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Fluorescent detection of target proteins *via* a molecularly imprinted hydrogel†

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Proteins are typically separated by an immune reaction, such as an enzyme-linked immunosorbent assay, and are detected by selective fluorescent labeling. This has potential for complicated procedures and the denaturation of proteins by labeling, and is cost consuming. In this study, we propose a technique for the selective separation and detection of a target protein using a molecularly imprinted hydrogel (PI gel) with fluorescent monomers. We focused on 8-anilino-1-naphthalenesulfonic acid (ANS), where the fluorescence intensity is easily changed by the interaction with proteins, and successfully synthesized the ANS monomer and a poly(ethylene glycol) (PEG) conjugated ANS monomer. The PI gel with the ANS monomers using bovine serum albumin (BSA) as a template showed the selective adsorption of BSA and the fluorescence intensity increased due to the adsorption of BSA.

1. Introduction

The selective detection of proteins is significantly important for the diagnosis of diseases,[1–4] the detection of bacterial pathogens and biological weapons,[5–8] the development of new medicines[9,10] and environmental screenings.[11,12] In general, fluorescent and luminescent labeling of target proteins are employed for highly sensitive detection.[13–15] For the selective capture of target proteins, the enzyme-linked immunosorbent assay (ELISA) is widely applied in various fields.[16–18] Using this typical method, we can achieve highly selective separation and sensitive detection of target proteins. On the other hand, we usually face several problems, including the denaturation and aggregation of proteins due to labeling procedures, the cost consumption owing to biomolecules such as antibodies and the relatively complicated protocols. In this study, we focus on using a molecularly imprinted hydrogel, introducing a specific fluorescent moiety, in which the fluorescent intensity is sensitively changed by the interaction with certain proteins, for the effective detection of target proteins without any complicated labeling and antigen–antibody reactions.

Molecular imprinting is one of the greatest methods to obtain artificial molecular recognition ability. As is well known, a molecular recognition site can be constructed by a lock-and-key system in molecularly imprinted polymers (MIPs) and MIPs have been applied in a great number of fields.[19–22] In the most recent decade, the target molecules have included biomolecules, especially proteins. Differently from a typical MIP for small molecules, aqueous solvents should be used for holding the natural structure of the proteins and a relatively hydrophilic polymer matrix should be used. Also, to avoid the containment of the target protein into a highly crosslinked polymer, flexible polymers have to be employed. Considering these requirements, many studies on the selective adsorption/separation and detection of proteins have been reported.[23–27] Similarly to these excellent works, we have reported protein imprinted hydrogels (PI gels) using poly(ethylene glycol) (PEG) based hydrogels.[28–30] PEG is widely applied in biotechnologies due to its low mobility factor and effective excluded volume. Based on these characteristics, PEG-based hydrogels can suppress the non-specific adsorption of proteins and have been applied in a variety of adsorption media.[31–33] In our previous study, we focused on the selective detection of the target protein, lysozyme, and a fluorescent monomer was employed for the detection. However, in this case, we only achieved a decrease of the fluorescence intensity in response to the concentration of the adsorbed lysozyme.

In this study, we employed 8-anilino-1-naphthalenesulfonic acid (ANS) as a fluorescent dye for the detection of the target protein bovine serum albumin (BSA). ANS demonstrates strong

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fluorescence in organic solvents and is quenched in water, and ANS strongly interacts with certain proteins based on the hydrophobicity, resulting in the alteration of its fluorescence intensity.[34,35] Here, we synthesized a new ANS-based monomer to incorporate into PI gels. Furthermore, the PEG-introduced ANS monomer was also synthesized for the effective hydrophobic interaction between ANS and BSA. The prepared hydrogels were evaluated by their adsorption selectivity for proteins and their fluorescence changes.

2. Experimental

2.1. Chemicals

Acetone, dichloromethane (CH_2Cl_2), triethylamine, toluene, ethyl acetate (EtOAc), methanol (MeOH), sodium sulfate (Na_2SO_4), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (Tris), sodium bisulfite ($NaHSO_3$), potassium persulfate ($K_2S_2O_8$), trifluoroacetic acid (TFA), acetonitrile (ACN), ammonium acetate and sodium tetraborate decahydrate were purchased from Nacalai Tesque (Kyoto, Japan), acetic anhydride, pyridine, phosphorus pentachloride (PCl_5), sodium hydroxide (NaOH), bovine serum albumin (BSA), 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (AIYP), ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) from Wako Pure Chemical Industries (Osaka, Japan), 8-anilino-1-naphthalenesulfonic acid (ANS), allylamine, dimethylaminopropyl methacrylamide (DMAPMA), 2-(diethylamino)ethyl methacrylate (DEAEMA), acrylamide (AAm), *N*-isopropylacrylamide (NIPAAm) and diethylene glycol bis(3-aminopropyl)ether from Tokyo Chemical Industry (Tokyo, Japan), and 4-vinylbenzyl trimethylammonium chloride (VBTMAC), concanavalin A (Con A) and β-lactoglobulin (β-lg) from Sigma-Aldrich Japan (Tokyo, Japan). Polyethylene glycol 600 dimethacrylate (14G) and polyethylene glycol 1000 diacrylate (23G′) were kindly donated by the Shin-Nakamura Chemical Co. Ltd. (Wakayama, Japan).

2.2. Instruments

A Nicolet iS5 ATR (Thermo Fisher Scientific Inc., Yokohama, Japan) was employed for the FT-IR analysis. The liquid chromatography-tandem mass spectrometer (LC-MS/MS) was the LCMS-8030 (Shimadzu Co., Kyoto, Japan). UV-2450 (Shimadzu) was utilized as a UV-visible spectrophotometer. NMR was carried out using a JNM-ECZ600R spectrometer (JEOL, Tokyo, Japan). For photo polymerization, LUV-16 365 nm (AS ONE, Tokyo, Japan) was used as a handy UV lamp at 254 or 365 nm. An IX71 (Olympus, Tokyo, Japan) was used as a fluorescent microscope with LUCPlanFLN 10×/0.30 NA and U-MWU2 (excitation 330–385 nm, emission 420 nm). For the protein analysis, typical reversed-phase LC was employed with a LC-10A system (Shimadzu) using the following conditions: a linear gradient of 30% B to 70% B for 15 min (A: 0.1% TFA in water, B: 0.1% TFA in ACN); flow rate, 0.5 mL min^{-1}; temperature, 35 °C; column, YMC-Pack Pro C18 (150 mm × 4.6 mm i.d.); detection, UV 214 nm.

2.3. Synthesis of the ANS monomer

According to previous reports,[36,37] the ANS monomer was synthesized (Scheme S1†); Briefly, 6.68 mmol ANS was dissolved in a mixture of acetic anhydride (20 mL) and pyridine (10 mL), and then the solution was refluxed at 100 °C for 2 h. The products were washed with acetone and dried in a vacuum. Acetyl ANS was reacted with PCl_5 and separated out in iced water. The obtained ANS chloride (4.16 mmol) was dissolved in CH_2Cl_2 (100 mL) with 4.20 mmol allylamine and 4.20 mmol Et_3N, then the mixture was stirred under ambient conditions for 16 h. The products were purified by silica-gel chromatography with toluene/EtOAc = 85/15 as a mobile phase. Finally, the acetyl ANS monomer (0.13 mmol) was reacted with MeOH/5 M NaOH aq. for hydrolysis. The final products were purified by silica-gel chromatography with toluene/EtOAc = 85/15.

2.4. Synthesis of the PEG–ANS monomer (Scheme S2†)

0.55 mmol ANS chloride and 0.55 mmol diethylene glycol bis(3-aminopropyl) ether were dissolved in chloroform and reacted at 40 °C for 16 h. The products were purified by reverse-phase column chromatography with COSMOSIL (R) 140C18-OPN (Nacalai Tesque) using MeOH/water = 7/3. PEG–ANS (0.17 mmol) and 0.17 mmol acrylic acid were dissolved in chloroform with EDC (0.17 mmol)/DMAP (0.17 mmol) and stirred under ambient conditions for 16 h. The final products were purified by silica-gel chromatography with EtOAc as the mobile phase.

2.5. Fluorescent characterization of the ANS based monomers

To examine the fluorescent reaction of both monomers, *i.e.*, the ANS monomer and the PEG–ANS monomer, the alteration of the fluorescence intensity was evaluated. Each monomer (50 μM) in 10 mM Tris–HCl buffer (pH 7) was mixed with certain amount of BSA (0, 10.20, 30, 40, 50 μM), followed by measuring the fluorescence intensity of each sample (excitation at 370 nm, emission at 482 nm) 1 h later.

2.6. Preparation of the hydrogels and adsorption test

The PI gel and non-imprinted gel (NIP gel) were prepared as follows. The crosslinker, functional monomers, ANS monomers, template (BSA) and initiator were mixed with the aqueous solution in a polypropylene tube. After obtaining homogeneous solutions, the mixtures were degassed by N_2 bubbling and then the mixtures were poured into a Pyrex® tube for radical polymerization. The prepared hydrogels were cut into 2 mm thickness. The repeatability of the sliced gel pieces was thoroughly confirmed in our previous study,[30] so we treated each gel as having the same weight. The sliced hydrogel pieces were washed with 1 M NaCl aq. (4 times), 1 mM Tris–HCl aq./MeOH = 7/3 (3 times) and 1 mM Tris–HCl aq. to remove the template and unreacted reagents. The washing process was also optimized in our previous study.[30] Here, we optimized the solvent of the adsorption test in advance. Because of the detection limit of the proteins, the protein concentrations were fixed at 4.0 mM. Furthermore, the NaCl concentrations (1.0,

10.0, 100.0 mM) and pH variations (4.0, 7.0, 10.0) were evaluated to obtain the highest adsorption amount of BSA using a typical NIP gels. Finally, the adsorption of the proteins was carried out with 4.0 mM BSA (or other proteins) in 10 mM NaCl/1.0 mM Tris–HCl buffer (pH 7.0) using a piece of each hydrogel. After soaking for 16 h, the fluorescent intensity of the hydrogels was measured and the supernatant was analyzed by HPLC to estimate the amount of adsorbed proteins. The adsorption ratio and imprinting factor (IF) were defined as follows:

$$\text{Adsorption ratio} = \text{amount of adsorbed protein (mol)/theoretical amount in PI (mol)}$$

$$\text{IF} = \text{Adsorption ratio of PI gel/Adsorption ratio of NIP gel}$$

3. Results and discussion

3.1. Synthesis and evaluation of the monomers

The FT-IR spectra of the ANS monomer and PEG–ANS monomer are shown in Fig. S1a and S2a.† For the ANS monomer, the FT-IR spectrum indicated specific absorptions based on the C=O of the acetyl group and the C=C of the vinyl group at around 1650 cm^{-1} and 3500 cm^{-1}, respectively. Furthermore, the final ANS monomer showed similar absorption due to C=C, with the absorption of the C=O based on acetyl groups disappearing. As an additional assessment of the product, ESI-MS was carried out. As shown in Fig. S1b,† while the molecular weight of the ANS monomer is 338, $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ and $[2\ M + Na]^+$ were confirmed. Similarly, the synthesized PEG–ANS monomer was examined by FT-IR and MS. The IR and MS spectra are shown in Fig. S2.† From the IR spectrum of the PEG–ANS monomer, the specific absorptions of the amide groups and vinyl groups were confirmed at around 1680 cm^{-1} and 1650 cm^{-1}, respectively. Additionally, according to the MS spectrum, while the molecular weight of ANS monomer is 597, $[M + H]^+$ and $[M + Na]^+$ were confirmed. These spectrometric results strongly supported the suitable syntheses of the ANS monomer and the PEG–ANS monomer. The ^{1}H-NMR results of both monomers are summarized in the captions of Schemes S1 and S2.†

To observe the fluorescent characteristics of these monomers, the fluorescence intensity of the monomers in the presence of BSA was evaluated. Fig. 1 shows the change of the intensity against the concentration of BSA. In comparison, as shown in Fig. S3,† BSA itself did not show any fluorescence intensity at each concentration. Additionally, the free BSA concentrations were not changed even after 16 h (Fig. S4-a†) and an accurate calibration curve was confirmed by LC evaluations (Fig. S4-b†). As we expected, an obvious response of the fluorescence intensity to the BSA concentration was confirmed for each monomer.

3.2. Adsorption selectivity of the hydrogels with the ANS monomer

To confirm the effect of polymerization, two types of PI gels were prepared; PI-a, by photo polymerization with AIYP, and PI-b, by redox polymerization with APS/TEMED (Table 1). The results of the adsorption of BSA and the fluorescence intensity with/without BSA are summarized in Fig. 2. Both PI gels showed a similar adsorption ratio while the fluorescent intensity was clearly changed only in PI-b. This result suggested that the long time (3 h) photo reaction in PI-a induced the photobleaching of

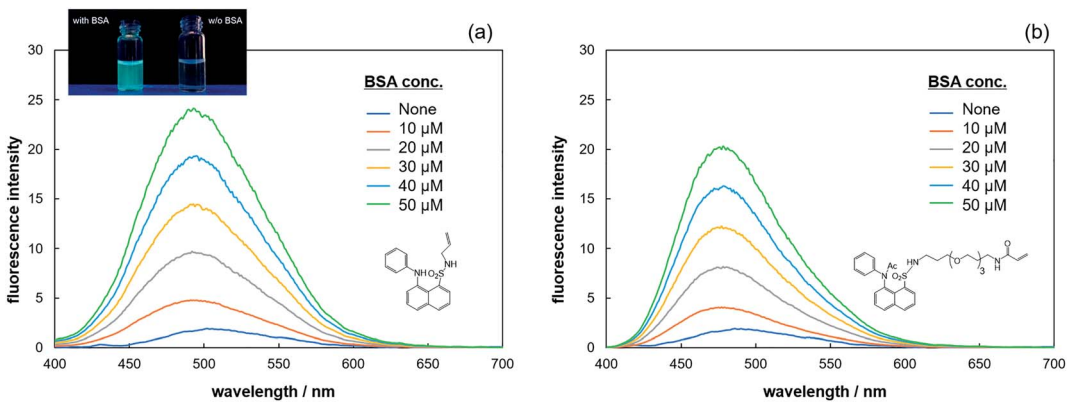

Fig. 1 Fluorescence intensity of the ANS-based monomers with different BSA concentrations. (a) ANS monomer with a photograph, (b) PEG–ANS monomer.

Table 1 Composition of the hydrogels with 14G and the ANS monomer

	Crosslinker 14G	ANS monomer	BSA	Initiator	Solvent
PI-a	225 μmol	0.1 μmol	0.1 μmol	AIYP 2.0 wt% *vs.* crosslinker	1.0 mM Tris–HCl buffer (pH 7.0) 2.4 mL,
PI-b				APS 25 μmol, TEMED 105 μmol	MeOH 0.6 mL
NI-b			—		

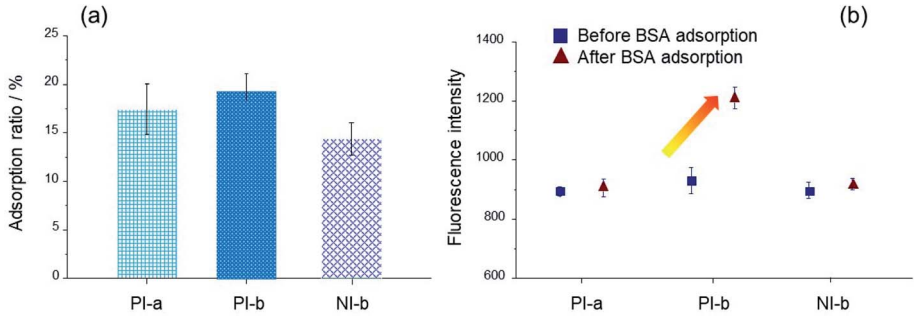

Fig. 2 Adsorption of BSA on the hydrogels. (a) Adsorption ratio of BSA for each hydrogel, (b) fluorescence intensity of each hydrogel with/without BSA.

the ANS moiety. Comparing PI-b and NI-b, the imprinted gel showed a higher adsorption ratio and fluorescence change due to the imprinting effect. However, even in the NI gel, relatively higher adsorption of BSA was observed. It seemed that the non-specific adsorption was caused by the hydrophobic interaction between the hydrogel itself and BSA. Therefore, we optimized the crosslinker and employed further functional monomers to enhance the imprinting effect.

3.3. Optimization of the functional monomer

To increase the amount of BSA adsorbed by the PI gels, AAm or NIPAAm, based on hydrogen bonding, and VBTMAC, based on ionic interaction, were employed as the additional functional monomers. Before checking the adsorption tests, we confirmed that each monomer showed no fluorescence intensity under excitation exposure. Furthermore, to suppress the non-specific hydrophobic interaction, the more hydrophilic 23G′ was utilized as the crosslinker. The compositions of the hydrogels are summarized in Table 2. As shown Fig. 3, the basic monomer, VBTMAC, worked effectively for the adsorption of BSA, while hydrogen bonding did not contribute to the imprinting effect. On the other hand, in spite of the higher alteration of the fluorescence intensity in PI-e, the adsorption selectivity was pretty low. It seemed that an excessive amount of VBTMAC randomly interacted with BSA, so that the relatively non-specific interaction occurred. Then, the amount of VBTMAC was optimized for PI-f and PI-g. By decreasing the amount of VBTMAC, the adsorption selectivity was clearly improved. In particular, in PI-g, higher adsorption selectivity was observed with obvious alteration of the fluorescence intensity.

3.4. PI gels using the PEG–ANS monomer

As shown in Fig. 3, the alteration of the fluorescence intensity was not clear, although higher imprinting factors were observed

Table 2 Composition of the hydrogels with 23G′ and the functional monomers[a]

	23G′	ANS monomer	Functional monomer	BSA	Initiator	Solvent
PI-c	143 μmol	0.5 μmol	AAm 11.7 μmol	0.1 μmol	APS 25 μmol TEMED 105 μmol	1.0 mM Tris–HCl buffer (pH 7.0) 2.4 mL, acetonitrile 0.6 mL
PI-d			NIPAAm 11.7 μmol			
PI-e			VBTMAC 11.7 μmol			
PI-f			VBTMAC 0.5 μmol			
PI-g			VBTMAC 2.5 μmol			

[a] 23G′, polypropylene glycol 1000 diacrylate; AAm, acrylamide; NIPAAm, *N*-isopropylacrylamide; VBTMAC, 4-vinylbenzyl trimethylammonium chloride.

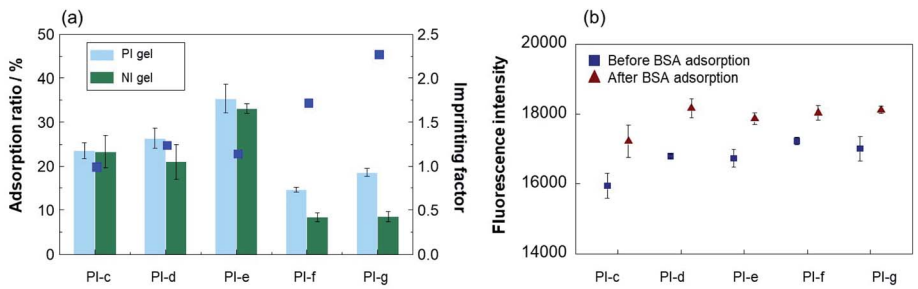

Fig. 3 Adsorption of BSA on PI gels with the functional monomers. (a) Adsorption ratio of BSA for each hydrogel, (b) fluorescence intensity of each hydrogel with/without BSA.

Table 3 Composition of the hydrogels with the PEG–ANS monomer

	23G′	ANS monomers	VBTMAC	BSA	Initiator	Solvent
PI-h	143 μmol	ANS monomer 0.5 μmol	—	0.1 μmol	APS 25 μmol, TEMED 105 μmol	1.0 mM Tris–HCl buffer (pH 7.0) 2.4 mL, acetonitrile 0.6 mL
PI-I		PEG–ANS monomer 0.5 μmol				
PI-j		ANS monomer 0.5 μmol	2.5 μmol			
PI-k		PEG–ANS monomer 0.5 μmol				

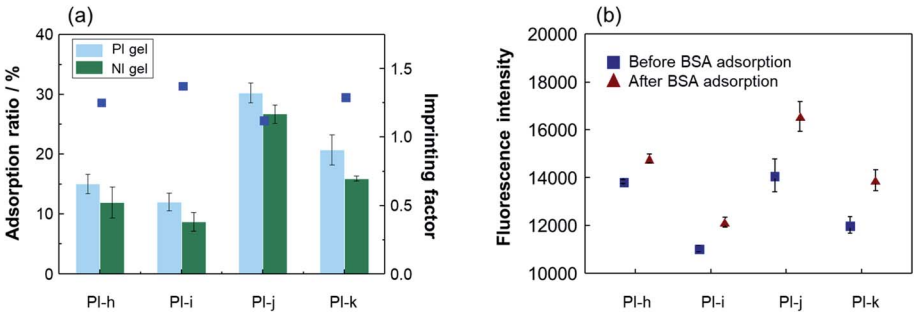

Fig. 4 Adsorption of BSA on PI gels with the PEG–ANS monomer. (a) Adsorption ratio of BSA in each hydrogel, (b) fluorescence intensity of each hydrogel with/without BSA.

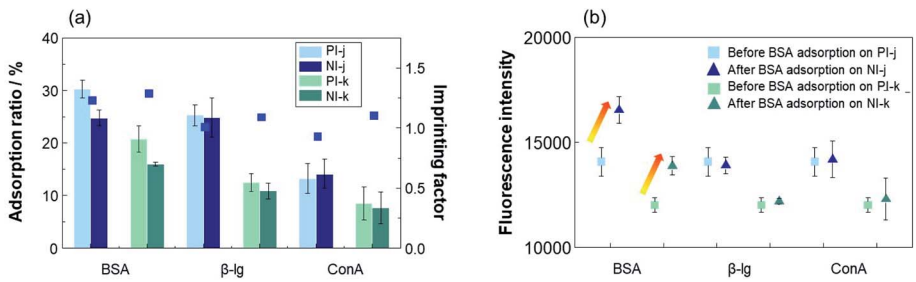

Fig. 5 Adsorption of proteins on hydrogels with the PEG–ANS monomer. (a) Adsorption ratio of each protein on PI-j and PI-k (b) fluorescent intensity of each hydrogel with/without BSA.

for PI-f and PI-g. Here, we anticipated that the fluorescent monomer, ANS monomer, did not reach the hydrophobic part of the template, BSA. Thus, we synthesized the new PEG–ANS monomer to improve the detection selectivity due to the fluorescence intensity. In fact, we expect that the PEG chain can contribute to increasing the accessibility of the ANS moiety to the inside of the hydrophobic part of BSA. The compositions of the prepared hydrogels are summarized in Table 3. As a comparison, gels with the ANS monomer were also prepared. The results of the adsorption tests and the fluorescence intensities are shown in Fig. 4.

According to Fig. 4a, a higher adsorption of BSA was observed in PI-j and PI-k due to the ionic interaction based on VATMAC, as expected. Furthermore, the absolute adsorption amount of BSA was slightly lower in the PI gels with the PEG–ANS monomer compared to the PI gels with the ANS monomer. Finally, we examined the adsorption selectivity of the prepared PI gels using other proteins, β-lactoglobulin (β-lg) and concanavalin A (Con A). The adsorption ratios and the changes to the fluorescent intensity are summarized in Fig. 5. In both PI gels, the selective adsorption of BSA was obviously confirmed, while the selectivity was not observed for β-lg and Con A at all. Similarly, the fluorescence changes were more clearly contributed to by the selectivity, and no alterations were confirmed for β-lg and Con A. These proteins are acidic and easily interacted with VBTMAC as well as BSA. However, the fluorescence alteration was observed only for BSA. The results suggested that the ANS moiety selectively interacted with the hydrophobic part only in BSA due to the imprinting effect. Additionally, the absolute adsorption of β-lg and Con A was much lower in PI-k. It seems that the PEG moiety provided the suppression of the non-specific interactions such as hydrophobic interactions. Thus, we finally propose the possibility that PI gels using PEG-introduced fluorescent monomers can be used for the selective adsorption and detection of target proteins.

4. Conclusion

In this study, we prepared PI gels in which the fluorescence intensity was changed by the interaction between the ANS

monomers and the target protein, BSA. After optimization of the gel preparation, including the crosslinkers, functional monomers, polymerization conditions and adsorption conditions, the newly prepared ANS monomer and PEG–ANS monomer effectively contributed to the selective adsorption and detection of BSA. In particular, the PI gel with the PEG–ANS monomer provided the possibility of suppressing non-specific interactions for other proteins. Further investigations for the efficient detection of the target proteins using similar protocols with additional fluorescent monomers are in progress.

Conflicts of interest

There are no conflicts to declare.

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