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Research Article

Synthesis and Characterization of Molecular Imprinting Polymer Microspheres of Cinnamic Acid: Extraction of Cinnamic Acid from Spiked Blood Plasma

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The molecular imprinting technique is used to create the molecularly imprinted polymers (MIPs) with higher binding capacity towards the template. In this research precipitation polymerization method with noncovalent approach was used to synthesize imprinted polymer microspheres. The polymerization reaction was conducted in a flask containing acetonitrile as a porogen, cinnamic acid as a template (T), acrylic acid (AA) as a monomer, divinylbenzene (DVB) as a cross-linker, and azobisisobutyronitrile as an initiator. The polymer particles were characterized by using SEM and FTIR. The rebinding efficiency was conducted by batch binding assay and the results were monitored by using HPLC. The batch binding results suggested MIP1 (T : AA : DVB, 1 : 6 : 20 molar ratio) is most suitable composition for the rebinding of cinnamic acid. The highly selective polymer (MIP1) was used for the extraction of cinnamic acid from human plasma. The extraction efficiency of imprinted polymer of cinnamic acid from spiked plasma was above 75%.

1. Introduction

Farlex Inc. (2003) stated that the cinnamic acid is a white crystalline weak organic acid and is insoluble in water and exists in two isomeric forms such as 3-phenylpropenoic acid and 3-phenylacrylic acid. The molecular formula of cinnamic acid is $C_9H_8O_2$. In addition, the cinnamic acid is an organic acid and is found in plants with lower toxicity and a broad spectrum of biological activities. Figure 1 shows the molecular structure of cinnamic acid.

The cinnamon or storax found in balsams is a source of cinnamic acid. Cinnamic acid is also used synthetically and produced as perfumery compounds. De et al. [1] claimed that the cinnamic acid and its natural analogs are used for treatment of cancer, maintaining youth, and promoting longevity and health. Jitareanu et al. [2] stated that the cinnamic acid and its derivatives consist of biological and pharmacological properties such as anti-inflammatory, antitumoral, and antidiabetic properties. The presence of phenolic and hydroxyl groups in cinnamic acid possesses an antioxidant property which provides several health benefits to human due to their strong free radical scavenging properties. These important properties of cinnamic acid lead us to prepare molecular imprinting polymers. These imprinted polymers can be used in the extraction of cinnamic acid from natural products and also in biological samples such as urine, plasma, and serum.

First and foremost, the molecular imprinting technology is a technique for preparation of polymers with specific recognition properties for given compound, its analogs, or single enantiomer [3]. The molecularly imprinted polymers (MIPs) are highly cross-linked polymeric phases with predetermined selectivity properties. The molecular imprinting follows the concept by using the molecular template in a casting procedure to prepare the substrate-selective recognition sites in a matrix [4]. According to the Ramstrom and Yan [4], the template molecule is first allowed to form solution interaction or bonds with functional element before locking-in of this

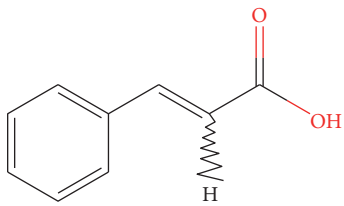

Figure 1: The molecular structure of cinnamic acid.

interaction or bonds leads to the formation of a matrix with a recognition site selective for the template. Subsequently, the prepolymerization mixture is irradiated with UV light or exposed to heat or sunlight in order to initiate polymerization [3]. Therefore, a spontaneous complex will be formed, while the strength of which depends on the properties of the solvent used and degree of complementarity of chemical functionalities in a template with those in functional monomers [5]. Yan and Kyung [3] stated that the imprinted polymer with a permanent memory for imprint species is formed after polymerization and removal of the template molecule, which allows the resultant polymer selectively to rebind an imprint molecule from a closely related compound. As a result, a specific cavity or imprint of MIP will be formed which is sterically and chemically complementary to the template molecule [5]. The molecular imprinting targets are able to create the artificial recognition cavities within synthetic polymers [6]. The molecularly imprinted polymers (MIPs) use the functionality of target molecule (template) by forming the specific interactions with matrix during polymerization in order to assemble its own recognition site [7]. Moreover, Silva et al. [7] explained that the receptors are capable of withstanding harsher condition (temperature, pH, and pressure). The low cost of MIPs and large amounts of manufacturing with good reproducibility are the reasons for MIPs being applied in diverse workplaces [7].

The synthesis of molecular imprinting polymer microspheres is relatively simple and results in a high binding capacity compared to monoliths prepared by bulk polymerization. Hence, the molecularly imprinting polymer microspheres of cinnamic acid are introduced in this research.

The process of producing MIP allows the functional monomers and template molecules to form complexes in solution firstly. After that, monomer, template, and addition of cross-linker are polymerized by freezing the functional groups at the specific location effectively. Molinelli [8] claimed that the last step of MIP is template removal from the polymer matrix and leaving a binding site ideally complementary in size, shape, and functionality to templated analyte.

2. Materials and Methods

2.1. Chemicals and Reagents. The chemicals and reagents are as follows: cinnamic acid (Merck Chemicals), acrylic acid (AA), divinylbenzene, DVB (Merck Chemicals), acetonitrile (Mallinckrodt Chemicals), azo-bis-isobutyronitrile, AIBN (R&M Chemicals), ice cube, nitrogen gas, methanol, MeOH (R&M Chemicals), acetic acid (J.T. Chemicals), distilled water, chloroform (QReC Chemicals), isopropanol, $CH_3CHOHCH_3$ (EMSURE Chemicals), hexane (HmbG Chemicals), ethanol, EtOH (HmbG Chemicals), ammonium sulfate (HmbG Chemicals), and acetone (HmbG Chemicals).

Table 1: Ratios of template to functional monomer to cross-linker used in MIPs and NIPs.

MIP	Ratio		
	Template	Monomer	Cross-linker
MIP1	1	6	20
MIP2	1	4	20
MIP3	1	6	28
NIP	—	6	20

2.2. Equipment. Bath sonicator (Model Branson 2510), centrifuge (Model Hettich EBA20), shaker (Model N-Biotek 101MT), magnetic stirrer (Model Bante MS300), water bath (Model Memmert W350T), high-performance liquid chromatography (HPLC) (Model Shimadzu LC-20), scanning electron microscope (SEM) (Model JEOL JSM-6390LA), Fourier transform infrared spectroscopy (FTIR) (Model Thermo Scientific Nicolet iS10).

2.3. Preparation of MIPs and NIP Microspheres. The MIPs for cinnamic acid were prepared by noncovalent approach [9]. The molar ratio of template, functional monomer, and cross-linker is given in Table 1 for the synthesis of different MIPs and NIP. Firstly, the template was added to a conical flask containing 75 mL of acetonitrile. This was followed by the addition of monomer, cross-linker, and an initiator into the reaction flask. After that, the mixture was sonicated for 15 min and then purged with nitrogen gas in an ice water bath for 15 min. The conical flask was sealed under this atmosphere to prevent any oxidation. The reaction mixture was immersed in the water bath at 60°C for first 2 hours and later temperature was raised and set at 80°C for 4 hours in order to complete the polymerization process. The produced polymer particles were extracted out by using the centrifugation at 5000 rpm for 10 min. The template was removed by washing the MIPs successively in the mixture of methanol and acetic acid (9 : 1, v/v) until the template was not detected by HPLC at 270 nm. Latter polymer particles were washed with acetone in order to remove the acetic acid from the polymer matrix. The MIPs were finally dried at 60°C for 6 hours in an oven. The same procedure was followed for the preparation of NIP without the use of template molecule in the synthesis.

2.4. Batch Binding Assay. The adsorption test was conducted in acetonitrile in order to evaluate the binding capacity of MIPs and NIP [10]. In adsorption step, 0.4 g of imprinted polymer particles was added to the 30 mL of acetonitrile with cinnamic acid (0.5 mmol, 74 mg) in the conical flask [9]. The mixture reaction was then agitated on a shaker and sample of the template was collected at different time intervals (0, 30, 60, 90, 120, and 180 min) at room temperature (25°C). The concentration of free cinnamic acid in the filtrate was

determined by using the HPLC. The degree of extraction of cinnamic acid was calculated by using the following equation:

$$\text{Extraction } (\%) = \frac{C_i - C_f}{C_i} \times 100\%, \quad (1)$$

where C_i and C_f are the concentrations of cinnamic acid in the solution before and after the extraction.

The HPLC was conducted by using the C18 column (250 × 4 mm, 5 μm) with the mobile phase consisting of acetonitrile, distilled water, and acetic acid in the ratio of 60 : 39.5 : 0.5, v/v/v, respectively. The flow rate was set at 0.6 mL/min with UV detection at 270 nm with run time at 6.5 min and injection volume was set at 20 μL.

2.5. Regeneration of MIPs and NIP. The adsorbed cinnamic acid was then desorbed from MIPs by washing with a mixture of methanol and acetic acid (9 : 1, v/v) three times in the centrifuge [9]. The nonimprinted polymers were treated in the same way as MIPs.

2.6. Extraction Procedure for Blood Plasma. 3 mL of drug-free fresh human blood was centrifuged at 2000 rpm for 15 min in order to extract the plasma from blood. Consequently, an immiscible layer was formed and then plasma was extracted. After that, the obtained plasma was spiked with 30 mL of acetonitrile containing 100 ppm of a cinnamic acid. In the spiked plasma sample, 0.4 g of selected polymer (MIP1) was added and after that samples were collected as followed in batch binding analysis. The concentration of free cinnamic acid in the filtrate was determined by using the HPLC and the degree of extraction of cinnamic acid was calculated by (1), while the NIP was treated in the same way.

3. Results and Discussion

Most of the MIPs are synthesized by the bulk polymerization followed by the process of crushing, grinding, and sieving to small particle size from macroporous polymer. In order to overcome these drawbacks of bulk polymerization, the polymer microspheres can be obtained during the polymerization. The copolymerizing monovinyl functional monomers with cross-linkers and grafting the polymer particles by functional polymer layers via the precipitation polymerization are able to prepare the functional polymer microspheres [11]. As a result, the spheres formed are uniform and in larger amounts via the precipitation polymerization process with excess of solvent/porogen.

In this research AA was used as a monomer, DVB as a cross-linker, and acetonitrile as a solvent/porogen. It has been reported that a porogen must be aprotic and does not compete to bind with the template molecule.

In the course of polymerization a noncovalent approach was adopted for the synthesis of polymer particles. According to Vasapollo et al. [12], the noncovalent approaches are often adopted for the preparation of MIPs due to the formation of a complex, which is simple, and the flexibility in terms of available functional monomers that can be applied to any types of templates. Yan and Kyung [3] stated that the noncovalent approach has high-affinity binding site since it incorporates multiple functional monomers which result in a number of binding interactions and impart greater affinity and selectivity to the site.

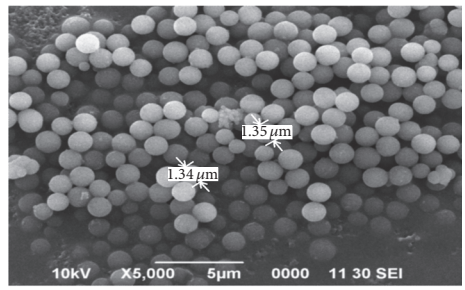

Figure 2: SEM image of imprinted polymer.

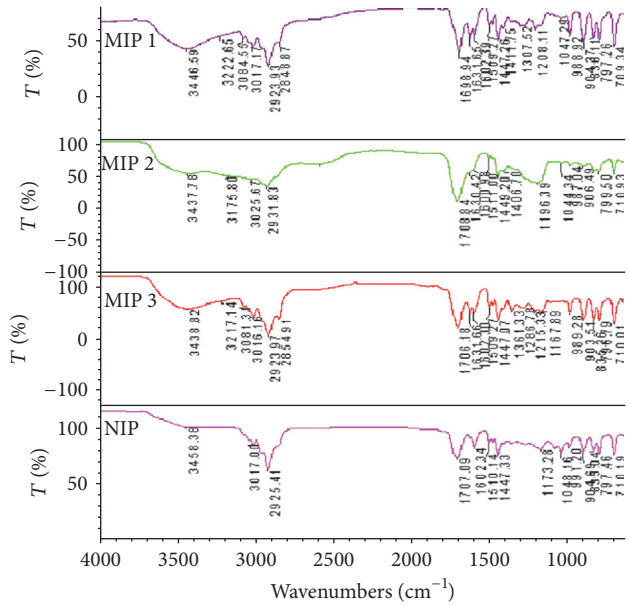

Figure 3: FTIR spectra of MIPs with template in comparison with NIP.

3.1. Scanning Electron Microscope (SEM). The morphology (shape and size) of imprinted polymers was studied under SEM at the magnification of 5000x. The SEM image (Figure 2) depicts uniform shape and size of polymer particles were achieved. This is due to the advantage of precipitation polymerization method and the favorable uniform size distribution between the interaction of monomer and template [9].

3.2. Fourier Transform Infrared Spectroscopy (FTIR). Chemical structure of MIPs and NIP was examined by using the fourier transform infrared spectroscopy (FTIR) (Figure 3). The IR spectra of MIPs with template are little different from NIP because of the existence of cinnamic acid as a template in the polymer matrix of MIPs. A strong broad peak at ~3500–3200 cm^{-1} attributed to the vibration mode of O-H stretch was observed in the IR spectra of the MIPs with template (cinnamic acid). Besides that, a sharp band at ~1631 cm^{-1} indicated the presence of C=C of alkene in the spectrum

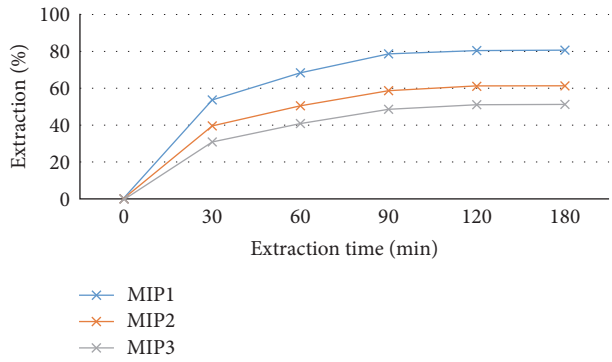

Figure 4: Cinnamic acid extraction efficiency depending on the extraction time among MIPs.

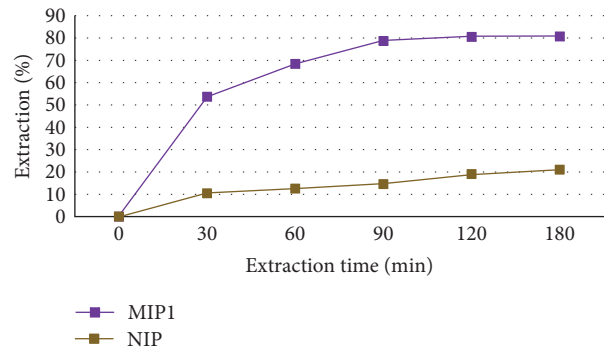

Figure 5: Cinnamic acid extraction efficiency depending on the extraction time between MIP1 and NIP.

of MIPs with template. A strong sharp peak at ~1320 cm^{-1} observed in the MIPs with template indicated the presence of C-O in the polymer matrix, while the disappearance of these peaks in the MIPs after the process of washing proved that the template, cinnamic acid, was completely removed from the polymer matrix. There are also strong peaks located at 2923.93 cm^{-1}, 2931.83 cm^{-1}, 2923.97 cm^{-1}, and 2925.41 cm^{-1} wavelength of MIP1, MIP2, MIP3, and NIP polymers, respectively. These peaks are due to the C-H stretching from alkane functional group.

A strong peak at ~1715–1690 cm^{-1} attributed to the vibration mode of C=O was observed in the IR spectra of the MIPs as well as NIP [9]. This strong peak indicated the presence of monomer (acrylic acid) that established an interaction with template in MIPs. The functional monomer is important for binding interaction in molecularly imprinting technology. In addition, a sharp band at 1047 cm^{-1} represents the C=C (alkene) of AA in the polymer matrix. The other absorption peaks observed in the polymer structure including the C=C stretch of aromatic compounds ranging from 1450 to 1600 cm^{-1} indicate the existence of benzene ring that revealed the presence of cross-linker (DVB) in the polymer matrix. The CH_2 bending at 1447.07 cm^{-1} to 1449.20 cm^{-1} indicated the presence of alkane group in MIPs and NIP. The strong peaks between ~1000 and 850 cm^{-1} attributed to the bending mode of =C-H were observed and also peaks between 860 and 660 cm^{-1} are due to the aromatic C-H bending vibrations because of DVB. The cross-linker (DVB) basically provides the mechanical strength to polymer and enhances the stability of recognition sites in a polymer matrix [13].

3.3. Batch Binding Assay. Batch binding process provides information regarding the optimization of various experimental conditions including composition of polymer matrix. This assay is used to choose a highly selective polymer for the target analyte. For a better MIP, it is crucial to have a higher affinity and rebinding efficiency towards the target substrate and a greater number of recognition sites.

The results shown in Figure 4 clearly depict that MIP1 (80.7%) has highest removal/binding efficiency as compared to MIP2 (61.3%) and MIP3 (51.3%). This can be conferred that MIP1 has got complimentary binding sites with the cinnamic acid. The increase in the concentration of monomer will increase the possibility of binding sites. However, the cross-linker is generally used to increase the mechanical stability of polymers. This can be observed from Figure 4 that increase in concentration of DVB will also affect the binding sites.

Figure 5 shows the competitive binding efficiency between MIP1 and NIP of the same composition except template is not involved in the synthesis of NIP. The binding efficiency of NIP (21.0%) is very low as compared to the MIP1 (80.7%). This may be due the lack of binding sites/cavities within the polymer matrix of NIP [9].

3.4. Regeneration of MIPs. The regeneration of MIPs is also important in this research in which selective washing procedure (methanol : acetic acid, 9 : 1) is used to remove the rebounded template. Different rinsing agents would result in a different extraction efficiency on cinnamic acid. The mixture of methanol and acetic acid (9 : 1, v/v) was used to remove the cinnamic acid from the MIP1. After the regeneration it was found that the removal efficiency was above 75% in MIP1.

3.5. Binding Assay of Cinnamic Acid from Human Plasma. Cinnamic acid is crucial for the antidiabetic activity from the compounds of cinnamon. Cinnamic acid is able to lower the glucose level and enhance the glucose tolerance in a human body. Therefore, cinnamic acid was first spiked in human plasma and then extracted with the most selective polymer MIP1. The extraction efficiency of MIP1 (75%) from the spiked plasma sample was higher as compared to NIP (20%). In this way these polymer particles can act as promising sorbents for the extraction of cinnamic acid from biological samples as well as in natural products.

4. Conclusion

Precipitation polymerization is able to produce uniform size and shape of MIP microspheres. In this research uniform shape and size of imprinted polymer particle for cinnamic acid were produced. The optimum ratio (1 : 6 : 20, template : monomer : cross-linker, resp.) of synthesized polymer was able to rebind about 80.7% of cinnamic acid as compared to other imprinted and nonimprinted polymers. These polymer particles have successfully extracted cinnamic acid

from human plasma. The extraction efficiency of imprinted polymer of cinnamic acid from spiked plasma was above 75%. The important applications of these polymer particles could be promising materials for solid-phase extraction and column packing materials and for drug delivery system.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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