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Effective Recognition on the Surface of a Polymer Prepared by Molecular Imprinting Using Ionic Complex

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ABSTRACT: To achieve selective recognition of water-soluble ionic compounds, we developed an effective preparation method that can construct specific recognition sites on a cross-linked polymer surface using ionic complexes of functional monomers and template molecules. The specific ionic complexes used for selective recognition were attached on the surface of cross-linked polymer pores via a reaction between ionic complexes and unreacted vinyl groups on the polymer. In this paper, we show that ionic complexes can attach to the polymer surface by determining the number of unreacted vinyl groups on the cross-linked base polymer. The prepared polymers had greater effective adsorption ability than normally prepared molecularly imprinted polymer because there were no dead sites in the cross-linked network. Furthermore, according to chromatographic evaluations involving frontal analyses, the prepared polymer has selective recognition ability based on the interval recognition of ionic compounds. The adsorption isotherm also suggests selectivity for the template molecule on the prepared polymer. Finally, the paralytic shellfish poison saxitoxin was selectively extracted using the prepared polymer.

Introduction

Selective separation and concentration technologies are very important for the purification and/or quantitative analysis of toxins, drugs, biological, materials, and chemicals. However, common techniques for separating and concentrating these compounds are not optimal from the viewpoints of sensitivity and selectivity because the compounds are affected by various contaminants. Therefore, new separation and concentration techniques are required.

Molecular imprinting[1,2] is an attractive method for obtaining selective recognition. As selective molecular recognition sites can be easily constructed on cross-linked polymer using this technique, it has been applied in various fields such as those of separations and sensors.[3−5] However, in using this technique, a real molecule is required as the template molecule, and thus the recognition of rare naturally occurring compounds and/or highly toxic compounds is difficult.[6] Additionally, the recognition sites are heterogeneous because an excessive amount of functional monomer compared with the amount of template is used. Moreover, many dead sites not involved in adsorption are constructed using this technique because the imprinted sites form not only on the surface but also deep inside the cross-linked polymer network where it is difficult for solvents to reach. As another important problem, the technique cannot be applied to hydrophilic compounds such as water-soluble alkaloids because common molecularly imprinted polymers (MIPs) are prepared with nonpolar organic solvents.[7−9]

To resolve these problems, we have reported alternative molecular imprinting techniques including fragment imprinting[10−14] and interval immobilization,[15,16] especially for the effective determination of environmental toxic compounds. Recently, we have reported interval immobilization for the selective adsorption of the shellfish paralytic poison saxitoxin (STX).[17] STX has many (about 15) analogues, each having two guanidine groups the same distance apart.[18−21] Postcolumn labeling, as another example, is one of the most frequently used analysis methods.[19,22−24] However, these methods are not suitable for the quantitative analyses of natural samples because of the presence of many contaminants in the samples and the presence of ghost peaks in the obtained chromatograms. Consequently, there is the need for a pretreatment procedure involving selective concentration. In our previous note,[17] we presented application data concerning the selective adsorption of an STX analogue using a newly prepared polymer medium.

In this paper, we describe fundamental studies concerning the newly developed preparation method for the selective adsorption of STX described in our previous note. From determination of the remaining unreacted vinyl groups on the base polymer, chromatographic evaluation, and investigation of the adsorption isotherm, we demonstrate the formation of recognition sites on the surfaces of cross-linked polymer pores via interval immobilization.

Experimental Section

Chemicals. Ethylene glycol dimethacrylate (EDMA) as the cross-linking agent was purchased from Wako Chemicals (Osaka, Japan) and distilled under vacuum to remove the polymerization inhibitor. Tributylamine, 2,2′-azobis(2,4-dimethylvaleronitrile) (ADVN) as a radical initiator, *p*-styrenesulfonic acid sodium salt (SSA), and other solvents including chloroform, toluene, tetrahydrofuran (THF), high-performance liquid chromatography (HPLC) grade methanol (MeOH), and HPLC grade acetonitrile (MeCN) were also purchased from Wako Chemicals. α,α′-Dichloro-*p*(or *m*)-xylene-4,4′-bis(chloromethyl)biphenyl, tetra-*n*-butylammonium chloride (TBA), and benzyltributylammonium chloride (BTBA) were purchased from Tokyo Kasei (Tokyo Japan). Decarbamoylsaxitoxin (dcSTX) was purchased from the National Research Council of Canada.

Synthesis of Template Molecules. α,α′-Dichloro-*p*-xylene (2.0 g, 11.4 mmol) was dissolved in 50 mL of MeCN. Tributylamine (8.0 mL, 33.7 mmol) was added slowly and stirred into the solution. After the addition of tributylamine, the mixture was stirred and refluxed at 100 °C for 24 h under a nitrogen atmosphere. The formation of the intended compound was confirmed by thin layer chromatography. After the reaction was complete, the reaction mixture was separated by silica gel column chromatography (chloroform/MeOH, v/v = 9/1) to isolate 4-(tributylammonium−methyl)-benzyltributylammonium chloride (TBTA). In a similar way, (3-tributylammonium-methyl)benzyltributylammonium (*m*-TBTA) and 4,4′-bis(tributylammonium−methyl chloride)biphenyl (BTAB) were also synthesized. The structures of these template molecules were

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confirmed by ^{1}H nuclear magnetic resonance (NMR): ^{1}H NMR (TBTA), 400 MHz ($CDCl_3$): δ 0.97 ppm (t, 18H), 1.34 ppm (sext, 12H), 1.77 ppm (m, 12H), 3.13 ppm (quint, 12H), 4.53 ppm (s, 4H), 7.60 ppm (s, 4H). ^{1}H NMR (*m*-TBTA), 400 MHz ($CDCl_3$): δ 0.99 ppm (t, 18H), 1.44 ppm (sext, 12H), 1.72 ppm (m, 12H), 3.41 ppm (quint, 12H), 5.13 ppm (s, 4H), 7.55 ppm (t, 1H), 7.85 ppm (d, 2H), 8.75 ppm (s, 1H). ^{1}H NMR (BTAB), 400 MHz ($CDCl_3$): δ 1.08 ppm (t, 18H), 1.47 ppm (sext, 12H), 1.87 ppm (m, 12H), 3.25 ppm (quint, 12H), 4.64 ppm (s, 4H), 7.66 ppm (d, 4H), 7.91 ppm (d, 4H).

Synthesis of Ionic Complexes. SSA and template molecules as nonimprinted polymers were dissolved in water, with the molar ratio between SSA and template molecules being in the range of 2−6:1, and extracted with chloroform via the phase transfer effect of the molecular assembly formed. After the removal of chloroform, transferred ionic complexes were obtained. The obtained ionic complexes were confirmed by ^{1}H NMR. Here, although SSA was not soluble in organic solvent, each ionic complex easily dissolved in organic solvent because of the formation of a complex.

Preparation of Polymer Particles. To prepare separation media for interval immobilization, first we prepared the BASE polymer particles by two-step swelling and polymerization.[25,26] Polystyrene seed particles were prepared by emulsifier-free emulsion polymerization.[27,28] The polymerization was carried out at 50 °C for 24 h using EDMA as a cross-linker, chloroform as a porogenic solvent, and 2.0 wt % ADVN as a radical initiator. After the polymerization procedures, the polymer particles were washed with pure water, MeOH, and THF to remove porogenic solvent and unreacted compounds. The washed particles were dried on a membrane filter at room temperature. The polymer particles were about 5.0 μm in diameter with excellent size uniformity.

In this study, we used two methods for preparing polymer particles: the mixed method and the dispersion method. The mixed method is a commonly used copolymerization technique comprising swelling and polymerization steps. Here, each ionic complex was mixed with porogenic solvent, and the ionic complexes were incorporated into polymer matrix. The procedures for the newly developed dispersion method were as follows. BASE polymer particles (2.0 g) were dispersed into $MeOH/H_2O$ (v/v = 1/1), and the ionic complex (0.038 mmol) of the template and SSA were added with the radical initiator. The mixture reacted and was refluxed at 70 °C for 24 h. In addition, BLANK and BTBA-P as control polymers were prepared without template molecules and had only an ionic group in their structures. For the preparation of BLANK and BTBA-P, which did not have interval immobilized SO_3^-, the ionic complex (0.076 mmol) of TBA or BTBA and SSA was used. Although BLANK and BTBA-P were prepared as control polymers, we examined the difference in the retention effect on SO_3^- from altering the cavity. The cavities around SO_3^- groups are much larger for BTBA-P because the molecular volume of BTBA is larger than that of TBA.

In this method, the ionic complex attaches only on the surface of the polymer pore;[29] the scheme for interval immobilization is shown in Figure 1. All polymer particles prepared using the two methods were washed with MeOH and THF and dried under vacuum. The compositions of polymer particles are summarized in Table 1.

HPLC Evaluation of Polymer Particles. For HPLC evaluations, the polymer particles were packed into stainless steel columns (150 mm × 4.6 mm i.d.), and the selective recognition for each template molecule was determined. First, MeOH/1.0 M aqueous sodium chloride (1/1 v/v) was eluted to remove the template molecules. For the actual HPLC evaluations, MeOH/0.4 M aqueous sodium chloride (7/3 v/v) was used as mobile phase, and the void volume was estimated using the retention time of acetone. The retention factor k' as an evaluation index is defined as follows:

$$k' = (V_{\text{retention volume of solute}} - V_{\text{void volume}})/V_{\text{void volume}}$$

Additionally, to examine the binding capacity of polymers prepared using the dispersion method, frontal analyses were conducted using BTBA and TBTA solutions with constant concentrations as mobile phases.

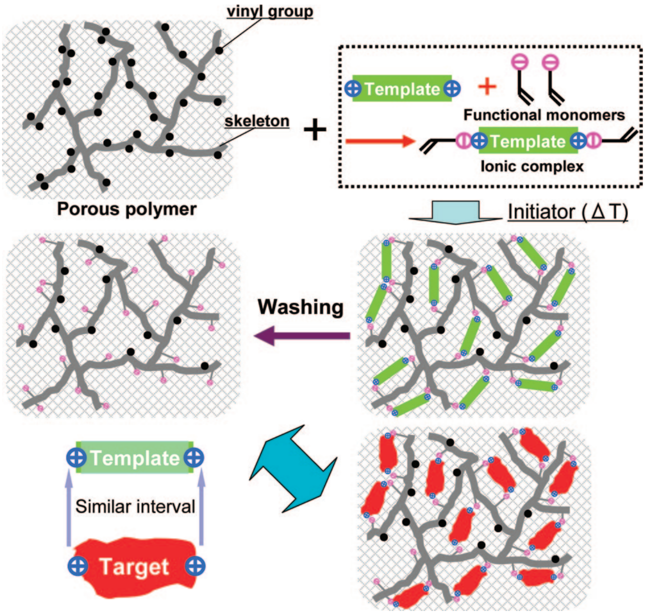

Figure 1. Schematic concept of polymer preparation. The black dots indicate the remaining vinyl groups, and the skeleton shows the cross-linked polymer.

Table 1. Abbreviations and Preparation Methods of Polymers

abbreviation	ionic complex	method
BASE		
BLANK (M)	TBA−SSA	mixed method
TBTA-P (M)	SSA−TBTA−SSA	mixed method
BLANK (D)	TBA−SSA	dispersion method
BTBA-P (D)	BTBA−SSA	dispersion method
TBTA-P (D)	SSA−TBTA−SSA	dispersion method
BTAB-P (D)	SSA−BTAB−SSA	dispersion method

Quantitative Determination of Unreacted Vinyl Groups in BASE Polymer for Calculation of the Distance between Vinyl Groups. 10 μL of bromine was dispersed in 1000 μL of carbon tetrachloride in tubular vials, and then BASE polymer (0−75 mg) was added to the vials. The mixtures were stirred at room temperature to react the bromine with free vinyl groups on the BASE polymer. After 12 h, the mixtures were filtered to remove the polymer particles. The filtrates were then diluted 20-fold with carbon tetrachloride, and the absorbance of the solution was measured by spectrophotometry (415 nm). Finally, the number of remaining unreacted vinyl groups on the BASE polymer was determined from the analytical curve.

Adsorption Isotherm. 1.0 mM−0.25 μM of TBTA solution (in $MeCN/H_2O$, v/v = 7/3) was prepared in a tubular vial. Each polymer particle (10 mg) prepared using the dispersion method was dispersed in a vial. The vials were stirred at 25 °C for 12 h, and the supernatants after centrifuge were analyzed by HPLC electrospray-ionization mass spectrometry to determine the free TBTA concentrations. From the determined free TBTA concentrations and amounts of bound TBTA on polymer, a Scatchard plot[8,9,30,31] was prepared, and the binding constant and number of binding sites were estimated.

Solid-Phase Extraction of STX Analogue. Blue mussel (10.0 g) was crushed and extracted with $MeOH/H_2O$ (v/v = 1/1) (200 mL).[32] After centrifugation, the supernatant was collected and the solvent was removed under vacuum. The residue was redispersed in $MeCN/H_2O$ (v/v = 7/3) (100 mL), and the extract was spiked with authentic dcSTX (the concentration of dcSTX was 1.5 μM). Each type of polymer particle (BLANK or TBTA-P (0.5 g)) was packed into a glass cartridge, and conditioning was carried out with MeOH and water. The extract containing dcSTX (5.0 mL) was passed through each cartridge, and

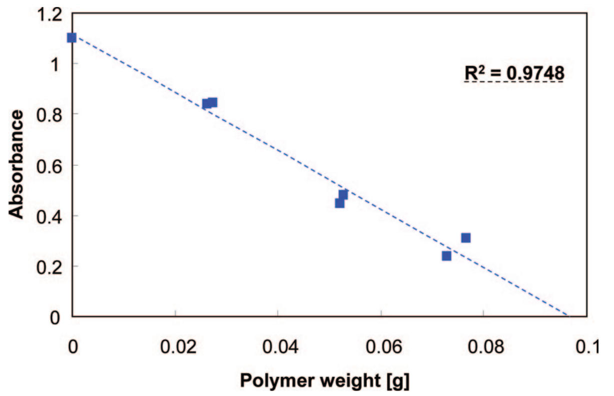

Figure 2. Analytical curve for the polymer weight and absorbance of free Br_2.

Table 2. Abbreviations of Templates and Distances between Ionic Groups

templates or ionic complex	distance N^+ to N^+ (Å)	distance of vinyl groups (Å)
TBTA	7.835	
m-TBTA	6.889	
BTAB	11.86	
SSA−TBTA−SSA		27.52
SSA−BTAB−SSA		30.98
saxitoxin	7.069	

the fractions in the extract were analyzed by liquid chromatography/mass spectrometry (LC/MS). Additionally, the adsorbed dcSTX on the TBTA-P cartridge was collected with 1.0 M ammonium carbonate aqueous solution (1.0 mL).

Equipment. Chromatographic data were acquired using an LC system (Shimadzu Co., Japan) comprising an LC-10 AD pump, an SPD-M10A photodiode array detector, and a CTO-10AC column oven. LC/MS was carried out using an LC/MS system (Shimadzu) equipped with an LC-MS2010 detector. NMR detection and spectrophotometry were carried out with a 400 MHz UltraShield (Bruker) and UV-1700 (Shimadzu).

Results and Discussion

Determination of Remaining Amounts of Vinyl Groups. As shown in Figure 1, the remaining unreacted vinyl groups are very important in interval immobilization. If there are few vinyl groups, the ionic complexes cannot react on the surface of the porous polymer. Therefore, we determined the remaining amounts of vinyl groups using bromine. As mentioned in the Experimental Section, 0−75 mg of BASE polymer reacted with a certain amount of bromine solution, and then the absorbance of bromine in supernatants was measured. As a classical reaction, bromine can easily react with free vinyl groups. From the results in Figure 2, the concentration of the remaining vinyl groups is estimated as 1.97×10^{-3} mol/g. Therefore, if the vinyl groups are distributed uniformly around the polymer particles, the distance between vinyl groups is estimated as 13.14 Å. Additionally, the results suggest that 19.5% of vinyl groups of the cross-linker did not react and remained in the polymer matrix.

The distances between ionic groups of template molecules and vinyl groups of each ionic complex are given in Table 2. Each distance was calculated for a stable structure in a vacuum using CambridgeSoft Chem3D version 9.0. According to the estimated distance between vinyl groups and the data in Table 2, it is proposed that two vinyl groups of each ionic complex containing the template molecule can attach onto the polymer because the distance between two the vinyl groups is greater than the estimated distance between vinyl groups on the BASE polymer.

Table 3. Retention Time of Each Solute in Polymer Columns[a]

	solutes			
polymer	acetone	TBTA	*m*-TBTA	BTAB
BASE	4.53	3.79	3.85	6.56
BLANK (M)	4.58	4.23	4.41	7.43
TBTA-P (M)	4.60	4.27	4.33	7.22
BLANK (D)	4.40	4.45	4.57	8.15
TBTA-P (D)	4.62	8.34	8.74	15.9

[a] LC conditions: column size: 150 mm × 4.6 i.d.; mobile phase: MeCN/0.4 M $NaCl_{aq}$ (v/v = 7/3); flow rate: 1.0 mL/min; temperature: 30 °C; detection: photodiode array.

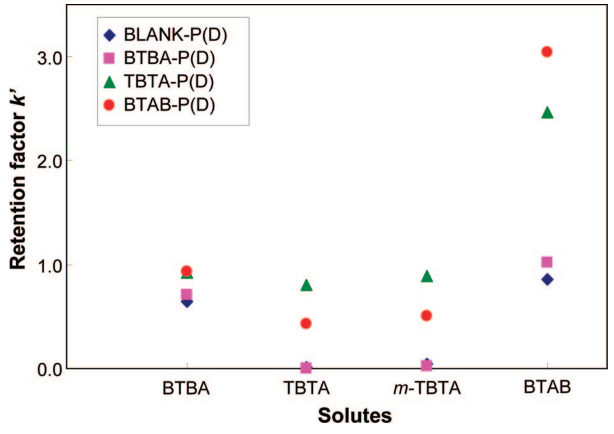

Figure 3. Retention factor of each solute in polymer columns. LC conditions were the same as those listed in Table 3.

Chromatographic Evaluations. To investigate the availability of interval immobilization based on the dispersion method, columns packed with each type of polymer particle were investigated under the same mobile phase conditions. The retention times of the solutes in the prepared columns are summarized in Table 3. From a comparison with the results for the BASE polymer, it is suggested that the ionic groups (SO_3^-) arise from ionic complex incorporated from the other polymer because the retention time of ammonium clearly increased. Furthermore, in comparing the two preparation methods, the retention of ammonium solutes is greater for the polymer prepared using the dispersion method. These results suggest that the ionic groups (SO_3^-) worked effectively on the polymers BLANK (D) and TBTA-P (D) because the functional groups were only on the pore surface whereas there were a large number of dead sites hidden in the network of the polymer prepared by common copolymerization. Here, the yields for BLANK (M) and TBTA-P (M) were almost equivalent. Therefore, we propose that the dispersion preparation method presented in this study is more effective in introducing functional groups to the surface of porous polymers.

The selectivity of each polymer prepared using the dispersion method was evaluated. The retention factors of solutes in prepared columns are presented in Figure 3. In these analyses, aqueous NaCl solution was used as mobile phase because the retentions of the ammonium solutes were too high to measure. As shown in Figure 3, selective retention was observed for each imprinted polymer. The potential retention for BTAB was larger than that for any other solute because of higher hydrophobicity. However, the retention factor for TBTA was higher on TBTA-P than it was on BTAB-P. Similarly, BTAB was much retained on BTAB-P. These results strongly suggest that the interval immobilization of SO_3^- worked effectively for selective recognition. Interestingly, the retention behavior of *m*-TBTA was almost the same as that of TBTA. This suggests that the distance from N^+ to N^+ on *m*-TBTA was sufficiently close to that on TBTA so that *m*-TBTA was also retained at the recognition

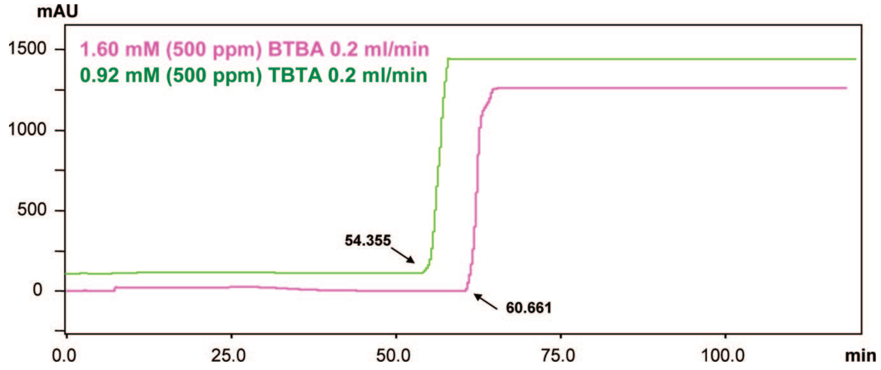

Figure 4. Frontal peaks of BTBA or TBTA on TBTA-P (D). LC conditions: column size: 150 mm × 4.6 mm i.d.; flow rate: 0.2 mL/min; mobile phase: 1.60 mM (500 ppm) BTBA or 0.92 mM (500 ppm) TBTA in MeOH/H_2O (v/v = 7/3); temperature: 40 °C; detection: photodiode array.

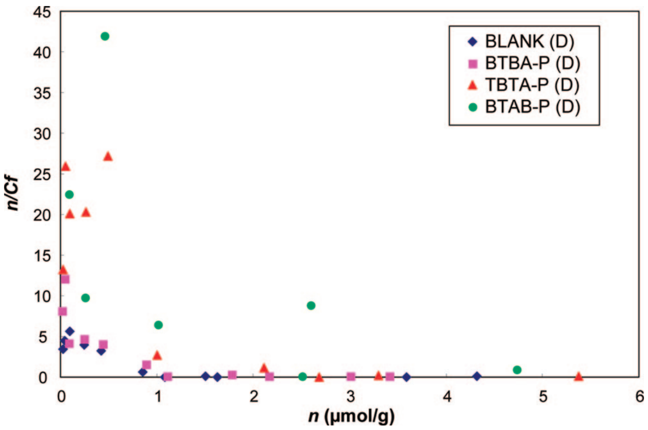

Figure 5. Scatchard plot (n/Cf vs n) for TBTA on TBTA-P (D).

sites of TBTA-P (D). The results also suggest that there were selective recognition sites on each polymer.

Figure 4 shows the results of frontal analyses using TBTA-P (D) with TBTA or BTBA solution having a constant concentration as mobile phase. The results indicate two points of interest: 58.9% of functional monomer (feed) participated in the retention of cationic solute, and 98.5% of attached SO_3^- groups participated in the two-point recognition of TBTA. These results clearly suggest there was selective two-point recognition for TBTA-P (D).

The chromatographic evaluations suggest that the novel dispersion preparation method for the selective recognition of ionic compounds is more useful than the commonly used copolymerization, and selectivity is achieved using this method.

Adsorption Isotherm. Scatchard analyses have been frequently carried out for the estimation of the association constant K_a and number of binding sites N. Here, the Scatchard plots were constructed using

$$n/Cf = -K_a n + K_a N$$

where Cf is the concentration of free solutes in the supernatant from the batch adsorption analyses and n is the number of solute particles bound on the solid state. This evaluation is based on the Langmuir model. The homogeneity of recognition sites is seen from the linearity of the plots. The Scatchard plots for each polymer using TBTA solution are shown in Figure 5. In addition, the estimated association constant and number of binding sites are summarized in Table 4. K_a and N can be estimated from the low- and high-concentration areas in the Scatchard plots.

Table 4. Association Constant and Number of Binding Sites for Each Polymer

	association constant K_a (M^{-1})		number of binding sites N	
polymer	high concn	low concn	high concn	low concn
BLANK (D)	7.74×10^5	4.59×10^6	2.1	0.97
BTBA-P (D)	1.18×10^6	8.23×10^6	1.67	1.06
TBTA-P (D)	1.58×10^6	2.24×10^7	2.76	1.12
BTAB-P (D)	1.20×10^6	1.34×10^7	6.11	1.42

As seen in Table 4, the association constant of TBTA at low concentration was higher for TBTA-P (D) than for any other polymer. Therefore, TBTA-P (D) potentially has selective recognition sites. Furthermore, from the Scatchard plots, we can see that recognition sites for TBTA-P (D) were more homogeneous compared with those for the other polymers. The broad distribution of plots indicates the heterogeneity of the binding sites, especially those on BTAB-P (D).

Adsorption isotherms based on the batch adsorption analyses suggest that the novel dispersion preparation for molecular imprinting effectively provides selective recognition for a template molecule having ionic functional groups.

Application of the Concentration of STX. As discussed above, the newly prepared polymer is effective in selectively recognizing water-soluble ionic compounds. Therefore, we attempted the selective concentration of STX using the prepared polymer. As shown in Table 2, the distance between guanidine groups is similar to the distance from N^+ to N^+ on TBTA (the structure of STX is shown in Figure 6). Therefore, we expect that TBTA-P (D) can also be used for selective separation and/or concentration of STX analogues. From the batch adsorption and solid-phase extraction from blue mussel extracts, it has been suggested that TBTA-P (D) has the ability to selectively recognize STX analogues. As shown in our previous study,[17] the selectivity for STX analogues is higher on TBTA-P (D) than it is on BLANK (D) or BTAB-P (D). Solid-phase extraction analyses have indicated the STX analogue can be completely removed from blue mussel extracts, and the concentrated STX

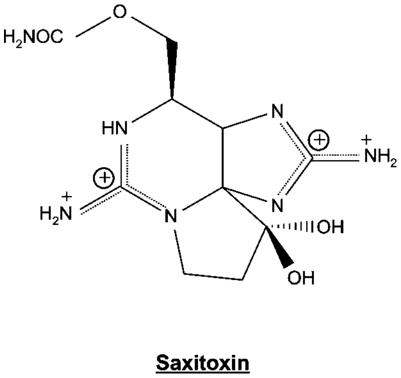

Figure 6. Structure of saxitoxin.

analogue can be completely recovered using 1.0 M ammonium carbonate aqueous solution. Further details were reported in our previous study.

Thus, the polymer prepared using our new dispersion technique can be used for the selective concentration of natural toxins. The recognition ability for STX depended on the distance between ionic groups on template molecules. Therefore, we expect that the dispersion technique described in this paper will be used for the preparation of selective separation media for a large number of water-soluble ionic compounds such as natural toxins, physiologically active substances, and biomolecules.

Conclusion

We reported a novel effective preparation method for the selective recognition of water-soluble ionic compounds using an alternative molecular imprinting technique. Using this technique, sites that recognize the distance between specific ionic groups in the target compounds can be placed on the surfaces of polymer pores by the addition reaction of ionic complex of templates and functional monomers. We examined the possibility of vinyl groups remaining after the reaction with ionic complex and the basic selectivity of each type of polymer particle. Moreover, one of prepared polymers was used for the selective recognition of paralytic shellfish poison, saxitoxin, using the similar distances between ionic groups of the template molecule and saxitoxin. In future works, we will apply our preparation method for the detection of several ionic compounds.

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