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Review

Monodispersed, molecularly imprinted polymers as affinity-based chromatography media☆

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Abstract

This review article deals with preparation methods for spherical and monodispersed molecularly imprinted polymers (MIPs) in micrometer sizes. Those methods include suspension polymerization in water, liquid perfluorocarbon and mineral oil, seed polymerization and dispersion/precipitation polymerization. The other methods are the use of beaded materials such as a spherical silica or organic polymer for grafting MIP phases onto the surfaces of porous materials or filling the pores of silica with MIPs followed by dissolution of the silica. Furthermore, applications of MIP microspheres as affinity-based chromatography media, HPLC stationary phases and solid-phase extraction media, will be discussed for pharmaceutical, biomedical and environmental analysis.
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Keywords: Molecularly imprinted polymer; Monodispersed MIP; MIP microsphere; Affinity-based media; Pharmaceutical analysis; Environmental analysis; Bioanalysis

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1. Introduction

Molecular imprinting techniques are very attractive because specific recognition sites for a target molecule could be easily molded in synthetic polymer networks [1–10]. The prepared molecularly imprinted polymers (MIPs) have been utilized as chromatographic media, sensors, artificial antibodies and

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catalysts [1,2]. Typically, MIPs were prepared by a bulk polymerization method, where the resultant monoliths had to be crushed, ground and sieved to produce microparticles for their applications [1]. When MIPs could be used as affinity-based chromatography media, HPLC stationary phases or solid-phase extraction (SPE) media, it is desirable to prepare the spherical and monodispersed beads.

To prepare spherical and monodispersed MIPs, several new polymerization methods have emerged. Typically, spherical MIPs were prepared by conventional suspension polymerization, where water is used as a continuous phase to suspend a droplet of pre-polymerization mixtures (template molecule, functional monomer, crosslinker and initiator) in the presence of a stabilizer or surfactant [1,4]. However, the size of the droplet formed is different depending on the conditions used. Therefore, the MIPs prepared by suspension polymerization are different in sizes (a few to a few hundred micrometers) and are always polydispersed [4]. It is thought that water is to weaken the non-covalent interactions such as hydrogen bonding and electrostatic interactions between a template molecule and functional monomer [4,11,12]. Water soluble template molecules and monomers would also be lost due to partitioning into the aqueous phase. Furthermore, a stabilizer or surfactant, which is required for the formation and stabilization of droplets, could interfere with interactions between a template molecule and functional monomer. Attempts to make MIP microspheres by suspension polymerization in water have led to only very poor recognition [4,11,12].

Recently, two new suspension polymerization techniques based on droplets of pre-polymerization mixtures formed in liquid perfluorocarbon [11] or mineral oil (liquid paraffin) [12] have been developed. These liquids were used as a continuous phase instead of water. The advantages of the former method are that liquid perfluorocarbons are immiscible with almost all organic solvents, and that many combinations of monomers and crosslinkers and a wide range of solvents, employed for bulk polymerization, could be utilized [11]. However, it is required to synthesize perfluoro polymeric surfactants. On the other hand, a suspension polymerization technique using mineral oil as a continuous phase comprises the formation of droplets of pre-polymerization mixtures directly in mineral oil by vigorous mixing followed by transformation of the droplets into solid spherical beads by photo polymerization [12]. It is interesting that no stabilizers or surfactants were required for the droplet formation. However, neither chloroform, nor dichloromethane, nor toluene, which is generally used as a porogen in molecular imprinting, could be used with mineral oil because these solvents are miscible with mineral oil. Both methods give polydispersed beads as well as suspension polymerization in water.

The second method for the preparation of MIP microspheres is seed polymerization, typically multi-step swelling and polymerization [13], as shown in Fig. 1. Uniformly-sized polystyrene seed particles were utilized as the shape template. The seed particles were swollen by microemulsion droplets containing an activating solvent (i.e., dibutylphthalate) and further swollen by pre-polymerization mixtures. After completion of swelling, monodispersed MIPs could be prepared by photo or thermal polymerization. The advantages of the method are as follows: it is easy to prepare monodispersed MIPs and to perform in situ modification. However, interactions between a template molecule and functional monomer could be interfered since water is used as a continuous phase.

The third method is dispersion/precipitation polymerization. In dispersion polymerization, primary particles swell in a polymerization medium and the polymerization proceeds in the particles, resulting in the formation of spherical beads, while in precipitation polymerization, primary particles do not swell in a polymerization medium and the polymerization takes place in the medium, leading to irregularly shaped and polydispersed particles [14]. However, a sharp distinction between dispersion and precipitation polymerization does not exist [10,14]. A crucial difference between the bulk polymerization and dispersion/precipitation polymerization techniques is the volume of a polymerization medium used. The latter requires larger volumes of the medium than the former. The excess of a polymerization medium may hamper interactions between a template molecule and functional monomer.

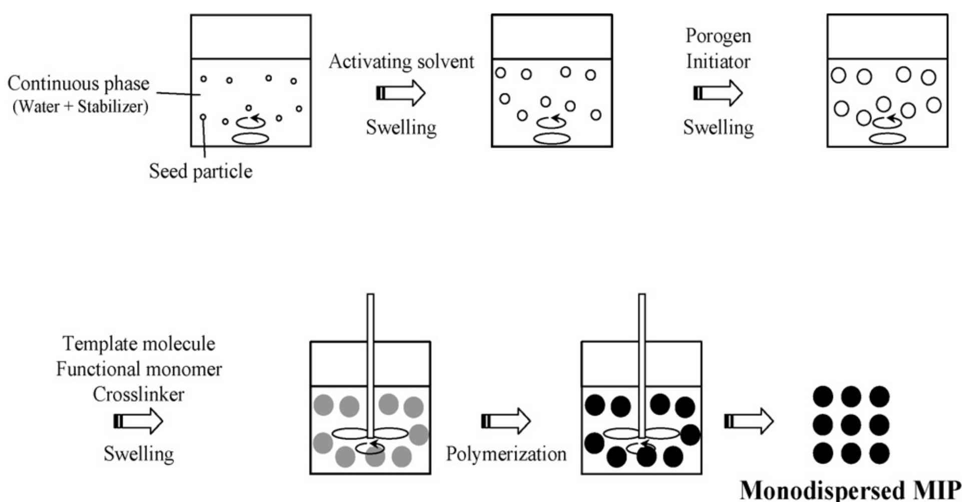

Fig. 1. Preparation method of monodispersed MIP by multi-step swelling and polymerization.

The other methods are the use of beaded materials such as a spherical silica or organic polymer for coating MIP phases onto the surfaces of porous materials or filling the pores of a silica with MIPs followed by dissolution of the silica [4,10]. The former is a grafting imprinting, where thin films of MIP phases were grafted on the surface of porous materials. The latter is a hierarchical imprinting, where a template molecule is immobilized to a solid support, and the support is dissolved after polymerization and thus sacrificed. The prepared MIPs by both methods are monodispersed.

This review article deals with preparation methods of spherical and monodispersed MIPs in micrometer sizes. Application of MIP microspheres as affinity-based chromatography media, HPLC stationary phases and solid-phase extraction (SPE) media, will be discussed for pharmaceutical, biomedical and environmental analysis.

2. Suspension polymerization

2.1. Suspension polymerization in water

Since water hampers hydrophilic interactions between a template molecule and functional monomer in non-covalent molecular imprinting, suspension polymerization using water as a continuous phase has been applied for a covalent molecular imprinting technique [4,10]. A MIP for L-histidine (L-His) was prepared by suspension polymerization of *N*-methacryloyl-L-His-copper(II)-L-His and ethylene glycol dimethacrylate (EDMA) as a monomer-template and crosslinker in covalent molecular imprinting [15]. After polymerization, the template, L-His, was removed form the resultant polymer, thus getting the MIP for L-His as shown in Fig. 2. Separation of His enantiomers on the MIP was attained by HPLC. Furthermore, the MIP was

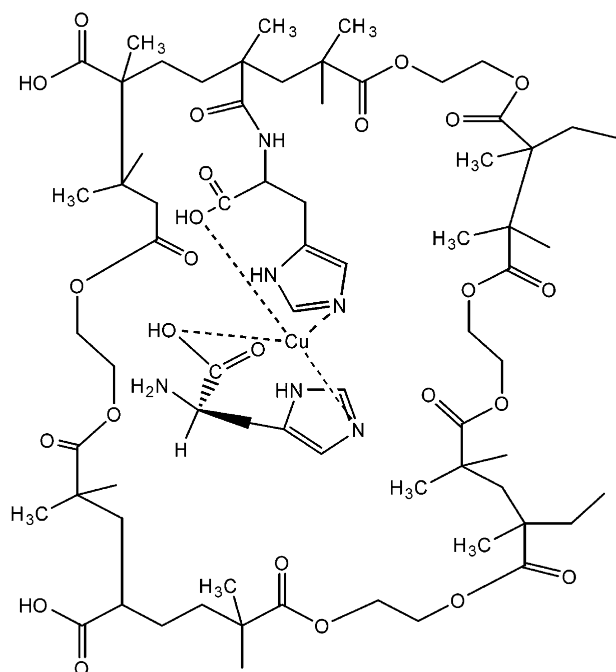

Fig. 2. Schematic representation of L-histidine template formation [15].

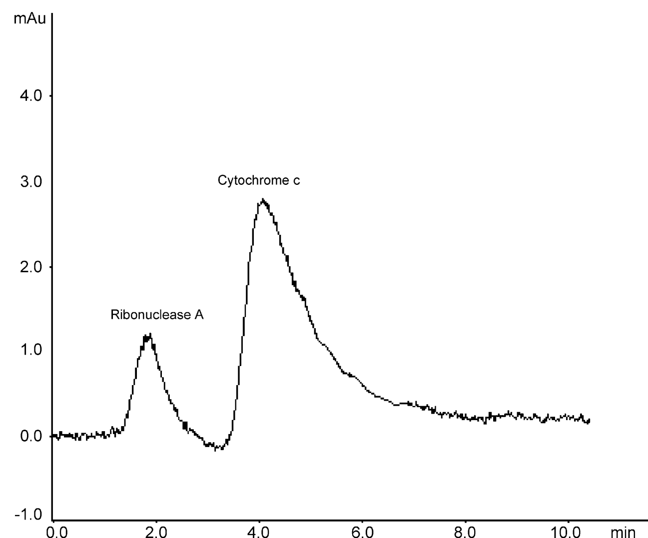

Fig. 3. Separation of cytochrome *c* and ribonuclease A on MIP for L-histidine. Column size, 50 mm × 5 mm i.d.; mobile phase, 0.05 M NaH_2PO_4 buffer containing 0.1 M imidazole; detection, 254 nm [15].

applied for recognition of cytochrome *c* and ribonuclease A (surface His exposed proteins) as shown in Fig. 3. In a hydro-organic mobile phase, a MIP for benzo[a]pyrene prepared by suspension polymerization in non-covalent molecular imprinting showed similar affinity with that prepared by bulk polymerization, when divinylbenzene (DVB) and 4-vinylpyridine (4-VPY) were used as a crosslinker and functional monomer, respectively [16]. This result suggests that water used as a continuous phase does not show any obvious effects on the affinity of the benzo[*a*]pyrene-imprinted 4-VPY-*co*-DVB polymers compared to that of the bulk polymer [16]. Strong hydrophobic interactions could compensate for the weakening of electrostatic interactions in this system [16,17].

As HPLC stationary phases or SPE media, spherical MIPs are superior to irregular ones prepared by bulk polymerization in their performance. Therefore, MIPs for some compounds have been prepared by suspension polymerization in water in non-covalent molecular imprinting. Though a MIP for trimethoprim gave less selectivity and adsorptive capacity than that prepared by bulk polymerization, it was successfully used to enrich and separate trimethoprim in human urine and pharmaceutical tablets [18]. A MIP for 4-aminopyridine was prepared by using methacrylic acid (MAA) and EDMA as a functional monomer and crosslinker, respectively [19]. The prepared MIP was applied for separation of 4-aminopyridine and 2-aminopyridine, whose pK_a values are 9.26 and 6.67, respectively. In a mobile phase at pH <5.5, 4-aminopyridine was eluted prior to 2-aminopyridine, while in a mobile phase at pH >5.5 the former gave a lager retention factor than the latter. This result suggests that ionic and hydrophobic interactions could work for recognition of 4-aminopyridine on the MIP. A MIP for matrine was prepared using MAA and EDMA as a functional monomer and crosslinker, respectively [20]. The prepared MIP could be used for selective SPE of matrine and oxymatrine from a traditional Chinese medicinal plant.

Recently, a MIP for dibutylmelamine, which is used as a pseudo-template molecule, was prepared [21]. The prepared MIP was successfully used for SPE of atrazine in a model sample spiked with a mixture of herbicides. It is well known that the leakage of a template molecule from the MIP affects on accuracy and precision of the assay in the case of ultra-trace analysis [7]. It comes from remainder of a trace amount of the template molecule in the resultant MIP. This problem has been overcome by imprinting a structurally related analogue (pseudo-template molecule) and combining with chromatographic separations [7]. Similarly, a MIP for amobarbital was prepared and used as selective SPE sorbents for extracting phenobarbital from human urine and medicines [22]. Furthermore, MIPs for bisphenol A (BPA) and BPA-d_{16} were prepared using 4-VPY and EDMA as a functional monomer and crosslinker, respectively [23]. The selectivity factor, which is the ratio of the retention factor (k) on a MIP to that on a non-imprinted polymer (NIP), k_{MIP}/k_{NIP}, was used to evaluate the molecular recognition ability of the MIPs. The selectivity factors for BPA on the MIPs for BPA and BPA-d_{16} were 4.45 and 4.43, respectively. Almost the same imprinting effect for BPA was obtained with both MIPs. This is due to that the shape and functionality of BPA and its deuterated form are almost the same with one another. Two phenolic groups of BPA could interact with pyridinyl groups of BPA- and BPA-d_{16}-imprinted 4-VPY-*co*-EDMA polymers. The latter polymers were used for selective extraction of BPA in river water samples as SPE sorbents followed by determination of BPA in liquid chromatography–mass spectrometry (LC–MS) [23]. These results reveal that the combined use of isotope imprinting and MS detection seems useful for MIP-based extraction of a target analyte in the case of its ultra-trace analysis.

2.2. Suspension polymerization in liquid perfluorocarbon

A MIP for *tert*-butoxycarbonyl-L-phenylalanine (Boc-L-Phe) was prepared in perfluoro(methylcyclohexane) using MAA, EDMA or tris(hydroxymethyl)propane trimethacrylate (TRIM) and chloroform as a functional monomer, crosslinker and porogen, respectively, by photo polymerization [11]. The MIP gave near baseline resolution for Boc-Phe enantiomers, compared with that prepared by bulk polymerization under similar conditions. An acrylate polymer with perfluorocarbon and poly(oxyethylene) ester groups as a surfactant was used for a stabilizer. The average bead size could be controlled between about 5 and 50 μm by varying the amount of the stabilizing polymer [11]. By subtle changes of surfactant design the MIP for Boc-L-Phe could be prepared using chloroform, dichloroethane or toluene as a porogen by photo or thermal polymerization, resulting in the baseline separation of Boc-Phe enantiomers [24]. Furthermore, MIPs for propranolol (PRP) and morphine were directly polymerized under UV light in SPE cartridges, resulting in a rapid and automatable process that requires no transfer or manipulation of the MIP microspheres [25].

N-(Benzyloxycarbonyl)-α-L-aspartyl-L-phenylalanine methyl ester (α-L,L-ZAPM) is a useful precursor for aspartame, artificial sweetener. The byproduct, *N*-(benzyloxycarbonyl)-β-L-aspartyl-L-phenylalanine methyl ester (β-L,L-ZAPM), was chromatographically removed from the product in the chemical synthesis using the MIP for β-L,L-ZAPM prepared by suspension polymerization in perfluorocarbon [26].

MIPs for carbaryl and 1-naphtol gave selective and reversible binding to carbaryl and 1-naphtol, respectively [27]. A 1:1 mixture of carbaryl- and 1-naphtol-imprinted polymers was used as a pretreatment column for extraction of carbaryl and its metabolite (1-naphtol) in complex matrices (rat plasma and apple homogenate) in column-switching HPLC.

2.3. Suspension polymerization in mineral oil

A MIP for PRP was prepared by suspension polymerization in mineral oil [12,28]. MIP microspheres of 1–100 μm in diameter were obtained with almost quantitative yield by optimizing experimental conditions. Though the MIP was applied for a radioligand binding assay of PRP in human serum in a 96-well format, no application as chromatographic media has been tried yet. However, this polymerization method is a promising candidate for the preparation of MIPs as HPLC stationary phases and SPE media.

3. Seed polymerization

A multi-step swelling and polymerization method was first applied for the preparation of a MIP for diaminonaphthalene using MAA and EDMA as a functional monomer and crosslinker, respectively [13]. The MIP was applied for the separation of diaminonaphthalene isomers using acetonitrile as a mobile phase by HPLC. MIPs for β-estradiol [29–31], BPA [30,32–34], catechin gallate [35] were prepared using 4-VPY or 2-vinylpyridine (2-VPY) as a functional monomer and applied for specific recognition of each template molecule using a hydro-organic mobile phase or organic mobile phase. When an organic mobile phase was used, hydrogen bonding interactions between a template molecule and pyridinyl groups on the polymer worked for recognition of the template molecule in addition to its shape recognition [35]. On the other hand, in addition to those interactions hydrophobic interactions seem important in a hydro-organic mobile phase [35]. It is noteworthy that MIPs prepared by multi-step swelling and polymerization could work well in a hydro-organic mobile phases [31,33,35]. Since the monomer phase in the pre-polymerization step must be water-saturated, it is thought that the prepared MIPs could work well in a water rich mobile phase [4]. Similarly, MIPs for clenbuterol [36], cinchona alkaloids [37] and atropine [38] were prepared using MAA or acrylamide as a functional monomer and evaluated using a hydro-organic mobile phase. In addition to its shape recognition, ionic and hydrophobic interactions could work for recognition of each template molecule [37,38], when MAA was used as a functional monomer.

It has been reported that porogenic solvents, which produce macroporous structures into a crosslinked polymer network, can be somehow imprinted onto crosslinked polymer matrix [39]. The crosslinked polymers were prepared using *o*- or *p*-xylene as a porogenic template without use of a functional monomer [40]. The prepared polymer (MIP for *o*- or *p*-xylene) was applied

for separations of tetra-chlorinated dibenzo-*p*-dioxins (TCDDs). TCDDs having chlorine atoms at *ortho* positions of phenyl rings were selectively retained on the MIP prepared with *o*-xylene as a porogenic template, while TCDDs having chlorine atoms at *para* positions of phenyl ring were selectively retained on the MIP prepared with *p*-xylene [40]. These results indicate that *o*- or *p*-xylene used as the porogenic template is imprinted and that by their shape recognition the positions of chlorine atoms on TCDDs could be recognized.

Furthermore, MIPs for chiral templates such as (*S*)-naproxen [41–43], (*S*)-ibuprofen [44], (*S*)-PRP [45,46], *d*-chlorpheniramine [47], *d*-brompheniramine [47,48], (+)-nilvadipine [49,50], *N*-protected L-amino acids [51–53] and (−)-ephedrine [54] were prepared by multi-step swelling and polymerization. The baseline or near baseline resolution of enantiomers was attained using a hydro-organic mobile phase or organic mobile phase. The MIPs for (+)-nilvadipine were prepared with 4-VPY, 2-VPY, MAA or 2-(trifluromethyl)acrylic acid as a functional monomer or with no use of a functional monomer [49]. In a hydro-organic mobile phase, the (+)-nilvadipine-imprinted 4-VPY-*co*-EDMA polymers gave the highest resolution for nilvadipine among the prepared MIPs. ^{1}H NMR and molecular modeling studies suggested a one-to-one hydrogen-bonding-based complex formation of (+)-nilvadipine with 4-VPY in chloroform [49]. In addition, enantioseparation of nilvadipine was attained on the (+)-nilvadipine-imprinted EDMA polymers without use of a functional monomer. These results reveal that the (+)-nilvadipine-imprinted EDMA polymers could recognize the template molecule by its shape recognition and that in addition, hydrophobic and hydrogen-bonding interactions seem to play important roles in the retention and chiral recognition of nilvadipine on the 4-VPY-*co*-EDMA polymers in a hydro-organic mobile phase. By optimizing chromatographic conditions such as column temperature and flow rate, the baseline separation of nilvadipine enantiomers was attained with a short analysis time and with a column efficiency comparable to commercially available chiral stationary phases based on a protein such as ovomucoid or α_1-acid glycoprotein, as shown in Fig. 4 [49].

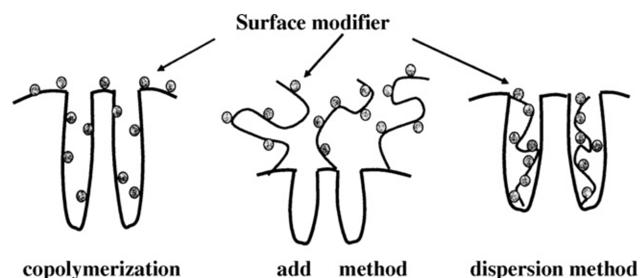

Fig. 5. Schematic image of the surface-modified polymer [57].

By one step swelling and polymerization, MIPs for sulfamethazine [55] and metsulfuron-methyl [56] were prepared. The size uniformity of the prepared MIPs was almost the same with those prepared by multi-step swelling and polymerization.

The excellent features of multi-step swelling and polymerization techniques are easy to modify the surface of prepared MIPs. MIPs prepared by bulk polymerization have little chance of an additional modification. However, multi-step swelling and polymerization can afford another functionality to the prepared MIPs. Two surface modification methods have been tried: one is an addition method and the other is a dispersion method [57]. The former was performed by addition of polar monomers such as a mixture of glycerol monomethacrylate (GMMA) and glycerol dimethacrylate (GDMA) after initiation of the polymerization step in the multi-step swelling and polymerization method (i.e., in situ surface modification), while the latter was performed by dispersion of MIPs in a solvent including a mixture of GMMA and GDMA or methacrylic acid 3-sulfopropyl (MAS). As shown in Fig. 5, the former method could modify the outer surface or macropores (>50 nm), while the latter method could mainly modify the mesopores (2–50-nm diameter), not affecting the macropores and micropores (<2 nm) [57].

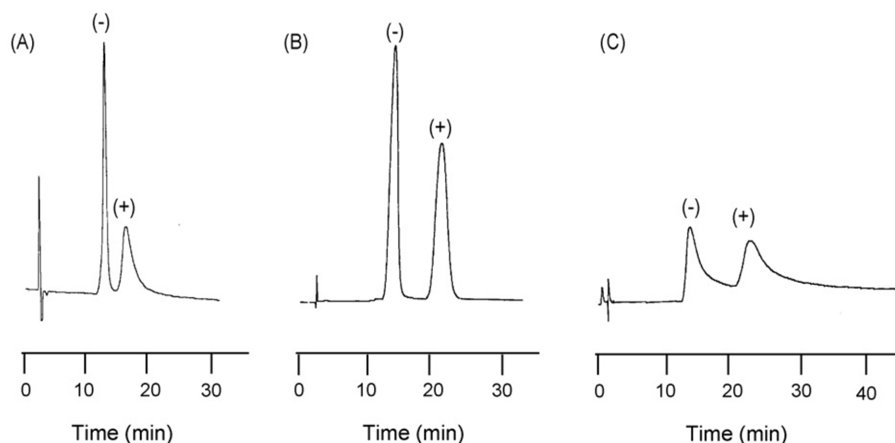

Fig. 4. Comparison of enantioseparation of nilvadipine on MIP for (+)-nilvadipine (A) with ovomucoid (B) and α_1-acid glycoprotein (C) columns. HPLC conditions for (A): column size, 100 mm × 4.6 mm i.d.; column temperature, 70 °C; mobile phase, 20 mM sodium phosphate buffer (pH 4.0)–acetonitrile (55:45, v/v); flow rate, 0.5 mL/min. HPLC conditions for (B): column size, 150 mm × 4.6 mm i.d.; column temperature, 25 °C; mobile phase, 10 mM sodium phosphate buffer (pH 4.7)–ethanol (80:20, v/v); flow rate, 1.0 mL/min. HPLC conditions for (C): column size, 100 mm × 4.0 mm i.d.; column temperature, 25 °C; mobile phase, 30 mM sodium phosphate buffer (pH 6.5)–2-propanol (92.5:7.5, v/v); flow rate, 0.9 mL/min. For all experiments, the loaded amount was 0.5 μg and the detection wavelength was 236 nm [49].

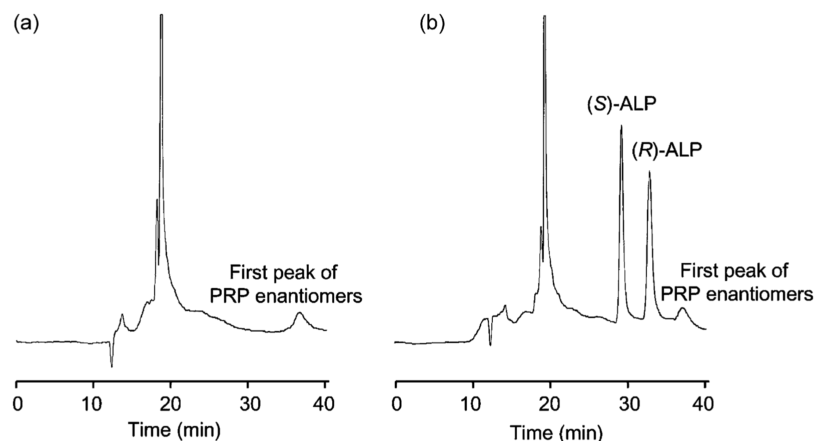

Fig. 6. Chromatograms of a rat plasma sample (a) and a rat plasma sample spiked with alprenolol (ALP) (b) using RAM-MIP for propranolol (PRP) as a pretreatment column. Pretreatment conditions: pretreatment column, RAM-MIP (10 mm × 4.0 mm i.d.); mobile phase, 20 mM phosphate buffer (pH 5.8)–acetonitrile (80:20, v/v, pH 6.2) at 1.0 mL/min for 7 min; column temperature, 35 °C; concentration of racemic ALP, 200 ng/mL; injection volume, 50 μL. Analysis conditions: analytical column, ULTRON ES-PhCD (150 mm × 6.0 mm i.d.); mobile phase, a mixture of 30% mobile phase A [20 mM phosphate buffer (pH 3.2)–acetonitrile (95:5, v/v, pH 3.3)] and 70% mobile phase B [20 mM phosphate buffer (pH 3.2)–acetonitrile (70:30, v/v, pH 3.7)]; time program, 70% mobile phase B at 0 min, 70% mobile phase B at 36 min, 100% mobile phase B at 36.01 min, 100% mobile phase B at 41.00 min and 70% mobile phase B at 41.01 min. Flow rate, 0.7 mL/min; column temperature, 35 °C; detection, fluorometry (excitation and emission wavelengths, 279 and 305 nm) [60].

Using an addition method, restricted access media (RAM)-MIPs for (*S*)-naproxen [58,59] and PRP [60], whose surfaces are modified with a mixture of GMMA and GDMA, were prepared. Similarly, RAM-MIPs for *p-tert*-butylphenol [57,61] and 4,4-methylenebisphenol (MBP) [57,61] were prepared for the purpose of ultra-trace analysis of BPA. As mentioned in previous Section, leakage of template molecules would prevent ultra-trace assays of a target analyte. Thus, MIPs were prepared using a structurally related analogue as a pseudo-template molecule. Another way to overcome the template-leakage problem is by combined use of isotope imprinting and MS detection as described in previous Section. The prepared RAM-MIP for BPA-d_{16} was used for direct injection analysis of BPA in serum or river water samples combined with column switching LC–MS [62,63]. The RAM-MIP for propranolol was used for selective extraction of alprenolol (ALP) from biological samples combined with column-switching HPLC [60]. Fig. 6 shows that ALP is separated from PRP and ordinary components in plasma samples, and that ALP could be enantioseparated on a chiral HPLC column.

Using a dispersion method, the MIP for MBP modified with MAS, which is an ionic monomer, was prepared and utilized for ultra-trace assays of BPA in river and lake water samples [57,61]. The MIP modified with MAS could effectively remove the interferences in those samples, compared with that modified with a mixture of GMMA and GDMA. Since the major interferences such as water soluble oligomers of humic materials in environmental water samples were negatively charged, these could be repulsively excluded from the surface-modified polymers, which have a sulfopropyl group of MAS [61].

4. Dispersion/precipitation polymerization

A MIP for pentamidine was prepared using MAA as a functional monomer, EDMA as a crosslinker and 2-propanol–water as a porogen in situ in the chromatographic column by dispersion polymerization [64,65]. Pentamidine was more strongly retained on the MIP for pentamidine than that for benzamide, reference polymer. This could be due to the difference in the number of potential interaction sites: pentamidine is able to bind complimentarily to the sites by two strong ionic interactions of pentamidine with carboxyl groups on the MIP for pentamidine, while one ionic interactions of pentamidine are only possible on that for benzamide. Thus, the selective enrichment of pentamidine in urine samples was attained with the absorption and desorption of pentamidine by changing mobile phase pH. Pentamidine in urine samples was absorbed to the MIP for pentamidine using a mobile phase of pH 5. After washing out the ordinary components of urine using a mobile phase of pH 9, pentamidine was desorbed from the column using a mobile phase of pH 3 [65].

In general, precipitation polymerization gave MIP aggregates or microspheres of one or a few hundred nanometers in diameter [66]. Recently, it is found that matching the solubility parameter of the developing polymer network to that of the porogenic solvent(s) is particularly important in precipitation polymerization. Copolymerization of DVB in a mixture of acetonitrile and toluene gave monodispersed microsheres. This approach was used to prepare monodispersed theophylline-imprinted polymers of about 5 μm in diameter using MAA as a functional monomer [66]. The prepared MIP could be applied for HPLC separation of theophylline. Similarly, the MIP microspheres for thiabendazole (ca. 3.5 μm in diameter) [67], PRP (ca. 3.5 μm) [68], β-estradiol (ca. 3 μm) [69] and (*S*)-nicotine (ca. 3.5 μm) [70] were prepared using DVB and a mixture of acetonitrile and toluene as a crosslinker and polymerization medium, respectively. The MIP for (*S*)-nicotine has been applied for separation of nicotine enantiomers and selective removal of nicotine from cigarette smoke extracts [70]. Fig. 7A and B shows chromatograms of nicotine enantiomers on the NIP and

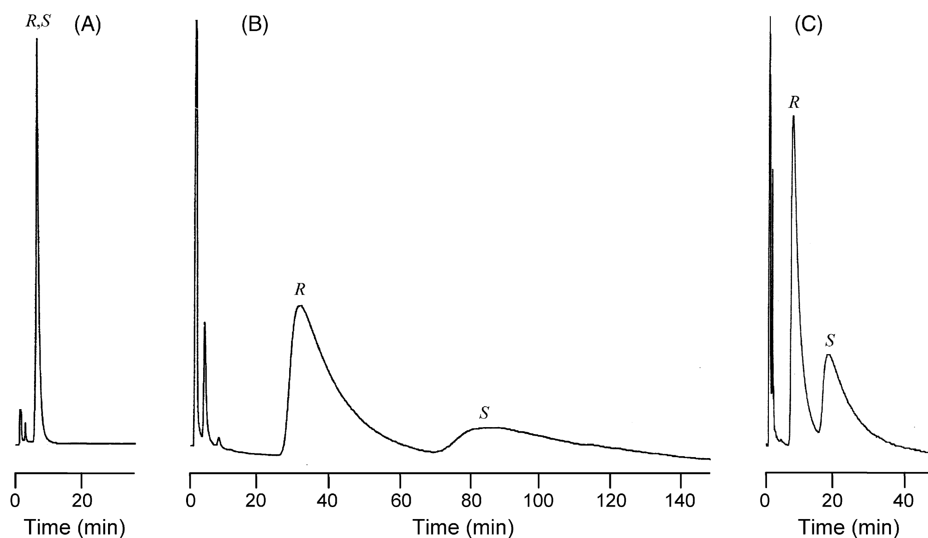

Fig. 7. Chromatograms of nicotine enantiomers on NIP (A) and MIP for (*S*)-nicotine (B and C). HPLC conditions: column size, 50 mm × 2.0 mm i.d.; column temperature, 25 °C for (A) and (B) and 70 °C for (C); mobile phase, 20 mM sodium phosphate buffer (pH 7.9)–acetonitrile (50:50, v/v) for (A) and (B) and 20 mM sodium phosphate (pH 6.0)–acetonitrile (35:65, v/v) for (C); flow rate, 0.2 mL/min for (A) and (B), and 0.25 mL/min for (C); detection, 254 nm; loaded amount, 500 ng for (A) and (B), and 125 ng for (C). Separation parameters: k_R and k_S, 4.3; α, 1.0 for (A), k_R, 27; k_S, 72; α, 2.7; Rs, 1.1 for (B) and k_R, 8.9; k_S, 22; α, 2.5; Rs, 1.1 for (C) [70].

MIP for (*S*)-nicotine. On the MIP, nicotine enantiomers were well resolved and the (*S*)-antipode was eluted later. By optimizing chromatographic conditions such as mobile phase pH, column temperature and flow rate, the baseline separation of nicotine enantiomers could be achieved with a short analysis time, as shown in Fig. 7C. Recently, the effect of a crosslinking monomer on the final size of MIPs was examined varying the ratio of DVB and TRIM [71]. The size of MIP beads for PRP ranged from 2.4 μm to 130 nm with an increase of a feed ratio of TRIM to DVB. This result suggests that by changing the ratio of two crosslinkers the MIP sizes could be altered from micrometer to nanometer sizes in precipitation polymerization.

Using the other crosslinkers and polymerization media in precipitation polymerization, monodispersed MIPs for fenuron (EDMA and toluene as a crosslinker and polymerization medium, respectively) [72], sulfasalazine (EDMA and a mixture of acetonitrile and toluene) [73] and BPA (TRIM and acetonitrile) [74] were prepared. The MIP sizes were ∼1 μm for fenuron and BPA, and ca. 4 μm for sulfasalazine. The MIPs for BPA could selectively separate BPA from its structurally related compounds. Fig. 8a and b shows chromatograms of phenol, *p*-nitrophenol, diethylstilbestrol, hexoestrol, bisphenol C and BPA on the MIP and NIP, respectively.

5. Others

5.1. Grafting imprinting

Thin films of MIPs were grafted to the surface of silica gels or organic polymers containing polymerizable double bonds. The surface of a wide pore silica activated with propylmethacrylate was grafted with thin layers of polymers imprinted with a template-monomer, phenyl-2,3,4,6-bis-*O*-(4-vinylphenylboronyl)-α-D-mannopyranoside [75]. The baseline separation of (4-aminopropylphenyl)-α-D,L-mannopyranoside was attained on the MIP by HPLC. Propylmethacrylate-activated silica particles were grafted by copolymerization with a metal-chelating monomer, Cu^{2+}-[*N*-(4-vinylbenzyl)-imino]diacetic acid, and EDMA in the presence of a metal-cordinating imidazole template [76]. The MIP was

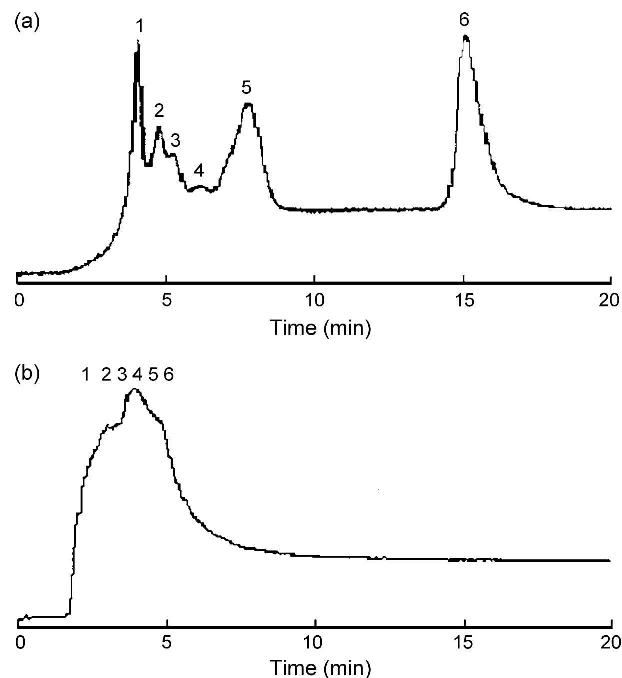

Fig. 8. Chromatograms of phenol (1), *p*-nitrophenol (2), diethylstilbestrol (3), hexoestrol (4), bisphenol C (5) and bisphenol A (6) obtained by using 70% methanol for 13 min then 100% methanol for 7 min as a mobile phase. (a) MIP for bisphenol A and (b) NIP column. HPLC conditions: flow rate, 1 mL/min; injection volume, 10 μL; analytes' concentrations, 0.2 mmol/L each in acetonitrile [74].

applied for separation of mono- or bis-imidazole compounds. Similarly, thin films of a MIP for L-Phe was grafted to propylmethacrylate-activated silica gels [77]. The MIP could resolve racemic Phe or Tyr, but could not resolve racemic Trp, Ala, Leu or Ile. This could be explained by rebinding of the L-enantiomer proceeding through chelation of the metal ion, in addition to which aromatic side chain fits into a cavity that selects for both the size and shape of this group [77].

The surface of TRIM containing residual double bonds was grafted with thin layers of a MIP for Boc-L-Phe [78]. The separation of Boc-Phe enantiomers was attained on the MIP with better chromatography performance than that prepared by conventional bulk polymerization. MIPs for phenol were synthesized by surface grafting on glycidyl methacrylate-*co*-EDMA polymers in covalent molecular imprinting [79].

The problem of the above technique is the presence of initiator in solution. Therefore, it is difficult to control the thickness of the polymer layer. Moreover, the maximum density of grafted polymer chains is limited due to kinetic and steric factors. To overcome these problems, the initiators were immobilized on the surface of silica gels or organic polymers, which allow the synthesis of thin film MIPs. First, immobilized azo initiators, which decompose to give two active radicals, were introduced onto wide pore silica gels or organic polymers [80]. However, solution polymerization could not be avoided due to one point attachment of the initiators. A MIP for L-Phe anilide (L-PA) grafted as thin films (ca. 0.8 nm as average film thickness) on a silica with 10 nm average pore diameter showed the highest column efficiency, and gave the theoretical plate numbers (N) for the imprinted enantiomer of ca. 700 m^{-1} and those for the antipode of ca. 24000 m^{-1}. On the other hand, the MIP prepared by conventional bulk polymerization exhibited N for L-enantiomer of ca. <700 m^{-1}, while those for D-enantiomer were <2000 m^{-1}. These results indicate the merit of MIP microspheres compared with conventional irregular ones [80]. However, because of its site heterogeneity and slow desorption, the template peak exhibited only a slight sharpening [80–82].

Next, immobilized dithiocarbamate initiators (initiator-chain transfer agents, iniferters), where the mobile radicals formed upon decomposition are stable and propagating chains, were used for the preparation of a MIP for L-PA [83], propazine [84] and pyrimethanil [85]. Furthermore, the last MIP was successfully applied to selective SPE of pyrimethanil in wine [86]. However, the iniferter-grafted MIPs are inferior to azo-grafted ones in the chromatographic mode. To address this problem, the azo-initiated grafting was performed by addition of the chain transfer agent [so-called RAFT (reversible addition-fragmentation chain transfer)], 2-phenylprop-2-yldithiobenzoate, as shown in Fig. 9 [87]. By this method, the MIP for L-PA grafted on a silica as thin homogeneous films (1–2 nm in thickness) was prepared. Fig. 10A and B shows separations of D,L-PA by normal and reversed-phase modes, respectively, on the columns packed with the MIP for L-PA prepared in the presence or absence of a RAFT agent. By addition of the RAFT agent, higher retentivity and enantioselectivity for L-PA were obtained [87].

5.2. Hierarchical imprinting

Spherical, monodispersed MIP beads could be easily prepared by hierarchical imprinting. Fig. 11 shows schematic representation of hierarchical imprinting. It was carried out as

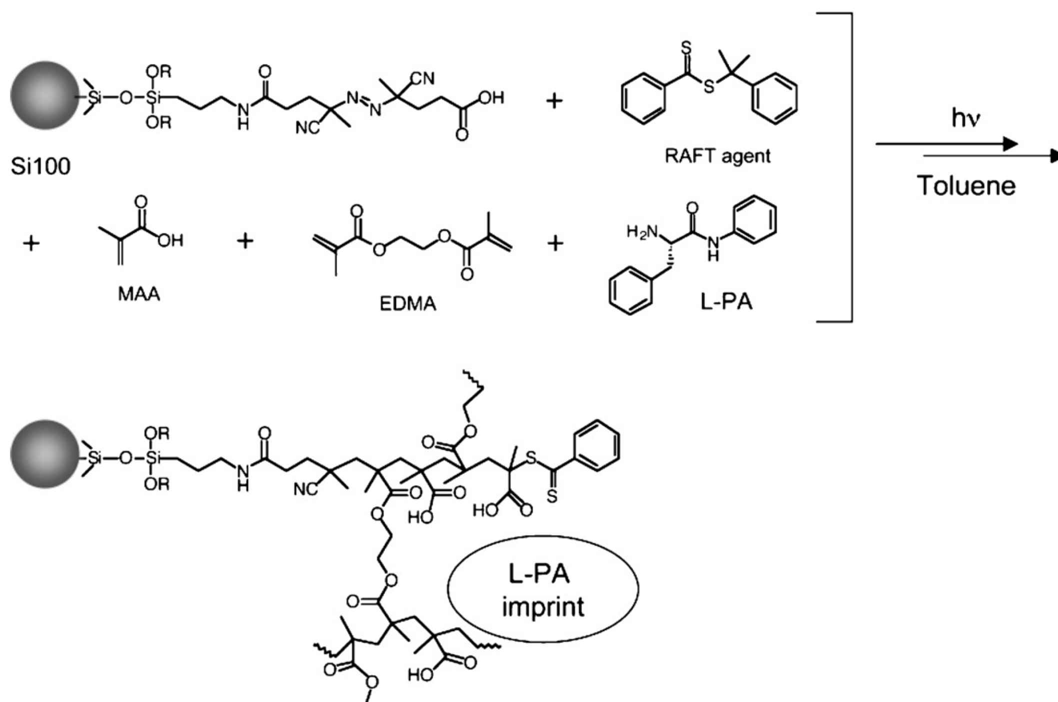

Fig. 9. Schematic representation of the grafting of L-phenylalanine anilide (L-PA) imprinted polymer films from porous silica supports controlled by addition of a RAFT agent [87].

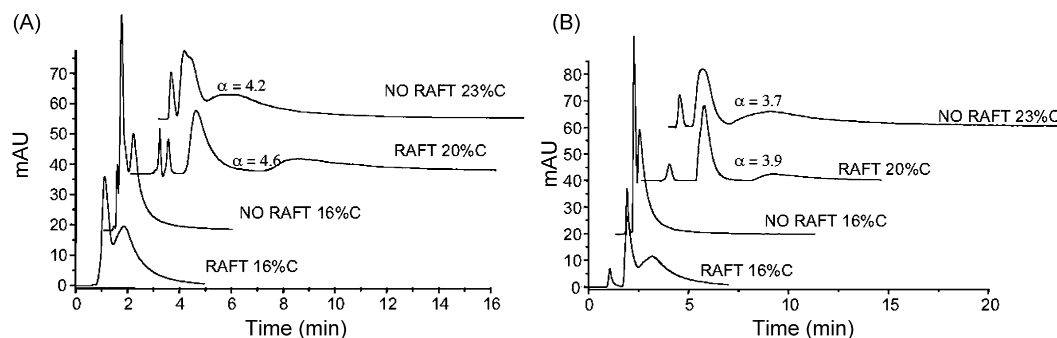

Fig. 10. Elution profiles of D,L-PA (10 nmol) injected on columns packed with MIPs prepared in the presence or absence of a RAFT agent. Mobile phase, acetonitrile–H_2O–acetic acid (92.5:5:2.5, v/v/v) for A and acetonitrile–0.01 M sodium acetate buffer (pH 4.8) (70/30, v/v) for B [87].

follows: (1) immobilization of a template (theophylline) on a solid support (for example, silica gels), (2) polymerization in the solid support and (3) removal (dissolution) of the solid support [88]. The prepared MIP could rebind with theophylline, but not its analogues, theobromine and caffeine. Since no porogen was used in this technique, all imprinted binding sites could be located at, or close to, the surface of the pores and interactions between a template and functional monomer could not be hampered [88].

Similarly, MIPs for *l*-isoproterenol [89], 9-ethyladenine and triaminopyrimidine [90], dipeptide [91,92] and BPA [93] were prepared. The prepared MIP for 9-fluorenylmethyloxycarbonyl (FMOC)-Phe-OH, preferentially retained *N*-protected Phe derivatives, including the dipeptide FMOC-Phe-Gly-OH, with ca. five times larger retention factors than that prepared using soluble FMOC-Phe-OH as a template molecule. The MIP for dipeptide, H-Phe-Gly-OH, could recognize the heptadecapeptide (nociceptin), whose amino acid sequences are H-FGGFTGARKSARKLANQ-OH, two-times more strongly than the MIP for H-Gly-OH [92].

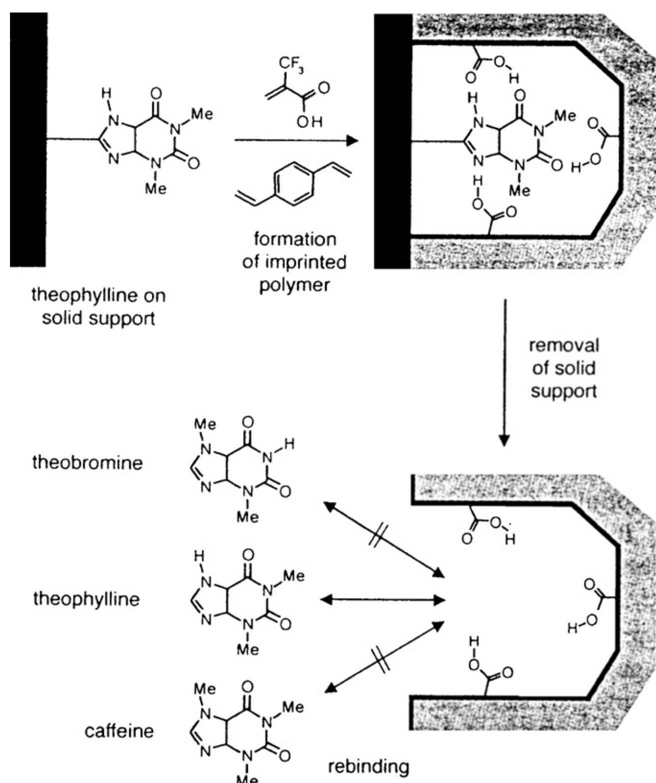

Fig. 11. Schematic representation of hierarchical imprinting [88].

6. Conclusion

Preparation methods for spherical and monodispersed MIPs in micrometer sizes have been described in this review article. Those methods include suspension polymerization in water, liquid perfluorocarbon and mineral oil, seed polymerization and dispersion/precipitation polymerization. The other methods are the use of beaded materials such as a spherical silica or organic polymer for grafting MIP phases onto the surfaces of porous materials or filling the pores of silica with MIPs followed by dissolution of the silica.

Spherical and monodispersed MIPs are superior to irregular particles prepared by bulk polymerization in their chromatographic performance as HPLC stationary phases or SPE media. However, the MIP microspheres gave less or comparable retentivity and selectivity compared with conventional MIPs prepared by bulk polymerization. In the case of suspension polymerization in water and seed polymerization, water used as a continuous phase interfered with interactions between a template molecule and functional monomer. In dispersion/precipitation polymerization, the excess of a polymerization medium could hamper interactions between a template molecule and functional monomer. Furthermore, in grafting imprinting, thin films of MIP phases resulted in low capacity.

MIP microspheres were successfully applied for pharmaceutical, biomedical and environmental analysis as affinity-based chromatography media, HPLC stationary phases and solid-phase extraction media. Especially, for applications of biomedical and environmental analysis, surface-modified MIPs prepared by multi-step swelling and polymerization could efficiently remove interferences in those samples. Monodispersed, MIPs and surface-modified MIPs seem useful as affinity-based chromatography media.

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