

Molecularly Imprinted Polymers with Signaling Function Based on the UV–Vis Spectral Change by Diastereoselective Binding Events

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Signaling cinchonidine-imprinted polymers were prepared using a polymerizable iron(III) porphyrin and/or methacrylic acid as functional monomer(s), and evaluated by chromatographic tests, Scatchard analysis, and spectroscopic analysis. An imprinted polymer prepared with an equimolar amount of the iron(III) porphyrin monomer to cinchonidine and six equivalents of methacrylic acid gave the strongest binding ($K_a = 5.7 \times 10^5$ M^{-1}) and highest selectivity ($\alpha = 14.6$) for cinchonidine against its diastereomer, cinchonine. Thus, it was concluded that the iron(III) porphyrin and methacrylic acid residues worked cooperatively at the imprinted binding site. There is also the added advantage that the porphyrin provides a signaling function proportional to the chiral binding event of cinchonidine. A linear relationship was observed between the UV–vis absorbance of the imprinted polymer and the logarithm of cinchonidine concentrations, indicating that the polymer may be used as a selective cinchonidine sensing material. Moreover, various metalloporphyrins can be used as functionalized monomers in order to prepare functional MIPs for a wide range of target compounds capable of coordination with such metalloporphyrins, even if the target compounds have no characteristic UV–vis absorption or fluorescence.

Molecularly imprinted polymers (MIPs) are attractive materials due to the elimination of complicated molecular designs, ease of syntheses, effective molecular recognition, and high selectivity for target molecules.[1–7] MIPs are formed by complexes comprised of a target molecule with a functional monomer(s) that are fixed by polymerization with appropriate crosslinking agent(s). Removal of the target molecule leaves cavities complementary to the target molecule produced in the resulting polymer matrices. MIPs have excellent stability due to the high level of crosslinking, and can be used as artificial receptors without the restrictions that bio-macromolecules possess.

Although molecular imprinting was first reported in 1931,[8] the number of papers regarding MIPs has only rapidly increased in the last decade. Two major applications of MIPs have been reported: as stationary phases for liquid chromatography, and as sorbents for solid-phase extraction and binding assays. In recent years, MIPs have been investigated for the incorporation of functions in addition to molecular recognition, which will allow MIPs to play an important role in the construction of tailor-made sensors toward desired molecules.[9–15]

We have reported that MIPs having binding sites with zinc(II) porphyrins residues exhibit fluorescence quenching by the selective and strong binding of 9-ethyladenine[16] and cinchonidine (CD).[17] The porphyrins can coordinate to various metal ions to give metalloporphyrins[18] that exhibit various functions that play important roles in functionalized MIPs. The chemical and physical properties of metalloporphyrins strongly depend on the kinds and valences of coordinated metal ions. The zinc(II) porphyrin-based MIPs have been utilized as fluorescence sensors based on fluorescence quenching; however, the fluorescence of porphyrins is limited to zinc(II) and magnesium(II), so that the use of metalloporphyrins does not always work as a fluorescent probe due to the lack of fluorescence by other metalloporphyrins.

Metalloporphyrins have characteristic UV–vis spectra that are independent of the coordinated metal ions, in which specific Soret band and Q bands in long-wavelength regions can be utilized for sensing based on the coordination of axial ligands. Herein, we report on signaling CD imprinted polymers, using a combination of two types of monomers, chloro[5,10,15,20-tetrakis(4-methacryloyloxyphenyl)porphyrinato]iron(III) (Scheme 1) as a signaling monomer and methacrylic acid (MAA) as a stereo-determining monomer.

Experimental

Chemicals. Cinchonidine (CD) and cinchonine (CN), chloroform, pyridine, methacrylic acid, and ethylene dimethacrylate (EDMA) were purchased from Wako Pure Chemical Industry (Osaka, Japan). Other reagents and solvents were obtained from commercial sources and used without further purification.

Preparation of the Iron(III) Porphyrin Monomer. 5,10,15,20-Tetrakis(4-hydroxyphenyl)-21*H*,23*H*-porphinato iron(III) chloride **1** was prepared using a mixture of 5,10,15,20-tetrakis(4-hydroxyphenyl)-21*H*,23*H*-porphine (0.5 g, 0.7 mmol), iron(II) chloride tetrahydrate (3 g, 15 mmol), and 2,6-lutidine (3 mL, 26 mmol) in DMF, which was stirred at 95 °C for 2 h under nitrogen. The solution was cooled to room temperature, then added to 5% hydrochloric acid, and the resulting precipitate was collected and washed with water. The crude iron complex was purified by

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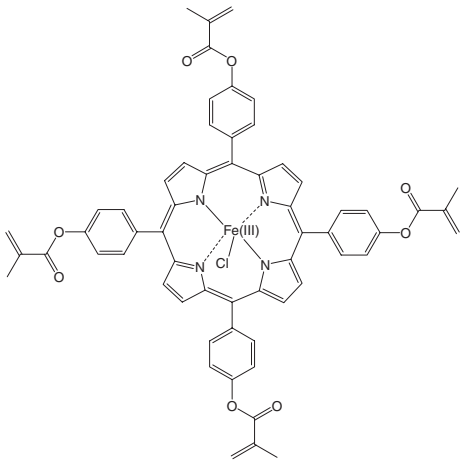
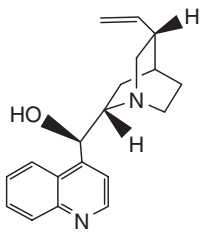
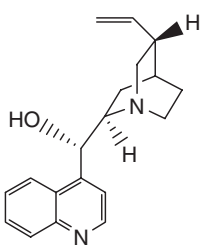
silica-gel chromatography (ethyl acetate/hexane, 2:1, v/v) to yield **1** as a violet solid (0.3 g, 0.4 mmol, 59%). UV/vis (CH_2Cl_2) λ_{max} (relative intensity) 418 nm (1.000), 512 nm (0.058), 572 nm (0.044), 609 nm (0.024), 648 nm (0.016). Subsequently, **1** was treated with methacryloyl chloride to obtain chloro[5,10,15,20-tetrakis(4-methacryloyloxyphenyl)porphyrinato]iron(III) **2** (Scheme 1). A solution of methacryloyl chloride (0.78 mL, 8 mmol) in diethyl ether (18 mL) was added dropwise to a solution of **1** (0.3 g, 0.4 mmol) and triethylamine (1.12 mL, 8 mmol) in diethyl ether (24 mL) under a nitrogen atmosphere. The resulting solution was stirred for 8 h, washed with water (50 mL × 3), and then dried over $MgSO_4$. After removing diethyl ether, the residue was purified by silica-gel chromatography (ethyl acetate/hexane, 4:1, v/v). The obtained solid was dissolved in benzene, and the solution was reprecipitated with hexane to yield **2** as a violet solid (0.3 g, 0.3 mmol, 75%). UV/vis (CH_2Cl_2) λ_{max} (relative intensity) 419 nm (1.000), 518 nm (0.053), 553 nm (0.045), 593 nm (0.030), 651 nm (0.033).



The iron(III) porphyrin monomer **2**



Cinchonidine (CD)



Cinchonine (CN)

Scheme 1.

Polymer Preparation. Compositions of pre-polymerization mixtures for the synthesis of CD-imprinted polymers (IP) and pyridine-imprinted polymers (RP) as reference polymers are listed in Table 1. The pre-polymerization mixture was placed in a glass tube and purged with nitrogen. The resulting mixture was polymerized by heating at 50 °C for 15 h in the dark.

Chromatographic Study. The obtained polymer was ground and sieved to yield polymer particles of 32–63 μm. The polymer particles were slurried with $CHCl_3$/MeCN (1:1, v/v) and packed into a stainless steel column (100 mm × 4.6 mm i.d.). The packed particles were washed with methanol/acetic acid (7:3, v/v) to remove the template and unreacted compounds. A mixture of CH_2Cl_2/acetic acid (99:1, v/v) was used as an eluent at 1.0 mL/min. The template and related compounds (Scheme 1), namely CD, CN, pyridine, and quinoline, were injected independently in triplicate. The retention factors were calculated by the equation, $k' = (t_R - t_0)/t_0$, where t_R is the retention time of a sample and t_0 is the retention time of a marker (acetone). Separation factors were calculated by the equation, $\alpha = k'_{CD}/k'_{CN}$, where k'_{CD} is the retention factor for CD and k'_{CN} is the retention factor for CN, respectively. Chromatographic studies were conducted with a Gilson HPLC system consisting of a 234 auto injector (sample: 5.0 mM, 10 μL), 305+306 pumps and a 117 UV detector (280 nm).

Scatchard Analysis. Polymer particles (size: less than 32 μm) were washed with methanol/acetic acid (7:3, v/v). The polymers (3.0 mg) were suspended into CH_2Cl_2 solutions containing various amounts of CD (1.5 mL, 5.0 to 2000 μM) in vials. After the polymer suspensions were rotated for 15 h at 25 °C, an aliquot (1 mL) of each CD-incubated suspension was taken and dried in vacuo. The residue was dissolved in CH_3CN (1 mL) and the concentration of free CD ([CD]), was determined with the Gilson HPLC with a reverse-phase column (TSK gel ODS-80Ts, TOSOH Corporation, Japan). The eluent was 0.1 M ammonium acetate buffer (pH 6.0)/CH_3CN (1:1, v/v). Each vial was determined in

Table 1. Composition of Pre-polymerization Mixture (mmol)

Polymers	Cinchonidine	Pyridine	**2**	MAA
IP(P1)	0.22	—	0.22	—
IP(M4)	0.22	—	—	0.88
IP(M6)	0.22	—	—	1.32
IP(P1M2)	0.22	—	0.22	0.44
IP(P1M4)	0.22	—	0.22	0.88
IP(P1M6)	0.22	—	0.22	1.32
RP(P1)	—	0.22	0.22	—
RP(M4)	—	0.22	—	0.88
RP(M6)	—	0.22	—	1.32
RP(P1M2)	—	0.22	0.22	0.44
RP(P1M4)	—	0.22	0.22	0.88
RP(P1M6)	—	0.22	0.22	1.32

EDMA (ethylene dimethacrylate) 14.7 mmol, ADVN (2,2′-azobis(2,4-dimethylvaleronitrile)) 0.06 mmol, $CHCl_3$ 7.75 mL.

triplicate. The amount of CD bound to polymers (B) was calculated by subtracting [CD] from the initial CD concentration. Scatchard analysis was based on the equation, $B/[\mathrm{CD}] = (B_{\max} - B)K_a$, where K_a is an association constant and $B_{\max}$ is the apparent maximum number of binding sites, was performed from the binding data.

Spectroscopic Analysis. Polymer particles (size: less than 32 μm, 2.0 mg) were placed into a vial tube, and various concentrations of a CD or CN solution in CH_2Cl_2 (2 mL, 0 to 1000 μM) were incubated for 15 h at 25 °C. The suspensions were transferred to a quartz cell and UV–vis spectra were measured with a JascoV-560 UV/VIS spectrophotometer (Japan) equipped with an integral sphere (ISV-469).

Results and Discussion

CD-imprinted polymers were prepared with **2**, MAA, or a combination of both **2** and MAA (Fig. 1). After preparing the imprinted polymers and corresponding reference polymers, chromatographic tests were performed using stainless steel columns packed with these polymers. The retention factors (k') in the imprinted and reference polymers for CD, CN, pyridine, and quinoline are listed in Fig. 2. Pyridine was used as the template instead of CD for preparing reference polymers, because radical polymerization did not proceed without a ligand capable of coordinating to **2**. The retention factors of CD in the CD-imprinted polymers were significantly higher compared to those on the reference polymer. These results reveal that the imprinting of CD effectively proceeded and the binding sites suitable for CD were induced in the imprinted polymer matrices during the imprinting process. Retention times of the corresponding diastereomer CN on the CD-imprinted polymers were weak, indicating that the binding sites are complementary to the structure of CD in terms of the steric and functional topography.

IP(P1) showed less retention and no diastereoselectivity. This polymer was prepared with equimolar amounts of CD and **2**, where CD may form a 1:1 complex with **2** in a pre-polymerization mixture. In this case, the polymer would bind CD at one point, resulting in a low affinity (Fig. 2). On the other hand, IP(M4) and IP(M6), which were prepared using only MAA as the functional monomer, showed strong binding to CD, and the separation factors increased when more MAA was used (Table 2). Because the use of MAA induced the diastereoselectivity to the polymers, MAA may be referred to as a "stereo-determining monomer".

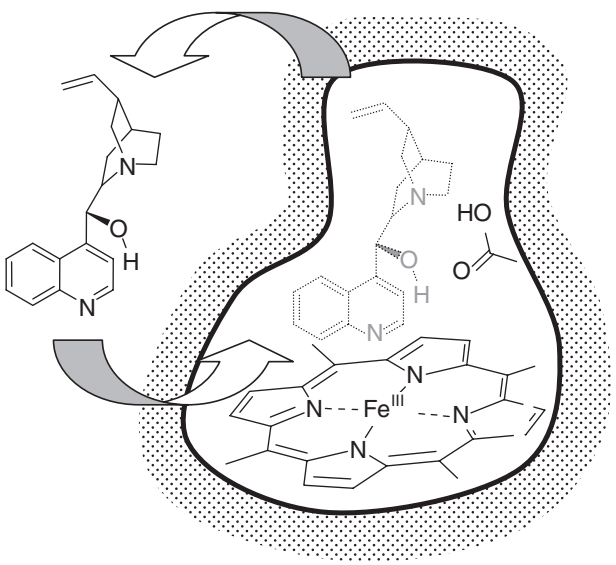


Fig. 1. Schematic representation of molecular imprinting of CD with the cooperative use of MAA and **2** as the functional monomers.

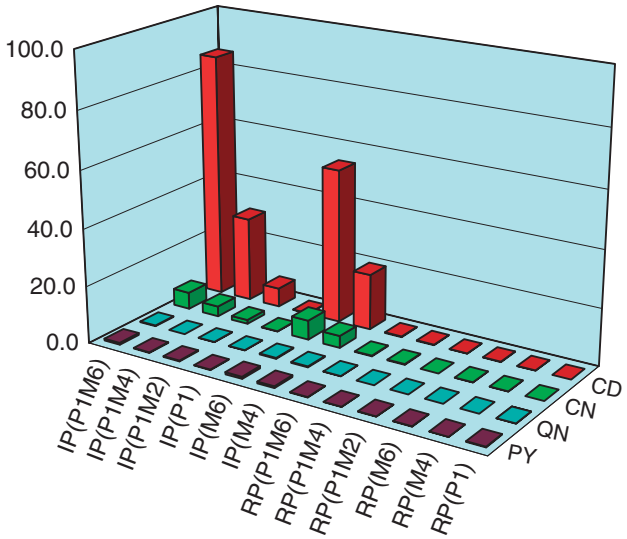


Fig. 2. Retention factors (k') on the polymers prepared. A mixture of CH_2Cl_2/acetic acid (99:1, v/v) was used as the eluent at 1.0 mL/min, and the detection was carried out at 280 nm. Retention factors (k') were calculated by an equation, $k' = (t_R - t_0)/t_0$, where t_R is the retention time of a sample and t_0 is the retention time of a marker. CD: cinchonidine, CN: cinchonine, QN: quinoline, PY: pyridine.

Table 2. Separation Factors for CD and CN Given by the Imprinted Polymers Prepared

Polymers	Separation factor $\alpha = k'_{CD}/k'_{CN}$
IP(P1)	1
IP(M4)	4.7
IP(M6)	7.8
IP(P1M2)	4.8
IP(P1M4)	8.3
IP(P1M6)	14.6

k'_{CD} and k'_{CN} represent retention factors for CD and CN shown in Fig. 2.

When both MAA and **2** were used as functional monomers, the resulting imprinted polymers, IP(P1M2), IP(P1M4), and IP(P1M6), strongly retained CD, and the retention factors for CD increased with the amount of MAA used. The poor retention by IP(P1) was greatly improved by cooperative work of MAA and **2** in the binding site and this cooperativity enhanced the affinity of CD. The increase in the retention factor was accompanied by an increase in the separation factor as MAA was increased (Table 2). In the pre-polymerization stage, the formation of a ternary complex of CD, **2**, and MAA was possible,

because bases such as imidazole and pyridine are known to coordinate to iron(III) porphyrins.[18] Thus, the quinoline-nitrogen of CD would coordinate to the iron(III) center and the hydroxy group of CD would interact with the carboxyl group of MAA by hydrogen bonding. After polymerization, these interactions would be memorized in the polymer matrices, resulting in binding sites that are complementary to the stereochemistry of CD (Fig. 1). The results presented here reveal that the MAA residues in IP(P1M6) were more suitably assembled for chiral recognition in a binding site than those in IP(M6), due to the fixation of CD by coordination bonding by the iron(III) porphyrin during the polymerization. As a result, IP(P1M6) could recognize the stereochemistry of CD more precisely (k'_{CD} and α in IP(P1M6): 85.9 and 14.6), compared to the corresponding MAA-based polymers (k'_{CD} and α in IP(M6): 54.1 and 7.8).

Particles of the imprinted polymers were suspended in known concentrations of CD solutions to perform batch re-binding tests, and Scatchard analyses were performed from the obtained binding isotherms. A typical binding isotherm and a Scatchard plot are shown in Fig. 3. All of the Scatchard plots indicated the typical shape, reported in most cases for non-covalent imprinting systems; two straight lines were fit corresponding to the high and low affinity sites. According to the Scatchard equation, the association constant (K_a) and the maximum number of binding sites (B_{max}) were estimated from the inclinations and the intercepts of the lines in the low concentration range (Table 3).

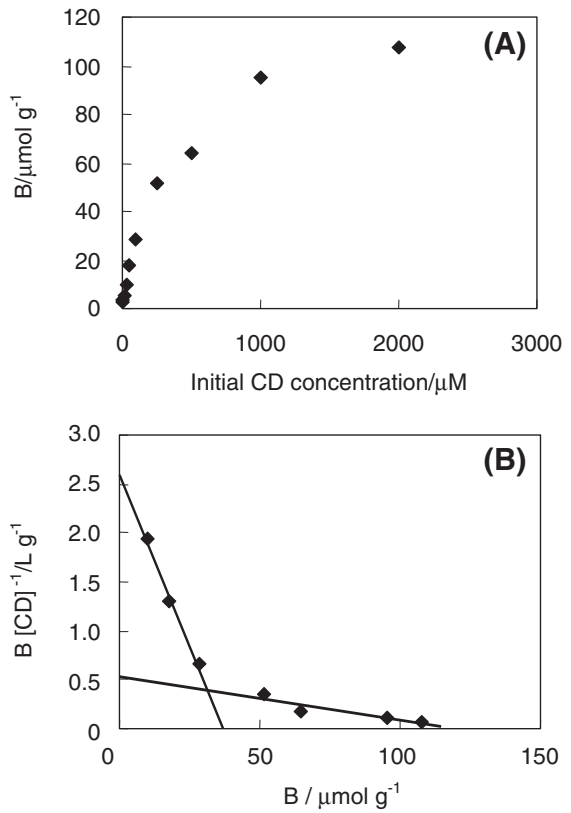


Fig. 3. Typical binding isotherm (A) and Scatchard plot (B) of IP(P1).

Table 3. Association Constants (K_a) and Maximum Numbers of Binding Sites (B_{max})

Polymers	K_a/M^{-1}	$B_{max}/\mu mol\ g^{-1}$
IP(P1)	7.0×10^4	37
IP(M4)	2.4×10^5	43
IP(M6)	3.7×10^5	42
IP(P1M2)	1.1×10^5	33
IP(P1M4)	3.6×10^5	41
IP(P1M6)	5.7×10^5	37

The values of K_a and B_{max} were determined from the Scatchard plots: $B/[CD] = (B_{max} - B)K_a$.

The values of K_a for the higher affinity sites (in the low concentration range) increased with the amount of MAA used as the functional monomer. The order of the K_a values was consistent with the results in chromatographic tests, meaning that regardless of the equilibrium state (batch re-binding tests), or non-equilibrium state (chromatographic tests), IP(P1M6) presented the highest affinity for CD ($K_a = 5.7 \times 10^5\ M^{-1}$). Because equilibria of the hydrogen-bonding complex between CD and MAA exist, the use of six equivalents of MAA was effective for the formation of high affinity binding sites in the resulting polymer network owing to a displacement of the equilibria toward complex association; however, more MAA may result in non-specific binding. The maximum numbers of higher affinity sites (B_{max}) were almost identical in all polymers, indicating that the binding sites corresponding to the high affinity sites in each polymer were generated on the basis of CD added.

We observed absorbance changes of the imprinted polymers suspended in known concentrations of a CD solution by using an integral sphere-equipped spectrophotometer. The UV–vis spectral change given by the iron(III) porphyrin residues of IP(P1M6) suspended in CD or CN solutions (0–1000 μM in CH_2Cl_2) are shown in Fig. 4. Beyond 400 nm, the visible absorbance is only derived from the iron(III) porphyrin residues in the polymer network, because CD and CN are optically transparent in this region, and the absorbance at 572 nm (Q-band) of IP(P1M6) and IP(P1) was plotted against concentrations of CD or CN incubated (Fig. 5). The absorbance of IP(P1M6) showed a larger increase with the concentrations of CD incubated than with those of CN incubated. In contrast, the absorbance changes showed an identical manner in IP(P1). Therefore, in the case of IP(P1M6), the iron(III) porphyrin residue could be located with MAA as a reporter of the binding events in the high affinity binding sites and provide the signaling function as well as stabilizing the complex of CD and MAA to develop the chiral selectivity.

The present study demonstrates that two functional monomers that have different functional groups can be suitably assembled in the polymer matrix using the template molecule. This means that one can construct cavities in synthetic polymers with a desirable assembly of multiple functional monomers; for example, an adjacent assembly of a polymerizable Lewis acid as a catalytic site and a hydrogen bonding monomer for binding site construction using an appropriate template molecule may lead to the preparation of a new type of artificial enzymes.

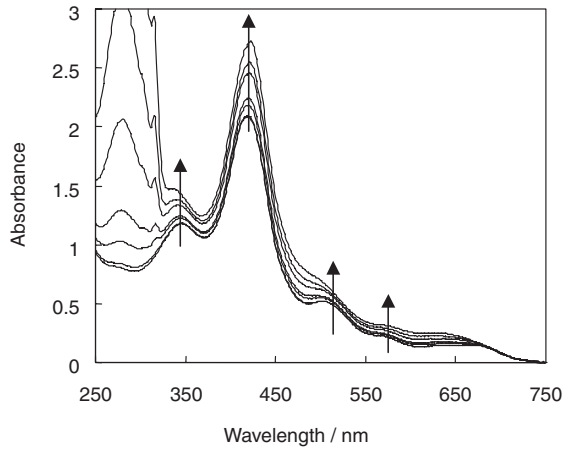


Fig. 4. UV–vis spectra of IP(P1M6) with various concentrations of CD (from bottom to top: 0, 10, 50, 100, 250, 500, and 1000 μM in CH_2Cl_2, respectively).

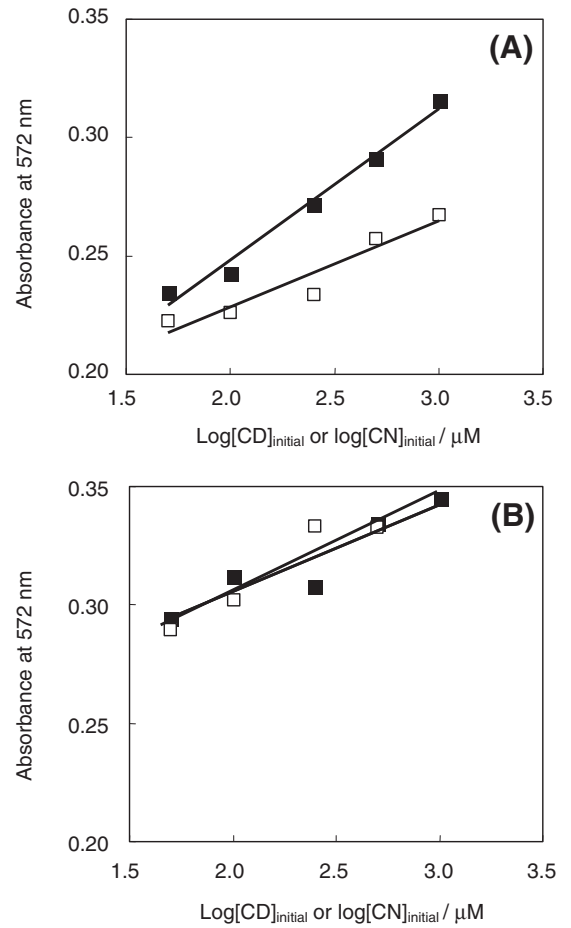


Fig. 5. Response of IP(P1M6) (A) and IP(P1) (B) in the presence of CD (■) or CN (□). $[CD]_{initial}$ and $[CN]_{initial}$ are the initial concentrations of CD and CN added.

In conclusion, a specific MIP for CD was prepared by the combined use of MAA and **2** as functional monomers. UV–vis spectra of the iron(III) porphyrin residues in the imprinted polymer displayed changes in the presence of CD, based on the coordination of the quinoline nitrogen to the iron(III) porphyrin. The relationship between the absorbance and the logarithmic concentrations of the incubated CD was linear, indicating that the binding events could be quantitatively read out by the spectral change. Since many compounds to be analyzed have no characteristic absorbance or fluorescence, the present readout system for the binding events based on the UV–vis absorption change of metalloporphyrins enables us to perform specific detection for many target compounds. The characteristic UV–vis spectra of metalloporphyrins, namely Soret band and Q bands, are independent of the kind of central metal ions; therefore, various metal ions other than iron(III) can be applied to the formation of metalloporphyrins, which can be used as functionalized monomers in order to prepare functional MIPs for a wide range of compounds capable of binding the metalloporphyrins.

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