

# The Preparation of D-Phenylalanine Imprinted Microbeads by a Novel Method of Modified Suspension Polymerization

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**Abstract** Molecularly imprinted polymeric microbeads (MIPMs) were prepared by the suspension and modified suspension polymerization methods using D-phenylalanine as the template, methacrylic acid as the functional monomer, ethylene glycol dimethacrylate as the cross-linker, toluene as the porogen, polyvinyl alcohol as the stabilizer, and sodium dodecyl sulfate as the surfactant. The addition of a surfactant to the conventional suspension polymerization mixture decreased the mean particle size of the MIPMs and increased the adsorption selectivity. For the modified suspension polymerization method, the mean particle size of the MIPMs was smaller than the particle size of MIPMs prepared via conventional suspension polymerization. Moreover, the adsorption selectivity improved considerably compared to the adsorption selectivities of MIPs reported previously.

**Keywords:** molecularly imprinted polymeric microbeads, phenylalanine, modified suspension polymerization, enantioselective separation, molecular recognition

## INTRODUCTION

Molecular imprinting is a technique for preparing synthetic polymers that contain predetermined selective binding sites for a desired template. Most of the studies on molecularly imprinted polymers (MIPs) have been carried out with irregularly shaped particles prepared by the conventional bulk polymerization method, which requires crushing and sieving processes [1,2]. The conventional preparation procedures are not only time and labor consuming, but the particles they produce are characterized by a wide size distribution along with huge amounts of fine waste. Also, the grinding process may be detrimental to some of the recognition sites [3-5].

Spherical MIPs have been synthesized in an attempt to overcome the inadequacies of bulk polymerization. Polymerization methods used to prepare spherical MIPs include: suspension [5], dispersion [6], precipitation [7], miniemulsion [8], multi-steps swelling [9], imprinted polymer grafting (silica or acrylate) [10], and wet phase inversion [11-13]. The resulting MIPs from these methods can be used directly after preparation and template removal. Suspension polymerization is one of the best methods for obtaining MIPMs in terms of recognition site formation, avoidance of bead agglomeration, and simplification of an otherwise laborious procedure; however, the resulting particles are of a diverse and wide size distribution [5,14-18]. The particle size of MIP beads prepared by conventional suspension polymerization has

been in the range of 400~450 [5] and 63~140  $\mu\text{m}$  [14]. Similarly, the imprinted beads of bovine serum albumin prepared by inverse-phase seed suspension polymerization were approximately 230  $\mu\text{m}$  [15]. Zhang *et al.* prepared MIP beads via seed swelling and suspension polymerization with a resulting average particle size of 60.5~255 [16] and 135  $\mu\text{m}$  with a major distribution between 120~140  $\mu\text{m}$  [17]. The particle size of MIP beads prepared by suspension polymerization can be controlled to a size range 5~50  $\mu\text{m}$  by varying the amount of the stabilizer [4]. More recently MIP beads in a size range of 1~100  $\mu\text{m}$  were synthesized by the suspension polymerization method using mineral oil as the stabilizer [18].

The adsorption selectivity of conventionally prepared MIPs is reported to be low. In a batch system, the adsorption selectivity of phenylalanine imprinted beads prepared by suspension polymerization was 1.12 [5], and for imprinted polymer beads of amino acid derivatives prepared by the surface imprinting technique the selectivities were 1.2 for *N*-benzyloxycarbonyl-glutamic acid (Z-Glu), 0.94 for Z-Asp, and 0.97 for Z-Gln [19]. Similarly, in a batch adsorption experiment the Z-Glu imprinted membrane prepared by the dry-phase imprinting technique had an adsorption selectivity of 1.2 [20].

In the present study, D-phenylalanine (D-Phe) imprinted polymeric microbeads were prepared by the suspension and modified suspension polymerization methods. Here we propose a novel modified suspension polymerization method that utilizes a surfactant in a conventional suspension polymerization mixture. We created this method to prepare MIPMs with a small particle size and a narrow size distribution (a few micrometers), as well as effectively capture water-soluble template mole-

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**Table 1.** Manufacturing conditions and characteristics of D-Phe imprinted P(MAA-co-EGDMA) microbeads

MIPM	Manufacturing conditions		Mean particle size ( $\mu\text{m}$ )	Characteristics of MIPMs		
	Amount of SDS* (g/L)	Stirring speed (rpm)		Adsorption selectivity ( $\alpha$ )		
				pH 2	pH 4	pH 6
MIPM-1		425	28	1.28	1.15	1.21
MIPM-2	3.123	425	15	1.25	1.12	1.17
MIPM-3	6.246	425	7.5	1.32	1.24	1.27
MIPM-4	12.592	425	7.0	1.31	1.22	1.27
MIPM-5	6.246	600	2.38	1.32	1.24	1.27

Adsorption conditions: MIPMs: 0.6 g; Phe racemate solution concentration: 100 mg/L; volume: 5 mL; pH 2, 4, and 6; time: 1 h; temperature: 25°C; shaking speed: 150 rpm.

\*SDS concentration was g SDS per liter of reactant solution for polymerization.

cules such as amino acids and proteins in the organic phase droplet during polymerization, thus increasing the adsorption selectivity of the MIPMs. The proposed method was investigated at various surfactant concentrations and stirring speeds. The enantioselective separation of phenylalanine (Phe) from the aqueous racemate solution was also evaluated to determine possible applications of the prepared microbeads.

## MATERIALS AND METHODS

### Materials

All chemicals used in these experiments were of reagent grade. Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), trifluoroacetic acid (TFA), D-phenylalanine (D-Phe), and L-phenylalanine (L-Phe) were obtained from Sigma-Aldrich (USA). Polyvinyl alcohol (PVA) was supplied by Yakuri Pure Chemicals Co. (Japan), 2,2'-Azo-bis-isobutyronitrile (AIBN) was from Junsei Chemical Co., Ltd. (Japan), and sodium dodecyl sulfate (SDS) was from Fluka (Switzerland).

### The Preparation of D-Phe Imprinted Polymeric Microbeads by the Suspension Polymerization Method (MIPM-1)

MIPM-1 was prepared by the suspension polymerization method using three solutions. Solution-1: D-Phe (1 mmol, 0.116 g) was mixed with MAA (4 mmol, 0.34 mL) and toluene (3 mL), followed by the addition of acetic acid (0.6 mL) and TFA (0.4 mL) in order to dissolve D-Phe in the organic phase. The mixture was stirred for 10 min at room temperature and then further stirred for 30 min at 0°C using an ice-bath. Solution-2: AIBN (0.15 g) was dissolved in toluene (2 mL) and EGDMA (20 mmol, 3.77 mL) at room temperature for 10 min with continuous stirring. Solution-3: PVA (3 g) was dissolved in hot distilled water (150 mL), stirred at 80–85°C for 30 min, and then cooled to room temperature. Each solution mixture was stirred separately for 2 h at room temperature before polymerization. Finally all three solutions were

combined in a three-neck double jacket glass vessel, purged with N<sub>2</sub> for 5 min, and stirred at 425 rpm for 60 min at room temperature. Polymerization was carried out at 60°C for 24 h with continuous stirring under N<sub>2</sub> atmosphere.

### The Preparation of D-Phe Imprinted Polymeric Microbeads by the Modified Suspension Polymerization Method (MIPM-2)

For the modified suspension polymerization method (MIPM-2), Solution-1 and Solution-2 were prepared in the same manner as for MIPM-1. Solution-3 consisted of PVA (3 g) dissolved in hot distilled water (130 mL). It was stirred at 80–85°C for 30 min and then cooled to room temperature. For Solution-4 0.5 g of SDS (3.123 g/L of reactant solution for polymerization) was dissolved in distilled water (20 mL) at room temperature. Organic Solution-1 and Solution-2 were combined, while aqueous Solution-3 and Solution-4 were combined. Again each solution mixture was stirred separately for 2 h at room temperature before polymerization. The other polymerization steps were the same as for MIPM-1. For MIPM-3 and MIPM-4 the preparation procedures were completed in the same manner as for MIPM-2, however, 6.246 and 12.592 g/L of SDS, respectively, were added, and stirring occurred at 450 rpm (Table 1). Similarly, MIPM-5 was prepared with 6.246 g/L of SDS and stirred at 600 rpm.

After polymerization the MIPMs were washed three times with distilled water containing 5% ethanol to remove the unreacted monomers, the initiator, and the porogen. This was followed by another washing with distilled water to remove the stabilizer and surfactant.

### Removal of the Template

Template molecules (D-Phe) were removed from the MIPMs matrix by washing with a 5% (v/v) acetic acid solution for 3 h followed by a washing with distilled water for 1 h. These alternating washing were repeated three times. The residual acetic acid was removed from the MIPMs by washing with an excess of distilled water until

the pH of the aqueous solution increased to the pH of the distilled water.

### Characterization of the MIPMs

Micrographs of the platinum-coated polymer samples were taken with a field-emission scanning electron microscope (FE-SEM) (S-4300; Hitachi Co., Japan) to determine their morphology, surface topography, and particle size.

The dried polymers were mixed with KBr pellets at room temperature and FT-IR spectra of the polymer samples were recorded using a Perkin Elmer FT-IR spectrophotometer (Spectrum GX & AutoImage, USA).

### Batch Adsorption

The MIPMs (0.6 g) were added to 5 mL of 100 mg/L Phe racemate solution and shaken for 1 h at 25°C and 150 rpm. After adsorption, the sample was centrifuged at 13,000 rpm for 3 min and filtered through a nylon membrane (0.45  $\mu\text{m}$ , Alltech).

The adsorption selectivity ( $\alpha$ ) of the D-Phe imprinted polymeric microbeads was obtained using the following formula:

$$(\alpha) = \{(D\text{-Phe})/(L\text{-Phe})\}/\{[D\text{-Phe}]/[L\text{-Phe}]\}$$

where (D-Phe) and (L-Phe) are the amounts of Phe adsorbed on the polymer, and [D-Phe] and [L-Phe] denote the concentrations in the solution after adsorption, as defined in the literature [11-13].

### Determination of the Phe Concentration

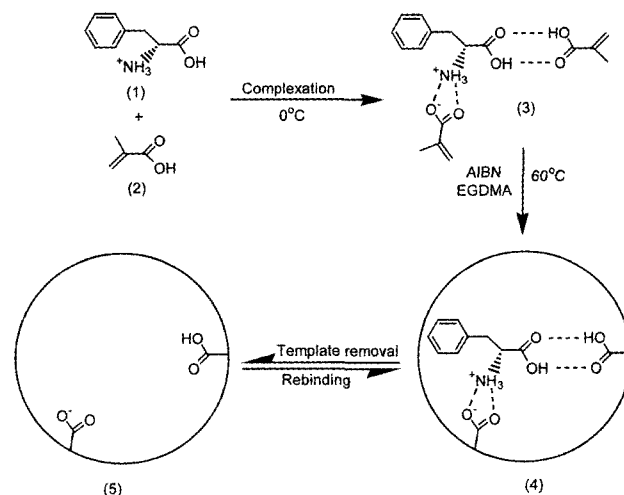
The D-Phe and L-Phe concentrations of the sample solution were determined using HPLC (Younglin M930, Korea) with a TSKgel Enantio L1 column (4.6 mm id  $\times$  250 cm). Copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.2 g/L) was used as the mobile phase at a flow rate of 1 mL/min. The absorbance of the substrate solution was monitored at 254 nm by a UV detector.

## RESULTS AND DISCUSSION

### Recognition Sites

The morphology and particle size of the prepared MIPMs were determined using FE-SEM. The particles of the prepared MIPM-1 were spherical, and upon more detailed examination at higher magnifications the surfaces of the microbeads were found to be of a porous nature (data not shown, similar to Fig. 5). The mean particle size of MIPM-1 was 28  $\mu\text{m}$  as shown in Table 1.

The possible mechanism for the formation of recognition sites in the prepared MIPMs is depicted in Fig. 1. The non-covalent interactions between the carboxylic and amino groups of D-Phe and the carboxylic groups of



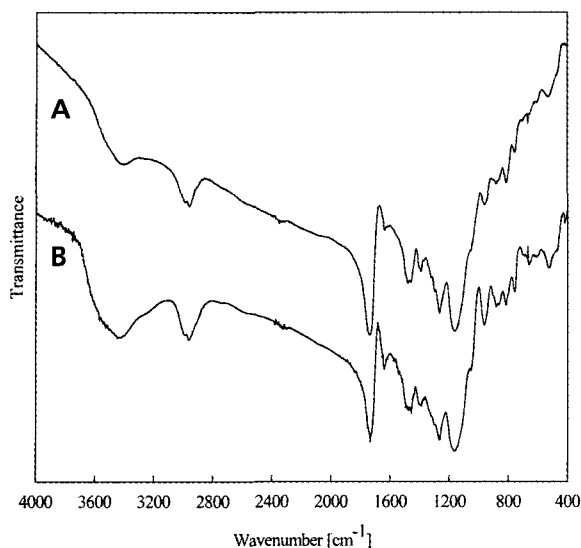
**Fig. 1.** The schematic representation of the molecular imprinting of D-Phe in the P(MAA-*co*-EGDMA). (1) D-Phe (template), (2) MAA (functional monomer), (3) template-functional monomer complex, (4) D-Phe imprinted polymer after co-polymerization of MAA and EGDMA (cross-linker) in the presence of AIBN (initiator) at 60°C, (5) post template extraction and remaining recognition sites complementary to the template.

MAA are established in the complexation step, and the template molecules are imprinted on the cross-linked polymer matrix during polymerization. The free carboxylic groups derived from MAA at the recognition sites in P(MAA-*co*-EGDMA) can then selectively bind the template enantiomer from its racemate solution or analogues.

The chemical functionalities of the D-Phe imprinted microbeads were indirectly determined by FT-IR spectroscopy. After template removal the remaining free carboxylic groups, which were components of the complex between the template molecules and the functional monomers, constitute the recognition sites [11-13]. As shown in Fig. 2, the free carboxyl groups were detected at 3,446  $\text{cm}^{-1}$  from the FT-IR spectra for the non-imprinted and D-Phe imprinted P(MAA-*co*-EGDMA) polymers. A weaker free carboxyl peak was observed for the non-imprinted P(MAA-*co*-EGDMA) and a stronger peak observed for the D-Phe imprinted P(MAA-*co*-EGDMA) polymers. This indirectly confirms the formation of recognition sites in the D-Phe imprinted P(MAA-*co*-EGDMA) polymer due to the existing large population of free carboxyl groups after the template removal [11-13].

### Adsorption Selectivity

The selective separation of amino acids is well documented in the literature [3,21-23]. D-Phe imprinted poly(acrylonitrile-*co*-acrylic acid) and poly(acrylonitrile-acrylic acid-acryl amide) polymers that were prepared by the wet phase inversion method showed inverse adsorption selectivity. This was due to the aromatic ring from D-Phe in the backbone of the polymer, which was formed by the coupling reaction between D-Phe and acrylic acid during the polymerization. As observed in the previous



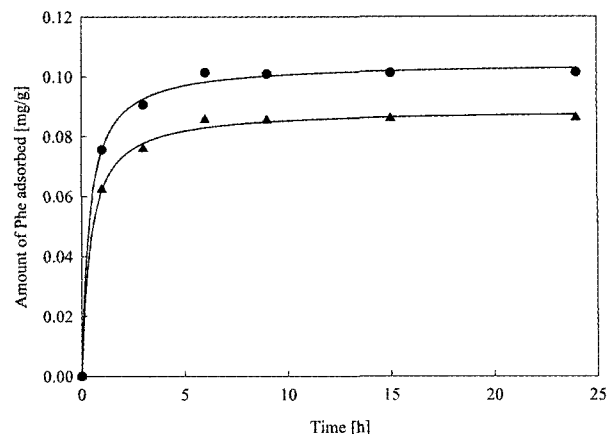
**Fig. 2.** The FT-IR spectra of P(MAA-co-EGDMA) microbeads prepared by the suspension polymerization method (MIPM-1); (A) non-imprinted; (B) D-Phe imprinted (after template removal).

studies [11,13,24], the presence of this phenyl group in the recognition site hinders the approach of D-Phe. In the present study, no coupling reaction was observed and thus the prepared D-Phe imprinted P(MAA-co-EGDMA) microbeads showed selectivity towards the template molecule in the racemate solution.

The uptake capacity of an adsorbate usually increases with the adsorption time until equilibrium is reached. Fig. 3 shows the adsorption kinetic curves of Phe on MIPM-1. Phe adsorption increased with time during the first five hours after which equilibrium was attained. The adsorption selectivity was evaluated as a function of the Phe racemate solution concentration in the range of 25 to 1,000 mg/L at a pH of 2, as shown in Table 2. The adsorption selectivity decreased from 1.28 to 1.19 with increasing concentrations of the Phe racemate solution. The affinity of conventional MIPs usually decreases with the template concentration, which can be explained by heterogeneous binding sites on the imprinted polymer surface; one is selective or has a high affinity and the other is non-selective or has a low affinity [25]. At the low template concentration range, the adsorption on the selective binding sites is stronger than on non-selective binding sites.

### Reusing MIP Microbeads

The regeneration of the adsorbent is likely to be the key factor in improving process economics. We performed adsorption and desorption experiments to determine whether the Phe bound to the MIPM-1 can be desorbed, and the MIPM-1 reused for selective adsorption. Phe adsorbed on MIPM-1 was desorbed with 5% (v/v) acetic acid solution and distilled water. The microbeads were then reused for another batch adsorption/desorption cycle



**Fig. 3.** The adsorption kinetic curves of D- and L-Phe onto D-Phe imprinted P(MAA-co-EGDMA) microbeads prepared by the suspension polymerization method: D-Phe (●); L-Phe (▲). Adsorption conditions: MIPM-1: 0.6 g; Phe racemate solution concentration: 100 mg/L; volume: 5 mL; pH 2; time: 1–24 h; temperature: 25°C; shaking speed: 150 rpm.

up to five times (Fig. 4). More than 98% of the adsorbed Phe was desorbed from MIPM-1 and the microbeads were reused without any loss in their adsorption capacity and selectivity, even after five consecutive batch adsorption/desorption cycles. Usually, however, the adsorption capacity and selectivity of the MIPs decreases with repeated batch adsorption/desorption cycles [12,14,15,26]. The demonstrated reusability of D-Phe imprinted P(MAA-co-EGDMA) microbeads over several batch adsorption/desorption cycles, without loss of adsorption capacity and selectivity, is a key advantage of this polymerization method.

### Particle Size Reduction with a Surfactant

A FE-SEM photograph showed that all the D-Phe imprinted microbeads prepared by the modified suspension polymerization method were spherical (Fig. 5). The mean particle size of MIPM-2 was 15  $\mu\text{m}$ , which indicates that the addition of SDS decreased the mean particle size of the D-Phe imprinted polymeric microbeads compared to those prepared without SDS (Table 1). MIPM-3 was prepared with the addition of 6.246 g/L of SDS and a further decrease in the mean particle size was observed (7.5  $\mu\text{m}$ ) (Table 1). Similarly, with 12.592 g SDS/L the mean particle size was 7  $\mu\text{m}$ , as shown by MIPM-4. This indicates that the amount of SDS has a pronounced effect on the mean particle size. The decrease in particle size with a surfactant may be due to the formation of very small organic phase droplets surrounded by the surfactant during polymerization. The stirring speed was also effective in reducing the particle size. MIPM-5 was prepared in the same manner as MIPM-3; however, the stirring speed was increased to 600 rpm. The resulting mean particle size was found to be 2.38  $\mu\text{m}$  (Table 1).

Compared to the MIPs prepared by the conventional suspension polymerization [5,14,18], the inverse-phase

**Table 2.** The Effects of Phe concentration on the adsorption selectivity of D-Phe imprinted P(MAA-co-EGDMA) microbeads prepared by suspension polymerization

Phe concentration (mg/L)	Adsorption selectivity ( $\alpha$ )
25	1.28
50	1.28
100	1.28
200	1.27
500	1.22
1,000	1.19

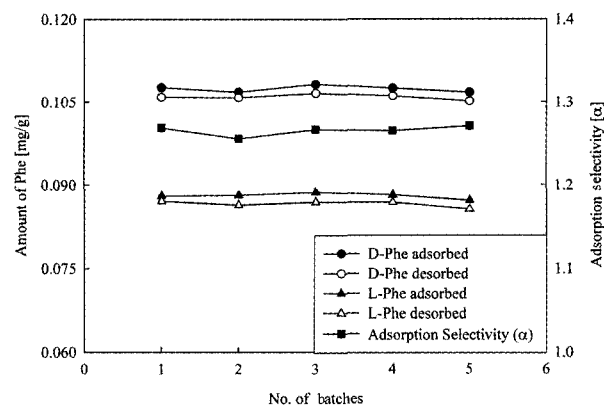
Adsorption conditions: MIPM-1: 0.6 g; Phe racemate solution concentration: 25~1,000 mg/L; volume: 5 mL; pH 2; time: 6 h; temperature: 25°C; shaking speed: 150 rpm.

seed suspension polymerization method [15], and the seed swelling and suspension polymerization method [16,17], the D-Phe imprinted polymeric microbeads (MIPM-5) prepared in the current study had a much smaller mean particle size (2.38  $\mu\text{m}$ ), with more than 45% in a size range of 2 to 3  $\mu\text{m}$ . This may be due to the addition of a surfactant, which has a tendency to produce micelles. It is widely acknowledged that the size of micelles is much smaller than the size of suspension droplets [8].

### Increased Adsorption Selectivity with a Surfactant

The FT-IR spectra of MIPMs prepared by the modified suspension polymerization method were similar to the spectra of MIPM-1. This suggests that the mechanism for recognition sites formation in the MIPMs prepared by the modified method was similar to that for MIPM-1. Also, for those MIPMs prepared by the modified suspension polymerization method, a weaker free carboxyl peak was observed for the non-imprinted micro beads and a stronger free carboxyl peak for the D-Phe imprinted P(MAA-co-EGDMA) MIPMs (data not shown, similar to Fig. 2).

At a pH of 2 the adsorption selectivity of D-Phe imprinted polymeric microbeads prepared by the modified suspension polymerization method increased from 1.25 to 1.32. This increase in selectivity was in line with the results found for increased surfactant amounts (3.123 to 6.246 g/L). Although no remarkable change occurred in selectivity with a lower SDS addition of 3.123 g/L, increasing the amount of surfactant to 6.246 g/L increased selectivity (Table 1). With a further increase in SDS to 12.592 g/L the adsorption selectivity remained almost the same as with 6.246 g/L *i.e.*, 1.31. Therefore, 6.246 g/L of SDS was determined to be the optimal amount of surfactant required. The resulting increase in adsorption selectivity with increasing surfactant amounts may be due to the fact that SDS surrounds the organic phase droplets contain the template molecules during polymerization; thus, the aqueous-soluble template molecules cannot easily dissolve into the aqueous phase. This produces an effective imprinting of the template, which in turn forms a



**Fig. 4.** Repeated batch adsorption/desorption experiments using D-Phe imprinted P(MAA-co-EGDMA) microbeads prepared by the suspension polymerization method (MIPM-1).

large population of selective binding sites and a strong affinity for the template molecules. When the D-Phe template molecule was dissolved in the aqueous solution before polymerization, and the complexation step performed in the aqueous phase, D-Phe imprinted P(MAA-co-EGDMA) microbeads showed no adsorption selectivity (data not shown).

As previously mentioned, a decrease in the particle size with increased stirring speed was observed. However, as shown in the case of MIPM-5, the adsorption selectivity remained unaffected (Table 1). This suggests that the template molecules were effectively contained in the organic phase droplets during polymerization, even at a high stirring speed. Thus the adsorption selectivity was not changed significantly with stirring speed.

The adsorption selectivity of 1.32 that was obtained in this study is superior compared to the selectivity of MIPs prepared by the suspension polymerization method [5], the surface imprinting method [19], and the MIP membrane technique [20]. However, it is comparable to 1.3 and 1.34, respectively, for sulphur mustard [27] and D-Phe [2] imprinted polymers prepared by the bulk polymerization method.

### The Effect of pH

It has been shown that the adsorption selectivity of MIPs varies in accordance with the pH of the solution [11-13,25]. We studied the effect of pH on the adsorption selectivity of D-Phe imprinted microbeads at pHs of 2, 4, and 6 (Table 1). The general trend of the adsorption selectivity with respect to the substrate solution pH was pH 2 > pH 6 > pH 4. This may be attributed to a charge change in the functional ligand found at the recognition sites [11]. In this study the prepared MIPMs recognized D-Phe with both COOH and COO<sup>-</sup>, as depicted in Fig. 1. According to simple calculations using the Henderson-Hasselbach equation, 79% of the carboxyl groups of Phe existed as COOH and only 21% existed as COO<sup>-</sup> at a pH of 2, while in the polymer matrix nearly all of the carboxyl groups of MAA (99.8%) existed as COOH. At a pH

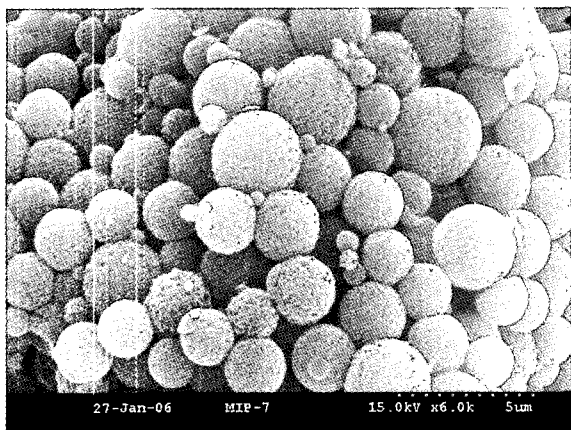


Fig. 5. FE-SEM photograph of D-Phe imprinted P(MAA-co-EGDMA) microbeads prepared by the modified suspension polymerization method; (MIPM-5,  $\times 6,000$ ).

of 4, 96% of the carboxyl groups of Phe were  $\text{COO}^-$  and 82.37% of the carboxyl groups of MAA were  $\text{COOH}$ . At a pH of 6, 98% of the carboxyl groups of Phe were  $\text{COO}^-$  and 95.5% of the carboxyl groups of MAA were  $\text{COO}^-$ . The amino groups of Phe remained as  $\text{HN}_3^+$  even at a pH of 6. During the rebinding experiment the best print form of the template molecule and higher interactions with the polymer matrix increased the adsorption selectivity. Thus, the prepared MIPMs showed higher adsorption selectivity at a lower pH. At a pH of 4, template molecules with a shape complementary to the recognition site could not bind to the polymer matrix due to the repulsive force caused by the like-charge from  $\text{COO}^-$  in the template molecule and polymer matrix. At a pH of 6 the interaction between  $\text{HN}_3^+$  of the Phe and  $\text{COO}^-$  of the polymer matrix seemed to be effective. These selective adsorption phenomena are attributable to the hydrogen bonding and ionic interactions between the template molecule and MIPMs at low and high pH, respectively.

## CONCLUSION

In this study we have described a novel method for the preparation of MIPMs using modified suspension polymerization and a surfactant. The addition of a surfactant decreased the particle size and size distribution, and improved the adsorption selectivity without causing any modifications to the main polymer structure. The adsorption selectivity of the MIPMs was very susceptible to changes in the pH of the Phe racemate solution and found to be greater at a lower pH. The selective adsorption phenomena seem to be attributable to hydrogen bonding or to ionic interactions between the template molecule and the MIPMs at low and high pH, respectively. The advantages of this proposed method compared to previously reported methods include spherical-shaped polymers and smaller particle sizes, as well as microbeads with narrow size distributions and improved adsorption selectivity. Moreover, the method is straightforward, eas-

ily modifiable for scaling up and automation, requires no expensive chemicals or equipment, and can be utilized with almost all of the commonly used porogens. The MIPMs prepared by this proposed method are anticipated to be useful in a broad range of applications, as well as vastly facilitate future developments in molecular imprinting.

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