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# Synthetic cinchonidine receptors obtained by cross-linking linear poly(methacrylic acid) derivatives as an alternative molecular imprinting technique

Jun Matsui [a,b,c], Norihito Minamimura [a], Kenji Nishimoto [a], Katsuyuki Tamaki [a], Naoki Sugimoto [a,b,c,*]

[a] *Faculty of Science and Engineering, Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan*
[b] *High Technology Research Center, Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan*
[c] *Frontier Institute for Biomolecular Engineering Research (FIBER), Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan*

**Abstract**

A molecular imprinting approach to construct synthetic receptors was examined, wherein a linear pre-polymer bearing functional groups for intermolecular interaction with a given molecule is cross-linked in the presence of the molecule as a template, and subsequent removal of the template from the resultant network-polymer is expected to leave a complementary binding site. Poly(methacrylic acid) (PMAA) derivatized with a vinylbenzyl group as a cross-linkable side chain was utilized as the pre-polymer for the molecular imprinting of a model template, (−)-cinchonidine. Selectivity of the imprinted polymer was evaluated by comparing the retentions of the original template, (−)-cinchonidine and its antipode (+)-cinchonine in chromatographic tests, exhibiting a selectivity factor up to 2.4. By assessment of the imprinted polymers in a batch mode, a dissociation constant at 20 °C for (−)-cinchonidine was estimated to be $K_d = 2.35 \times 10^{-6}$ M (the number of binding sites: $4.54 \times 10^{-6}$ mol/g-dry polymer). The displayed affinity and selectivity appeared comparable to those of an imprinted polymer prepared by a conventional monomer-based protocol, thus showing that the pre-polymer, which can be densely cross-linked, is an alternative imprinter for developing template-selective materials. (−)-Cinchonidine-imprinted polymers were prepared and assessed using the pre-polymers bearing different densities of the vinylbenzyl group and different amounts of the cross-linking agent to examine the appropriate density of the cross-linking side chain that was crucial for developing the high affinity and selectivity of the imprinted polymers.


*Keywords:* Molecular imprinting; Cinchonidine; Poly(methacrylic acid)

## 1. Introduction

Many analytical systems have effectively utilized biomolecules, such as enzymes and antibodies. These biological macromolecules display a highly specific recognition of their ligands due to complementary binding sites that are constructed by the elaborate folding of polypeptides. Inspired by biological systems, attempts have been made for obtaining synthetic polymers having binding sites complementary to a given molecule by molecular imprinting [1]. A typical protocol of molecular imprinting can be summarized as follows: a monomer having an interaction with a target molecule, i.e., a functional monomer, is selected as the imprinter; the functional monomer is mixed with the target molecule as a template to form complexes by covalent or non-covalent bonding; polymerization is conducted with a cross-linking agent to construct a three-dimensional network-polymer; and the template molecule is removed from the cross-linked polymer to realize a binding site complementary to the template molecule. While use of the functional monomers have been extensively studied for synthesizing molecularly imprinted polymers (MIPs), only a few polymers have been examined as an imprinter such as amylose cross-linked with cyanuric chloride [2], chitosan cross-linked with epichlorohydrin [3], poly(4-vinylpyridine) cross-linked with diboromobutane [4], proteins and synthetic polymers precipitated in organic solvents [5], thermo-responsive acrylamide copolymers cross-linked with S–S bonds [6], and oligopeptides embedded in a polymeric membrane [7]. Although these MIPs exhibited many

* Corresponding author. Tel.: +81-78-431-4341; fax: +81-78-435-2539.
*E-mail address:* sugimoto@konan-u.ac.jp (N. Sugimoto).

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attractive features in their synthesis and performance, the specificity was moderate compared to functional monomer-based MIPs especially when organic molecules were the targets, probably due to no cross-linking or a deficient degree of cross-linking. However, it is quite important to further examine linear polymers as imprinters because it is potentially useful for attaining template—imprinter complexes upon multiple hydrogen bonding/electrostatic interactions even in polar, protic media as observed in biomolecular systems [8].

We postulated that linear polymers should work as imprinters that can be cross-linked by radical polymerization, which is recognized as the most successful chemical reaction for constructing MIPs [1b]. In this study, linear pre-polymers bearing side chains for interaction with a template molecule (a carboxyl group) and for cross-linking (a vinyl group) was tested as imprinters using conventional systems for the preparation and assessment of the (−)-cinchonidine-imprinted polymer (pre-polymer-based imprinting for developing functional macromolecules that work in aqueous media or hydrophilic organic solvents is also being studied [9]), in a protocol in which the pre-polymers are cross-linked in the presence of a template molecule (Fig. 1). A cinchona alkaloid (−)-cinchonidine (for cinchona alkaloids in asymmetric catalysis, see [10]; for cinchona alkaloids as anti-malaria drugs, see [11]) and poly(methacrylic acid) (PMAA) derivatives were employed as a model template molecule and pre-polymers, respectively, to examine the proposed imprinting protocol because the imprint of (−)-cinchonidine using a carboxylic monomer, methacrylic acid had been already studied [12,13]. Pre-polymers as imprinters were obtained by simple modification of a commercially available polymer, poly(methacrylic acid), with cross-linkable vinylbenzyl group, to demonstrate that the present strategy can be generally applied to utilize various ordinary linear-polymers for molecular imprinting. Pre-polymers with different vinyl group densities, co-used with different amounts of the cross-linker, were examined for the MIP synthesis to explore the importance of the cross-link for imprinting the template molecule.

## 2. Experimental

### 2.1. Materials and instruments

Acetic acid, 2,2′-azobis(isobutironitrile) (AIBN), chloroform, (−)-cinchonidine, (+)-cinchonine, dimethyl sulfoxide (DMSO) and ethylene glycol dimethacrylate (EDMA) were purchased from Wako Pure Chemicals (Osaka, Japan). 4-Vinylbenzyl chloride, 4-methylbenzyl chloride and 1,8-diazacyclo-[5.4.0]-7-undecene (DBU) were purchased from Aldrich. Acetonitrile and methanol were obtained from Kanto Chemical (Tokyo, Japan). Chloroform, DMSO and EDMA were purified before use by standard procedures [14]. Poly(methacrylic acid) was obtained by acidifying an aqueous solution of poly(methacrylic acid sodium salt) (Aldrich, average $M_w$: ca. 6500, average $M_n$: ca. 4000). The obtained precipitate was thoroughly rinsed with water, dried in vacuo, and purified by reprecipitation in acetone/ethanol. Conversion of a carboxylate anion to a carboxyl group was confirmed by FT-IR (C=O, 1698 $cm^{-1}$). The chromatographic experiments were performed at 30 °C with a Waters HPLC system consisting of an Alliance 2690 Separations Module and a 2487 UV-absorbance detector. For reversed phase HPLC, a Waters XTerra RP18 column (5 μm, 4.6 mm × 150 mm) was used with acetonitrile−water as the eluent.

### 2.2. Preparation of linear pre-polymers

A typical procedure for preparing the pre-polymer PP1 (see Fig. 1 and Table 1): Into 54 ml of DMSO were added PMAA (1.8 g, –COOH: 21 mmol), 4-vinylbenzyl chloride (5.25 mmol), 4-methylbenzyl chloride (12.6 mmol), and 1,8-diazacyclo-[5.4.0]-7-undecene (2.71 g, 17.85 mmol). The mixture was stirred at 40 °C for 2.5 h, then poured

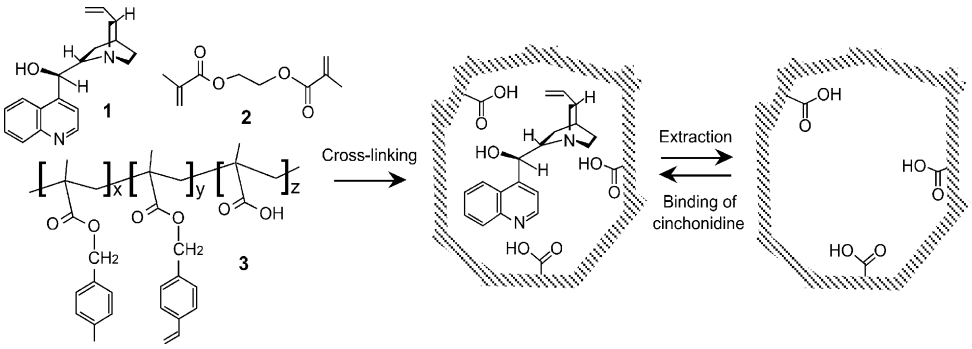


Fig. 1. Schematic representation of molecular imprinting utilizing linear pre-polymers for synthesizing (−)-cinchonidine-imprinted polymers: (1) (−)-cinchonidine as the template species; (2) ethylene glycol dimethacrylate (EDMA) as the cross-linker; (3) pre-polymers. Six kinds of pre-polymers, PP1–PP6, were prepared and used for synthesizing the imprinted polymers. For the ratios of *x*, *y* and *z* of each pre-polymer (see Table 1). The thick dashed lines represent a polymer network constructed by pre-polymers and EDMA.

Table 1
Pre-polymers prepared in this study as precursors of molecularly imprinted polymers

| Pre-polymer | 4-VBC[a] | 4-MBC[a] | $x$[b] | $y$[b] | $z$[b] |
|---|---|---|---|---|---|
| PP1 | 5.25 (0.25) | 12.60 (0.60) | 0.24 | 0.57 | 0.19 |
| PP2 | 10.50 (0.50) | 7.35 (0.35) | 0.47 | 0.32 | 0.21 |
| PP3 | 12.60 (0.60) | 5.25 (0.25) | 0.58 | 0.22 | 0.2 |
| PP4 | 14.70 (0.70) | 3.15 (0.15) | 0.65 | 0.14 | 0.21 |
| PP5 | 15.75 (0.75) | 2.10 (0.10) | 0.72 | 0.09 | 0.19 |
| PP6 | 17.85 (0.85) | 0 | 0.77 | 0 | 0.22 |

[a] The amount (mmol) of the reagents used with 21 mmol of poly(methacrylic acid) for preparing the corresponding pre-polymers. The parenthesized figures are the molar ratios of the reagents to poly(methacrylic acid) (21 mmol) used for the preparation.
[b] Ratios of three kinds of side chains, *x*, *y* and *z* were determined by quantitating unreacted 4-vinylbenzyl chloride and methylbenzyl chloride by reversed phase HPLC. For structures of the pre-polymers (see Fig. 1).

into water to precipitate the product. The product was exhaustively washed with water, with acetic acid–methanol, then with methanol. After drying under reduced pressure, the product was purified by reprecipitation in hexane/THF. Identification was made by $^1$H NMR (DMSO-$d_6$) and FT-IR: 0.69, 0.92 (s, –$CH_3$), 1.5–2.4 (–$CH_2$–), 2.3 (s, Ph–$CH_3$), 4.8 (s, O–$CH_2$–Ph), 5.2, 5.7 (d, –CH=$CH_2$), 6.6 (t, –CH=$CH_2$), 7.0–7.3 (–$C_6H_4$–), 12.4 (–COOH); 1730 $cm^{-1}$ (C=O), 1630 $cm^{-1}$ (C=C). Other pre-polymers, PP2–PP6, were prepared in the same fashion using the corresponding amounts of 4-vinylbenzyl chloride and 4-methylbenzyl chloride as summarized in Table 1.

### 2.3. Synthesis of molecularly imprinted polymers (MIPs)

A typical procedure for preparing the imprinted polymer IP1 (Table 2): 9.3 mg (32 μmol) of the template (−)-cinchonidine was dissolved in 7.5 ml of chloroform in a screw-capped glass tube. Into the solution were added 475 mg of PP1, 2.55 g of the cross-linking agent EDMA and 26.5 mg of the polymerization initiator AIBN. After nitrogen gas was sparged into the mixture for 5 min, the glass tube was sealed and kept in a water bath at 60 °C for 5 h. The obtained polymer was crushed and washed by Soxhlet extraction with methanol (recovery of (−)-cinchonidine >90%). After being dried, the polymer was ground in a mortar to pass through a 63 μm sieve. Fine particles were removed using a 32 μm sieve. The resulting particles were used for the chromatographic studies. Other imprinted polymers, IP2–IP6, were similarly prepared using PP2–PP6, respectively (Table 2). The non-imprint blank polymers, BP1–BP6, were prepared without the addition of the (−)-cinchonidine template. Imprinted polymers, IP4a–IP4e, were prepared using the pre-polymer PP4 with the addition of different amounts of the template. The imprinted polymers, IP4(200), IP4(75), IP4(50) and IP4(0), and their corresponding non-imprint polymers were also prepared by cross-linking PP4 with different amounts of EDMA.

Table 2
Imprinted (IP) and non-imprint blank (BP) polymers prepared and examined in this study

| Polymer | Template (μmol)[a] | Pre-polymer (mg)[a,b] | Cross-linker (mmol) |
|---|---|---|---|
| IP1 | CD (60) | PP1 (536) | 13 |
| IP2 | CD (60) | PP2 (506) | 13 |
| IP3 | CD (60) | PP3 (529) | 13 |
| IP4 | CD (60) | PP4 (496) | 13 |
| IP5 | CD (60) | PP5 (564) | 13 |
| IP6 | CD (60) | PP6 (467) | 13 |
| BP1 | None | PP1 (536) | 13 |
| BP2 | None | PP2 (506) | 13 |
| BP3 | None | PP3 (529) | 13 |
| BP4 | None | PP4 (496) | 13 |
| BP5 | None | PP5 (564) | 13 |
| BP6 | None | PP6 (467) | 13 |
| IP4a | CD (15) | PP4 (496) | 13 |
| IP4b | CD (30) | PP4 (496) | 13 |
| IP4c | CD (120) | PP4 (496) | 13 |
| IP4d | CD (240) | PP4 (496) | 13 |
| IP4e | CD (480) | PP4 (496) | 13 |
| IP4(200) | CD (60) | PP4 (496) | 26 |
| IP4(75) | CD (60) | PP4 (496) | 9.75 |
| IP4(50) | CD (60) | PP4 (496) | 6.5 |
| IP4(0) | CD (60) | PP4 (496) | 0 |
| BP4(200) | None | PP4 (496) | 26 |
| BP4(75) | None | PP4 (496) | 9.75 |
| BP4(50) | None | PP4 (496) | 6.5 |
| BP4(0) | None | PP4 (496) | 0 |

CD: (−)-cinchonidine.
[a] The amounts of the template and the pre-polymers are those used with 13 mmol of the cross-linker, ethylene glycol dimethacrylate (EDMA).
[b] The amounts of the pre-polymers were adjusted for each pre-polymer to contain 0.6 mmol of carboxylic group.

### 2.4. Chromatographic assessment of the imprinted polymers

The polymer particles were slurried in chloroform–acetonitrile (1:1, v/v) and packed in stainless-steel column tubes (150 mm × 4.6 mm i.d.). The columns were washed with methanol–acetic acid (7:3, v/v). The column was then washed with acetonitrile–acetic acid (99:1, v/v) until a stable baseline was obtained. Chromatographic measurements were conducted using acetonitrile–acetic acid (99:1, v/v) as the eluent at a flow rate of 1.0 ml/min at 30 °C and the detection was made at 280 nm. The injection volume was 20 μl and the sample concentration was 0.5 mM. Each sample was independently injected. A capacity factor ($k'$) was calculated using the equation, $k' = t_R/(t_R - t_0)$, where $t_R$ is the retention time of (−)-cinchonidine or (+)-cinchonine and $t_0$ the retention time of the void marker, acetone. The selectivity factor, $k'_{cd}/k'_{cn}$, was used for evaluating the selectivity, where $k'_{cd}$ and $k'_{cn}$ are the capacity factors of (−)-cinchonidine and (+)-cinchonine, respectively.

### 2.5. Estimation of affinity of the imprinted polymers

The polymer particles were washed with methanol–acetic acid (7:3, v/v) then methanol in a stainless-steel column. The removal of the template was monitored by reversed phase HPLC, and no extraction of the template was finally confirmed. After drying in vacuo, the polymer (10 mg) was immersed in a 1.0 ml acetonitrile solution of (−)-cinchonidine of various concentrations ranging from 5.0 μm to 2.0 mM at 20 °C. After the incubation for 18 h, the sample tubes were centrifuged. Aliquots of the supernatant were taken and analyzed by HPLC to quantify the concentration of free (−)-cinchonidine, $F$. The amount of (−)-cinchonidine bound to the polymer, $B$, was calculated by subtracting $F$ from the initial (−)-cinchonidine concentration. Three independent batches were tested for each concentration and the quantification by HPLC was done in triplicate for each batch. Three independent batches were prepared and tested for each concentration and the quantification by HPLC was done in triplicate for each batch. The average data were used for subsequent analysis. For the Scatchard analysis, $B/F$ is plotted versus $B$ according to the equation, $B/F = (B_{max} - B)/K_d$, where $K_d$ is the equilibrium dissociation constant and $B_{max}$ the apparent maximum number of binding sites. Estimation of $K_d$ and $B_{max}$ were also performed using the commercially available software, LIGAND [15].

## 3. Results and discussion

### 3.1. Design of linear functional pre-polymer

Poly(methacrylic acid) was employed as a precursor of the pre-polymers because the methacrylic acid monomer was known to be one of the most promising functional monomers based on the previous molecular-imprinting studies [1]. Pre-polymers were designed to possess both carboxyl and 4-vinylbenzyl groups (Fig. 1). The former group was expected to form complexes with the model template, (−)-cinchonidine during the cross-linking process via hydrogen bonding and/or electrostatic interaction for composing binding sites complementary to the template. The latter was expected to engage in the cross-linking process for anchoring the carboxyl groups of the pre-polymers in the resultant polymer networks. Pre-polymers were prepared by partial esterification of PMAA with 4-vinylbenzyl chloride and 4-methylbenzyl chloride in the presence of DBU [16]. To investigate the effects of the cross-linkable 4-vinylbenzyl group density on the potency of the pre-polymers for developing affinity and selectivity, four kinds of pre-polymers bearing different 4-vinylbenzyl group densities were prepared using various concentrations of 4-vinylbenzyl chloride (Table 1). To provide a fair comparison, 4-methylbenzyl chloride was simultaneously used for the preparation to make the six pre-polymers, PP1–PP6, bear the same densities of carboxyl group. A molecularly imprinted polymer was synthesized using each pre-polymer independently. (−)-Cinchonidine was employed as a model template species for assessing the imprinting protocol utilizing pre-polymers, because it was known as a steady protocol that (−)-cinchonidine is imprinted by sole use or cooperative use of a methacrylic acid monomer [12,13]. Molecular imprinting was conducted using the pre-polymers, the amount of carboxyl groups in which was approximately 2.5–40 times greater than that of (−)-cinchonidine. The excess carboxyl group was expected to convert (−)-cinchonidine to the complex species with the pre-polymers in the pre-polymerization mixture, allowing most of the (−)-cinchonidine to be involved in the imprinting process. Non-imprint blank polymers were identically synthesized without adding the template species, and were compared to the corresponding MIPs for assessing the binding property induced by the imprint effects.

### 3.2. Retention ability of the imprinted polymers induced by addition of the template

The imprinted polymers were prepared using different amounts of the template, and were assessed by chromatography to confirm that the addition of the template was effective for inducing affinity to the resultant cross-linked polymers. The retention properties of seven kinds of imprinted polymers, IP4, IP4a–IP4e, and the blank polymer, BP4, are summarized in Table 3. All the imprinted polymers, which were prepared in the presence of the template, exhibited a longer retention of the template than BP. Furthermore, the imprinted

Table 3
Assessment of retention property the imprinted polymers prepared using less amount of cross-linker

| Polymer | Template (μmol) | Template:–COOH (pre-polymer)[a] | Retention factor | | Selectivity factor $k'_{cd}/k'_{cn}$ |
|---|---|---|---|---|---|
| | | | Cinchonidine ($k'_{cd}$) | Cinchonidine ($k'_{cn}$) | |
| BP4 | 0 | – | 0.65 | 0.69 | 0.95 |
| IP4a | 15 | 1:40 | 3.8 | 2.6 | 1.4 |
| IP4b | 30 | 1:20 | 6.6 | 3.2 | 2.1 |
| IP4 | 60 | 1:10 | 8.8 | 3.6 | 2.4 |
| IP4c | 120 | 1:6.7 | 3.7 | 2.2 | 1.7 |
| IP4d | 240 | 1:5.0 | 2 | 1.3 | 1.5 |
| IP4e | 480 | 1:2.5 | 0.91 | 0.62 | 1.5 |

[a] Molar ratio.

polymers retained the original template, (−)-cinchonidine, significantly longer than its antipode (+)-cinchonine, proving that the addition of the template was effective for developing selective binding sites. The longest retention and the highest selectivity were marked by IP4, which was prepared by employing the template and the carboxyl side chain at a molar ratio of 1:10. This confirms the assumption that excess carboxyl groups are necessary for imprinting the template. Using the smaller amounts of the template, however, the capacity factor of cinchonidine, $k'_{cd}$, significantly decreased with reduced selectivity. This could be due to a smaller number of binding sites produced by the template and a larger number of carboxyl groups that was not engaged in complexation with the template and may have formed non-specific adsorption points. One the other hand, too many template molecules would interfere with the multiple-point interaction based complexation between the template and the pre-polymer, which is believed to be the origin of the affinity and selectivity of the MIPs.

### 3.3. Assessment of imprinted polymers by comparison with non-imprint polymers

Evaluation of the cross-linked polymers, prepared using different pre-polymers, was chromatographically conducted. The retention characteristics of the imprinted polymers, IP1–IP6, and the non-imprint polymers, BP1–BP6, are shown in Fig. 2. The imprinted polymers, IP2–IP6, showed a considerably long retention of the template, as compared to their corresponding non-imprinted polymers, BP2–BP6, respectively. Furthermore, the retention appeared to be selective to the template species. Selectivity factors, the ratios of the capacity factor of the template to that of the antipode, marked by the imprinted polymers ranges from 1.5 to 2.4. The (−)-cinchonidine-imprinted polymer conventionally prepared with the MAA monomer has recently been reported to exhibit a separation factor of 2.02 [13]. Although

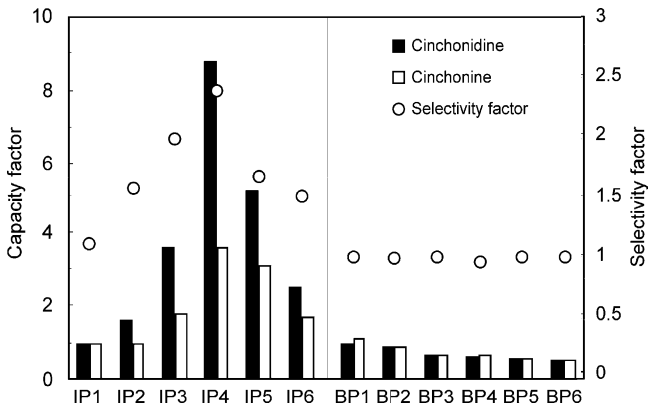


Fig. 2. Retention behaviors of (−)-cinchonidine-imprinted (IP) and non-imprint blank (BP) polymers: filled bar, capacity factor for (−)-cinchonidine ($k'_{cd}$); blank bar, capacity factor for (+)-cinchonine ($k'_{cn}$); circle, selectivity factor as $k'_{cd}/k'_{cn}$.

direct comparison may not be made because of the inconsistent conditions for the synthesis and the chromatography, the pre-polymer-based protocol appeared to be useful for synthesizing polymers selective for the template molecule.

While IP4 marked the highest selectivity among the four MIPs, IP1 displayed a poor retention and selectivity that was comparable to the corresponding non-imprint polymer BP1. Because all the imprinted polymers bear almost the same density of carboxylic residues, these results can be conceived to reflect the potency of the pre-polymers for developing affinity and selectivity. An apparent difference among the imprinted polymers is the density of the vinylbenzyl group introduced into the pre-polymers that is expected to participate in the cross-linking with EDMA. It has been known that sufficient cross-linking is essential to preserve the alignment of carboxyl groups adjusted by the molecular imprinting procedure [17]. Therefore, the shorter retention without a significant selectivity observed in IP1 could be accounted for by the unsuccessful molecular imprinting due to a lack of cross-linking. As seen in IP5 and IP6, however, the imprinted polymers prepared with the higher density of vinylbenzyl groups exhibited a shorter retention. These results suggest that an exceedingly cross-linked polymer network could cause less accessible carboxylic moieties.

While the selectivity for (−)-cinchonidine was clearly observed in the imprinted polymers, the corresponding non-imprint blank polymers exhibited no selective retention behaviors, as shown in Fig. 2. Furthermore, the capacity factors are considerably smaller than those marked by the corresponding imprinted polymers. A trend can be seen such that the blank polymers prepared with pre-polymers bearing a lower density of vinyl groups showed a longer retention of the template. These results support the assumption that dense cross-linkable side chains in pre-polymers could result in too tight a polymer network into which the substrate can not easily penetrate (in the case of polymer gels prepared with only a small amount of cross-linking agents, it has also been reported that the increasing concentration of the cross-links results in a decrease of the non-imprint gels' affinity. The affinity of the imprinted gel, however, is affected by the cross-link concentration in a different manner, as compared to our highly cross-linked imprinted polymers [18]). Thus, it is again highlighted that a compromise is well made in IP4 between the rigidity for maintaining the complementary structure and the flexibility for allowing reversible binding of the template to the binding sites.

Assessment of the imprinted polymers was also conducted in the batch mode. Saturation experiments were conducted on IP1, IP4, IP6 and BP4 [19]. Scatchard plots (not shown) for the imprinted polymers exhibited non-linear profiles as commonly observed in batch tests of previous imprinted polymers [13,20], suggesting that the binding sites are heterogeneous in terms of affinity. In the analysis of the binding data by LIGAND [15], a theoretical curve based on a two-site model fit the closest to the actual binding data, whereas a one-site model was suited for analyzing the data

Table 4
Dissociation constants ($K_d$) and maximum number of binding sites ($B_{max}$) of the imprinted and non-imprint polymers

| Polymer | High-affinity sites | | Low-affinity sites | |
|---|---|---|---|---|
| | $K_d$ (M) | $B_{max}$ (mol/g) | $K_d$ (M) | $B_{max}$ (mol/g) |
| IP1 | $(3.96 \pm 0.60) \times 10^{-6}$ | $(4.47 \pm 0.30) \times 10^{-6}$ | $(3.44 \pm 0.33) \times 10^{-4}$ | $(2.12 \pm 0.045) \times 10^{-5}$ |
| IP4 | $(2.35 \pm 0.69) \times 10^{-6}$ | $(4.54 \pm 0.63) \times 10^{-6}$ | $(1.30 \pm 0.22) \times 10^{-4}$ | $(1.61 \pm 0.59) \times 10^{-5}$ |
| IP6 | $(4.07 \pm 0.71) \times 10^{-6}$ | $(6.08 \pm 0.51) \times 10^{-6}$ | $(2.71 \pm 0.45) \times 10^{-4}$ | $(1.76 \pm 0.063) \times 10^{-5}$ |
| BP4 | – | – | $(1.11 \pm 0.17) \times 10^{-4}$ | $(1.38 \pm 0.99) \times 10^{-5}$ |

A one-site model was applied to analysis of BP4.

of BP4. This suggests that carboxylic moieties were randomly located in the blank polymers and is consistent with the no selective retention by the blank polymers. The dissociation constants ($K_d$) and the theoretical numbers ($B_{max}$) of the high-affinity binding sites are summarized in Table 4. As expected, the imprinted polymer IP4, which exhibited the longest retention of the template, showed the highest affinity to the template. Selectivity, observed in the chromatography, was also confirmed by the difference in the affinity between (−)-cinchonidine and (+)-cinchonine; the dissociation constant for (+)-cinchonidine was $1.03 \times 10^{-5}$ M. Affinity ($K_d$) of the low-affinity binding site ($1.30 \times 10^{-4}$ M) was quite similar to that of the blank polymer, suggesting that the low-affinity binding site consisted of one carboxylic moiety. The other imprinted polymers, IP1 and IP6, exhibited significantly lower affinities, as compared to IP4, which was reflected in the chromatographic results. The lower affinity of IP1 supports the assumption that the density of the vinylbenzyl side chains in PP1 was not sufficient to freeze the location of the carboxylic moieties guided by the template. Although the lower affinity of IP6 is inconsistent with the parallel discussion, it can be speculated that the self-organized location of the carboxylic moieties could be strained and unsuitably immobilized by cross-links too dense.

### 3.4. Effects of cross-linker on affinity of the imprinted polymers

As discussed in the previous paragraph, the density of cross-links was found to be crucial for developing affinity and selectivity. Therefore, the amount of the cross-linker was examined. Imprinted polymers, IP4(200), IP4(75), IP4(50) and IP4(0), were prepared using different amounts of the cross-linker which were 200, 75, 50 and 0% of the original amount used for preparing IP4, respectively. The retention properties of IP4(200), IP4, IP4(75) and IP4(50) are summarized in Table 5. IP4(0) was chromatographically not examined because the polymer was too soft to be ground and packed in an LC column. Therefore, the dissociation constant and the theoretical number of binding sites were estimated on IP4, IP4(50) and IP4(0), as summarized in Table 6. In the chromatographic results, a trend can be seen both in the imprinted and the non-imprint polymers that less of the cross-linker resulted in a longer retention of the template. This is attributed to the fact that polymers prepared with a lower amount of the cross-linker contain more carboxylic moieties in a unit weight, which is supported by the saturation test; the number of binding sites in IP4(50) is about 1.7 times greater than that of IP4(100). Although the decreasing affinity was marked by IP4(50) and IP4(0), selectivity was still exhibited by IP4(50). Speculating from the results of the saturation test, IP4(0) could be as selective as IP4(50). Using no cross-linker has the merit of producing imprinted polymers with a high density of active sites that is required for practical applications. On the other

Table 5
Assessment of affinity of the imprinted polymers prepared by cross-linking PP4 with use of different amounts of the cross-linker EDMA

| Polymer | Capacity factor | | Selectivity factor $k'_{cd}/k'_{cn}$ |
|---|---|---|---|
| | Cinchonidine ($k'_{cd}$) | Cinchonidine ($k'_{cn}$) | |
| IP4 | 8.8 | 3.6 | 2.4 |
| IP(200) | 2.8 | 2 | 1.4 |
| IP4(75) | 11.7 | 4.3 | 2.7 |
| IP4(50) | 15.4 | 4.7 | 3.3 |
| BP4 | 0.65 | 0.69 | 0.95 |
| BP(200) | 0.16 | 0.16 | 1.0 |
| BP4(75) | 1.1 | 1.1 | 1.0 |
| BP4(50) | 2 | 1.9 | 1.0 |

Table 6
Assessment of retention property the imprinted polymers prepared using less amounts of the cross-linker

| Polymer | High-affinity sites | | Low-affinity sites | |
|---|---|---|---|---|
| | $K_d$ (M) | $B_{max}$ (mol/g) | $K_d$ (M) | $B_{max}$ (mol/g) |
| IP4 | $(2.35 \pm 0.69) \times 10^{-6}$ | $(4.54 \pm 0.63) \times 10^{-6}$ | $(1.30 \pm 0.22) \times 10^{-4}$ | $(1.61 \pm 0.59) \times 10^{-5}$ |
| IP4(50) | $(6.11 \pm 1.62) \times 10^{-6}$ | $(7.81 \pm 1.19) \times 10^{-6}$ | $(4.54 \pm 0.91) \times 10^{-4}$ | $(5.16 \pm 0.30) \times 10^{-5}$ |
| IP4(0) | $(6.45 \pm 1.01) \times 10^{-6}$ | $(3.75 \pm 0.40) \times 10^{-5}$ | $(4.69 \pm 0.62) \times 10^{-4}$ | $(2.22 \pm 0.069) \times 10^{-4}$ |

hand, focusing upon the aim of developing a high affinity, the cross-linker appeared crucial in the case of molecular imprinting with the pre-polymer PP4 (although it was not conducted in this study, a comprehensive investigation on the density of a vinylbenzyl group in the pre-polymer, the amount of the cross-linker and the amount of the template should be made by a high-throughput screening protocol to determine the optimal conditions for each molecular imprinting system [21]).

## 4. Conclusions

Linear pre-polymers were found to be potentially useful for synthesizing substrate-selective polymers by molecular imprinting, and the affinity and selectivity of the resultant MIPs appeared to be comparable to those of MIPs synthesized in the conventional fashion using a methacrylic acid monomer. A merit of pre-polymers over functional monomers would be that various functionalities can be introduced into a single pre-polymer molecule by modification of the parent linear polymer, e.g., poly(methacrylic acid), and that such pre-polymers may produce MIPs showing multiple functions, e.g., multiple-point interactions, catalytic activity and sensing. Such design and synthesis could also be conducted by mixing multiple functional monomers having different roles [21–23]. Taking the monomer-based tactics into account, however, a functional group having no strong interaction with a template molecule cannot be located near the template molecule, while it would be possible in principle, adopting the pre-polymer-based strategies, to locate any functional group connected to linear polymers complexed with the template molecule. The present study showed that the simple derivatization of the commercially available polymer was a convenient approach to prepare pre-polymers bearing multiple side chains which were assigned for interacting with the template molecule, masking carboxyl groups, and cross-linking the pre-polymers.

Additional merits will be expected from pre-polymers as compared to the monomer-assembly-based imprinting. For instance, linear polymers would be entropically favorable, especially when a template is a macromolecule, to form simultaneous multiple bonds with a template molecule that are essential for constructing highly specific binding sites [24], and could provide reaction fields such as a hydrophobic space for hydrogen bonding/electrostatic interaction in aqueous media [8]. Synthesis of MIPs utilizing these advantages is ongoing in our laboratory [9].

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