Uniformly Sized Molecularly Imprinted Polymers for on-line Concentration, Purification, and Measurement of Nimodipine in Plasma

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ABSTRACT: Uniformly sized molecularly imprinted polymers (MIPs) for nimodipine have been prepared in an aqueous system by multi-step swelling and polymerization method, utilizing 4-vinylpyridine (4-VPY) or methacrylic acid (MAA) as a functional monomer, ethylene glycol dimethacrylate (EDMA) as a cross-linking agent. Scanning electron microscopy was used to identify the structure features of the obtained polymers. Further, the influences of some chromatographic conditions were examined to explore the possible recognition mechanism. The results reveal that stable molecularly imprinted polymeric microspheres with good size monodispersity were obtained, and the polymer beads showed specific recognition for the template molecule and some other dihydropyridine calcium antagonists (DHPs). Besides hydrophobic interaction, the molecular shape complementation of DHPs and the MIPs seems to play an important role in the retention and recognition of DHPs. The Scatchard analysis showed that two kinds of binding sites existed in the MIPs. The MIPs was then used as a high-performance liquid chromatography (HPLC) separation medium to simultaneously concentrate and purify nimodipine in plasma. The results reveal that the obtained MIPs could be used for on-line concentration, purification, and measurement of nimodipine in biological samples.
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Key words: nimodipine; molecular imprinting; purification and measurement; on-line; HPLC

INTRODUCTION

The molecularly imprinted technique, introduced in 1972 by Wulff and Sarhan[1] and much advanced by the work of Mosbach and Arshady[2] in the 1980s, is a way to produce polymers with specific selectivity of target or template molecules, which is called molecularly imprinted polymers (MIPs). Because of chemical stability and high recognition ability, MIPs are increasingly used as selective support in liquid chromatography,[3,4] capillary electrophoresis,[5,6] and solid-phase extraction,[7–9] and used as biosensors[10,11] or artificial antibodies.[12] Generally, MIPs preparation methods are classified into two catalogues, i.e., covalent and noncovalent methods. In noncovalent method, which is now frequently used, MIPs can be prepared by bulk polymerization,[13] *in situ* polymerization,[14] suspension polymerization,[15] precipitation polymerization,[16] and multi-step swelling polymerization.[17] Compared with other methods, the multi-step swelling polymerization method, established by Hosoya et al.,[18] is advantageous, because it allows facile preparation of uniformly sized and monodispersed particles and also because it is suitable for preparing HPLC packing materials.[19]

Dihydropyridine calcium antagonists (DHPs) constitute a fundamental family of calcium antagonists. They are widely used for the treatment of hypertension and angina pectoris. Because of low concentration and interior interference, tedious liquid-liquid or solid-phase extraction procedures are routinely needed to concentrate and purify the drug from biological samples before HPLC analysis.[20]

In our previous study, MIPs for (*S*)-nilvadipine, another DHPs, were prepared, which showed encouraging specific recognition ability for (*S*)-nilvadipine and enantioseparation of nilvadipine racemate was achieved.[21] In this paper, uniformly-sized MIPs for nimodipine were prepared by a multi-step swelling and polymerization method, utilizing 4-vinylpyridine (4-VPY) or methacrylic acid (MAA) as a functional monomer, ethylene glycol dimethacrylate (EDMA) as a cross-linking agent. The recognition mechanism of the MIPs was investigated by chromatography and isothermal adsorption experiments. The obtained MIPs were then used as an HPLC stationary phase to on-line concentrate and purify nimodipine in plasma samples.

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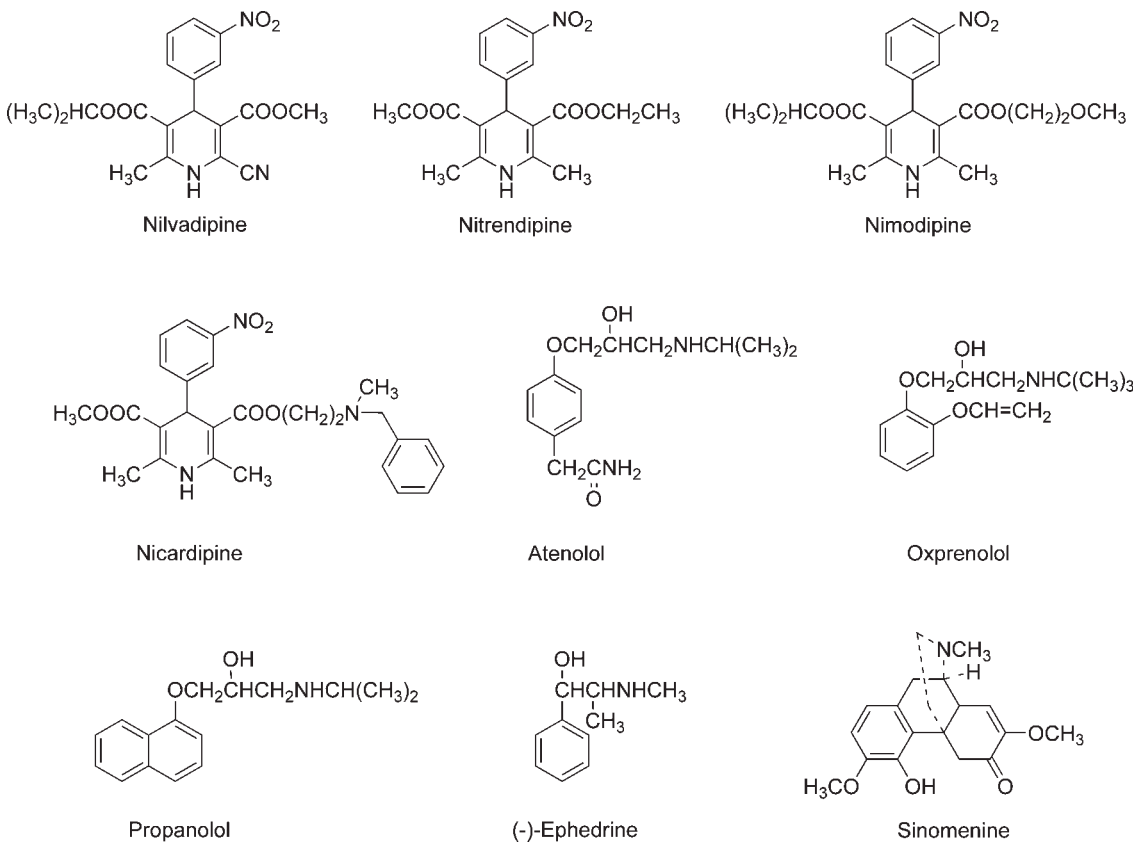

Figure 1 Structures of compounds used in this study.

EXPERIMENTAL

Reagents and materials

Nimodipine was purchased from Pharma Chemical Reagent Plant (Ruicheng, China). Nitrendipine, nicardipine, oxprenolol, propranolol, sinomenine, and ephedrine were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nilvadipine were obtained from Fujisawa Pharmaceutical (Osaka, Japan). The structures of compounds used in this study are illustrated in Figure 1. Methacrylic acid (MAA) was purchased from Tianjin Chemical Reagent Plant (Tianjin, China) and 4-vinylpyridine (4-VPY) were obtained from Sigma-Aldrich (Milwaukee, USA). MAA and 4-VPY were redistilled before use to remove polymerization inhibitor. Ethylene glycol dimethacrylate (EDMA), also obtained from Sigma-Aldrich (Milwaukee, USA), was purified with 10% aqueous sodium hydroxide and water, and dried over anhydrous magnesium sulfate before use. 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Shanghai No. 4 Reagent Factory (Shanghai, China). Acetonitrile and methanol were of HPLC grade from Tianjing Chemical Reagent Factory (Tianjin, China). All the other reagents were of analytical grade. Water was freshly prepared by distillation thrice prior to use. Heparinized plasma, separated by blood centrifugation at 4000 r min^{-1} for 20 min at 4°C, was obtained from New Zealand rabbits (Xi'an Jiaotong University Experimental Animal Center, China).

Preparation of the MIPs

MIPs for nimodipine (NM-MIPs) were prepared by a multi-step swelling and polymerization method, according to the literature[21] with slightly changes. Briefly, a water dispersion of 1.5 mL of uniformly sized polystyrene seed particles was admixed with a microemulsion prepared from 0.48 mL of dibutyl phthalate as an activating solvent, 0.02 g of sodium dodecyl sulfate and 10 mL of water by sonication.

The first-step swelling was carried out at room temperature for 15 h with stirring at 125 rpm until oil micro-drops completely disappeared. A dispersion of 0.25 g of AIBN as an initiator, 5 mL of toluene or chloroform as a porogenic solvent, 10 mL of 4.8% polyvinyl alcohol aqueous solution as a dispersion stabilizer, and 12 mL of water was added to the dispersion of swollen particles.

The second-step swelling was carried out at room temperature for 2 h with stirring at 125 rpm. According to the preparation scheme, shown in

TABLE I
The Preparation Conditions for Nimodipine Imprinted Polymers and Nonimprinted Polymers

Column no.	Polymer no.	Template/ (mmol)	Functional monomer/(mmol)	Cross-linkers/ (mmol)	Porogenic solvents/(mL)
P_1	NM-MIP_1	NM/2	MAA/6	EDMA/25	Toluene/5
P_2	NM-NIP_1		MAA/6	EDMA/25	Toluene/5
P_3	NM-MIP_2	NM/3	MAA/6	EDMA/25	Toluene/5
P_4	NM-NIP_2		MAA/6	EDMA/25	Toluene/5
P_5	NM-MIP_3	NM/2	MAA/6	EDMA/25	Chloroform/5
P_6	NM-NIP_3		MAA/6	EDMA/25	Chloroform/5
P_7	NM-MIP_4	NM/3	4-VPY/6	EDMA/25	Toluene/5
P_8	NM-NIP_4		4-VPY/6	EDMA/25	Toluene/5

Table I, a dispersion of nimodipine as a template, 25 mmol of EDMA (5 mL) as a cross-linking reagent, and MAA or 4-VPY as functional monomers were added in sequence. 10 mL of 4.8% polyvinyl alcohol aqueous solution and 13 mL of water was added to the dispersion of swollen particles.

The third-step swelling was carried out at room temperature for 2 h with stirring at 125 rpm. After the third-step swelling, the polymerization procedure started at 50°C under nitrogen stream with stirring at 125 rpm for 24 h.

After polymerization, the dispersion of polymer particles was poured into 200 mL of methanol and the supernatant was discarded after sedimentation of the particles. The polymer particles were re-dispersed into methanol, and this procedure was repeated thrice in methanol, once in water, and twice in tetrahydrofuran, subsequently. The resulting 3–5 μm polymer particles were collected using a glass filter, washed with tetrahydrofuran and dried at room temperature.

Nonimprinted polymers (NIPs) were similarly prepared without addition of the template molecule nimodipine at equal pace.

Test of the morphologies of the MIPs

The morphologies of the prepared microspheres were analyzed by using an *S*-570 scanning electron microscope (HITACHI, Japan) at 20keV.

Chromatographic evaluation of the MIPs

Preparation of the MIPs-stationary HPLC column

The obtained polymers were packed into a stainless-steel column (50 × 4.6 mm, i.d) by a slurry packing technique using methanol-2-propanol (2 : 1, V/V) as the slurry solvent and methanol as the packing solvent to evaluate the chromatographic characteristics. And then MIPs-stationary HPLC column was online washed extensively by a mixture of methanol : acetic acid (4 : 1, V/V) to remove the templates until stable baseline was achieved.

Chromatographic conditions and evaluation parameters

The HPLC system consisted of a Spectra P200 pump, a Spectra 100 UV detector (Thermo Electron, USA), and a N3000 Chromatographic software (Zhejiang Information Research Center, China). The mobile phase was prepared by mixing sodium phosphate buffer solution (PBS) and methanol or acetonitrile. The pH value and concentration of PBS and the volume ratio of methanol or acetonitrile to PBS were illustrated in table notations and figure captions. The flow rate was maintained at 0.5 mL min^{-1} unless otherwise stated. Detection of DHPs was performed at the wavelength of 236 nm, sinomenine and ephedrine at 256 nm, and β-accepter antagonists at 210 nm. The column temperature was maintained at 25°C unless otherwise stated by a HT-230A column heater (Fuji Electric, Japan).

The retention factors (k) were determined by the formula $k = (t_R - t_0)/t_0$, where t_R is the retention time of a given specie and t_0 is the retention time of acetone, a void marker. The separation factors (α) were calculated from the equation $\alpha = k_1/k_2$, where k_1 and k_2 were the retention factors of nimodipine and another drug tested on the HPLC column with the MIPs as stationary phase. The selectivity factors(S) were calculated from the equation $S = k_{MIPs}/k_{NIPs}$, in which the k_{MIPs} and k_{NIPs} were the retention factors of nimodipine on the molecularly imprinted and nonimprinted polymers, respectively.

Preparation of samples

Samples of DHPs and other compounds were prepared by using methanol as solvent to a concentration of about 1 mg mL^{-1} and diluted to a concentration of 0.1 mg mL^{-1} with water before use. A 10 μL aliquot of the samples solution was injected onto the column.

Adsorption isotherms and scatchard analysis of the MIPs

Preparation of nimodipine standard solution

Nimodipine of 2.0923 g was accurately weighed into 100 mL volumetric flask, dissolved in chloroform, and diluted to 100 mL, 50 mmol L^{-1} nimodipine stock solution was obtained. Then the varied concentration of nimodipine standard solution 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mmol L^{-1} were gotten by diluting 0.2, 0.6, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 mL of the stock solution to 100mL with chloroform as solvent.

Adsorption isotherms

Approximate 20 mg dried polymers of NM-MIP_4 or NIPs were weighed into a 10 mL conical flask. First, added 0.5 mL methanol, and sonicated; then mixed with 5 mL of nimodipine standard solution; finally, the conical flask was oscillated by an HZ-881S action shaker (Taicang City Scientific Instruments Factory, China) in a water bath for 16 h at 25°C. Then the mixture was filtrated through a microporous membrane of 0.22 μm and the nimodipine concentration in the filtrate was measured by a SP-2102 UV spectrophotometer (Shanghai Spectrum Instruments. China) at wavelength of 236 nm. The reference nimodipine solution was prepared by diluting 1 mL of standard nimodipine solution of varied concentrations to 25 mL, and directly detected absorbance at wavelength of 236 nm.

The amount of nimodipine bound to the polymers was calculated by subtracting the concentration of free nimodipine in the filtrate from the initial nimodipine in the reference solution. The calculation equation of the amount of equilibrium: $C = (A \times C_0)/A_0$ and $Q = (C_0 - C) \times 5/(20 \times 10^{-3})$, where Cand C_0 are the concentration of nimodipine in the filtrate and in its reference solution, respectively; A andA_0 are the absorbance of the filtrate and its reference solution, respectively. Q(μmol g^{-1}) is the amount of nimodipine bound to the polymers. The Scatchard equation $Q/C = (Q_{max} - Q)/K_d$ was used to estimate the binding parameters of the MIPs, where Q_{max} was the apparent maximum number of binding sites, K_d is the equilibrium dissociation constant.

Measurement of nimodipine in plasma

An amount of 20.0 mg nimodipine was dissolved in acetonitrile and diluted to 100 mL, affording a 0.2 mg mL^{-1} stock solution of nimodipine. Then the stock solution of nimodipine was diluted to various concentrations with acetonitrile. A 0.5 mL aliquot of plasma samples was spiked with 100 μL of the nimodipine standard solution at different concentration, and then 0.5 mL acetonitrile was added to remove protein by precipitation and centrifugation. After centrifugation, a series of quality control plasma samples with different concentrations of standard nimodipine, 5, 10, 20, 40, 80, and 160 μg mL^{-1} were prepared.

The MIPs-stationary HPLC column was prepared as above. A mixture of acetonitrile-sodium phosphate buffer solution (20 mmol L^{-1}, pH 6.0) (40 : 60, V/V) was used as the mobile phase. Other chromatographic conditions were same to that of the previously described.

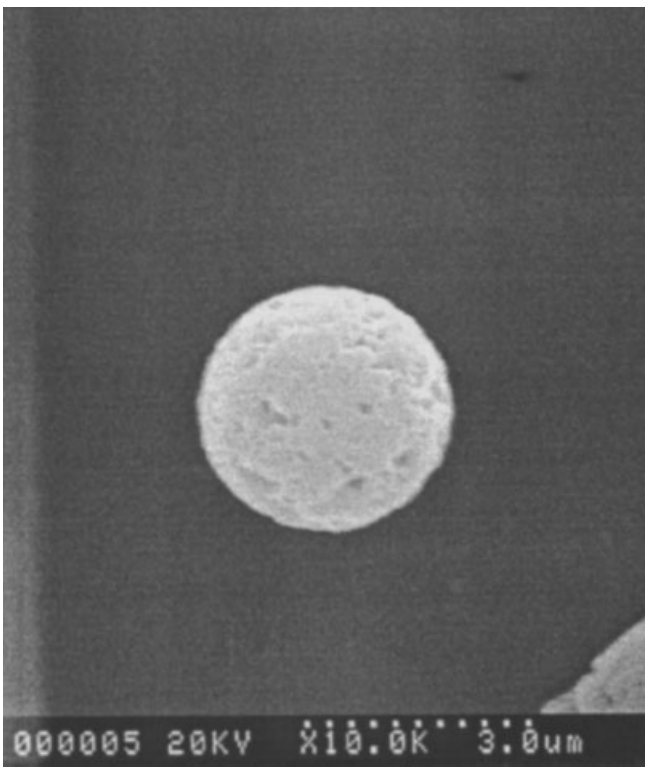

Figure 2 The scanning electron micrograph of the beads prepared by multi-step swelling and polymerization method. (×10,000).

RESULTS AND DISCUSSION

Morphology of polymers

Figure 2 is a scanning electron micrograph showing the morphologies of NM-MIPs prepared in this work when 4-VPY was used as a functional monomer. It can be seen from the image that spherical polymer beads with an approximate mean diameter of 4 μm were obtained, making them suitable for the HPLC stationary phase. Figure 2 also shows that many cavities were made in the surface of the beads, which was considered to be formed by removal of the porogens from the particles during the washing procedure. Such a porous structure provides

TABLE II
Retention Factors and Selectivity Factors of Nimodipine on the NM-MIPs Prepared Under Different Conditions

Column no.	k'_{MIPs}	k'_{NIPs}	S
P_1	8.53	2.30	3.71
P_3	9.75	2.30	4.24
P_5	7.38	2.40	3.08
P_7	8.78	2.30	3.82

HPLC Conditions: Mobile Phase: acetonitrile-sodium phosphate buffer solution (20 mmol · L^{-1}, pH 6.0) (40 : 60, V/V). Flow Rate: 0.5 mL · min^{-1}. Detection Wavelength: 236 nm.

recognition sites, and leads to large surface areas, thus contributing to high binding capacity.

Selectivity of NM-MIPs

In MIPs preparation, usually template molecules involves relative polar functional groups, such as carboxylic acid, hydroxyl, and/or amino groups, therefore approximate functional monomers are expected to interact with the functional groups of the template molecules through intermolecular interactions. However, In nimodipine structure, no obvious acidic or basic functional groups exist. Therefore, both acidic monomer MAA and basic monomer 4-VPY were tested according to the preparation schedule shown in Table I. The prepared MIPs were evaluated by chromatographic experiments to examine the effect of functional monomer type and amount on the retentivity and selectivity for nimodipine. The results, listed in Table II, showed that all of the obtained MIPs gave a high retention for the template molecule, which means either MAA or 4-VPY could be used as a functional monomer. When MAA used as the functional monomer, increasing template molecule amount could produce a higher retention factor. The selectivity factor indicates that the template molecule had much stronger retention on the MIPs than on the NIPs no matter MAA or 4-VPY was used as a functional monomer. This suggests that MIPs for nimodipine were successfully prepared by multi-step swelling and polymerization method, utilizing 4-VPY or MAA as a functional monomer, ethylene glycol dimethacrylate (EDMA) as a cross-linker.

The P_7 column was used to further investigate retentivity and selectivity of DHPs and some other compounds.

The Figure 3 shows that on P_7 column, selectivity factors for DHPs were much higher than for other tested compounds, e.g., β-receptor antagonist and alkaloids. It indicated that NM-MIPs were capable of specifically recognizing DHPs. The methanol ratio in mobile phase was changed to observe the influence of methanol on DHPs retentivity (data not showed). A typical hydrophobic behavior was observed. When pH value around four mobile phase used, with the amounts of methanol in PBS decreased, the retention of tested DHPs increased greatly except for nicardipine; when the amount of methanol was less than 58%, DHPs could hardly be eluted; when 100% methanol was used as the mobile phase, the tested DHPs tended to be eluted on the MIPs column. The results are indicative of the hydrophobic interactions playing an important role in the selective recognition procedure. Noticeably, the highest selectivity factors in the tested DHPs was obtained not from template molecule nimodipine but from nilvadipine, which suggests the molecular shape of nilvadipine might be better matched the spatial structure of the recognizing sites, because the recognition occurs based on the molecular shape.[22] This explanation could be strongly supported by the facts of nimodipine and nitrendipine with highly similar structures showing

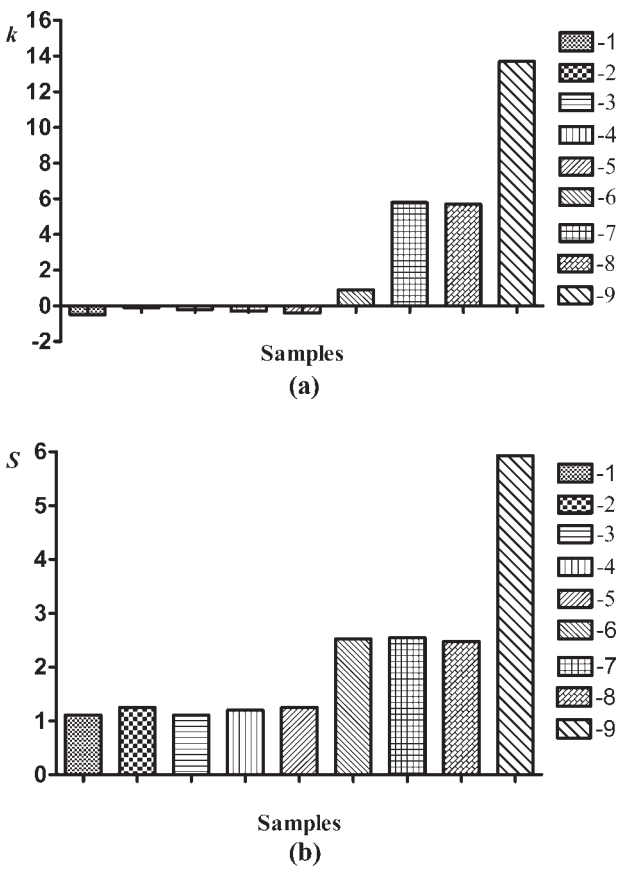

Figure 3 Selectivity of nimodipine imprinted polymers column. 1. atenolol, 2. oxprenolol, 3. propranolol, 4. sinomenine, 5. (−)-ephedrine, 6. nicardipine, 7. nimodipine, 8. nitrendipine, 9. nilvadipine. HPLC conditions: Column, MIPs-HPLC column (P_7, 100 × 4.6 mm, i.d.); Flow rate, 0.5 mL min^{-1}; coulmn temperature, 25°C; Injected amount, 0.5 μg; Mobile phase, methanol-20 mmol L^{-1} sodium phosphate buffer (pH 4.0) (70 : 30, V/V); Detection wavelength, 1-3, 210 nm; 4-5, 256 nm; 6-9, 236 nm.

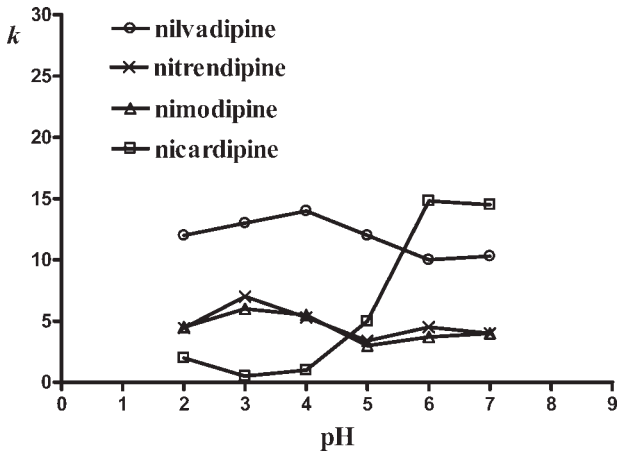

Figure 4 Influence of mobile phase pH on retention of the MIPs. HPLC conditions: Column, MIPs-HPLC column (P_7, 100 mm × 4.6 mm, i.d.); Flow rate, 1.0 mL min^{-1}; Coulmn temperature, 25°C; injected amount, 0.5 μg; Mobile phase: methanol-20 mmol L^{-1} sodium phosphate buffer (65 : 35, V/V); Detection wavelength: 236 nm.

closer retention characteristics. Above all, P_7 showed highly specific recognition for nimodipine and its analogous, and could be utilized in aqueous media. So it can be expected to apply for screening and concentrating the DHPs analogous from complex media.

Influence of the mobile phase pH on retentivity of the MIPs

Figure 4 shows the effect of mobile phase pH on DHPs retention factors. There were no good recipient groups of proton in the structures of the tested DHPs except nicardipine. Although the pH value of mobile phase varied from 2 to 4, the retention factors of DHPs showed similar changing tendency. A slight decrease was observed below pH 3 for nimodipine and nitrendipine, whereas for nilvadipine and nicardipine was observed below pH 4. This could be explained by the protonation of pyridyl groups in the polymers, whose average pK_a value was reported to be blow 3.[23] Although the pH changed from 4 to 7, the quantity of electric charge of nimodipine, nitrendipine, and nilvadipine still kept in a small range, so the varied pH did not influence their retention factors much. However, with the increasing of pH from the range 4 to 6, the retention factor of nicardipine increased sharply. This tendency shows nicardipine was more sensitive to the variation of the pH in mobile phase because of its tertiary amine group. These results are indicative of the strong hydrophobic interaction between the MIPs and DHPs.

Determination of the MIPs binding parameters

Figure 5 shows the binding isotherms for nimodipine on the NM-MIP_4 and on its corresponding non-

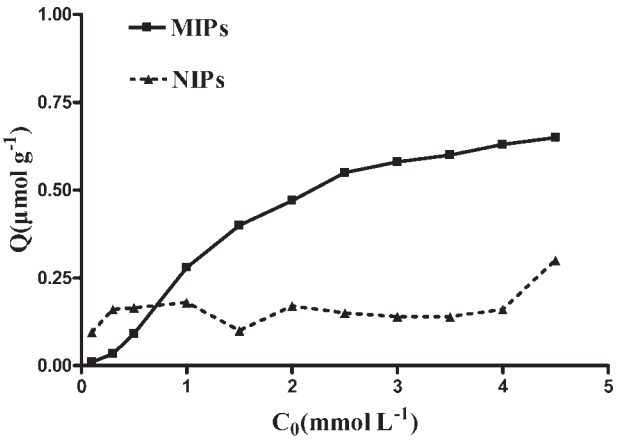

Figure 5 Binding isotherm of the polymers for nimodipine.

imprinted polymers. The binding amount increased gradually with the increasing of nimodipine concentration, but the binding amount on the MIPs was clearly larger than that on the nonimprinted polymer and this difference became enlarged with increasing of nimodipine concentration in the observed range. The obtained data were calculated according to the

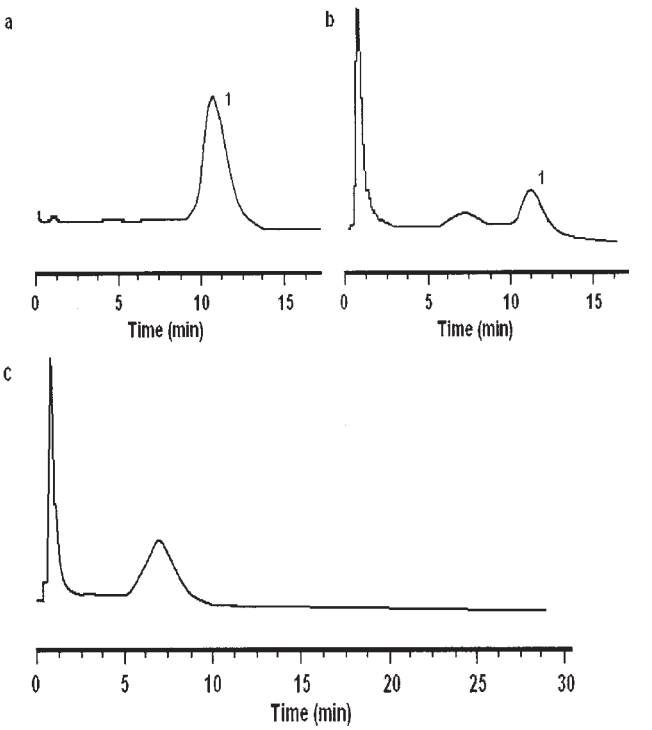

Figure 6 Chromatograms of nimodipine plasma samples. a. Nimodipine standard solution (0.16 mg mL^{-1}), b. Plasma sample spiked with nimodipine (40 μg mL^{-1}), c. Blank plasma, 1. nimodipine. HPLC conditions: Column, MIPs-HPLC column (P_7, 100 mm × 4.6 mm, i.d.); Flow rate, 0.5 mL min^{-1}; Coulmn temperature, 25°C; injected volume, 10 μL; Mobile phase: acetonitrile-20 mmol L^{-1} sodium phosphate buffer (pH 6.0) (40 : 60, V/V); Detecting wavelength: 236 nm.

TABLE III
Recovery and Precision Results of Nimodipine in Plasma

Injected amount ($\mu g \cdot mL^{-1}$)	Measured amount ($\mu g \cdot mL^{-1}$)	Recovery (%)	R.S.D. (%)
5.0	5.8	116.4	6.9
40.0	44.0	110.1	8.9
160.0	170.8	106.8	9.1

Scatchard equation. It was found that two distinct sections well fitting the equation were obtained with regression coefficient $r_1^2 = 0.9634$ and $r_2^2 = 0.9519$, respectively. It means Scatchard equation could be used to express the binding characteristics and there were two classes of binding sites in the NM-MIP$_4$. From Scatchard equation, the equilibrium dissociation constant $K_{d\ 1}$ and the apparent maximum number $Q_{max\ 1}$ of one section were estimated to be 1.606×10^{-5} mol L^{-1} and 79.75 μmol g^{-1}, respectively. In the same way, the equilibrium dissociation constant $K_{d\ 2}$ and the apparent maximum number $Q_{max\ 2}$ of another section were estimated to be 1.435×10^{-4} mol L^{-1} and 152.82 μmol g^{-1}, respectively.

Enrichment and determination of nimodipine in plasma

After the MIPs-HPLC column washed extensively, 10 μL of blank plasma sample, nimodipine standard solution and quality control plasma samples were injected onto the column. The Figure 6 shows that within 15 min, baseline separation of nimodipine could be obtained on the MIPs-HPLC column and no interference in plasma was observed, though the nimodipine peak was wide.

A standard calibration curve was plotted by using peak area (y axis) and concentration of quality control plasma [x axis ($\mu g\ mL^{-1}$)]. The linear equation was $y = 0.0804\ x + 0.9771$, with a correlation coefficient $r^2 = 0.9975$. It shows that a fine linear correlation relationship existed between the peak area and the concentration of nimodipine over the investigated concentration range.

According to the standard calibration curve, recovery and precision were calculated with three different concentrations of nimodipine, i.e., 5, 40 and 160 $\mu g\ mL^{-1}$. This procedure was repeated five times for each concentration. The results, shown in Table III, indicate that the NM-MIPs $_4$ could be used as a separation medium to concentrate, purify and determine nimodipine in biological samples.

CONCLUSIONS

A spherical molecularly imprinted polymer for nimodipine was prepared by a multi-step swelling and polymerization method in an aqueous system, using 4-VPY or MAA as the functional monomer and EDMA as the cross-linker agent. The obtained polymer beads had good size monodispersity. The chromatography and isothermal adsorption experiments showed that the nimodipine-imprinted polymers exhibited high-retentivity and selectivity for nimodipine and some other DHPs. Besides hydrophobic interaction, the molecular shape complementation of DHPs and the MIPs seems to play an important role in the retention and recognition of DHPs. The obtained MIPs could be used for on-line concentration, purification, and measurement of nimodipine in biological samples.

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