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Preparation of Avermectin Molecularly Imprinted Polymer Microspheres and Their Adsorption Properties

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In this study, avermectin molecularly imprinted microspheres have been prepared by high-temperature thermopolymerization under optimized conditions, employing avermectin as a template molecule, 4-vinylpyridine as a functional monomer, divinylbenzene as a crosslinking agent and azobisisobutyronitrile as an initiator. The interaction between functional monomers and template molecules was investigated by UV/visible and infrared spectroscopies and the adsorption properties of molecularly imprinted polymer and control polymer for avermectin were also comparatively analyzed. The results showed that the adsorption capacity of molecularly imprinted polymer for avermectin was significantly higher than that of the control polymer ($P < 0.01$), which indicated that the molecularly imprinted polymer microspheres prepared by this method had a specific adsorption effect on the template molecule and that the polymer microspheres could be used to detect and analyze avermectin residues.

Key Words: Adsorption, Avermectin, Microspheres, Molecularly imprinted polymer.

INTRODUCTION

Avermectins, a group of macrolides produced by *Streptomyces avermitilis*, contain eight natural components with similar structures. The compounds have significant repelling and killing efficacy on parasitic nematodes and arthropods in animals, thus allowing a reduction in the drug dosage of anti-worm agents from the mg kg^{-1} to the μg kg^{-1} level[1]. At present, avermectin, ivermectin, doramectin and eprinomectin are among the commercial drugs of this type and are considered as the most excellent and widespread anthelmintic agents for livestock[2,3]. During the last 20 years, the most outstanding results in research on parasitic agents have been achieved with these drugs because of their remarkable anthelmintic activity and high safety. Avermectin is soluble in most organic solvents, including chloroform, but has very low solubility in water (6-9 g L^{-1}). However, due to its extreme potency, it is toxic to freshwater aquatic life even at extremely low concentrations (for *Daphnia magna* it is toxic at 25 ng L^{-1}). With increasingly strict demands on the quality of animal products, almost every country has imposed limit regulations for veterinary drug residues in food. Therefore, avermectin residue in animal tissues has been one of the key monitoring objects in the research field of veterinary drug residues. Currently, there are several main residue-detecting methods, including HPLC[4,5], LC-MS-MS[6,7], immunity analysis technology[8] and immunoaffinity chromatography[9], each of which has its own advantages and disadvantages. Avermectin and similar drugs cannot be detected by gas chromatography because of their high molecular weight, involatility and low dosage in clinical use, whereas the sensitivity of thin-layer chromatography cannot meet the requirements for residue analysis. The sensitivity of LC-MS-MS can reach ng or even pg level and this technique is widely used in confirmatory analysis, but its popularization and application have been limited in China due to the relatively high cost of such tests. HPLC can adequately detect trace amounts of residues with good sensitivity and stability, high accuracy and a high level of specificity. However, it needs complex pretreatment and professional operators and incurs higher costs.

Molecularly imprinted polymer production is a technique that has been developed in recent years[10]. In the preparation of polymer microspheres by this approach, functional monomers are arranged around a template by covalent or non-covalent interactions between them. These functional monomers and cross-linkers are then co-polymerized. After removal of the

template, binding sites are left, which are complementary to the template in size, shape and functional groups. These binding sites exhibit a molecular recognition effect, greatly enhancing the affinity and selectivity of the molecularly imprinted polymer for its template[11]. Recently, molecularly imprinted polymers have attracted extensive attention and have been widely applied in many fields, such as solid-phase extraction, chemical sensors and artificial antibodies owing to their favourable selectivity, physical robustness and thermal stability, as well as low cost and easy preparation[12-14].

In this paper, we report the preparation of avermectin molecularly imprinted polymer microspheres, as well as an evaluation of their adsorption characteristics, with a view to detecting avermectin residues in a fast, sensitive and convenient way by combining this technique with solid-phase extraction and HPLC.

EXPERIMENTAL

A standard sample of avermectin (containing 92 % B1a) was obtained from DR company (Germany); methacrylic acid (MAA), methyl methacrylate, 4-vinylpyridine, 2-vinylpyridine and divinylbenzene (each HPLC grade) were purchased from the Sinopharm Chemical Reagent Co. Ltd.; acrylamide (AR grade) was obtained from Amresco; azobisisobutyronitrile (AIBN) was purchased from the Shanghai No. 4 Reagent and H.V. Chemical Co. Ltd.

UV/visible spectroscopic analysis of the interaction between template molecules and functional monomers

Screening of functional monomers: A 0.01 mmol L^{-1} solution of avermectin in acetonitrile, 0.04 mmol L^{-1} solutions of different functional monomers in acetonitrile and mixed solutions of avermectin and the functional monomers at the same concentrations (which were left to stand overnight after mixing) were first prepared. The UV/visible spectra of the respective solutions were then measured on a GZX-9023MBE UV/VIS spectrophotometer (Amersham Biosciences). The measured absorbencies of the functional monomers were added to that of avermectin to obtain the theoretical values for the mixed solutions and these were compared with the measured values obtained after mixing the two substances. The greater the difference was, the stronger the force between the two components was.

Optimization of the molar ratio between the template molecule and functional monomers: After determination of the UV/visible spectra of 0.05-0.18 mmol L^{-1} solutions of the functional monomers in acetonitrile and of mixed solutions of 0.01 mmol L^{-1} avermectin and the functional monomers with equivalent volumes but different concentrations (which were left to stand overnight at 4 ºC after agitation), the absorption maxima of the functional monomers at different concentrations and of the mixed solution after adding avermectin were compared. The greatest difference gave an indication of the optimum proportion.

Equilibration times for the interaction between the functional monomers and template molecules: A mixed solution was prepared at the optimum concentration ratio and its UV/visible spectrum was recorded at intervals of half an hour after agitation and mixing. The time at which the interaction between the functional monomers and template molecules reached equilibrium was determined from the temporal evolution of the spectrum.

Infrared spectroscopic analysis of the interaction between template molecules and functional monomers: Template molecule (0.01 mmol) and functional monomer (0.15 mmol) were mixed and dissolved in acetonitrile (2 mL) and the solution was left to stand overnight at 4 ºC after agitation and mixing. Appropriate standard samples of template molecules, 4-vinylpyridine, their mixtures and KBr were ground in an agate mortar, pressed into a thin section by a tablet machine, dried under an infrared lamp and analyzed on a BRUKER TGA-IR, TENSOR27 Fourier-transform infrared spectrometer (Thermo Electron Corporation).

Preparation of molecularly imprinted polymer: A standard sample of avermectin (0.067 mmol) was dissolved in a mixture of acetonitrile (16 mL) and chloroform (6 mL) containing 4-vinylpyridine (1 mmol) and the solution was left to stand overnight at 4 ºC after agitation and mixing at room temperature to ensure sufficient reaction of the avermectin with the functional monomers. Divinylbenzene (5 mmol) and azobisisobutyronitrile (50 mg) were added to the reaction products and the mixture was ultrasonically degassed for 5 min and purged with N_2 for 10 min. Thermal polymerization was then carried out at 60 ºC for 24 h after sealing in a constant-temperature water bath box. The vessel was then removed and cooled to room temperature, the precipitated polymerized product was collected by centrifugation (10000 rpm, 5 min) and then the polymerized molecularly imprinted polymer was collected after freeze-drying in vacuum for 12 h. Avermectin was not eluted from the molecularly imprinted polymer with a 9:1 (v/v) mixture of methanol and acetic acid, the baseline of the UV/visible spectrum was straight and the products were molecularly imprinted microspheres. The preparation method for control polymer was similar to that for the molecularly imprinted polymer, except that the template molecule was omitted.

SEM observation of molecularly imprinted polymer microspheres: A sample of molecularly imprinted polymer microspheres was fixed on the sample seat of a GZUS3400 SEM (Hitachi Company) by double-sided adhesive after freeze-drying *in vacuo*. SEM images were acquired after sputtering with gold.

Adsorption test: Samples of molecularly imprinted polymer (15 mg) and control polymer (15 mg) were added to 10 mL aliquots of a 0.1 mmol L^{-1} solution of avermectin and the UV/visible spectra of the respective solutions were measured after agitation by shaking for 12 h. The results were compared with the UV spectrum of a solution of avermectin in acetonitrile without molecularly imprinted polymer and control polymer. Comparison of the absorbencies allowed an appraisal of the adsorption capacities of molecularly imprinted polymer and control polymer for avermectin.

Statistical analysis: The obtained results were evaluated under F-test statistical analyses using the commercially available statistical package SPSS 13.0 (SPSS 13.0, 2005, SPSS Inc., Chicago, USA). The general linear model was used to

analyze the data of our experiments. A significant difference was declared at $P < 0.05$ (F-test) and an extremely significant difference was declared at $P < 0.01$ (F-test).

RESULTS AND DISCUSSION

Screening of functional monomers: UV/visible spectra were measured of a 0.01 mmol L^{-1} solution of avermectin in acetonitrile, 0.04 mmol L^{-1} solutions of the six different functional monomers, that is, methyl methacrylate, methacrylic acid, acrylic acid, acrylamide, 4-vinylpyridine and 2-vinylpyridine, in acetonitrile and of mixtures of avermectin with these respective functional monomers. The absorption maxima of avermectin and the respective functional monomers were added to give the theoretical values and then the measured values of the mixed solutions were subtracted from these theoretical values. The results are shown in Figs. 1 and 2. The results indicated that the largest difference between the measured and theoretical values was observed with 4-vinylpyridine. Hence, 4-vinylpyridine was used as a functional monomer for the preparation of avermectin molecularly imprinted polymer in the subsequent tests.

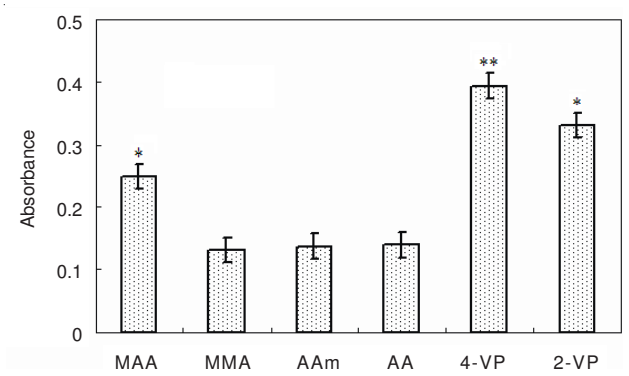

Fig. 1. Difference between the maximum theoretical absorption peaks and measured values of interaction between template molecule and different functional monomer

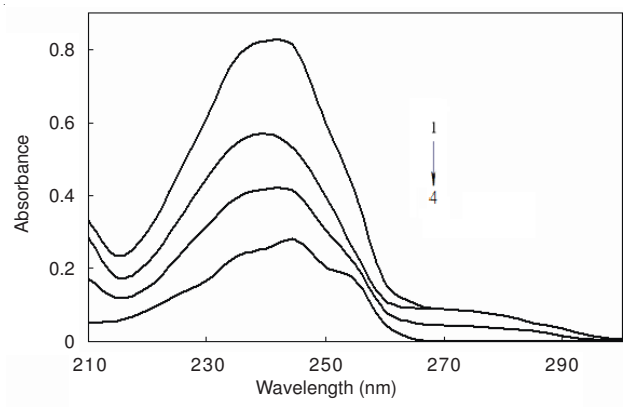

Fig. 2. Ultraviolet spectrogram of interaction between 4-vinylpyridine and AVM 1-4: theoretical absorbance values of 4-vinylpyridine +AVM, absorbance values of 4-vinylpyridine, tested absorbance values of 4-vinylpyridine +AVM and absorbance values of AVM

Optimization of the molar ratio between the template molecule and functional monomers: The difference was obtained by subtracting the UV/visible absorption maxima of 4-vinylpyridine at different concentrations from the absorption maxima of the mixed solution after the addition of avermectin and the results are shown in Fig. 3. The results indicated that the difference in the UV/visible absorption peaks of 4-vinylpyridine and the mixed solution increased with increasing concentration and their interaction reached a maximum when the molar ratio between 4-vinylpyridine and avermectin was 15:1. Thereafter, the difference in the UV/Vis absorption peaks began to decrease. Therefore, the optimal molar ratio between the template molecule and functional monomers was identified as 15:1 in this test.

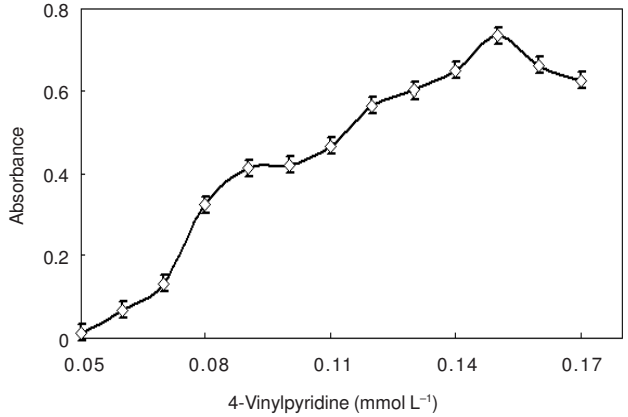

Fig. 3. UV absorption peaks difference between 4-vinylpyridine with different concentrations and mixed solution of avermectin and 4-vinylpyridine

Equilibration times for the interaction between the functional monomers and template molecules: UV/VIS spectra were recorded every 0.5 h after agitation of a mixture of a 0.05 mmol L^{-1} solution of avermectin in acetonitrile and a 0.75 mmol L^{-1} solution of 4-vinylpyridine in acetonitrile. The temporal evolution of the interaction between the functional monomers and template molecules is shown in Fig. 4. The results revealed that the functional monomers and template molecules began to interact immediately after mixing and that the absorbance of the mixed solution first decreased with time but then remained relatively stable after 3 h, indicating that the reaction had reached equilibrium.

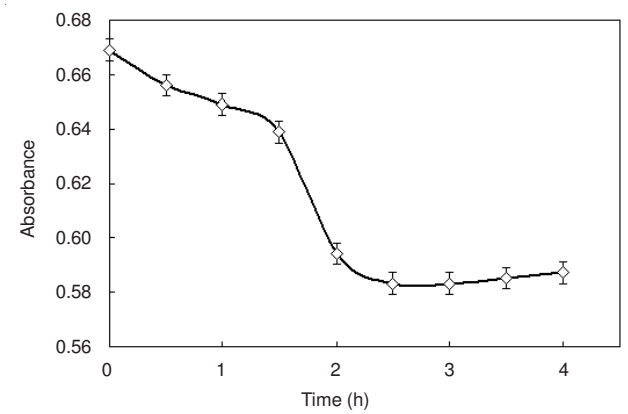

Fig. 4. Time curve of interaction between functional monomer and template molecule by ultraviolet scanning

Infrared spectroscopic analysis of the interaction between avermectin and 4-vinylpyridine: Infrared spectra of avermectin, 4-vinylpyridine and their mixture were recorded and the results are shown in Fig. 5. The characteristic band of avermectin in the C-H/O-H stretching region shifted from n = 3460 to n = 3426 cm^{-1} upon interaction, while the C-H stretching band of 4-vinylpyridine shifted from n = 3421 to n = 3426 cm^{-1}, which showed that avermectin and 4-vinylpyridine interacted after mixing and that a new group was generated.

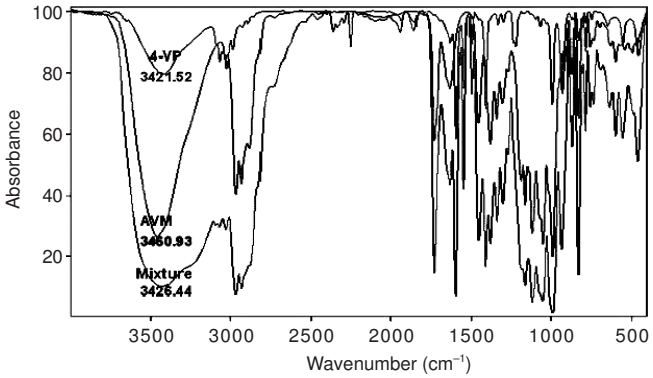

Fig. 5. Infrared spectrum analysis of interaction between avermectin and 4-vinylpyridine

SEM observation of molecularly imprinted polymer microspheres: Observation of molecularly imprinted polymer and control polymer by SEM revealed that the two polymers consisted of microspheres with particle sizes of 300-1000 nm, moderately narrow dispersity and rough surfaces with a porous morphology, which was beneficial for the interaction with the template molecules. The images are shown in Fig. 6.

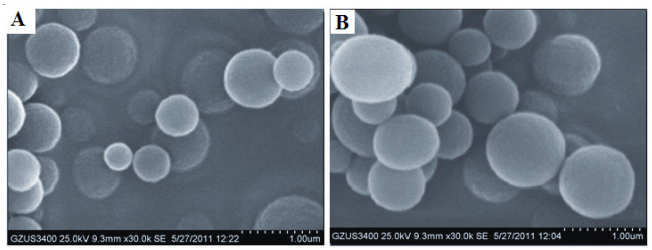

Fig. 6. SEM photos of molecularly imprinted polymer (a) and control polymer (b)

Static adsorption test of molecularly imprinted polymer microspheres: UV/VIS spectra were recorded of a 0.1 mmol L^{-1} standard solution of avermectin in acetonitrile and of the standard solution after adsorption on molecularly imprinted polymer and control polymer, respectively. The results shown in Fig. 7 reveal that the absorbance of the avermectin standard solution decreased in the presence of molecularly imprinted polymer and control polymer and that the absorbance of the standard solution after adsorption on the molecularly imprinted polymer was significantly lower than that after adsorption on control polymer.

The adsorption capacities of molecularly imprinted polymer and control polymer for the template molecules were calculated according to the UV spectrum of the AVM standard solution and the results are shown in Fig. 8. It can be seen that the adsorption capacity of the molecularly imprinted polymer microspheres for avermectin was 12.982 μg mg^{-1}, while the adsorption capacity of the blank group was 4.681 μg mg^{-1}. In addition, the adsorption on the molecularly imprinted polymer was significantly higher than that on control polymer and the difference was extremely significant ($p < 0.01$).

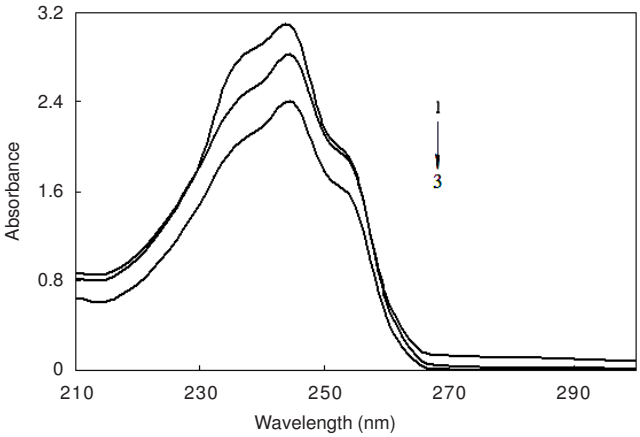

Fig. 7. UV spectrum scanning curves of 0.1 mmol·L^{-1} avermectin standard solution adsorbed by molecularly imprinted polymer and control polymer; 1-3: absorbance values of avermectin, absorbance values of control polymer and absorbance values of molecularly imprinted polymer

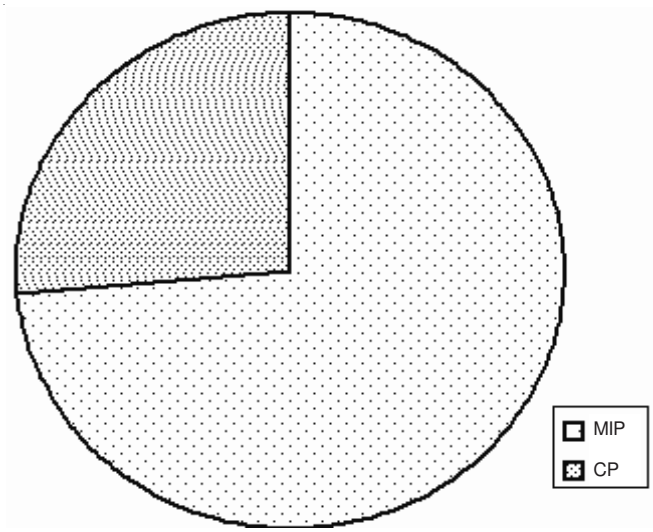

Fig. 8. Comparison of adsorption capacity of molecularly imprinted polymer and control polymer for avermectin

In this study, molecularly imprinted polymer microspheres were successfully prepared using avermectin as a template molecule, 4-vinylpyridine as a functional monomer, divinylbenzene as a crosslinking agent and acetonitrile and chloroform as solvents. The thermo polymerization was carried out at 60 ºC for 24 h. Comparison of the adsorption tests showed that the adsorption capacity of molecularly imprinted polymer was significantly higher than that of control polymer ($P < 0.01$), which indicated that the molecularly imprinted polymer presented specific binding sites. The interactions between the template molecule and functional monomers at different concentrations were analyzed by UV/VIS spectroscopy and the results showed that the interaction was strongest when the molar ratio between 4-vinylpyridine and avermectin was 15:1,

which is consistent with the study of Tom and Fosterb[15]. The preparation process of molecularly imprinted polymer involves thermal initiation at high temperatures and photo-initiation at low temperatures[16,17]. UV irradiation is commonly used in photo-initiation and the reaction can be operated at low temperature, which is beneficial for the formation of complexes between the template molecules and functional monomers. It is, therefore, generally recognized that the specific absorption of photo-initiation at low temperature is better for the template molecule than thermal initiation at high temperature. In this study, however, thermal initiation at high temperature was used to prepare avermectin molecularly imprinted polymer microspheres, considering that UV irradiation might destroy the molecular structure of avermectin.

One may speculate that avermectin and 4-vinylpyridine are highly likely to form a hydrogen bond based on the principle of interaction of these two substances and their molecular structures. The position of the hydrogen bond is shown by a dashed line between the molecules in Fig. 9.

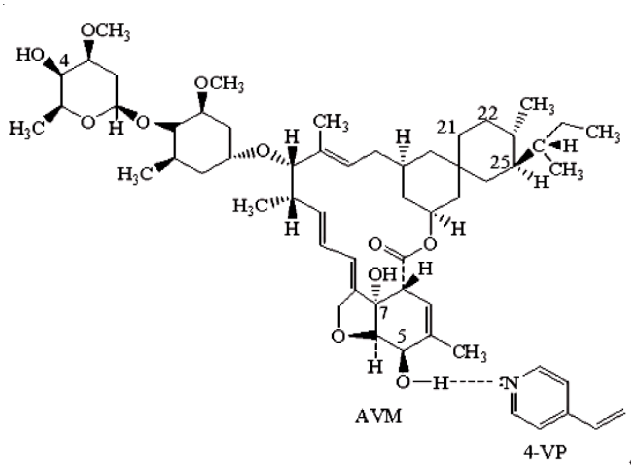

Fig. 9. Conjecture of the mechanism between 4-vinylpyridine and avermectin

FTIR results showed that the characteristic C-H/O-H stretching bands of avermectin and the C-H stretching bands of 4-vinylpyridine shifted in opposite directions towards an intermediate wave number upon mixing. The results indicated that the O-H bond was modified and that an N-H hydrogen bond was formed. According to previous studies[4], avermectin contains three hydroxyl groups. The secondary allylic hydroxyl group at C5 is the most reactive, followed by the secondary hydroxyl group at C4. The tertiary allylic hydroxyl group at C7 is sterically hindered, which limits its reactivity. The specific binding sites still need to be confirmed by NMR and X-ray diffraction analyses.

UV/VIS spectroscopy, FTIR and NMR are three kinds of analytical techniques for studying intermolecular force. Among them, UV/VIS spectroscopy, characterized by a simple device and easy operability, is used as the first choice for selecting the composition of molecular imprinting. In this study, functional monomers were selected by means of UV/VIS spectroscopy and the adsorption properties of molecularly imprinted polymer and control polymer for avermectin were determined. The particle size of the microspheres was 300-1000 nm, which could easily have caused obstruction in solid-phase extraction or on an HPLC stationary phase. The protocol for preparing molecularly imprinted polymer microspheres still requires further optimization in order to attain a significant increase in the particle size of the polymer microspheres. Ideally, a size of about 25 μm would be desirable, so that the particles can be deployed in solid-phase extraction and on-line HPLC detection. The ultimate goal is to apply the molecular imprinting technique to detect avermectin residues in a fast, sensitive and convenient way.

Conclusion

Among the six kinds of functional monomers studied, namely methyl methacrylate, methacrylic acid, acrylic acid, acrylamide, 4-vinylpyridine and 2-vinylpyridine, the molecular force between 4-vinylpyridine and avermectin has been identified as the strongest and the optimal molar ratio between them is 15:1. The time required for the interaction to reach equilibrium is 3 h. Thus, a standard sample of avermectin (0.067 mmol) and 4-vinylpyridine (1 mmol) were mixed and placed in a refrigerator at 4 °C for 3 h after agitation and mixing at room temperature. Divinylbenzene (5 mmol) and AIBN (50 mg) were then added to the reaction products. Thereafter, molecularly imprinted polymer was obtained by ultrasonic degassing for 5 min, purging with N2 for 10 min and thermal polymerization at 60 °C for 24 h. The particle size of the resulting polymer microspheres was in the range 300-1000 nm, the polydispersity was moderate and the adsorption of avermectin was significantly higher than that on control polymer ($P < 0.01$).

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