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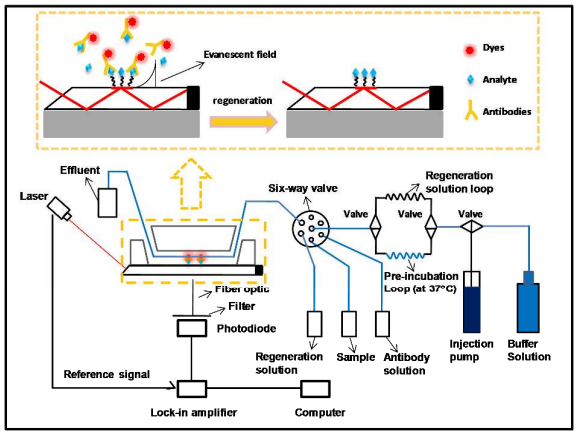

An integrated biosensor for sensitive and automatic detection of sulfadimidine in aqueous samples based on immunoassay and evanescent-wave fluorescence excitation

Highly sensitive detection of sulfadimidine in water and dairy products by means of an evanescent wave optical biosensor

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Abstract: During the past years, there has been an increasing demand in developing rapid biosensing technologies that could be performed outside the laboratory, for example on farms, near rivers, in food collection stations and store houses or in food production plants. Therefore, cost-effective and automatic detection methods are promising for onsite residual analysis in food and environmental monitoring. In this work, we propose an automatic, rapid, highly sensitive and reusable planar waveguide evanescent wave immunosensor (PWEI) to onsite determine sulfadimidine (SM2) in water and dairy products. PWEI is based on an indirect inhibition immunoassay that takes place at an optical transducer chip chemically modified with an analyte derivative. Fluorescence produced by labeled antibodies bound to the transducer is excited by the evanescent wave formed on the transducer surface and detected by photodiodes through a lock-in amplifier, which is reversely correlated with the analyte concentration. Each test cycle is fulfilled automatically in 15 min. The optical transducer chip of PWEI modified with the analyte derivative is robust and features with high reusability, which allows for over 300 times regeneration without sensitivity loss. Under the optimized conditions, the dose-response curve established for SM2 shows a low detection limit of 0.06 μg/L. The 50% inhibition concentration is 1.39 ± 0.08 μg/L with a linear working range from 0.19 μg/L to 10.10 μg/L. The cross-reactivity against the organic compounds structurally similar to SM2 is negligible. The recoveries of SM2 in all sorts of dairy products and natural water range from 80% to 107%. The PWEI is featured with portable dimensions of 42 cm × 50 cm × 24 cm in length × width × height and shows great prospects in the onsite measurement of SM2 in reality once in combination with the appropriate

pretreatment.

Keywords: Fluorescence immunosensor; Sulfadimidine; Evanescent wave; Optical waveguide; Onsite

1. Introduction

Sulfonamides (SAs) are commonly used for therapeutic and prophylactic purposes in animals[1], also as additives in animal feed, due to their low cost and effective as growth promoters[2]. Due to the toxicity of SAs and their abuse in practice, strict maximum residue levels (MRL) have been established, e.g. 25 μg/L in milk set by the Codex Committee of FAO/WHO[3] and 100 μg/L set by the European Council[4,5,6]. Sulfadimidine (SM2), also known as sulfamethazine, is one of SAs which has been widely used for the treatment and control of inflammation associated with *Bordetellubronchiseptica* infection in animals by feed medication at high level[7]. However, SM2 is also the most common contaminating antimicrobials in animal feed, generating potentially serious problems in human health, such as allergic or toxic reactions[1]. Therefore, to detect its abuse and residual in various water samples[8,9,10] and animal-food products[11,12] is increasingly demanding in recent years.

Traditional methods for the determination of SM2 in food and environmental samples include high-performance liquid chromatography (HPLC)[13,14], gas chromatography (GC)[15] and liquid chromatography-mass spectrometry (LC/MS)[16,17]. These methods are accurate, sensitive and specific; however, are also labor-intensive, expensive and need sophisticated instrumentation. During the past years, there has been an increasing demand in developing rapid biosensing technologies that could be performed outside the laboratory, for example on farms, near the river, in food collection stations and store houses or in food production plants. Therefore, cost-effective and automatic detection methods are promising for onsite abuse and residual analysis in food and environmental monitoring. Biosensors show remarkable advantages, including high specificity even in complex matrices and the potential for becoming cost-effective, portable and easy-to-use test devices[18]. For example, surface plasmon resonance (SPR)[19] and fluorescence polarization immunoassay (FPIA)[20] have been reported as alternatives to traditional methods for SAs determination with advantages of simple detection procedures, quick response and real-time monitoring. However, the sensitivities of SPR and FPIA are not superior enough for trace SM2

detection in aqueous samples. Moreover, SPR in real applications is limited to the regeneration of the receptor surface, which must at least be washed or even entirely replaced between analyses of different samples[21]. In this work, we propose an automatic and compact planar waveguide evanescent wave immunosensor (PWEI) to realize the rapid, highly sensitive, reusable and onsite determine SM2 in food and environmental monitoring. The principle of PWEI is based on immunoassay and evanescence wave formed on the surface of planer waveguide due to the total internal reflection propagation of incident light. As far as we know, this is the first work reporting the application of PWEI to determine SM2. Based on the developed PWEI platform, the method for SM2 detection is optimized and fully validated in terms of linearity, accuracy, precision, recovery and specificity in this work.

2. Material and methods

2.1 Material

Potassium dihydrogen phosphate, sodium phosphate dibasic, sodium chloride, potassium chloride, hydrochloric acid, sodium dodecyl sulphate (SDS), toluene and ethyl alcohol were purchased from Sinopharm Chemical Reagent Co. Ltd. 3-Mercaptopropyl-trimethoxysilane (MTS), N-(4-maleimidobutyryloxy) succinimide (GMBS), bovine serum albumin (BSA), sulfadimidine (SM2), sulfadiazine (SDZ), sulfamerazine (SM1) and sulfamethoxazole (SMX) were purchased from Sigma-Aldrich and stored at 4^{o}C. Cy5.5 and N-hydroxysuccinimide (NHS) ester were obtained from GE Healthcare Life Sciences. Monoclonal anti-sulfadimidine antibody and the analyte derivative - hapten conjugate of BSA-SM2 were purchased from Shijiazhuang Solarpex Biotechnology Co. Ltd. Labeling of the SM2-antibody with Cy5.5 was performed according to the method described by Mujumdar[22]. All chemicals were analytical grade if not specified and used as being received without further purification.

1000 mg/L SM2 stock solution was prepared by using methanol and stored at 4℃ before using. Phosphate buffered saline (10 mM PBS, pH 7.4) was prepared by DI water (18.2 MΩ cm). SM2 stock solution was diluted to be a series of concentration

levels by using 10 mM PBS buffer solution.

2.2 PWEI platform

The planar waveguide evanescence wave immunosensor used in this study has been described in detail in our previous literature[23] and presented in Fig. 1. Briefly, the pulse laser beam from a 635-nm pulse diode laser was directly coupled into one beveled edge face of a planar waveguide transducer and propagated along the transducer via total internal reflection. The evanescent wave generated at the surface of the waveguide interacted with the surface-bound fluorescently labeled target conjugate, and caused excitation of the fluorophores. The emitted fluorescence was collected by the high-numerical-aperture polymer fibers (NA = 0.46) located beneath the waveguide opposite to the chemical modified surface, and subsequently filtered by means of a band pass filter and detected by photodiodes through a lock-in amplifier.

BK7 glass was adopted as the planar waveguide transducer with refractive index of 1.52 and sizes of 60 mm×15 mm in area and 1.5 mm in length. One end face of BK7 glass was polished and beveled to be 45^{o} for light coupling. A thin layer of SiO_2 film (60 nm thickness) was coated on the waveguide chip surface by chemical vapour deposition method (Foxconn nanotechnology research center, Beijing). The planar waveguide was embedded into a rectangular teflon flow cell with sizes of 42 mm×2.0 mm in area and 50 μm in depth. All reagents were delivered by a flow delivery system operated with one peristaltic injection pump, a six-way valve and three one-way valves for liquid switch. A 1 mL regeneration solution loop was design to store the regeneration solution. A 1 mL pre-incubation loop was kept at $37^{\circ}C$ for incubating the mixture of the test sample and the Cy5.5-labeled antibody solution. The controls of fluid delivery system, data acquisition and processing were automatically performed by the built-in computer. As shown in Fig. 1B, the instrumented sizes of PWEI were 42 cm × 50 cm × 24 cm in length × width × height.

2.3 Surface chemical modification of waveguide chip

To make the waveguide chip to be biosensitive to the target, the SM2 derivative (SM2-BSA conjugate) was immobilized covalently on the chip surface as stepped as

follows. The waveguide chip was first cleaned by detergent and subsequently immersed in piranha solution (H_2SO_4:H_2O_2=3:1, v/v) for one hour, rinsed with DI water afterwards and then dried in nitrogen gas. Fig. 2 shows the chemical modification procedure of SM2-BSA onto the chip surface. Silanization of the chip surface was achieved by immersion in a 2% (v/v) MTS toluene solution for 2 h at room temperature. The silanized surface was rinsed with toluene and dried by using nitrogen gas. To immobilize SM2-BSA onto the surface of thiol-silanized chip, the waveguide chip was first immersed in 2 mM GMBS (in ethanol) solution for 1 h at room temperature, subsequently washed thoroughly with ethanol and DI water. And then the SM2-BSA conjugate was applied to the specific binding site on the chip surface for overnight at 4^{o}C. Finally, the non-specific binding was shielded by immersing the chip in BSA (2 mg/mL) for 1 h. The modified chip was stored at 4^{o}C before using.

2.4 Immunoassay procedures

All the measurements were performed by using a binding inhibition test format. The SM2 derivative (SM2-BSA) was immobilized on the chip surface and competed with the SM2 analyte in the test samples to bind the Cy5.5-labeled antibody. To optimize the Cy5.5-labeled antibody concentration, the binding inhibition immunoassay was carried out at antibody concentrations of 0.4 μg/mL, 0.6 μg/mL and 0.8 μg/mL, respectively. Firstly, 800 μL analyte standard solution was mixed with 200 μL Cy5.5-labeled monoclonal antibody solution and pre-incubated at 37°C for 4 min. After the binding reaction reached an equilibrium state, the mixture was pumped into the flow cell at a constant flow rate of 200 μL/min. Only the unbound antibodies were able to bind to the analyte derivative covalently bound on the waveguide surface. The binding process lasted 5 min when the excitation laser was turned off to avoid the strong photobleaching of the Cy5.5 dyes. After that, the waveguide chip was rinsed with 10 mM PBS buffer to remove the residual of Cy5.5-labeled antibodies unbound on the surface. And then the laser was turned on to generate evanescence wave on the chip surface, which was used to excite the fluorescence of bound Cy5.5 dyes. The fluorescent signal was recorded and reversely related with the analyte concentration

because the number of free antibodies able to bind to the surface was reduced. Finally, a regeneration process was performed by rinsing the chip surface with the SDS solution (0.5%, pH 1.9) for 5 min, allowing ready for a new test cycle.

All of the immunoassay processes were conducted automatically by the control system of PWEI embedded into a built-in computer. The total time for one test cycle was less than 15 min.

2.5 Data analysis

All sample fluorescent signals were normalized with the signal corresponding to a blank, i.e. signal obtained in the absence of analyte. The standard curve for SM2 detection was plotted against the logarithm of the concentrations of SM2 ranging from 0.001-1000 μg/L through a five-parameter logistic model as follows[18, 24]:

$$SS = \frac{A_1 - A_2}{1 + ([Ag]/[Ag_0])^p} + A_2$$

Where [Ag] was the SM2 concentration; *SS* was the signal strength of optical immunosensor; A_1 and A_2 were the maximum (blank signal, x→0) and minimum signal (background signal, x→∞) to the titration curve; $[Ag_0]$ was the SM2 concentration at the midpoint or inflection point (IC_{50}); and *p* was the slope of the tangent at the inflection point. Error bars indicated in the curves represented the relative standard deviation for three individual experiments.

2.6 Selectivity and recovery

In the selectivity experiment, three structural analogues of SDZ, SM1 and SMX were chose instead of SM2, respectively. The interference chemicals were processed in the same way as the SM2 standard solutions. The relative 50% inhibition values of the cross-reactivity (CR) were used to judge the selectivity of the sensing system via the following formula[25]:

$$CR(\%) = [IC_{50}(SM2)/IC_{50}(\text{structural analogue})] \times 100\%$$

The dairy samples included liquid milk, yoghourt and baby formula milk were bought from the local supermarket. For solid baby formula milk, 4 g sample was firstly dissolved into 20 mL 0.01 M PBS buffer at 80 °C for 5 min. 2 mL dissolved formula milk (or raw liquid milk/yoghourt), 3 mL acetonitrile and 15 mL 10% trichloroacetic

acid were added into a centrifuge tube and then spiked with SM2 standard solutions to final concentrations at two levels (1 μg/L and 5 μg/L). The mixture was centrifuged at 12,000 rpm for 5 min to precipitate protein and dissolve organic substances. Subsequently, 200 μL of the supernatant sample was diluted with 10 mM PBS buffer to 10 mL for PWEI detection. Three kinds of water samples including bottled water, lake water and wastewater were also chose for recovery experiments. No pretreatment for bottled water. Lake water and wastewater were filtered through a 0.22-μm membrane filter and subsequently stored at 4^{o}C for test. Samples were measured within 24 h of collection to avoid biodegradation. The calibration curve in the buffer solution was adopted to calculate the spiked SM2 concentrations in the recovery experiment due to the interferences caused by the pretreated milk matrix were negligible.

3. Results and discussion

3.1 Performance of PWEI for SM2 detection

The temporal fluorescence responses for different concentrations of SM2 in a test cycle are recorded in Fig. 3. An observable decrease of PWEI fluorescent response to SM2 was observed even when the SM2 concentration was decreased to 0.01 μg/L. When the SM2 concentration was increased to 1000 μg/L, a slight fluorescent signal was observed, indicating that the non-specific adsorption on the chip surface was negligible due to the immobilized BSA acting as the shielding agent. The result was further confirmed by the negligible fluorescent signal due to the input of 10 μg/mL Cy5.5 labeled BSA solution.

The antibody concentration is an important factor in immunoassays to strongly affect the detection limits and working ranges[24,26,27]. Therefore, the concentration of Cy5.5 labeled SM2-antibody was optimized to provide the calibration curve with the highest sensitivity. Immunoassay determinations of SM2 were carried out by using three Cy5.5 labeled SM2-antibody concentration levels of 0.4 μg/mL, 0.6 μg/mL, 0.8 μg/mL, respectively. The detection limit (LOD) of PWEI was calculated from the calibration curve as the analyte concentration providing a 10% decrease of the blank

signal[28]. The dynamic detection range of the test was defined by the analyte concentrations causing 20% and 80% inhibition of the maximum fluorescent signal.

The comparison between the standard curves for three antibody concentrations is shown in Fig. 4. The linearly quantitative SM2 detection range of the PWEI were 0.19–10.10 μg/L at 0.4 μg/mL Cy5.5-labeled antibody, 0.28–6.42 μg/L at 0.6 μg/mL Cy5.5-labeled antibody and 0.48–7.98 μg/L at 0.8 μg/mL Cy5.5-labeled antibody, respectively, as described by 20–80% inhibitory concentrations. The LOD values were determined to be respectively 0.06 μg/L at 0.4 μg/mL Cy5.5-labeled antibody, 0.13 μg/L at 0.6 μg/mL Cy5.5-labeled antibody and 0.22 μg/L at 0.8 μg/mL Cy5.5-labeled antibody. The Cy5.5-labeled antibody concentration of 0.4 μg/mL gave the highest detection sensitivity, also resulted in the reduced reagent costs; therefore, it was adopted in the subsequent experiments. Compared with other methods reported previously, such as LC-MS (8.2 μg/L)[17], ELISA (4.3 μg/L)[29], SPR (0.28 μg/L)[19], and FPIA (10 μg/L)[20], the proposed method showed a superior sensitivity for SM2 detection with the corresponding lowest LOD, which also met the strict MRL of 25 μg/L SAs in milk samples set by the FAO/WHO[6].

3.2 Regeneration and stability

Regeneration and stability of the biosensing surface element are important factor that may limit the reliability of an immunosensor[5]. Strong acid or alkaline buffer solution can be used to break the binding between the antibody and the analyte derivative covalently immobilized on the waveguide chip surface[30]. In our system, 0.5% SDS solution (pH 1.9) was pumped into the flow cell as the regeneration agent at a flow rate of 1 mL/min. The robustness of the PWEI method was checked by monitoring the fluorescent response of the same waveguide chip towards the same test sample over 50 test cycles (Fig. 5). Results showed that 50 repeated test cycles gave a relative standard deviation of 3.0%, indicating that a satisfactory regeneration performance was achieved by using 0.5% SDS solution (pH 1.9). Moreover, the waveguide chip was reused up to six times during one month, running approximately 300 test cycles without significant sensitivity loss.

3.3 Specificity

Specificity studies were performed by investigating the dose-response curves with three structural analogues of SDZ, SM1 and SMX as shown in Fig. 6. Comparing the IC_{50} values for the target and the cross-reacting compounds potentially present in the sample is a typical method to estimate the cross-reactivity of method[31]. The IC_{50} were calculated to be 1.39 μg/L, >10000 μg/L, >10000 μg/L and 48.21 μg/L for SM2, SMX, SDZ and SM1, respectively as shown in Table 1, which corresponded to the CR values of 100%, <0.01%, <0.01% and 2.74%, respectively.

Molecular modeling studies on the SAs structures have provided valuable insight into the principles of the cross-reactivity characteristics of anti-SM2 antibodies. As reported by the previous studies[32,33], the molecules of SA antibiotics have a characteristic bend around the tetrahedral -SO_2- grouping (see the common structure of SA antibiotics in Table 1), which would be the recognition part for the group-specific antibodies against SA antibiotics. However, the cross-reactivity of anti-SM2 antibody towards SMX and SDZ was negligible due to the CR values of <0.01%, which accorded with other previously reported studies[11, 34, 35]. The results indicated that the characteristic bend around the tetrahedral -SO_2- grouping was not the binding site of the antibody adopted on the PWEI platform. Moreover, a slight cross-reactivity towards SM1 was observed with the CR value of 2.74%. The possible reason was the adopted antibody specifically recognized the similar R-group of the structure of SM2 and SM1, i.e. there was only a methyl group difference between the two molecules. However, the interference from SM1 should be negligible in most cases because the interfering response signal towards SM1 is equal to the target signal only when the concentration of SM1 present is 35-fold higher than that of SM2. In a word, the CR values show that the selectivity of PWEI platform is convincing even in a complicated matrix containing other SA antibiotics.

3.4 Application to real samples

In order to investigate the accuracy of the proposed method, PWEI was applied to the measurement of SM2 in real test samples, including the diary products and water

samples. In the recovery experiment, the fluorescent signals were almost the same as that responding to the buffer solution before the test samples spiked with SM2. The detection performance of PWEI system for the spiked samples with SM2 is presented in Table 2. Results showed that the recoveries by means of the PWEI platform ranged from 80%~107%. Satisfactory variations were also demonstrated with the relative standard deviation of less than 17%. These results confirm that the proposed PWEI has the ability to be applied for the SM2 detection in real aqueous samples.

4. Conclusion and outlook

In this work, we proposed an automatic, rapid, highly sensitive and reusable immunosensor (i.e. PWEI) to determine SM2 in food and environmental monitoring. The analytical performance of PWEI was confirmed with a high sensitive detection of SM2 with IC_{50} of 1.39 ± 0.08 μg/L at the Cy5.5-labeled antibody concentration of 0.4 μg/mL. The detection limit of 0.06 μg/L satisfied the needs for monitoring of trace SM2 in dairy products and all sorts of water samples. The whole test cycle was automatically fulfilled in 15 min. Its cross-reactivity against the organic compounds structurally similar to SM2 was negligible in most cases. Results for real spiked dairy and water samples showed satisfactory recovery ratios ranging from 80% to 107%. Moreover, the PWEI was featured with portable dimensions of 42 cm × 50 cm × 24 cm in length × width × height. All above-mentioned results show great prospects for the application of PWEI for highly-sensitive, rapid and onsite detection of SM2 in reality once the appropriate pretreatment can be supplied. Through adopting other antibodies, the proposed PWEI is a universe platform for the detection of other pollutants in both environmental aqueous samples and milk products. It also offers the ability for the simultaneous detection of the multiple trace pollutants in test samples, such as by covalently immobilizing variable antigen derivatives at the different total reflection points on the waveguide chip surface or using the different sized quantum dots to label different antibodies for recognizing different trace pollutants.

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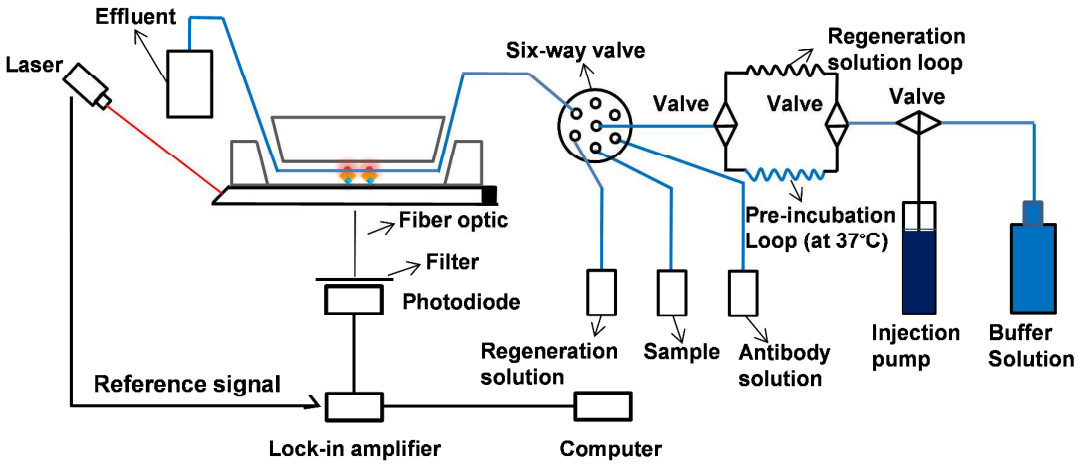
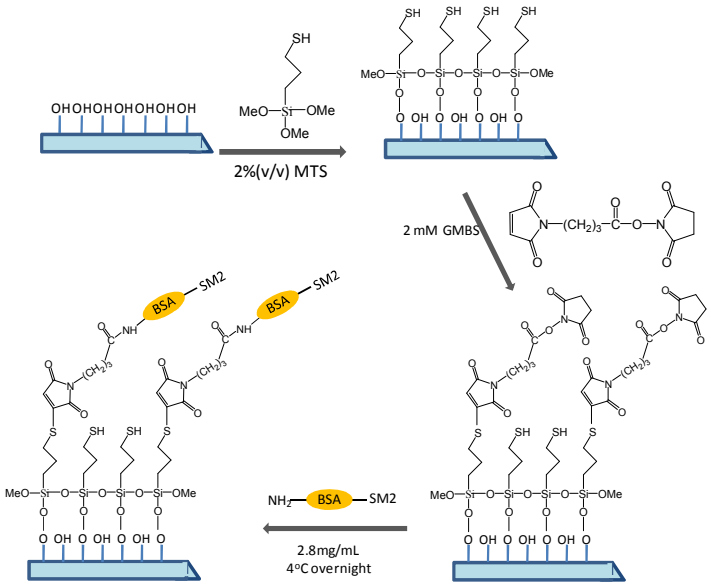


(A)

(B)

Fig. 1 (A) Schematic set-up of planar waveguide evanescence wave immunosensor (PWEI) platform and (B) Instrumentized Photograph of PWEI



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374 **Fig. 2** Schematic diagram of immobilizing the hapten conjugate of SM2-BSA onto the

375 waveguide chip surface

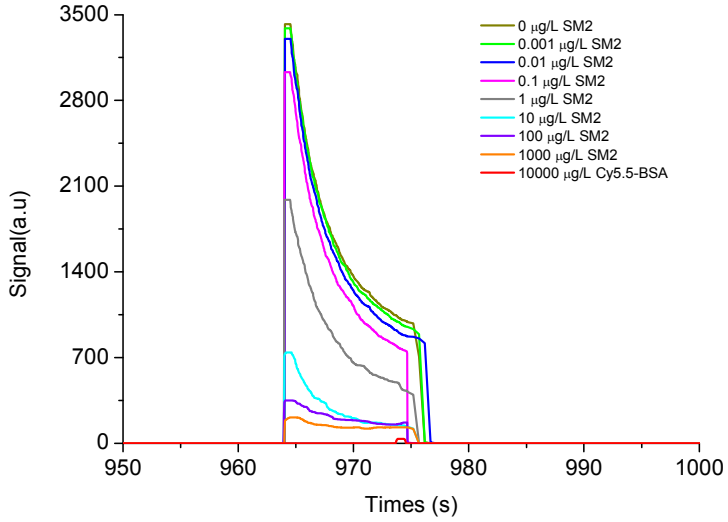


Fig. 3 Temporal fluorescence responses for different concentrations of SM2 at 0.4 μg/mL Cy5.5 labeled SM2-antibody and Cy5.5 labeled BSA without SM2 in a test cycle

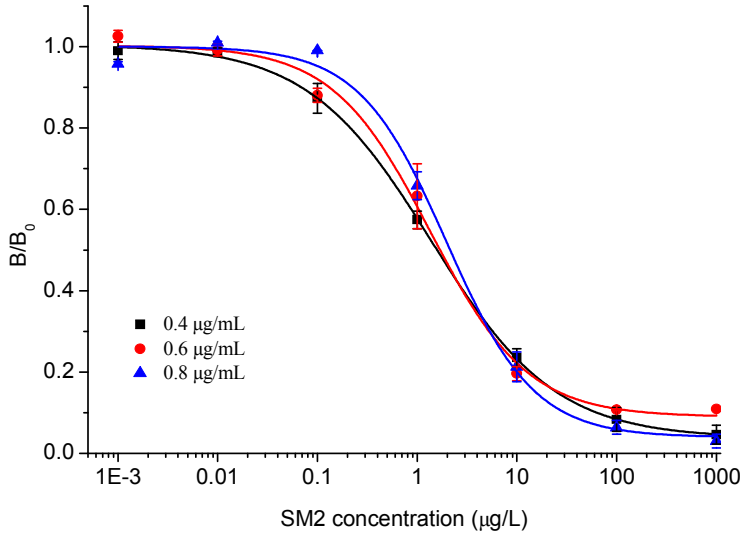
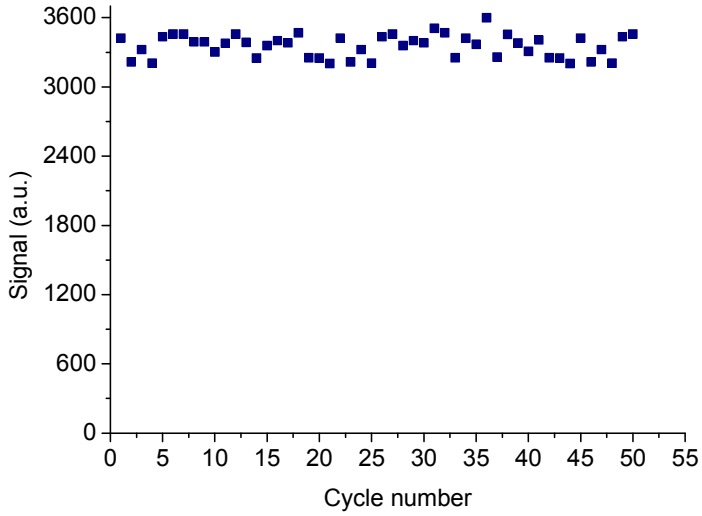


Fig. 4 Standard curves at three different concentrations of Cy5.5-labeled antibody of 0.4 μg/mL, 0.6 μg/mL and 0.8 μg/mL, respectively. Error bars represent the standard deviations for three individual experiments



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387 **Fig. 5** Regeneration of fluorescent signals towards 0.4 μg/mL Cy5.5 labeled

388 SM2-antibody on the PWEI platform

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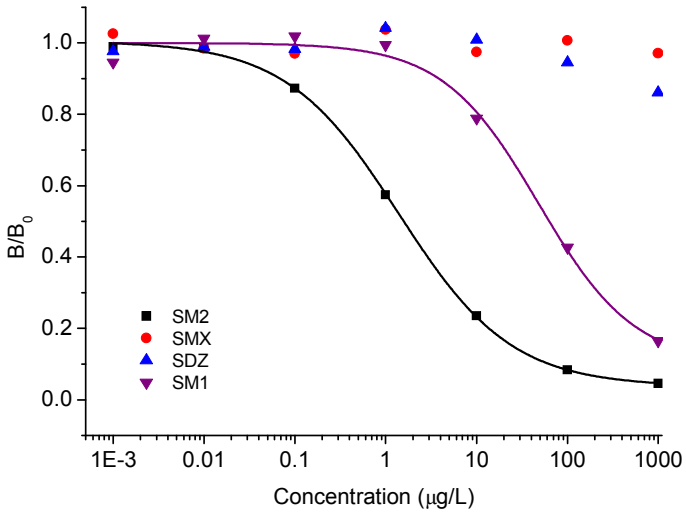

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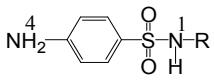
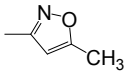
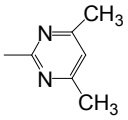
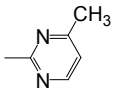
391 **Fig. 6** Standard curves and cross-reactivity of immunoassay on the PWEI platform

392 towards SM2, SMX, SDZ and SM1 in 10 mM PBS buffer solution

393

394 **Table 1** Structures and cross-reactivity of PWEI towards SM2 and other structural

395 analogues

Compounds	Commom structure 		
	R=	IC_{50} μg/L	CR %
Sulfadimidine (SM2)		1.39	100
Sulfamethoxazole (SMX)		＞10,000	＜0.01
Sulfadiazine (SDZ)		＞10,000	＜0.01
Sulfamerazine (SM1)		48.21	2.74

396

397 **Table 2** Recovery of PWEI towards SM2 in dairy and water samples (n = 3, mean ±
398 SD)

Samples	Spiked (μg/L)	Found (μg/L)	Recovery (%)	RSD%
Liquid milk	1.00	0.93 ± 0.09	93	10
	5.00	4.71 ± 0.47	94	10
Yoghurt	1.00	1.02 ± 0.08	102	8
	5.00	4.15 ± 0.08	83	9
Baby formula	1.00	1.01 ± 0.10	101	10
	5.00	4.92 ± 0.20	98	4
Bottle water	1.00	1.07 ± 0.05	107	5
	5.00	5.19 ± 0.87	104	17
Lake water	1.00	0.80 ± 0.03	80	4
	5.00	4.40 ± 0.62	88	14
Wastewater	1.00	1.03 ± 0.04	103	4
	5.00	4.19 ± 0.91	84	2

399