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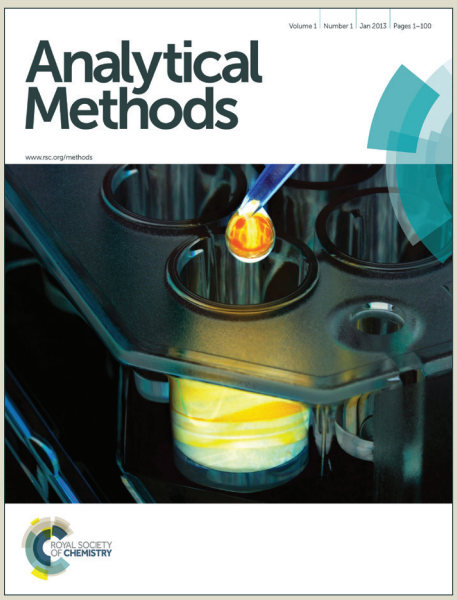

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ARTICLE

Direct fluorescent quantification of sulfadiazine from quenching of novel functional monomer based molecularly imprinted polymer

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Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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Novel fluorescent, molecularly-imprinted polymers (MIPs) were synthesized to detect sulfadiazine (SDz), an antibiotic used in animals that produce food for human consumption. Radical polymerization between a novel fluorescent monomer 7-Acryloxy-4-methylcoumarin, ethylene glycol dimethacrylate and methacrylic acid, in the presence of SDz as a molecular template, afforded the target MIPs. The photoluminescent properties of these MIPs were studied and found to exhibit stable, enhanced fluorescence emission. The fluorescence emission of both the MIPs and non-imprinted polymers (NIPs) were quenched in the presence of SDz and similar analogues. Significantly, the MIPs were more sensitive than the NIPs for the SDz analyte, confirming that molecular imprinting imparted selectivity. The fluorescence quenching ratio of the MIPs exhibited a linear decrease with increasing concentration of SDz in the range 1.0–40 μmol L^{-1} with a detection limit of 0.48 μmol L^{-1}. Importantly, the MIPs proved successful in determining SDz concentrations in spiked milk samples and showed superior recovery from 85.73% to 101.37%.

Introduction

The development of optical sensors is key to analytical research.[1] However, the design and synthesis of materials with the ability for molecular recognition remains a major challenge in sensor development. Recently, molecularly-imprinted polymers (MIPs) have gained attention for their ability to bind target analytes selectively. MIPs are usually prepared by copolymerization of functional monomers and crosslinkers in the presence of the target analytes, which act essentially as template molecules. These template molecules imprint specific binding sites in the polymer network and are subsequently removed by washing. MIPs display several advantages over biological receptors, including stability, low cost and enhanced mechanical properties,[2-4] making them promising tools for chemosensors,[5] separation,[6-7] catalysis[8] and drug delivery[9].

For assay purpose, complex sample processing schemes were always indispensable to guarantee a satisfied result. Regarding the sample treatment, fluorescence-based chemosensing has a remarkable advantage over other detection methods due to its detection simplicity as well as high sensitivity. The fluorescent materials have been synthesized and applied in many areas, especially as direct biological probes. Fluorescent MIPs are a relatively recent development but they have gained increasing attention owing to a high sensitivity and high selectivity.[10-19] MIPs are synthesized traditionally using non-fluorescent monomers. However, their use is restricted to fluorescent analytes. Furthermore, the concentration of the analytes can be determined only after chromatographic separation.[18, 20-24] Fluorescent MIPs have been prepared by incorporating fluorescent monomers, including 4-methylamino-N-allylnaphthalimide,[11] N-ally-4-ethylenediamine-1,8-napthalimide,[9] acrylamidofluorescein,[25] 2-acrylamidoquinoline[26] and Zinc(II) protoporphyrin[27]. The efficacy of a fluorescent MIPs sensor is determined using fluorescence quenching or enhancing with response to the amount of analytes. Introducing a florescent reporter into the MIPs in the chemical synthesis was shown to greatly enhance the sensitivity of the MIPs sensor without sacrificing the selectivity.

Antibiotics are commonly added to animal feed. Sulfonamides are widely employed as they are inexpensive and show wide-spectrum antimicrobial activity.[28-29] However, the use of sulfonamides has serious side effects, including toxicity, allergic reactions, emiction and hematopoiesis turbulence. Bacterial drug resistance can occur after long-term use. Additionally, sulfonamides are detrimental to the environment, even at low concentrations.[30-32] Sulfonamides accumulate in animal bodies and are discharged to the environment through excrement. These antibiotics can enter the food chain and affect human health. The European Union has established a maximum residue limit of 100 ng/g for sulfonamides in foods of animal origin, including meat, milk and eggs.[33] Currently, sulfonamides are detected using chromatographic methods (HPLC, LC/MS, GS, and TLC)[28, 34-38], capillary electrophoresis[39], electrochemical detection[40] or

chemiluminescence sensors[41]. While these techniques are accurate, some lack sensitivity and/or selectivity and require expensive equipment or complex procedures to purify samples.

Fluorescent MIPs are required to detect non-fluorescent analytes directly using simple spectroscopic methods. Fluorescent monomers are a prerequisite in synthesizing fluorescent MIPs, yet few have been reported to the best of our knowledge. Furthermore, the fluorescent monomers that have been reported were synthesized by complex and multi-step procedures. In this study, we demonstrate the synthesis of a novel fluorescent functional monomer 7-Acryloxy-4-methylcoumarin (AOMC) *via* a simple, one-step esterification. Radical polymerization of AOMC, crosslinkers ethylene glycol dimethacrylate (EGDMA) and co-monomer methacrylic acid (MAA) in the presence of SDz afforded the desired MIPs. Fluorescence quenching experiments confirmed the MIPs selectivity towards the target analyte SDz. Furthermore, SDz was successfully detected in milk samples spiked at low concentration, confirming the MIPs were sensitive and selective to trace analytes in real samples.

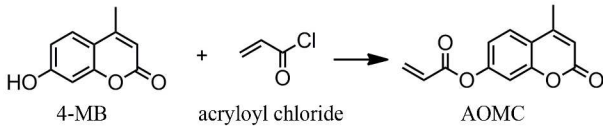
Experimental

Materials and reagents

4-Methylumbelliferone (4-MB), acryloyl chloride and EGDMA were purchased from J&K Scientific Ltd. (Beijing, China). Ethanol, tetrahydrofuran (THF), sodium chloride, sodium bicarbonate, MAA, 2,2'-azobisisobutyronitrile (AIBN), acetonitrile (ACN) and N,N-dimethylformamide (DMF) were obtained from Sinoreagent Chemical Reagent Co., Ltd. (Beijing China). SDz, sulfamethazine (SMz), sulfamethoxazole (SMx), benzene sulfonamide (BS), and dapsone (DPs) were supplied by Sigma-Aldrich (Shanghai, China). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All chemicals were analytical grade and used as received without any further purification.

Synthesis of AOMC

4-MB (0.5285 g, 3 mmol) was dissolved in THF (30 mL), then sodium bicarbonate$_{(aq)}$ (25.2g/L, 30mL) was added into the system. Acryloyl chloride (0.73 mL, 9 mmol) was added drop wise and the mixture was stirred for 3 h in an ice-water bath. The resultant precipitate was washed with saturated NaCl$_{(aq)}$, filtered to remove excess impurities and dried under vacuum at 60 °C for 24 h. The fluorescent monomer was obtained as a white floccule and stored at 4 °C prior to use. Reaction of 4-MB and acryloyl chloride yielded AOMC *via* one-step esterification (Scheme 1).

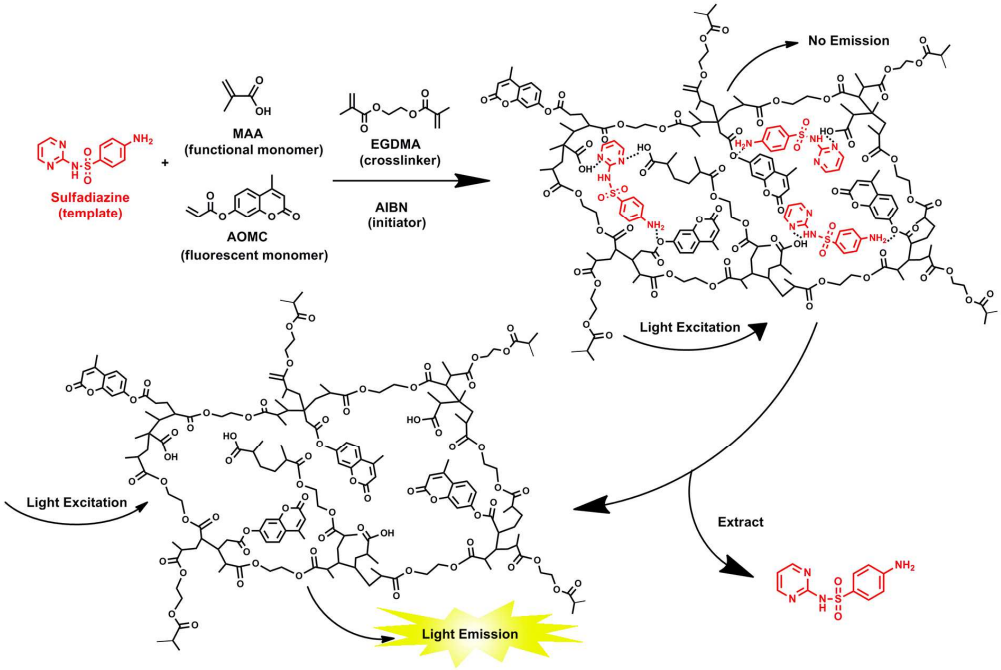


4-MB acryloyl chloride AOMC

Scheme 1. Synthesis of the fluorescent monomer AOMC.

Synthesis of MIPs and NIPs

AOMC (0.02302 g, 0.1 mmol), MAA (0.0344 g, 0.4 mmol) and SDz (0.02503 g, 0.1 mmol) were added to a mixture of ethanol (15 mL) and ACN (3 mL). The solution was shaken in a water bath at 25 C for 30 min. Subsequently, EGDMA (2.0 mmol) and AIBN (5 mg) were added. After purging with nitrogen gas for 10 min to remove oxygen, the system was heated to 65 °C for 24 h. The resultant MIPs were filtered then washed with methanol-acetic acid (9:1, v/v) to remove SDz, free monomers and crosslinkers. This was repeated until SDz absorption was no longer detected using UV-visible spectrophotometry (268 nm). The precipitated MIPs were dried at 70 °C under vacuum. For comparison, NIPs were prepared using the same procedure, however in the absence of the template molecules. The synthesis is presented in Scheme 2.



Scheme 2. Synthesis of the MIPs. Emissive and non-emissive states shown before and after the extraction processes.

Binding study

Both MIPs and NIPs (30 mg) were added to a series of SDz solutions of varying concentration in ACN (25 mL). The suspensions were agitated for 2 h at ambient temperature to allow binding of the analytes. These experiments were repeated with structural analogues of SDz (SMz, SMx, BS and DPs. Fig. 1).

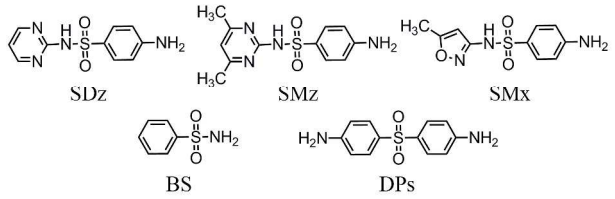


Fig. 1. Chemical structures of SDz and its analogues.

Characterization

Fluorescent measurements were performed using F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with a solid sample holder. ^{1}H NMR and ^{13}C NMR spectra (300 and 75 MHz for ^{1}H and ^{13}C, respectively) were recorded at room temperature on BRUKER DPX (Karlsruhe, Germany) in D_2O and $CDCl_3$, respectively. FT-IR spectra were recorded using the NEXUS-470 FT-IR spectrophotometer (Nicolet, Madison, WI); samples were dispersed in KBr pellets. MS spectra were recorded using a JEOL JMS-700 mass spectrometer.

After 2 h rebinding analytes, the filtrated MIPs and NIPs were dried at 70 °C under vacuum. MIPs or NIPs (10 mg) were added to the solid sample holder which fixed on the F-7000 spectrophotometer at a certain angle (45 °) and scanned. The excitation wavelength used was 334 nm with an emission range of 345-550 nm. Both excitation and emission slit widths were set at 2.5 nm and the photomultiplier tube voltage was 700 V.

Sample collection and pretreatment

Organic milk samples were purchased from www.benlai.com. Milk samples were diluted ten-fold using ACN, then placed in a 50 mL centrifuge tube. The solutions were centrifuged at 8000 rpm for 20 min. The supernatant was collected for analysis. And then, MIPs (30 mg) were added to the supernatant. The suspensions were agitated for 2 h at ambient temperature to allow binding of the analytes. After filtration, the MIPs were dried at 70 °C under vacuum. After all these procedures, the resultant MIPs were scanned using the F-7000 spectrophotometer.The organic milk was determined to be free of SDz. Milk samples to be analyzed were spiked with SDz at concentrations 1, 5, 10 and 20 μmol L^{-1}. There was no further pretreatment of the samples.

Results and discussion

Characterization of AOMC

Successful conversion was confirmed using ^{1}H NMR spectroscopy. As shown in Fig. 2, the ^{1}H NMR spectrum of 4-MB (D_2O): δ2.37–2.52 (3H, d), 6.12–6.13 (1H, s), 6.71 (1H,d), 6.78–6.82 (1H,d), 7.58–7.61 (1H,d), 10.51 (1H,s). ^{1}H NMR of AOMC (D_2O): δ2.45–2.52 (3H, d), 6.19–6.23 (1H, s), 6.40–6.50 (2H, d), 6.56–6.62 (1H, d), 7.23–7.27 (1H, d), 7.35–7.36 (1H, d), 7.82–7.86 (1H, d). The disappearance of the characteristic O–H singlet of 4-MB (10.51 ppm), and the concomitant appearance of vinylic peaks at 6.40–6.50 ppm (2H, d) and 6.19–6.23 (1H, s) indicated the successful synthesis of AOMC. In addition, ^{13}C NMR, MS spectra of the AOMC were list in Fig.S1.

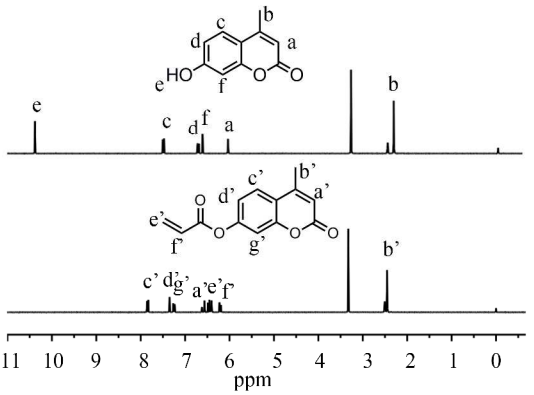


Fig. 2. ^{1}H NMR spectra of 4-MB and AMOC.

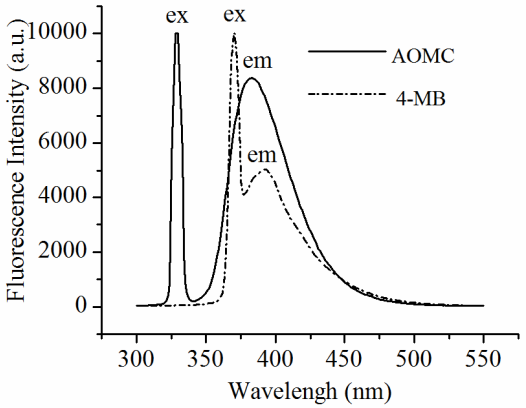


Fig. 3. Fluorescence excitation and emission spectra of AOMC and 4-MB (ex=excitation, em=emission).

The fluorescence emission behavior of AOMC was investigated and compared with 4-MB. The emission maxima for AOMC and 4-MB were 383 and 392 nm, respectively (Fig. 3). The fluorescence emission intensity of AOMC (8379) was 1.66 times greater than that of 4-MB (5046), which is attributed to the extended p-π-conjugation in AOMC.[42]

Characterization of MIPs

The SDz imprinted MIPs were synthesized by radical polymerization. Fluorescence quenching of the polymers was used to report SDz binding. The fluorescence quenching mechanism may be due to hydrogen bonding as illustrated in Scheme 2.

The successful synthesis of both MIPs and NIPs was confirmed using FT-IR spectroscopy (Fig. 4). Prior to removal of SDz, the IR spectrum of the MIPs preparations displayed a weak absorption at 1367 cm^{-1} (Fig. 4a) which is attributed to the SO_2-N asymmetric vibrations of SDz. This suggested the template SDz molecule was interacting with the MIPs through hydrogen bonds, corresponding to the results of ^{1}H-NMR (Fig. 2). After washing the MIPs to remove SDz, the SO_2-N asymmetric vibration disappeared (Fig. 4b) and was almost identical to that of NIPs (Fig. 4c). Furthermore, all spectra possessed the same absorption bands around 1730, 1260 and 1157 cm^{-1}, assigned C=O of carboxyl (MAA), C–O asymmetric and symmetric stretching vibration of ester (EGDMA), respectively.[43] The peaks at 3450, 1459, 1392 cm^{-1} correspond to aromatic ring stretching vibrations. Meanwhile, the adsorption band at 3433 cm^{-1} was attributed to the stretching vibration of O–H bonds from MAA.

These results confirmed that the MIPs were synthesized successfully and the template molecules were removed.

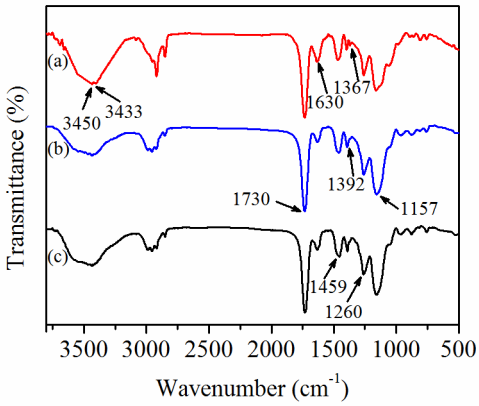


Fig. 4. FT-IR spectra of the MIPs (a) before and (b) after washing. The FT-IR spectrum of the NIPs is shown for comparison (c).

The fluorescence emission of both MIPs and NIPs was investigated (Fig. 5). The emission intensity of the MIPs increased dramatically (97% of NIPs) after removal of the SDz. Additionally, the emission profile remained unchanged. These results demonstrated that the template SDz molecules strongly and reversibly quenched the fluorescence of the MIPs. The fluorescence emission remained largely unchanged over a 90 day period at 4 °C (Fig. S2), demonstrating the long-term stability of these MIPs.

Barman *et al* reported that formation of H-bonds between the donors and acceptors can alter charge density, which leads to intramolecular charge transfer and causes fluorescence quenching.[44]

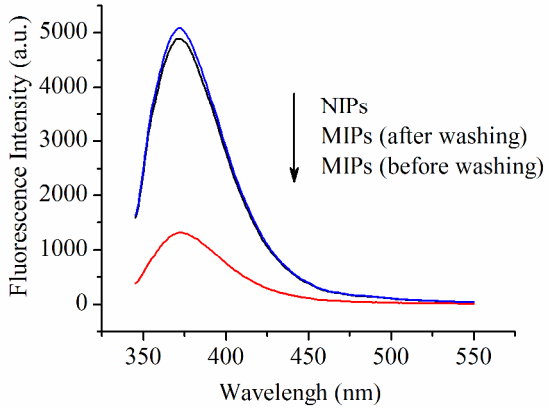


Fig. 5. Fluorescence emission spectra of MIPs (red) before and (black) after the removal of template. The fluorescence emission spectrum of the NIPs is shown for comparison (blue).

In our system, the AOMC and SDz molecules acted as the electron donor and electron acceptor, respectively. The template might accept the energy fluorescent monomer absorbed via hydrogen bond or release in the form of heat during collision. Thus, the quantum yield was reduced and fluorescence quenching was observed.

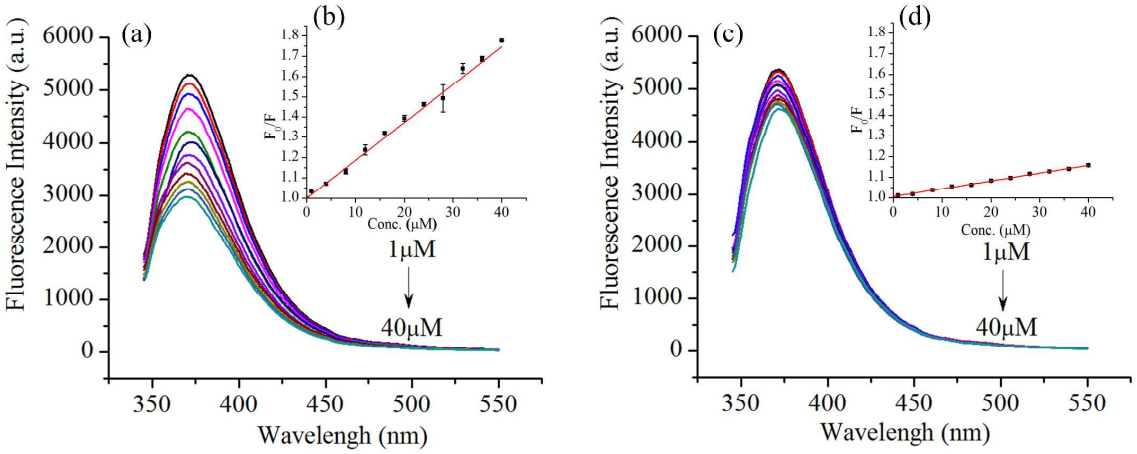


Fig. 6. Change in fluorescence emission of MIPs (a) and NIPs (c) when exposed to increasing concentration of SDz. Insets (b, d) show Stern–Volmer plots.

Determining SDz concentration using MIPs

The quenching efficiency of the MIPs *vs* the NIPs upon exposure to SDz was investigated further (Fig. 6). Both MIPs and NIPs exhibited a linear decrease in emission intensity (Fig. 6b and d, plotted as F_0/F vs [SDz]) in the presence of increasing concentrations of SDz. However, the MIPs displayed a greater quenching response to SDz than did the NIPs, confirming the MIPs selectivity for the template molecule.

The Stern–Volmer equation is given by:

$$F_0/F=1+K_{sv}[C]$$

where F_0 and F are the fluorescence intensity in the absence and presence of SDz, respectively, K_{sv} is the Stern–Volmer constant, and [C] is the quencher concentration. Linear Stern–Volmer relationships were observed for both MIPs and NIPs, with K_{sv} of 0.0192 and 0.0038 M^{-1}, respectively. The increased quenching efficiency of the MIPs further suggested selectivity for SDz because of the templating process.

The fluorescence quenching of the MIPs was sensitive at low concentrations (1–40 μM), with a limit of detection (LOD) of SDz determined to be 0.48 μM. These results indicated that fluorescent MIPs are highly sensitive and are suitable materials for the rapid determination of analytes concentration.

In order to investigate the binding kinetics of the MIPs, an equilibrium binding analysis was carried out (40 μmol L^{-1}). More than 75% binding was obtained after a shaking period of 40 min, and the adsorption equilibrium was achieved after 80 min(Fig. S3).

Selectivity of MIPs vs. NIPs towards sulfonamides

The selectivity of MIPs and NIPs for SDz was probed by exposing the polymers to sulfonamides with a similar structure to SDz, including SMz, SMx, BS, and DPs. The Stern–Volmer relationships for these additional analytes are shown in Fig. 7. The K_{sv} values obtained for the SDz analogues were significantly lower than that of SDz, consistent with a lower adsorption of the analogues (Fig. 7c). These results confirmed that the templating process imparted selectivity towards the target analyte. By contrast, the NIPs interact with analytes. by non-discriminative H-bonds due to the absence of a template molecule during its preparation. As a result, there was negligible difference in K_{sv} values for all five competitive analogues when the measurements were performed with the NIPs.

The imprinting factor (IF, $K_{sv,MIPs}/K_{sv,NIPs}$) gives a measure of the selectivity of MIPs towards a templated analyte compared with that of NIPs. The IF for SDz (5.05) was dramatically higher than those of SMz (0.87), SMx (0.90), DPs (0.96) and BS (2.20). Conversely, the NIPs did not exhibit any obvious difference in quenching after binding SDz or its structural analogues, consistent with the NIPs having no specific sites for the SDz analyte (Fig. 7c).

The selectivity of our MIPs for SDz was further investigated by binding competitions with the structural analogue BS. In these experiments, the concentration of SDz was held constant while the concentration of BS was increased. The change in fluorescence intensity was negligible when the BS:SDz ratio ($C_{BS/SDz}$) was increased (Fig. 7d). These results demonstrated that the MIPs exhibit high selectivity and specificity for SDz because of the imprinting sites present in the MIPs.

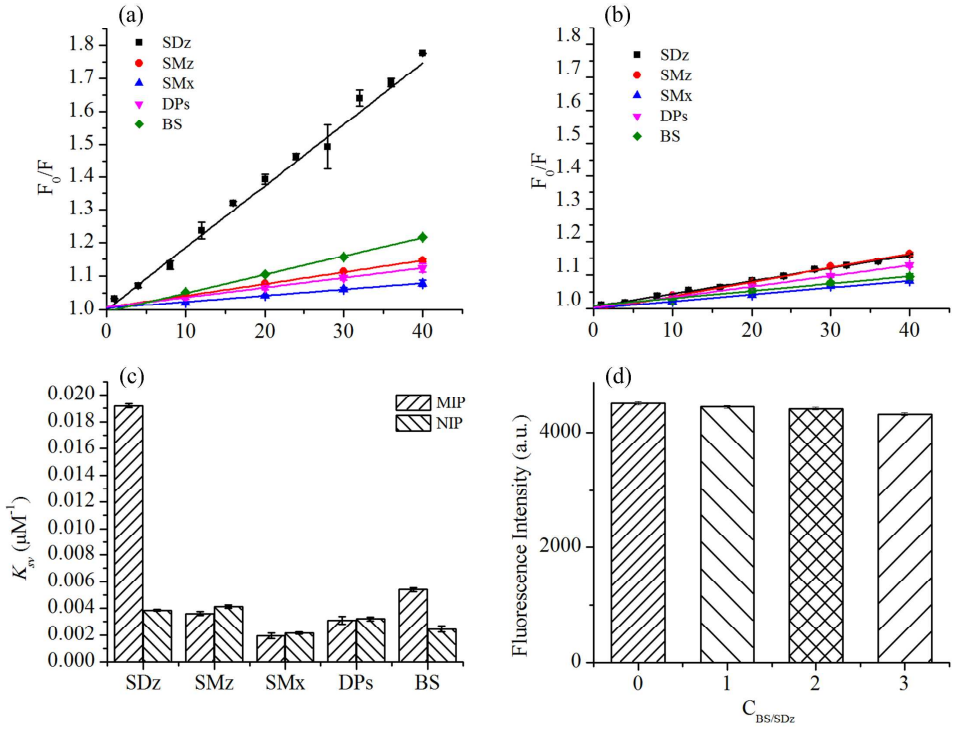


Fig. 7 Stern–Volmer plots for the MIPs (a) and the NIPs (b) when exposed to the analytes SDz, SMz, SMx, DPs and BS. Stern–Volmer quenching constant calculated for the MIPs and the NIPs (c). Fluorescence emission intensity of the MIPs observed after competitive binding assays with SDz and BS. The BS:SDz ratio ($C_{BS/SDz}$) is shown (d).

Regeneration of the MIPs

Regenerability was a key factor for an effective detection polymer. In this study, the regeneration of MIPs was tested by 8 consecutive loading, washing, and elution procedures. The recovery (RE) was performed by the relative fluorescence intensity, using the equation: RE= F_n/F_0. As shown in Fig. 8, In comparison with fresh MIPs, there was only a little change in the fluorescence intensity after eight rebinding cycles. This demonstrates the good reusability of these MIPs for the detection of SDz.

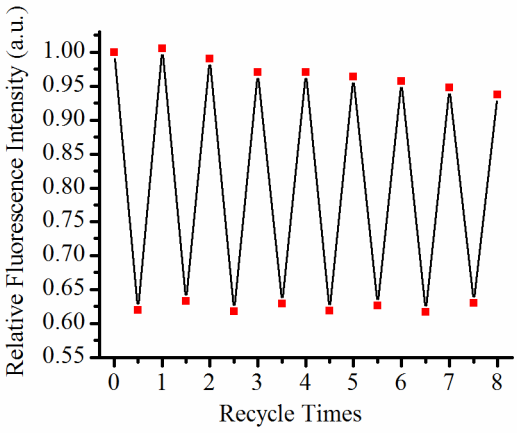


Fig. 8. Regeneration of MIPs

Detection of SDz in milk samples using the fluorescent MIPs

Identification and quantification of sulfonamides in biological samples typically involve complex and time-consuming procedures. Several pretreatment methods have been described, including solid-phase extraction[45], liquid-liquid extraction[46] and solid-phase micro extraction[34]. The fluorescent MIPs developed in this work can detect and quantify sulfonamides without additional processing but with high specificity and sensitivity.

To determine if the MIPs could detect and quantify SDz in complex biological media, whole and low fat milk samples were spiked with increasing concentrations of SDz. A blank recovery study was conducted using milk spiked with SDz at concentrations of 1, 5, 10, 20 μmol L^{-1}. The concentration of SDz adsorbed by the MIPs was calculated using the Stern–Volmer equation determined for the MIPs, $(F_0/F= 0.0192C+1.0004)$. The recovery (R) was calculated using the equation $R(\%)=C_f/C_i\times100$, where C_i is the concentration of SDz added and C_f is the concentration of SDz detected by the fluorescent MIPs. Excellent recovery values ranging from 96.24 to 101.37% were obtained for low fat milk samples (Table 1). Recovery values ranging from 85.73 to 101.11% were obtained for whole milk samples. The presence of fatty substances in whole milk may interfere with the detection of SDz, explaining the larger range observed. Nevertheless, these studies indicated that fluorescent MIPs can accurately determine the concentration of SDz in real samples.

Table 1 Recoveries and relatitive standard deviations (RSD, %, n=3) from milk samples.

Samples	Concentration of template (μmol L^{-1})		Recovery (%)	RSD(%)
	Amount added	Amount found (mean, n=3)		
Whole Milk	1	0.8573	85.73	4.48
	5	4.9642	99.28	2.68
	10	10.2107	101.11	0.66
	20	20.2121	101.06	1.81
Low-Fat Milk	1	0.9624	96.24	6.26
	5	4.9862	99.72	2.21
	10	10.0698	100.07	0.50
	20	20.2745	101.37	1.84

Conclusions

We have reported the synthesis of a novel fluorescent monomer, AOMC, which was used to prepare fluorescent MIPs. These MIPs were prepared using the sulfonamide SDz as a template molecule. Binding experiments demonstrated that the MIPs were specific towards the imprinted analytes. Additionally, it exhibited high sensitivity in SDz detection. Thus, fluorescent MIPs are expected to be excellent chemosensory materials for the rapid detection of target sulfonamides.

Acknowledgements

This work was supported by the Chinese National Scientific Foundation (21375146).

Notes and references

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† Electronic Supplementary Information (ESI) available: NMR data et al. See DOI:10.1039/b000000x/

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