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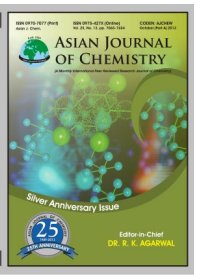

Synthesis and Evaluation of Cyromazine Molecularly Imprinted Polymeric Microspheres by Two-Step Seed Swelling Polymerization

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A novel cyromazine molecularly imprinted polymeric microspheres were prepared by two-step seed swelling polymerization method in aqueous system. The imprinting process was carried out using polystyrene particles as the seed, cyromazine as the template molecule, methacrylic acid as the functional monomer, ethylene glycol dimethacrylate as the cross-linker. Factors affecting the particle morphologies including emulsifier, dispersant, the water-oil ratio and *etc*., were optimized in order to obtain the molecularly imprinted polymeric microspheres with a final narrow particle diameter of 3-5 μm. The prepared molecularly imprinted polymeric microspheres were characterized and evaluated by scanning electron microscope, infrared absorption spectroscopy, mercury analyzer and equilibrium binding experiments, respectively. Scatchard analysis suggested cyromazine were recognized by the prepared molecularly imprinted polymeric microspheres with two classes of binding sites. The apparent maximum binding capacity and dissociation constant were calculated to be 1.25 μmol/g (Q_{max_1}) and 1.27 μmol/L (K_{d_1}) for high affinity site, whilst to be 2.84 μmol/g (Q_{max_2}) and 10.02 μmol/L (K_{d_2}) for low affinity site, respectively. The cyromazine molecularly imprinted polymeric microspheres synthesised in this method could be used as a stationary phase for the selective analysis of cyromazine in real samples by high performance liquid chromatography.

Key Words: Molecular imprinted polymer microspheres, Two-step seed swelling, Scatchard analysis, Cyromazine.

INTRODUCTION

Polymer-based stationary phases have been utilized for a variety of separations in high performance liquid chromatography and related technologies[1]. Especially, molecular imprinted polymeric microspheres (MIPMs) that exhibit high selectivity and affinity to template molecules are a rapidly growing research focus[2-5]. The imprinting process is performed by functional monomers and cross-linkers in the presence of a template molecule that corresponds to a certain target molecular. The subsequent removal of the imprint molecule reveals binding sites in the polymer network. The advantages of MIPMs compared to other technology possess higher sample load capacity, low cost and wide applications used as affinity-based chromatography media, HPLC/CEC stationary phases, solid-phase extraction media, *etc*[6-10].

Molecular imprinted polymeric microspheres can be produced using suspension polymerization in water, liquid perfluorocarbon and mineral oil, seed polymerization and dispersion/precipitation polymerization[11-13]. The other approaches are the use of beaded materials such as a spherical silica or organic polymer for grafting molecularly imprinted polymeric phases onto the surfaces of porous materials or filling the pores of silica with MIPs followed by dissolution of the silica. Molecular imprinted polymeric microspheres prepared by bulk polymerization have little chance of an additional modification[14,15]. For the seed swelling polymerization method, the two important requirements for the production of uniformly sized MIPMs as functional carries are the availability of uniformly sized seed particles and control of swelling and polymerization process.

A multi-step swelling and polymerization method was first applied for the preparation of a MIP for diaminonaphthalene using methacrylic acid (MAA) and ethylene glycol dimethacrylate (EDMA) as a functional monomer and crosslinker, respectively[16]. Tuncel prepared PS-MAA-DVB macroporous particles in the size range of 3-13 μm by two step polymerization and investigated the influence of seed particles on the size and morphology of final particles[17]. Wang and Liu[18] adopted a two step swelling procedure to synthesize mono-dispersed and highly cross-linked poly (St-divinylbenzene) particles with PS microspheres. Hua *et al*., prepared a uniform-sized MIPMs for phenobarbital by a two step swelling polymerization

method and the resulted MIPMs were packed into a stainless steel column to evaluate their chromatographic characteristics by HPLC[19]. Porous polystyrene-divinylbenzene (PS-DVB) microspheres of narrow size distribution in the size range of 3.9-4.4 μm were prepared using the identical method. Effects of swelling conditions such as swelling activator, temperature, the way of dispersion and dibutyl phthalate (DBP)/seed particle ratio on particle size distribution of the PS-DVB microspheres were investigated in detail[20]. Similar work was further studied to prevent the formation of deformed polymer particles during synthesis by a seeded polymerization method[21].

The purpose of this work is to prepare cyromazine (CYR) MIPMs using polystyrene seed particles by two step swelling polymerization. The first step is the activation of the seed particles by the absorption of an activating solvent and the subsequent step is the absorption of the monomer, template, porogens, *etc*. The schematic representative of preparation of MIMPs and their imprinting principle are shown, respectively (Fig. 1). The effect of these swelling variables on the monodispersity of the particles was discussed and better swelling and polymerization conditions for the preparation of monodisperse functional MIPMs were investigated, respectively. Moreover, the prepared MIPMs and NIPMs were evaluated by adsorption experiments, Scatchard analysis and characterized by SEM, FT-IR and mercury analyzer in detail.

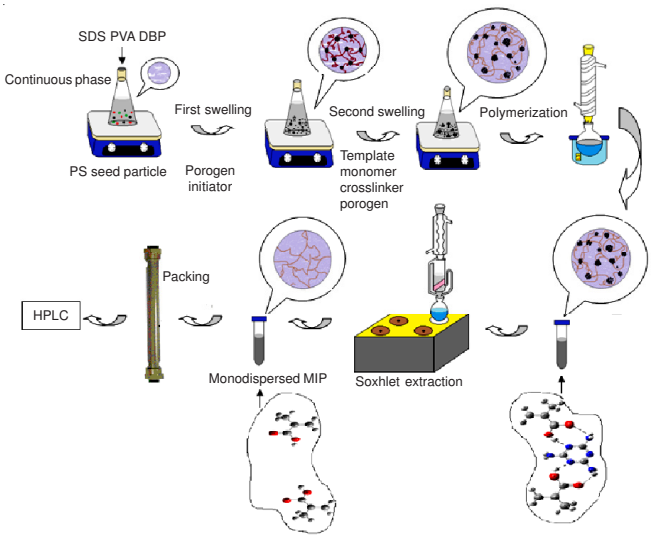

Fig. 1. Schematic representation of the preparation of MIPMs and their imprinting principle

EXPERIMENTAL

All mesurements were performed by a reversed-phase HPLC system from the Agilent Company (USA), containing a quaternary pump. TENSOR27 infrared spectrometer (BRUKER, Germany), PM-33-11 Poremasters (Quantachrome Instruments, Florida, USA), Quanta200 scanning electronic microscope (FEI, Hillsboro, Oregon, USA). UV-VIS-2100 Spectrophotometer (Beijing Rayleigh Analysis Instruments Limited Company, China). All chemicals used are of chromatographically grade and analytical grade. Distilled water was obtained from a super-purification system (Danyangmen Corp., Jiangshou, China). Cyromazine (99 %) was kindly provided by Hunan Agricultural University. MAA, EDMA, thiourea, 1-octanesulfonic acid sodium salt monohydrate, toluene, poly(vinyl alcohol) (PVA), citric acid, styrene, dibutyl phthalate (DBP), acetonitrile (ACN), 2,2-azobisisobutyronitrile (AIBN), cyclohexanol, sodium dodecyl sulfate (SDS), sodium dodecylsulfonate, potassium peroxydisulfate, tetrahydrofuran, acetone, acetic acid glacial and ammonia solution were purchased from Beijing Bailingwei Chemical Reagent Co. and Tianjin Chemical Reagent Co., China.

Preparation of monodisperse polystyrene seed particles: The polystyrene seed microspheres (PS) were prepared by emulsifier-free emulsion polymerization according to the following procedure: 20 mL styrene, 0.25 g sodium chloride and 200 mg of potassium peroxodisulfate were added to 250 mL of deionized water in a conical flask. The polymerization was carried out under nitrogen atmosphere at 75 ºC for 18 h. After the reaction, the obtained microspheres were washed repeatedly with deionized water and then centrifugated, the resulted solid was dried for later use.

Preparation of the uniform-sized molecular imprinted polymeric microspheres: The synthesis of MIPMs involves two step swelling and polymerization, the first step swelling was initially carried out by dissolving 0.1 g SDS and 0.3 g PVA with 60 mL deionized water in a 100 mL conical flask completely, then 0.74 g of polystyrene seed, 3 mL of DBP, 0.1 g AIBN and 3 mL of toluene were added. The mixture was stirred at room temperature until the emulsified organic phase was completely absorbed by the seed particles. When no oil drop was observed under an optical microscope, the first step of swelling was ended. The second step swelling was then done by dissolving 1 mmol cyromazine, 10 mmol MAA, 24 mmol EDMA, 3 mL toluene and 4 mL cyclohexanol in a 100 mL conical flask. This mixture was stirred (150 rpm) over night at room temperature. Then, this mixture was added into the first step swelling solution followed by continuous stirring. Until the oil droplets did not appear under the optical microscope, the second step was ended. The swelled solution was transferred into a sealed three-necked flask for polymerization and nitrogen was introduced into the flask to remove the dissolved oxygen, the whole polyreaction process was conducted at a constant temperature of 75 ºC for 12 h using an optimized stirring rate. The polymer emulsion obtained from the reaction was dispersed into 400 mL H_2O, the solution was boiled away for 10 min under stirring to break emulsion, then cooled to room temperature and centrifugated to obtain polymer microspheres. The above procedures were repeated for two times. The polymer microspheres was dried first and then extracted with methanol/acetic acid (9:1) in a Soxhlet extractor for 12 h, afterwards, washed with methanol for 6 h to remove the template molecules. The polymer was vacuum dried at 65 ºC for 24 h and put into a desiccator for later use. The NIPMs [non-imprinted (blank) polymer microsphere] preparation method was the same as the above, except that no template molecule was added during the preparation.

Steady-state binding experiments: In a 10 mL vial, 10 mg of MIPMs and NIPMs were mixed with 2.0 mL of 1-45 μmol/L of cyromazine standard solution, respectively, followed by a vibration for 24 h in a thermostat oscillator. After the vibration was completed, the mixed solution was moved into

a centrifuge tube and centrifugated for 10 min at 6,000 rpm. The supernatant was taken and diluted with methanol and brought to a proper concentration. Ultraviolent-visible spectrophotometer was used to determine the cyromazine concentration in the solution at adsorption equilibrium and the maximum binding capacity of the polymer for target molecule (Q_{max}) was calculated based on the change in concentration before and after the binding.

RESULTS AND DISCUSSION

The particle size of MIPMs and its distribution are related to various factors such as the type and amount of emulsifier, dispersant, the water-oil ratio, the stirring rate, porogen and *etc*. Herein, some factors influencing the shape and size of particles were investigated in detail by SEM. The final optimal recipes obtained for the synthesis MIPMs were shown for the two step seed swelling polymerization methods in Table-1. Monodispersed, well-defined and regular polymer particles were finally obtained under the optimal conditions with different sizes in the range of 1, 3-5, 3-5 μm, respectively (Fig. 2). Moreover, it could be visually observed that more pores were formed on the surface of MIPMs than that of NIPMs.

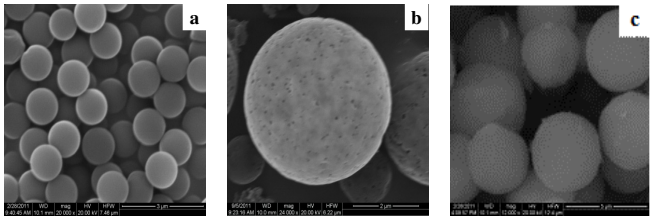

Fig. 2. SEM images of the prepared PS seed particles, NIPMs and MIPMs under the optimized conditions. Symbols a, b and c stand for PS seed particle, MIPMs and NIPMs

Infrared spectrum of MIPMs: The results of IR spectra of the monomer, template, cross-linker, seed particle, MIPMs and NIPMs were comparatively shown in Fig. 3. Obviously different IR spectra were obtained for each compound studied here. The strong absorption bands appeared at 1728 cm^{-1}, ascribed to C=O stretching vibration of EDMA molecule. The band at 1640 cm^{-1} of absorption bands was attributed to the C=C stretching vibration. The absorption bands for MIPMs appeared at 3550 cm^{-1} stand for O-H stretching vibration. It showed they contained the carbonyl groups in the cavity of cyromazine MIPMs, which interacted with the imprinting molecules by hydrogen bonds. In contrast, infrared spectrum of NIPMs was closed to that of MIPMs infrared spectrum, but some peak shapes appeared between 3650-3200 and 3100-2900 cm^{-1} are weaker or unobvious. This indicated that the surface of NIPMs had less free -OH groups. In addition, the strong absorption bands for EDMA and MAA at 1640 cm^{-1} appeared indistinctly in MPIMs, which approved that the full polymerization occurred between EDMA and MAA.

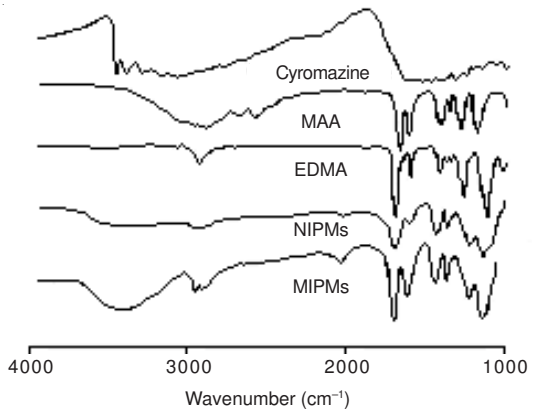

Fig. 3. IR spectra of EDMA, cyromazine, MAA, Seed, MIPMs and NIPMs

Pore structure analysis by mercury analyzer: Control of porogen is very important to stabilize the interactions between template and monomers. In general, pore sizes in the polymers can be classified into different size categories, span macropores (> 50 nm), mesopores (2-50 nm) and micropores (< 2 nm). Increasingly research interest lies in nanoporous materials having pores in the range less than 50 nm but importantly macroporosity above 50 nm provides much of the connective and mass transport in such systems[11,21,22]. In average, peaks for NIPMs in the distribution shifted slightly to lower pore sizes, compared with its relative MIPMs in Fig. 4 and there is a relatively large population of pores in the macroporous range for the obtained MIPMs. It is undoubted that the apertures and cavities volume of MIPMs were bigger than blank polymers from the comparative curves. Moreover, larger amount of macropores were obtained for MIPMs centred around at 100 nm than NIPMs around 80 nm. For MIPMs, most pores were formed in the range of 30-500 nm, whilst in the range of 10-200 nm for NIPMs. More macropores in the MIPMs showed that the addition of template in polymerization influenced the polymer structures and increase the pore size due to the removal of template.

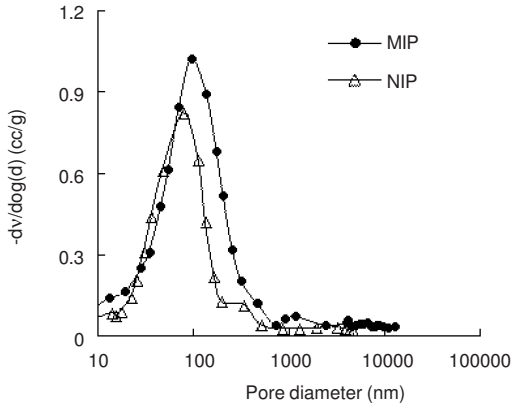

Fig. 4. Pore size distribution of MIPMs and NIPMs

TABLE-1
FINAL OPTIMAL RECIPES FOR THE PREPARATION OF CYROMAZINE MIPMS

First step of swelling					Second step of swelling				
SDS (g)	PVA (g)	PS (g)	AIBN (g)	Toluene (mL)	CYR (mM)	MAA (mM)	EDMA (mM)	Toluene (mL)	Cyclohex (mL)
0.1	0.3	0.74	0.1	3	1	10	24	3	4

Binding capacity of the MIPMs: Equilibrium adsorption experiments were done to evaluate the binding affinity of MIPMs and NIPMs for cyromazine. The results indicated that MIPMs had a higher binding capacity than NIPMs when cyromazine concentration was varied in the selected concentration range in Fig. 5 the binding capacity increased obviously with the increase of the initial cyromazine concentration for MIPMs, whilst slower increase was obtained for NIPMs. For example, when the initial concentration of cyromazine equal to 45 μmol/L, the binding capacity of MIPMs is 3.95 times than that of NIPM.

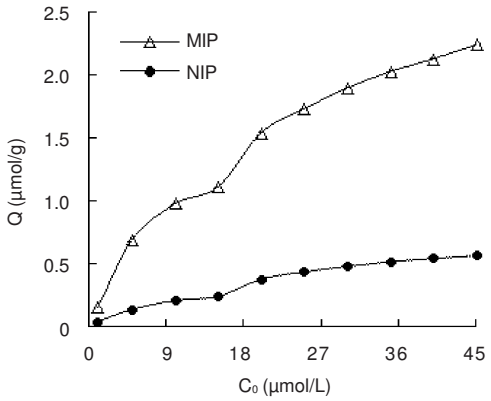

Fig. 5. Binding isotherm of the cyromazine (a) and Scatchard plots (b) for the prepared MIPMs and NIPMs

The data of the static absorption experiment was further processed with the Scatchard equation to estimate the binding parameters of the MIPMs and NIPMs. Scatchard equation was as follows: $Q/C = (Q_{max}-Q)/K_d$. Where, Q_{max} and K_d are maximum binding number and balance dissociation constants, respectively and C is the balance concentration of the template molecules. Based on the above equation, Q/C *versus* Q diagram was plotted (Fig. 6) and it can be seen from the Scatchard diagram that there was a nonlinear relationship between the Q/C and Q. It indicated that there were different binding sites in the MIPMs for cyromazine, but there was a good linear relationship in two evident parts at both ends of the diagram. This showed that there were two types of different binding sites in the MIPMs in the studied cyromazine concentration range. Linear fitting was conducted for the two parts separately and the following parameters were obtained from the slopes and intercepts of both straight lines. The binding sites with high (low) affinity and their largest binding amounts were comparatively listed for MIPMs and NIPMs in Table-2.

TABLE-2
DISSOCIATION CONSTANT (K_d) AND APPARENT MAXIMUM BINDING CAPACITY (Q_{max}) FOR BOTH BINDING SITES FOR MIPMS AND NIPMS

Polymers	Q_{max1} (μmol/g)	Q_{max2} (μmol/g)	K_{d1} (μmol/L)	K_{d2} (μmol/L)
MIPMs	1.25	2.84	1.27	10.02
NIPMs	0.35	0.98	6.71	29.26

Conclusion

Uniformly sized MIPMs for cyromazine were prepared by a two-step seed swelling polymerization method. Main factors influencing the size and shape of the formed MIPMs were optimized and the prepared polymers with a uniform size of 3-5 μm were characterized by SEM, FT-IR and MA. The adsorption capacity and Scatchard analysis showed that the MIPMs had high affinity and specificity for cyromazine, compared with NMIPs.

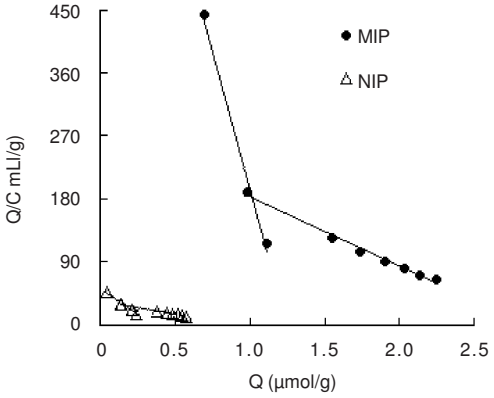

Fig. 6. Scatchard analysis of the prepared MMIPs and NIPMs

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