

Development of a one-step semi-homogeneous fluorescent method for multi-screening of sulfonamides in milk

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Abstract

In this study, sulfamethoxypyridazine was used as template to synthesize a type of magnetic molecularly imprinted microsphere based on surface imprinting technique, and tetraphenylethylene was used as fluorophore to synthesize a fluorescent tracer containing aggregation induced emission property. The two agents were combined to develop a semi-homogeneous fluorescent method for determination of sulfonamides. This method was simple (one sample-loading step) and rapid (7 min), its sensitivity was ultra-high (pg/mL level), and the microsphere could be reused for 6 times. Thanks to the ultra-high sensitivity, the milk sample could be assayed directly after 80-fold dilution, and the limits of detection for determination of the 21 SAs in milk were in the range of 0.08–3.98 ng/mL. Compared with fluorescein labeled fluorescent tracer, the sensitivities were improved for 19–671 folds. The analysis results for the unknown milk samples were consistent with a confirmational liquid chromatography mass spectrometry method. This is the first study reporting a semi-homogeneous competitive fluorescent method for determination of sulfonamides in food of animal origin based on magnetic molecularly imprinted microsphere and aggregation induced emission luminogen.

Introduction

During the past several decades, immunoassays have been widely used for screening of various contaminants in food samples [1], and monoclonal antibody and polyclonal antibody are the most commonly used recognition reagents in those immunoassays. For production of an antibody, many laboratory animals are required, the procedures are tedious, the times are long, and the costs are high. In addition, the antibodies are not reusable. Therefore, other novel recognition reagents are created and used for development of pseudo immunoassays, such as receptors [2, 3], molecularly imprinted polymers [4, 5], and aptamers [6, 7].

Among these recognition reagents, molecularly imprinted polymer can be regarded as a type of optimal antibody substitute. It is synthesized with a specific molecule as the template, and the formed cavities can specifically recognize and adsorb the template molecule. In addition, the synthesis of a type of molecularly imprinted polymer only needs several days, the process is simple, the cost is low, and the product is reusable. Therefore, molecularly imprinted polymers have been used to develop many pseudo immunoassays for detection of different analytes [4, 5], such as benzimidazoles [8], chloramphenicol [9], pentachlorophenol [10], progestins [11], and roxarsone [12]. Results show these methods usually achieve better performances than the related immunoassays.

Among the reported immunoassays and pseudo immunoassays, the competitive homogeneous fluorescent methods have several unique merits. First, this type of method is performed at “mix-and-read” mode, i.e. containing only one sample-loading step, so it is simple. Second, this kind of method does not require a substrate system and avoids the related incubation/washing steps, so it is rapid. Third, its signal sources can be different fluorophores, including fluoresceins [13], quantum dots [14], carbon dots [15] and upconversion materials [16]. So many competitive homogeneous fluorescent

(immuno)assays have been reported for the analysis of different analytes [17, 18]. Molecularly imprinted polymers have combined with the above fluorophores to synthesize different fluorescent sensors for the detection of various analytes [19], but a competitive homogeneous fluorescent method based on molecularly imprinted polymer and conventional fluorophore has not been reported so far. This is because the polymer is solid material and a washing step is required to remove the excessive fluorescent tracers (not at “mix-and-read” mode).

The conventional fluorophores based fluorescent methods mentioned above have an inevitable limitation: the sensitivity is usually low. This is because the quantum yields of conventional fluorophores are low and they can lead to aggregation-caused quenching phenomenon when they are at high concentration, which hinders the development of sensitive fluorescent method. So, the finding of new fluorophore that can avoid the above limitations and improve the method sensitivity is desirable.

During the past two decades, the aggregation-induced emission luminogens (AIEgens) have attracted the attentions of many analysts. As a class of novel fluorophores, AIEgens show ultra-high fluorescent signal at aggregation state but show weak or no fluorescent signal at solution state. So, they have been used to develop many sensitive fluorescent methods for the analysis of different analytes [20, 21]. As expected, AIEgens have also been used as signal sources to develop many fluorescent immunoassays for the analysis of different analytes [22], such as pesticide [23], toxin [24], bacteria [25], and veterinary drug [26]. However, a competitive homogeneous fluorescent (immuno)assay based on AIEgen has not been reported so far, which is because of an insoluble problem. For an AIEgen-based homogeneous immunoassay, even if the AIEgen labeled tracer binds with the antibody the fluorescent signal is still weak, which is because the antibody-tracer complexes are still at solution state, i.e. not achieving aggregation state to exhibit AIE effect.

As discussed above, it is difficult to develop a competitive homogeneous fluorescent method based on molecularly imprinted polymer and conventional fluorophore, and it is also difficult to develop a competitive homogeneous fluorescent immunoassay based on antibody and AIEgen. Molecularly imprinted polymer contains many binding cavities that can adsorb the target analyte and the related tracer, and this property provides a possibility to develop a semi-homogeneous competitive fluorescent method based on molecularly imprinted polymer and AIEgen labeled tracer. In the present study, a type of magnetic molecularly imprinted microsphere (MMIM) for sulfonamides (SAs) was synthesized, and an AIEgen labeled tracer for SAs was also synthesized. As shown in Fig. 1, the MMIM, the SAs and the AIEgen labeled tracer were mixed for competition, and then the fluorescent signal was read directly. This semi-homogeneous method was performed at “mix-and-read” mode, same as the typical assay formation of competitive homogeneous fluorescent immunoassay. Then, this method was optimized for determination of SAs in milk.

Experimental

Chemicals. Sulfaquinoxaline (SQ, 99.0%) and sulfamethoxypyridazine (SMP, 99.2%) were purchased from Dr. Ehrenstorfer (Ausburg, Germany). 2-methylbenzene-1-sulfonamide (MBS, 98.0%), sulfadimethoxine (SDM, 98.0%), sulfadoxine (SDM', 98.0%), sulfamonomethoxine (SMM, 98.0%), sulfamethazine (SM₂, 98.0%), mafenide hydrochloride (SML, 98.0%), acetyl sulfisoxazole (AS, 98.0%), sulfamethoxazole (SMZ, 98.0%), sulfanitran (SNT, 96.0%), sulfaguanidine (SG, 98.0%), sulfathiazole (STZ, 98.0%) and fluorescein isothiocyanate (FITC, analytical grade) were purchased from Shanghai Yuanye Biological Technology Company Limited (Shanghai, China). N-acetylsulfadiazine (ASZ, 98.0%) was purchased from Aladdin Biochemical Technology Company Limited (Shanghai, China). Sulfacetamide (SAA, 98.0%), sulfacetamide (SA, 99.8%), 6-aminopyridine-3-sulfonamide (APS, 97.0%), 2,5-dichlorosulfanilic acid (DCSA, 98.0%), succinylsulfathiazole (SST, 99.0%), phthalylsulfathiazole (PST, 95.0%), 3,5-dichlorosulfanilamide (DCS, 98.0%), 1-hydroxybenzotriazole (HOBT, analytical grade) and N,N-dicyclohexylcarbodiimide (DCC, analytical grade) were purchased from Macklin Biochemical Company Limited (Shanghai, China). Amino-modified tetraethylenebenzene (TPE, analytical grade) was obtained from XI'AN QIYUE Company Limited. (Xian, China).

Synthesis of magnetic molecularly imprinted microsphere (MMIM). The magnetic molecularly imprinted microspheres (MMIM) were synthesized as the following two steps. The first step was to synthesize Fe₃O₄-COOH nanoparticles according to a previous report [27]. Briefly, 0.54 g FeCl₃·6H₂O, 1.5 g sodium acrylate, and 1.5 g sodium acetate were dissolved in 20 mL ethylene glycol/diethylene glycol (50:50, v/v). This solution was heated in a Teflon-lined stainless-steel autoclave for 10 hours at 200 °C. The obtained particles were washed with ethanol and water for three cycles to obtain Fe₃O₄-COOH. The second step was to coat molecularly imprinted layer on Fe₃O₄-COOH surface based on the self-polymerization of dopamine. Briefly, 100 mg Fe₃O₄-COOH particles, 5 mg sulfamethoxypyridazine and 50 mg dopamine were put into 30 mL Tris-HCl solution to be stirred for 5 hours at 25 °C. The collected magnetic solids were washed with acetic acid/water (5:95, v/v) to elute the template molecules until sulfamethoxypyridazine was not detectable in the elution solution. Finally, the obtained MMIM was dried for later use. At the same time, a type of magnetic non-imprinted microsphere (MNIM) was also synthesized as described above but absence of the template sulfamethoxypyridazine.

Synthesis of AIEgen labeled fluorescent tracer. The carboxyl-modified tetraphenylethylene (TPE) was coupled with sulfacetamide (SAA) to synthesize the fluorescent tracer (TPE-SAA). Briefly, 0.5 mmol 4-(1,2,2-triphenylvinyl)benzoic acid, 120 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 80 mg 1-hydroxybenzotriazole, 70 mg N-hydroxysuccinimide and 6 mL N,N-dimethylformamide was added into a dry round-bottom flask. The mixture was stirred for 1 hour at room temperature under the protection of nitrogen stream. Subsequently, 0.5 mmol SAA was added, and the mixture was further stirred for 48 hours. The obtained crude product was purified by column chromatography to obtain TPE-SAA with the mixture of ethyl acetate and petroleum ether (3:1, v/v) as elute solution. At the same time, SAA was coupled with fluorescein isothiocyanate (FITC) to synthesize a controlled fluorescent tracer (FITC-SAA) according to the method described in our previous report [28].

Development of semi-homogeneous fluorescent method. The schematic representation of the semi-homogeneous competitive fluorescent method was shown in Fig. 1. Briefly, 50 μL MMIM suspension (0.5 mg/mL, containing 25 μg MMIM), 50 μL TPE-SAA (0.1 $\mu\text{g/mL}$) and 50 μL SAs solution were added into the wells of a microplate to be incubated for 7 min at room temperature. Then the microplate was put onto a multimode reader to read the fluorescence intensity (FI) values of each well ($\lambda_{\text{ex } 365}/\lambda_{\text{em } 480}$ nm). Finally, 200 μL methanol/acetic acid (9:1, v/v) was added into the wells to wash the suspensions for 5 cycles under the help of a 96-well bottom magnet, which is to elute the TPE-SAA molecules and the SAs molecules adsorbed on MMIM surface. The regenerated MMIM particles were used for the next assay.

The 21 SAs were assayed by the above method respectively. The FI/FI_0 values (FI_0 was the signal at zero concentration and FI was the signal at each concentration) at a series of concentrations were calculated that were used to develop the competitive inhibitory curve for each drug. Based on the curve, the half of inhibition concentration (IC_{50} , the drug concentration showing 50% of inhibition) and the limit of detection (IC_{10} , the drug concentration showing 10% of inhibition) were calculated. In addition, the controlled fluorescent tracer FITC-SAA was also used to perform the above method, and the IC_{50} and the IC_{10} values for these SAs were calculated respectively.

Determination of milk samples. Some blank milk samples were obtained from several healthy dairy cows known to be without use of SAs at the controlled dairy farm, and these samples were determined as negative samples by the following LC-MS/MS method. Five representative SAs were spiked into the blank milk samples at levels of 1, 10 and 100 ng/mL, and these spiked samples were diluted with water for 80-folds to be analyzed directly by the above method. The intra-day recovery at each concentration was the mean recovery of six repeat assays in one day, and the inter-day recovery was the mean recovery of duplicate assays in six successive days. Finally, 60 milk samples (25 samples from several local supermarkets and 35 samples from several local dairy farms) were analyzed as described above, and the detection results were confirmed by using a LC-MS-MS method [29].

Results and discussion

Assay principle. For development of an AIEgen-based detection method, the key point is to make the AIEgen molecules achieving aggregation state. The MMIM in the present study was synthesized by using surface imprinting method, and its surface contained many binding cavities that could provide the aggregation sites for the TPE-SAA molecules. For this method, the MMIM, the TPE-SAA and the analyte were mixed for incubation, and then the fluorescent signal was read directly without additional washing step. This was because only the TPE-SAA molecules aggregated on MMIM surface could exhibit fluorescent signal whereas the TPE-SAA molecules in solution showed negligible signal. For a negative sample, all the binding cavities were occupied by TPE-SAA molecules that achieve aggregation state and exhibit AIE effect, thus obtaining high FI value (Figure 1). For a positive sample, SAs molecules competed with TPE-SAA molecules to occupy the cavities, thus interfering the aggregation of TPE-SAA

and obtaining low FI value. The SAs concentration was negatively correlated with the FI value, so the analyte could be quantified based on the competitive inhibitory curve.

Characterization of MMIM. The MMIM was synthesized by coating a thin molecularly imprinted layer on Fe_3O_4 surface. The scanning electron microscope showed the Fe_3O_4 (mean diameter 217.2 nm) and the MMIM (mean diameter 223.4 nm) were all spheric particles, and the surface of MMIM was smoother than that of Fe_3O_4 (Figure 2A), indicating the surface of Fe_3O_4 was coated with a thin layer. The transmission electron microscope showed the MMIM was core-shell structured particle, and the molecularly imprinted layer was in the range of 6-10 nm (Figure 2B). These results proved the molecularly imprinted layer was coated on Fe_3O_4 surface. The Fourier-transform infrared spectroscopy showed the MMIM contained the characters of Fe_3O_4 (650.2 cm^{-1} from Fe-O lattice vibration), COOH ($1083.1, 2925.2, 1621.7$ and 1385.1 cm^{-1}) and dopamine (1291.2 cm^{-1} from the $\delta(\text{C-OH})$ bending vibration of self-polymerization of dopamine) (Figure 2C), indicating the MMIM was a composite. In addition, the result of vibrating sample magnetometer showed the MMIM was a type of magnetic material, and its magnetic intensity was weaker than Fe_3O_4 due to the coated molecularly imprinted layer (Figure 2D). In combination of the above results, it could be said the MMIM was synthesized.

Evaluation of MMIM. As shown in Figure 2E, the N_2 absorption/desorption experiments showed the MMIM and the MNIM all exhibited IV type isotherm curves, indicating they were all microporous materials. The BET surface area, the t-plot external surface area and the BJH surface area of MMIM (inserted in Figure 2E) were all higher than that of MNIM, indicating the MMIM contained more pores than the MNIM. After that, their adsorption properties for SAs and other drugs were evaluated. As shown in Figure 2F, the adsorption capacities of MMIM for SAs ($13.02\text{-}16.32\text{ }\mu\text{g/mg}$) were much higher than that for other drugs ($2.11\text{-}2.41\text{ }\mu\text{g/mg}$). In contrast, the adsorption capacities of MNIM for all of these analytes were comparably low ($2.06\text{-}2.70\text{ }\mu\text{g/mg}$). These results indicated the MMIM was a specific absorbent.

Characterization of TPE-SAA. The fluorescent tracer TPE-SAA was synthesized by coupling the carboxyl group of carboxyl modified TPE with the amino group of SAA (Figure 3A). The UV scanning showed the MMIM contained the characters of TPE and SAA (Figure 3B), indicating it was a new compound. The fluorescence scanning showed the excitation/emission spectra of TPE-SAA and TPE were identical and their excitation/emission wavelengths were same ($\text{Ex}_{365}/\text{Em}_{479}\text{ nm}$, Figure 3C), indicating the fluorescent properties of TPE-SAA and TPE were same. The liquid chromatography mass spectrometry showed the detected molecular weight of TPE-SAA (MS (ESI) m/z : 573.18414 ($[\text{M}+\text{H}]^+$), Figure 3D) was consistent with its theoretical molecular weight (572.6748), indicating the obtained product was the designed compound. In combination of the above results, it could be said the TPE-SAA was synthesized.

Evaluation of TPE-SAA. After synthesis, its fluorescent property was evaluated. Firstly, it was prepared with different solutions, and the results showed it emitted the highest fluorescent signal when dissolving in dimethyl sulfoxide (DMSO, Figure 3E). Secondly, it was prepared with the DMSO solutions containing

different proportions of water, and the fluorescent signals were compared. Results showed it emitted the highest fluorescent signal when the water percentage was 70% (Figure 3F). This was because all or most of TPE-SAA molecules were at molecule state (dissolving in DMSO) when the water percentages were low (0-50%), and all or most of its molecules were at aggregation state when the water percentages were high (60%-100%). The picture shown in Figure 3F also illustrated this point. The above results proved the TPE-SAA exhibited typical AIE property. In addition, the controlled fluorescent tracer FITC-SAA was also characterized by UV scanning and fluorescence scanning, and the results showed it was synthesized (Figure 3G and 3H).

Evaluation of assay feasibility. During the experiments, different concentrations of MMIM were mixed with different concentrations of TPE-SAA, and the fluorescent signals were measured. As shown in Figure 4A, the FI values when using high concentration of MMIM were higher than that when using low concentration of MMIM, which was because the high concentration of MMIM could adsorb more TPE-SAA molecules and exhibit stronger fluorescent signal. When the MMIM concentration was constant, the FI value increased as the concentration of TPE-SAA increased until the FI value increased to a plateau (Figure 4A). This meant the binding cavities on MMIM surface were saturated by the TPE-SAA molecules, and in such case the aggregation-caused quenching phenomenon did not occur. When the MMIM concentration was zero, the FI values of different concentrations of TPE-SAA were comparably low (Figure 4A), further proving its AIE property.

Then the MMIM and the TPE-SAA were mixed with six analytes to perform the assay respectively (three SAs and three other drugs showing structural similarity to SAs, 0 and 100 ng/mL). As shown in Figure 4B, the FI value of single TPE-SAA was negligible, and this was because the TPE-SAA molecules were at molecule state and did not exhibit AIE effect. In contrast, the FI values of MMIM+TPE-SAA mixtures (analytes 0 ng/mL) were all high (Figure 4B), and this was because the TPE-SAA molecules aggregated on MMIM surface and exhibit AIE effect. When adding the analytes (100 ng/mL), the FI values showed different changes. As shown in Figure 4B, the FI values decreased 78%-83.32% when the analysis of SAs, but showed negligible changes when the analysis of other drugs. This was because SAs molecules competed with the TPE-SAA molecules to occupy the cavities on MMIM surface and interfered with the aggregation of TPE-SAA molecules, but other drugs had no such competition ability. These results proved the MMIM and the TPE-SAA could be used to develop a specific detection method for SAs.

For comparison, the controlled tracer FITC-SAA was also used to perform the above experiments. As shown in Figure 4B, the FI value of single FITC-SAA was high, which was because its molecules in solution state could exhibit fluorescent signal. The MMIM+FITC-SAA mixtures also showed FI values (analyte 0 ng/mL), but the FI values were much lower than that when using TPE-SAA. The FI values also decreased when the analysis of SAs (100 ng/mL), but the decrease percentages (58.42%-59.9%) were much lower than the use of TPE-SAA. When the analysis of other drugs, the FI values also showed negligible changes. These results proved the MMIM and the FITC-SAA could also be combined for specific detection of SAs, but the sensitivity should be much lower than the use of TPE-SAA. In addition, a washing step was required to remove the not-adsorbed tracer molecules when using FITC-SAA, which

was because these not-adsorbed molecules in solution could also show fluorescent signal and lead to false result. The above results indicated the AIEgen-based fluorescent method should be more sensitive and simpler than the fluorescein based fluorescent method.

Optimization of assay parameters. During the experiments, the important assay parameters were optimized. Firstly, SAA (100 ng/mL) was mixed with different concentrations of MMIM (0.25-8 mg/mL) and TPE-SAA (0.025-0.4 μ g/mL) to perform the assay. As shown in Figure 5A, its highest inhibition ratio ($1-FI/FI_0$) was obtained when using 0.5 mg/mL MMIM and 0.1 μ g/mL TPE-SAA. Secondly, the MMIM, the TPE-SAA and four SAs (100 ng/mL) were mixed and incubated for different times (1-25 min). As shown in Figure 5B, the competition equilibrium was obtained when the mixture was incubated for 7 min. Thirdly, the reusability of the MMIM was evaluated. As shown in Figure 5C, the inhibition ratios of SAA (100 ng/mL) from three portions of MMIM were stable (>70%) when they were regenerated for 6 times, and then rapidly decreased (<60%). So these optimal conditions were used in the subsequent experiments.

Method performances. The 21 SAs were all determined by the optimized method. As shown in Table 1, the IC_{50} values for these drugs were in the range of 0.16-1.35 ng/mL, and the limits of detection (IC_{10}) were in the range of 1.0-49.74 pg/mL. For comparison, the two parameters for the 21 SAs when using the controlled fluorescent tracer FITC-SAA were also determined (IC_{50} 6.29-395 ng/mL, IC_{10} 7.33-852 pg/mL, Table 1). Calculated as IC_{50} , the sensitivities when using TPE-SAA were increased for 19-671 folds (Figure 5D), and the inhibitory curves of 3 representative SAs when using the two tracers also illustrated this point (Figure 5E). These results proved the method sensitivity when using TPE-SAA was much higher than that when using FITC-SAA. For the different enhancement factors, this maybe was because the competitive abilities of the two tracers with each drug were different. For the drug with high enhancement factor (MBS, 671-fold), the difference between the competitive abilities of TPE-SAA/MBS was much higher than that of FITC-SAA/MBS. For the drug with low enhancement factor (ASZ, 19-fold), the difference between the competitive abilities of TPE-SAA/ASZ and FITC-SAA/ASZ were not so large. As the results, the enhancement factors among different drugs showed large variations.

Table 1. Determination parameters for 21 SAs when using two fluorescent tracers.

Analyte	TPE-SAA		FITC-SAA	
	IC ₁₀ (pg/mL)	IC ₅₀ (ng/mL)	IC ₁₀ (pg/mL)	IC ₅₀ (ng/mL)
SQ	23.79	0.38	852.96	95.27
MBS	1.00	0.59	123.05	395.91
SDM	49.74	0.21	352.00	41.55
SDM'	21.0	0.77	47.94	38.77
SMP	1.07	0.17	85.30	52.93
SAA	15.25	0.47	286.06	72.25
SMM	1.00	0.68	18.89	15.10
SM ₂	16.64	1.35	25.49	44.12
SA	1.95	0.83	101.39	54.04
SML	2.29	0.25	10.47	19.19
APS	8.07	0.43	64.84	18.37
DCSA	1.36	0.41	20.72	11.99
SST	1.54	0.31	38.07	43.51
AS	1.43	0.16	20.62	10.87
PST	1.06	0.23	32.40	37.46
ASZ	1.36	0.75	84.12	14.29
DCS	6.21	0.60	16.08	12.56
SMZ	6.93	0.29	7.33	10.87
SNT	1.28	0.18	13.07	6.29
SG	2.29	0.47	7.96	70.28
STZ	1.09	0.21	9.27	14.32

Method application. The sample pretreatment process was important for a rapid screening method. In this study, the milk sample was diluted with water for 10-160 folds to be assayed directly. As shown in Figure 5F, the inhibitory curve of SAA spiked milk sample after 80-fold dilution was almost same as that of SAA standard, proving the milk impurities could be omitted when the milk sample was diluted for 80 folds. Consideration of the dilution factor, the limits of detection for determination of the 21 SAs in milk were in the range of 0.08-3.98 ng/mL. Then 10 representative SAs were spiked into blank milk samples

to be diluted and determined respectively. The intra-day recoveries (70.3%-106%) and the inter-day recoveries (73.5%-96.2%) were shown in Table 2.

Table 2. Recoveries of representative SAs from standards spiked blank milk samples (n=6).

Drug	Added (ng/mL)	Inter-assay recovery (mean±SD, %)	Intra-assay recovery (mean±SD,%)
SDM'	1	84.7±6.0	92.6±5.4
	10	85.8±7.3	89.0±6.0
	100	93.6±9.7	90.3±6.1
SMZ	1	76.1±10	81.6±7.0
	10	85.3±9.4	86.6±7.5
	100	84.9±7.6	85.7±8.3
SQ	1	76.6±12	93.0±5.1
	10	85.0±7.8	90.1±6.0
	100	87.8±9.7	88.3±6.6
SM ₂	1	99.3±9.6	89.7±8.6
	10	77.3±8.9	87.3±6.5
	100	88.4±6.1	91.9±7.0
PST	1	92.4±8.7	89.1±6.2
	10	86.5±8.4	83.5±7.5
	100	80.8±6.4	85.2±5.7
SAA	1	106±9.2	92.3±6.5
	10	93.4±8.2	90.4±7.2
	100	91.2±7.6	88.5±7.0
SA	1	92.5±11.3	82.2±8.2
	10	82.8±8.5	81.3±7.2
	100	83.2±6.4	79.3±6.3
SG	1	86.4±8.6	82.5±9.7
	10	76.6±8.2	96.2±8.2
	100	70.3±7.2	84.0±8.1
SMM	1	86.2±9.6	81.3±7.6
	10	81.4±7.3	77.3±7.1
	100	78.3±8.1	73.5±6.5

SMP	1	101±8.2	80.3±11.5
	10	94.6±7.9	76.2±8.8
	100	95.2±7.6	84.1±7.6

Finally, the 60 unknown milk samples were analyzed. Results showed one sample from a local dairy farm was determined as positive by the present method (17.5 ng/mL, expressed as SAA), and other 59 samples were all negative. The 60 samples were further determined by a LC-MS/MS method, and the results showed the above positive sample contained sulfamethazine (24.4 ng/mL, Figure S1) and other samples were all determined as negative samples. The above results meant the present method could be used as a practical tool for rapid screening of the 21 SAs in large number of milk samples. For the positive samples, the specific analyte and its actual residual level should be determined by an instrumental method.

Comparison with previous fluorescent (immuno)assays. The present method for the first time used MMIM as recognition reagent to develop a semi-homogeneous competitive method for determination of 21 SAs. There were several articles reporting the use of molecularly imprinted polymers as recognition reagents to develop pseudo immunoassays for detection of SAs [30-32], but these methods required the microplate-coating, blocking, sample-adding, incubating, substrate-adding and several cycles of washing steps. So they were not homogeneous or semi-homogeneous methods and had no comparability with the present method. In addition, these molecularly imprinted polymer based methods could detect at most 15 SAs, and their detection spectra were much narrower than the present method.

Table 3. Comparisons with the competitive homogeneous fluorescent (immuno)assays for detection of SAs.

Recognition reagent	Signal source	Analyte	Assay time	LOD (ng/mL)	Reusable?	Ref.
polyclonal antibody	FITC	4 SAs	<10 min	0.7-2.6	No	33
polyclonal antibody	FITC	1 SAs	---	0.2	No	34
monoclonal antibody	FITC	2 SAs	5 min	0.25-0.7	No	35
monoclonal antibody	FITC	3 SAs	10 min	0.9-3.1	No	36
ScFv	FITC	13 SAs	5 min	2.74-20.36	No	37
receptor	FITC	29 SAs	20 min	1.6-59	No	38
receptor	FITC	31 SAs	2 min	2.0-38.5	No	39
receptor	FITC	35 SAs	2 min	0.03-1.16	No	40
MMIM	TPE	21 SAs	7 min	0.08-3.98	Yes	This study

There were several antibody-based [33-37] and receptor-based [38-40] homogeneous fluorescent (immuno)assays reported for determination of SAs, and their results were summarized in Table 3 to compare with the present method. First, the MMIM could be reused for 6 times, whereas the recognition reagents in those methods were not reusable. Second, the present study used AIEGen as signal source, and other methods all used conventional FITC as signal source. Our experiments showed AIEGen was better than FITC, so the sensitivity of the present method was much higher than those methods. Third, the assay procedures and assay time of the present method were comparable to those methods. Fourth, the detection spectrum of the present method was broader than the antibody-based methods but narrower than the receptor-based methods. If an optimal template was used to synthesize the MMIM, the detection spectrum should be comparable to the receptor-based methods. Five, the milk sample could be analyzed directly in the present method only after a simple dilution because of the ultrahigh sensitivity, but other methods all required different sample pretreatments. General consideration of these points, this method was superior to those fluorescent methods. So it could be used for routine screening of SAs residues in large number of milk samples at the concentrations far below their maximum residue level (100 ng/mL).

Conclusions

Molecularly imprinted polymers have been used as the novel recognition elements for development of many analytical methods, and the aggregation induced emission luminogens have been used as the superior fluorescent signal sources to develop many immunoassays. This study for the first time used magnetic molecularly imprinted microsphere and tetraphenylethylene labeled fluorescent tracer to develop a novel semi-homogeneous fluorescent method for detection of sulfonamides in milk. Results

showed this method was simple, rapid, ultra-sensitive and reusable. This study broadened the applications of molecularly imprinted polymer and aggregation induced emission luminogen, and demonstrated the advantages of aggregation induced emission luminogen over the conventional fluorescein. Under the guidance of this study, more similar and advanced analytical methods based on the two reagents should be reported in the future.

Declarations

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Artificial intelligence

The authors declare that no artificial intelligence (AI) tools were used in the preparation of this manuscript.

Competing interests

The authors declare no competing interests.

Author contributions:

Su Han Wang: Methodology. Peng Lei Cui: Methodology. Jian Ping Wang: Writing – review & editing, Writing – original draft, Conceptualization.

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Figures

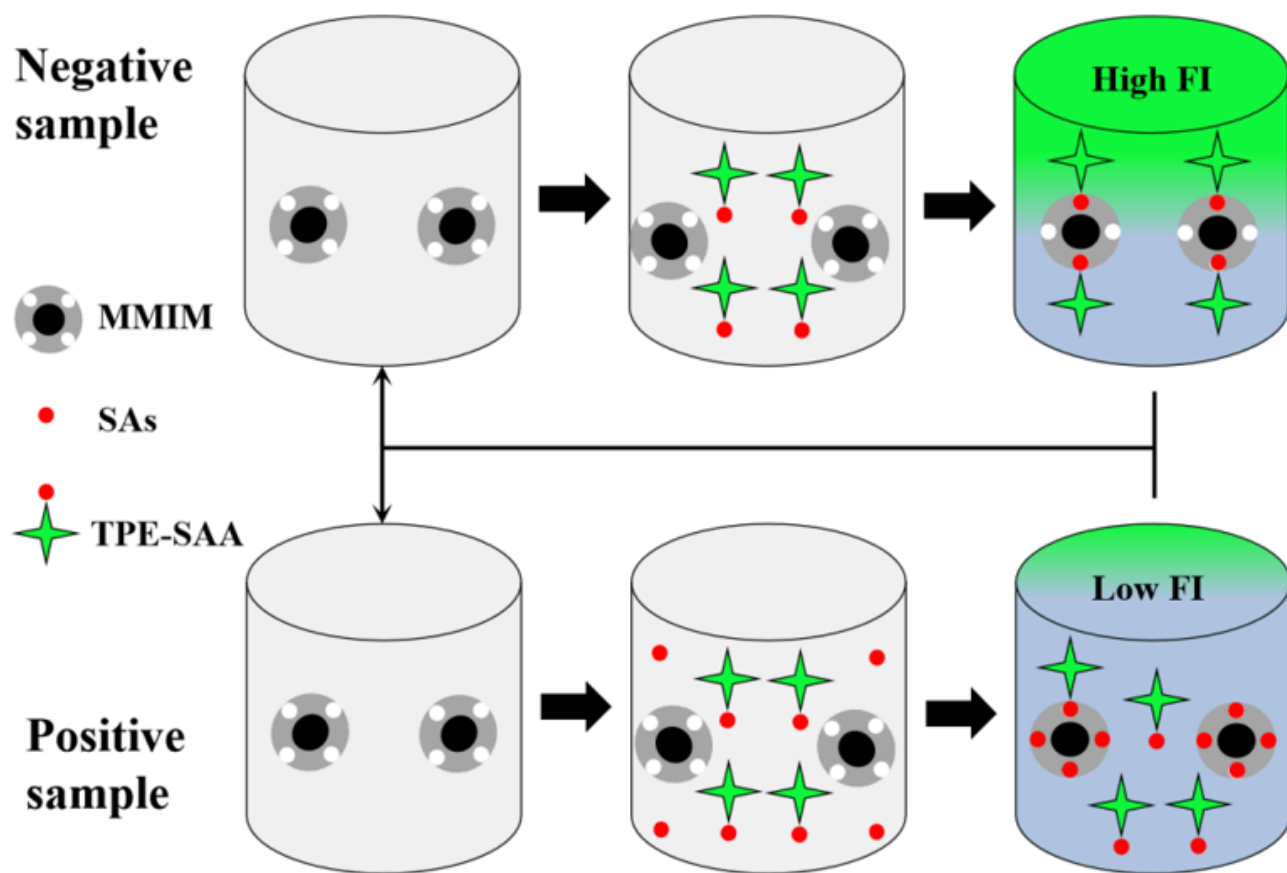


Figure 1

Schematic representation of MMIM and TPE-SAA based semi-homogeneous fluorescent method.

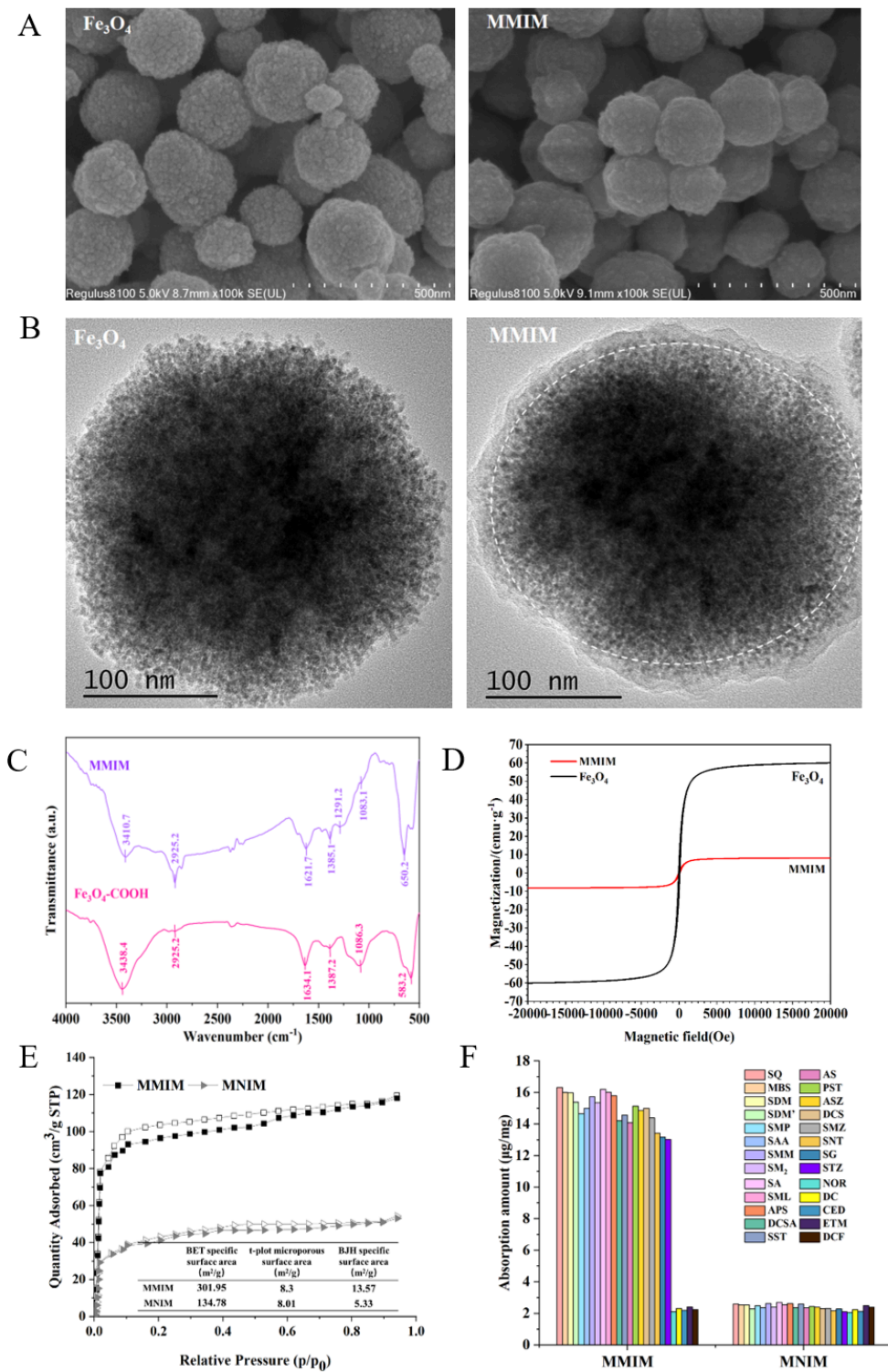


Figure 2

Characterization of MMIM by using (A) SEM, (B) TEM, (C) FTIR, and (D) VSM. (E) N₂ absorption/desorption experiment results of MMIM and MNIM. (F) Absorption capacities of MMIM and MNIM for SAs and other drugs.

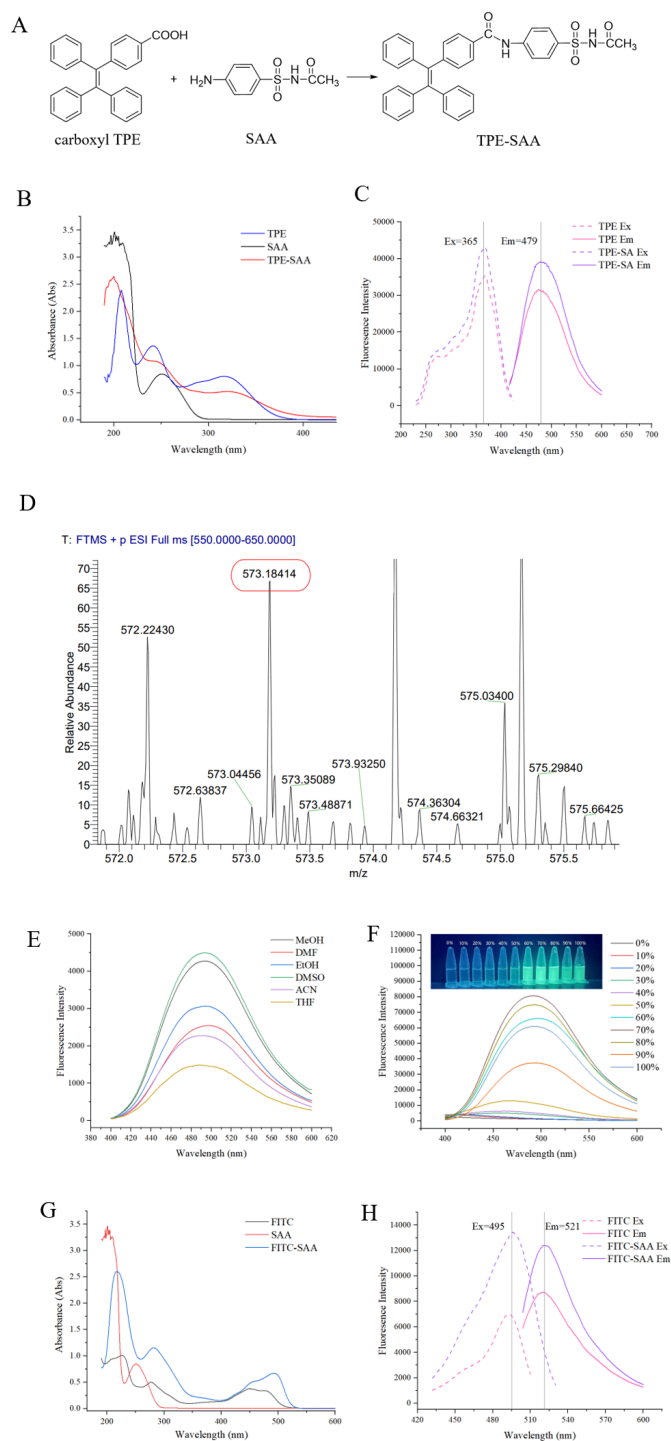


Figure 3

(A) Synthetic route of TPE-SAA and its (B) UV absorbance curve, (C) fluorescence spectrum, and (D) characterization result by using LC-MS/MS. (E) The emission spectra of TPE-SAA in different solvents and (F) its emission spectra when dissolving in DMSO solution containing different percentages of water (the inserted picture was obtained under a UV lamp at 365 nm). (G) UV absorbance curve and (H) fluorescence spectrum of FITC-SAA.

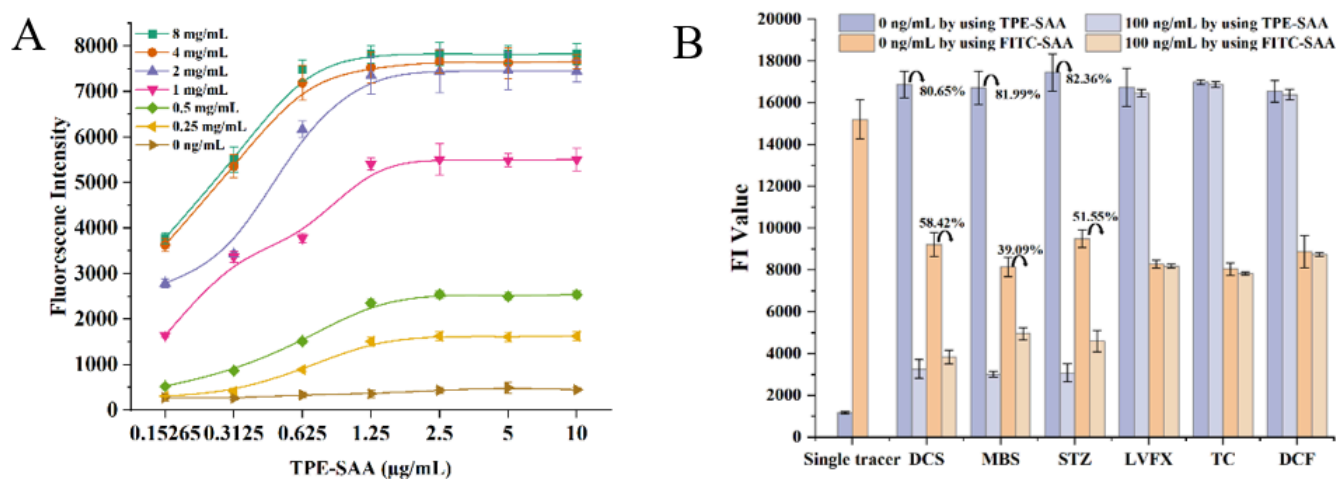


Figure 4

(A) FI values when mixing different concentrations of TPE-SAA and MMIM. (B) FI values when the analysis of SAs and other drugs by using the two fluorescent tracers (tracer concentration 0.1 $\mu\text{g/mL}$, MMIM 0.5 mg/mL; LVFX=levofloxacin, TC=tetracycline, DCF= diclofenac).

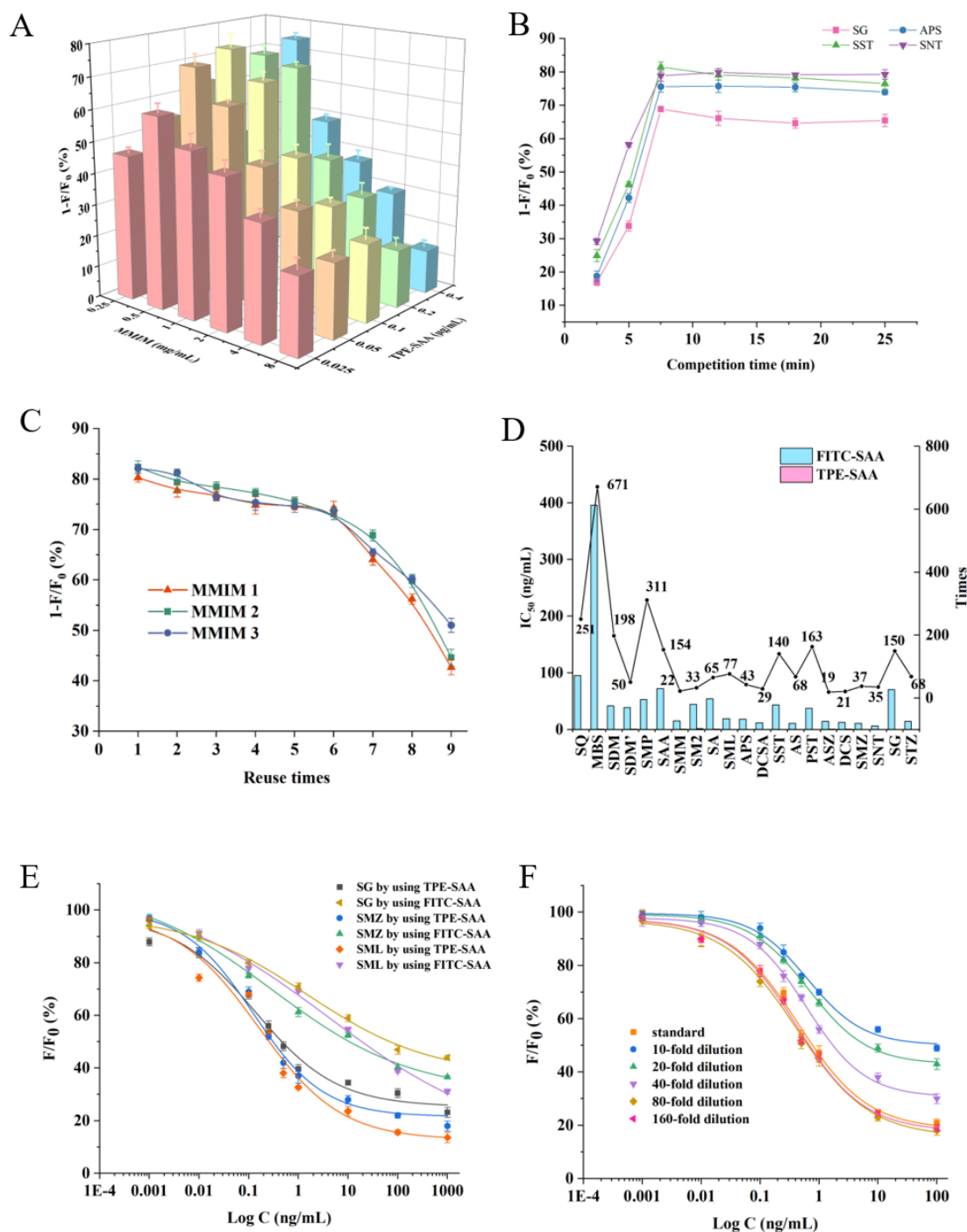


Figure 5

(A) Inhibition ratios of SAA (100 ng/mL) when using different concentrations of MMIM/TPE-SAA. (B) Inhibition ratios of SAs (100 ng/mL) at different competition times. (C) Inhibition ratios of SAA (100 ng/mL) when the MMIM was reused for different times. (D) IC_{50} changes of the 21 SAs when using the two fluorescent tracers. (E) Inhibition curves of three SAs when using the two fluorescent tracers and (F) that of SAA spiked milk sample at different dilutions when using TPE-SAA.

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