGTCCAAAGGCGCTGAACTG
P9	TCGCTAATCAGTTTTTGTTCGGCGGCCGC TGCAGAGACAGTGACCAGAGT
P10	CGGAGTCA GGCCCCGAGGCC AGGTCTTCTTCCGAAATCAGTTTTTGTTC

[a] The recognition sites for restriction endonucleases are underlined.

were used to amplify the sequence of scFv. The amplified AcGFP domains and the scFv domains were subjected to PCR splicing by overlap extension to assemble the AcGFP/scFv gene with a $(Gly_4Ser)_2$ linker. After purifying the amplified genes encoding the C-fluobody, we introduced a myc-tag at the C-terminus and *Sfi*I site at the N-terminus and C-terminus with primers P8–P10, respectively. Finally, the full-length C-fluobody fragments were separated and purified by 1% (w/v) agarose gel electrophoresis. Afterward, the synthesized C-fluobody gene was cloned to the pJB33 vector[26] using the introduced *Sfi*I sites and transformed into *Escherichia coli RV308*. The transformants were placed on Luria Bertani (LB) plates supplemented with 30 μg mL^{-1} chloramphenicol and incubated overnight at 37 °C. The positive clones were identified by PCR and confirmed by DNA sequencing. Finally, plasmids were extracted from the culture from the positive colonies and stored at −20 °C.

2.6 Expression and purification of recombinant fluobodies

The protocol of expression and purification of fluobodies was performed as described previously.[27] Briefly, a single colony harboring the plasmid encoding the positive fluobody fragment was grown overnight at 37 °C with shaking (200 rpm min^{-1}) in 10 mL 2xYT medium (16 g tryptone per L, 10 g yeast extract per L, and 5 g NaCl per L) containing chloramphenicol. A 1 mL aliquot of the culture was then inoculated into 100 mL of 2xYT medium containing chloramphenicol and incubated at 37 °C and 250 rpm min^{-1} until the OD_{600} reached 0.6 nm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM and incubated at 25 °C for 16 h.

The cells were harvested by centrifugation and resuspended thoroughly in 10 mL of precooled periplasmic cell extract buffer (30 mM Tris–HCl, 2 mM EDTA, 20% (w/v) sucrose, and 100 μg mL^{-1} lysozyme). After storing on ice for 30 min, cell debris was then removed by centrifugation, and the supernatant was suspended in 200 μL of SDS–PAGE loading buffer (60 mM Tris–HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 25% glycerine by wt, and 14.4 mMb-mercaptoethanol). The fluobodies prepared above were identified by SDS–PAGE and western blotting analysis, as previously published.[27] The large purification of fluobody was carried out by immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™HP column according to the instructions of GE Healthcare.

2.7 Measurement of fluorescence intensity

The fluorescence spectra of GFP/scFv (100 μL per well) equalized in PBS were measured using the SpectraMax M5 microplate reader from Molecular Devices (Downingtown, PA, USA). Excitation spectra were measured at 490 nm and emission spectra were measured at 530 nm by excitation.

2.8 FLISA protocol and traditional ELISA

A black microtiter plate (FluoroNunc, MaxiSorp, Roskilde, Denmark) was coated with the 4′-*O*-succinoyl-ABM–OVA conjugate at 4 °C overnight. The plates were washed three times with washing buffer, and then blocked with blocking buffer (300 μL per well) at RT for 1 h. For comparison, 50 μL of standard solutions of AVMs of various concentrations and 50 μL of fluobody solution (3.7 μg mL^{-1} in PBS) were added to each coated well, and the plates were incubated at 37 °C for 30 min. After washing the plate a further three times with PBS-T and adding 100 mL PBS to each well, the remaining fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 530 nm using a fluorescent microplate reader (MTP-600FE, Corona).

For ELISA, Costar high-binding enzyme immunoassay 96-well plates (Corning Inc., Shanghai, P.R.C.) were coated with the 4′-*O*-succinoyl-ABM–OVA conjugate at 4 °C overnight. The plates were washed three times with washing buffer, and then blocked with blocking buffer (300 μL per well) at room temperature (RT) for 1 h. Afterward, serial dilutions (50 μL per well) of the analyte were added, followed by addition of 50 μL per well of scFv at a previously determined concentration. Then, the plates were incubated for 1 h at RT followed by washing three times as described above. The anti-c-myc monoclonal antibody 9E10 (1 : 5000 in PBS) and the goat anti mouse IgG HRP conjugate (1 : 5000 in PBS) were added to wells and incubated at RT for 1 h. After washing the plates three times, the substrate solution was added and incubated at RT for 15 min before the enzymatic reaction was stopped by adding 2 M H_2SO_4 (100 μL per well).

The absorbance of each well was measured at 450 nm by the ELISA plate reader.

2.9 Curve fitting and cross-reactivity determination

The data were then fitted with the four parameter logistic equation using the Origin 8.0 software package (Microcal, Northampton, MA, USA). Competition curves were graphed by plotting the fluorescence values against the $\log_{10}$ of the analyte concentration, and the fluorescence values were fitted using a four parameter logistic equation in order to calculate the IC_{50} value. The cross-reactivity (CR) values were calculated according to the following equation: CR (%) = [IC_{50} (ABM)/IC_{50} (analyte)] × 100.

2.10 Sample preparation

Commercial milk samples were purchased from a local supermarket in Beijing, China. All the control samples were previously checked as AVMs free using the HPLC method at the National Reference Laboratory for Veterinary Drug Residue (Beijing, China). Sample pretreatment procedures were performed by a previously published method.[28] Briefly, aliquots (10 mL) of commercial pasteurized defatted cow milk were accurately pipetted into 50 mL polypropylene centrifuge tubes and fortified with AVMs. Samples were shaken on a vortex mixer for 20 s and left to stand at room temperature for 15 min to enable sufficient equilibration with the milk matrix. After the addition of 1 mL of sodium nitroprusside solution (0.36 M) and 0.5 mL of zinc sulfate solution (1 M), the samples were vortex-mixed for at least 2 min and then centrifuged at 12 000 rpm for 10 min. The clear supernatant was carefully removed and diluted ten times by PBS (20 mM, pH 7.0).

2.11 Analysis of field milk samples

Thirty milk samples were collected from retail outlets in Beijing. Each sample was divided into two portions: one was analyzed by the FLISA and another was analyzed by liquid chromatography-fluorescence detection (LC-FLD). The LC-FLD analysis of AVMs was adopted according to our previous report.[29] The spiked samples were extracted according to the method described by N. Campillo *et al.*

3 Results and discussion

3.1 Cloning and characterization of scFv and the fluobody genes

Total RNA from the hybridoma cell line 2C11 was used to clone the V_L and V_H domain cDNAs. These two genes encoding the variable domains were amplified by separate PCR primers. The V_L and V_H genes were assembled by SOE-PCR to generate the scFv gene with a flexible linker $(Gly_4Ser)_4$. PCR assembly and amplification of scFv produced an expected 747 bp fragment (Fig. 1). Then, the scFv and AcGFP gene were also assembled by SOE-PCR to generate fluobody genes (Fig. 1B) with a flexible linker $(Gly_4Ser)_2$ and restriction enzyme sites at both ends (*Sfi*I). The recombinant plasmid pJB33-fluobodies were identified by PCR and electrophoretic analysis (Fig. 1). The DNA sequencing

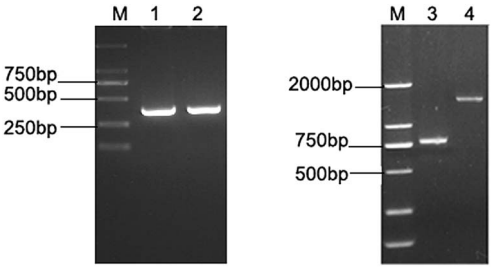

Fig. 1 PCR products of V_H, V_L, scFv and fluobody: lane 1, V_H; lane 2, V_L; lane 3, scFv; lane 4, fluobody; lane M, DNA molecular weight standards (DL-2000).

of plasmid pJB33-fluobodies also confirmed that the inserted DNA fragment was in accordance with the fluobody antibody gene. All results showed that the expression vector pJb33-fluobodies were successfully constructed.

3.2 Expression, purification and identification of the fluobodies

The soluble periplasmic proteins were extracted by cold osmotic shock and identified by SDS–PAGE and Western blotting (Fig. 2). The molecular weight of expressed fluobodies was expected to be ~60 kDa. For purification of the recombinant 6 × His-tagged antibody, we used a fast flow Ni-NTA agarose column and the eluted fraction was detected by SDS–PAGE and Western blotting (Fig. 2). The fluobodies were further characterized by indirect ELISAs and the results indicated that the specific binding characteristics to hapten 4′-*O*-succinoyl-ABM–OVA were retained in the constructed fluobodies (see ESI Fig. S1†).

3.3 Measurement of fluorescence intensity

The fluorescence intensity of the fluobody (100 μL $well^{-1}$) was analyzed at a protein concentration of 20–640 μg mL^{-1} using the SpectraMax M5 microplate reader at an emission wavelength of 490 nm and an excitation wavelength of 530 nm. The results suggest that the fluorescence characteristics of the expressed protein are not affected by the fusion of the 6 × His

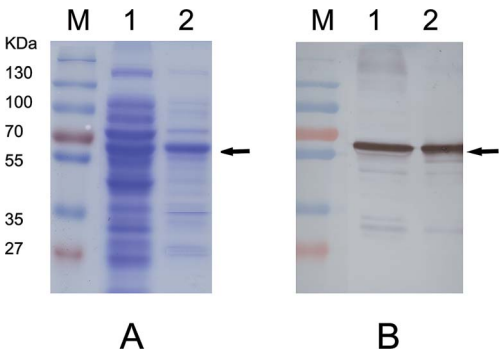

Fig. 2 SDS–PAGE (A) and western blotting (B) analyses of fluobody expression and purification. Lane M, protein molecular weight standards; lane 1, IPTG induced *E. coli strain RV308* total protein; lane 2, purification of fluobody.

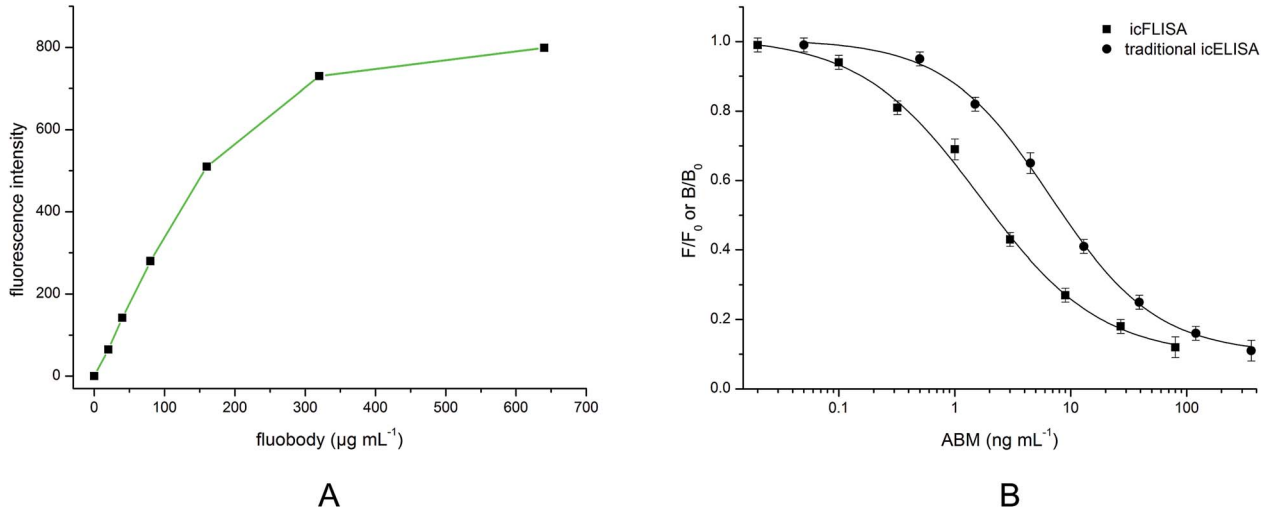

Fig. 3 (A) Measurement of fluorescence intensity. (B) Normalized standard curves for ABM of fluobody-based ciFLISA and scFv-based traditional ELISA.

tag to the N terminus and the scFv to the C terminus of AcGFP and can be applied for FLISA (Fig. 3A).

3.4 Comparison of the developed FLISA and traditional ELISA

A FLISA based on bifunctional fluobodies and a traditional ELISA were both developed to compare their specificities and sensitivities to the AVMs. The sensitivity of the FLISA for AVM, which was represented by IC_{50} values, was 2.13 ng mL^{-1} (Fig. 3B). The sensitivity of the FLISA using the fluobody was found to be higher than that in conventional ELISA (3.05 ng mL^{-1}).[10] The previous research supposed that the highly sensitive fluorescence of AcGFP detected by the fluorescent microplate reader contributed to the improvement in sensitivity and this is consistent with our results.[21,22] The cross-reactivity values for other AVMs in the optimized FLISA were all consistent with that in the traditional colorimetric ELISA method, while lower IC_{50} in the FLISA was obtained (Table 2). All these data showed that a one-step FLISA could be developed by using a fluobody instead of a monoclonal antibody.

Meanwhile, the use of the fusion protein can reduce the time required for immunochemical detection, since the bifunctional fusion protein used in the FLISA avoided the use of an enzyme-labeled secondary antibody and eliminated one washing step compared with conventional ELISA. The total reaction time for FLISA with the scFv–AcGFP fusion protein was about 70 min, which was a great improvement over the reported incubation time for ELISA based on mAb (>3 h).[10]

Table 2 Cross-reactivity (CR) profile and IC_{50} values to AVM compounds in the developed FLISA and traditional ELISA

	FLISA		ELISA	
Analyte	IC_{50} (ng mL^{-1})	CR[a] (%)	IC_{50} (ng mL^{-1})	CR[a] (%)
ABM	2.13	100.00	8.05	100.00
IVM	8.18	26.04	30.10	26.74
EMA benzoate	9.25	23.02	32.38	24.86
EPR	21.20	10.04	78.96	10.20
DOR	37.08	5.74	133.04	6.05

[a] CR% = IC_{50}, ABM/$IC_{50,cross\text{-}reactant}$ × 100%.

3.5 Matrix effect elimination

Because the complicated milk matrix may influence immunoassay performance, this must be simplified to attain the necessary sensitivity and satisfactory recovery. In this study, two sample pretreatment procedures were performed. Simple dilution: milk sample (0.5 mL) was directly diluted with PBS (20 mM, pH 7.0). Solvent extraction: sodium nitroprusside solution and zinc sulfate was used for extraction and protein precipitation in the milk sample, and then the extract was diluted 1 : 10 with PBS. It was found that after direct dilution (1 : 10) the blank milk still exhibited strong fluorescence signals which significantly affected detection. The second method exhibited hardly any fluorescence background after ten fold dilution with PBS after extraction compared to the first method. Fig. 4 shows the optimized FLISA standard curves performed in assay buffer and two sample matrixes, implying that the pretreatment procedures can obviously reduce the matrix effect in milk samples. Finally, the sodium nitroprusside solution and zinc sulfate solution were selected for method development.

3.6 Limit of detection and recovery test

The limit of detection (LOD) was calculated as the concentration corresponding to the mean value of 20 blank milk samples, plus three times the standard deviation. Among the 20 blank milk samples, the highest observed blank was 1.05 ng mL^{-1} for ABM. The LODs for IVM, EMA benzoate, EPR and DOR were 4.08, 1.25, 7.82 and 15.73 ng mL^{-1}, respectively. The LOD for ABM measurement in FLISA (1.05 ng mL^{-1}) was improved two fold compared to conventional ELISA (2 ng mL^{-1}). Because the

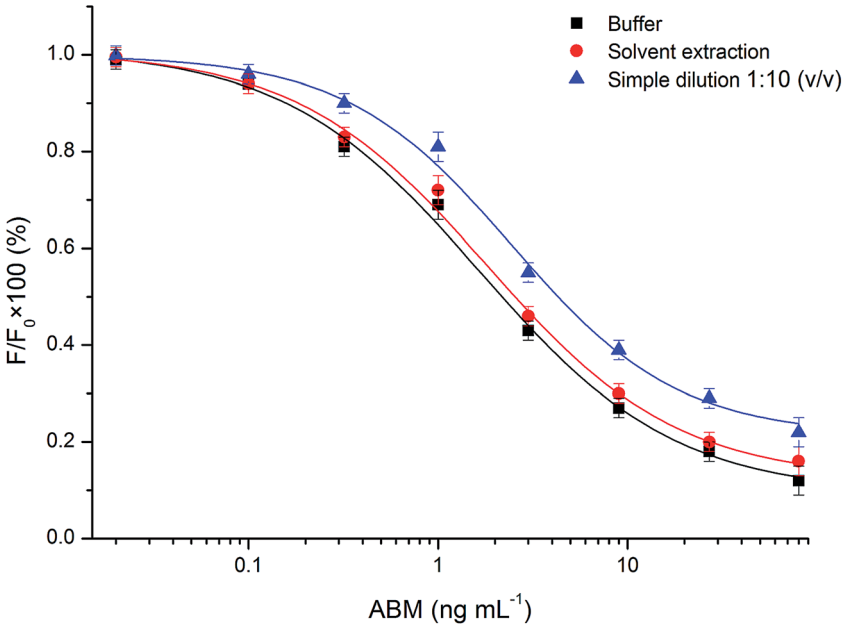

Fig. 4 Standard curves for the determination of ABM in PBS buffer, solvent extraction and 1/10 milk dilution.

use of ABM, DOR and IVM is not allowed in lactating species by the EU, the FLISA with higher sensitivity has more advantages than the traditional ELISA to screen them in milk. In addition, as the tolerance limit set by the EU for the EPR is 20 ng mL^{-1} in milk, the FLISA (7.82 ng mL^{-1}) developed here offers sufficient sensitivity for the determination of EPR compared with traditional ELISA (15.60 ng mL^{-1}).

The recoveries of 5 AVMs (ABM, IVM, EPR, DOR and EMA benzoate) in blank milk were determined to evaluate the accuracy of the developed FLISA. The precision of the FLISA method was assessed on the basis of the relative standard deviation (RSD). Intra-assay variation was measured using four replicates of three spiked concentrations. As shown in Table 3, recoveries of the five selected AVMs ranged from 84.1% to 115.3%, with the RSD values less than 10.2% in milk samples. The results showed good agreement between the spiking level and the concentration detected. Hence, it is suggested that this FLISA could be used for the determination of AVMs in field milk samples.

Table 3 Mean recovery and Relative Standard Deviation (RSD) for AVMs in milk

AVMs analytes	Added (ng mL^{-1})	Found (ng mL^{-1})	Recovery (%)	RSD (%)
ABM	1	1.1	110.0	5.3
	2	1.8	90.0	7.5
	5	5.4	92.6	6.3
IVM	5	5.8	116.0	10.2
	10	9.1	91.0	5.3
	20	17.1	85.5	8.3
EMA benzoate	5	4.4	88.0	5.4
	10	8.4	84.0	9.3
	20	23.2	116.0	6.2
EPR	10	12.3	123.0	3.2
	20	24.2	121.0	9.4
	40	38.4	96.0	4.5
DOR	15	16.7	111.3	8.8
	30	32.6	108.7	7.3
	60	68.3	113.8	5.2

3.7 Analysis of field milk samples

To confirm the accuracy and applicability of the FLISA in field milk samples, the samples were analyzed by the newly developed FLISA and the LC-FLD method, respectively. The results (Table 4) showed that the FLISA is able to screen for the positive samples (samples 7, 10 and 21) with the AVM residue levels above the LOD. Sample 21 was determined to be negative by FLISA because the residue level of EPR (2.4 ng mL^{-1}) was below the LOD of the method. The other samples free from AVMs were also determined and no analytes were detected in all the cases. Although the FLISA may underestimate the AVM residue concentrations because of its limited working range when compared to LC-MS/MS, this does not affect its usefulness as a

Table 4 Determination of field milk samples collected from retail outlets in Beijing by the FLISA and the LC-FLD method[a]

Samples	FLISA[b] (ng mL^{-1})	LC-FLD (ng mL^{-1})
S7	2.10	0.68 (ABM)
S10	16.50	8.41 (EPR)
S20	11.12	13.34 (EPR)
S21	ND	2.40 (EPR)
S1–S6, S8–S9, S11–S19 S22–S30	ND	ND

[a] Notes: ABM, abamectin; EPR, eprinomectin; ND, not detectable. [b] Each value was determined with three repeats.

screening tool because it will still indicate the presence of AVMs above their detection limits. All these results demonstrated that the FLISA can be used for rapid screening of AVM residues in food samples prior to chromatographic analysis.

4 Conclusions

In this study, the V_L and V_H genes of an anti-AVM antibody were cloned and assembled to generate an entire functional scFv. The scFv gene was then fused to the C-terminus of AcGFP (fluobody). After expression and purification, the functional fluobody was successfully used to develop a FLISA protocol for the detection of AVMs in milk samples. The sensitivity for AVM determination in FLISA with the fluobody was higher than that seen in conventional ELISA. Moreover, the use of the fusion protein can avoid the use of an enzyme-labeled secondary antibody and reduce the time required for immunochemical detection. These results indicated that the developed FLISA could be applied to detect AVMs in food samples.

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