

Effect of Template on Chiral Separation of Phenylalanine using Molecularly Imprinted Membrane in Aqueous Medium

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Summary: L-Phenylalanine (L-Phe) and D-Phenylalanine (D-Phe) imprinted poly [(acrylonitrile)-co-(acrylic acid)] membranes were prepared by wet phase inversion method for chiral separation. The chiral separation ability of molecularly imprinted copolymer membranes towards the underivatized D,L-Phe aqueous mixture was evaluated by ultrafiltration experiment. The novel membranes show continuous permselectivity but chiral resolution ability of L-Phe imprinted membrane was much better than that of D-Phe. It was observed that both membranes simultaneously, selectively reject, selectively adsorbed and selectively permeate solute. The achieved adsorption selectivities of L-Phe imprinted membrane [α_{Ads}]_L and D-Phe imprinted membrane [α_{Ads}]_D were 2.6 and 2.40 respectively. Permselectivity of L-Phe imprinted membrane [α_{Perm}]_L was 2.56 while D-Phe imprinted membrane's permselectivity [α_{Perm}]_D was 2.03. The rejection selectivities of L-Phe and D-Phe imprinted membranes were [α_{Rej}]_L=0.32 and [α_{Rej}]_D=0.28 respectively.

Keywords: amino acid; chiral resolution; molecularly imprinted membrane; molecular recognition; selectivity; separation.

Introduction

In chemical and biological processes the selective separation and recognition of specific target molecule is an important issue [1]. The optical resolution of racemates has been essential in the perfume production, pharmaceutical industry, food preparation, and so forth due to the harmful effect of one of the enantiomer of racemate mixture. The resolution of racemates is the primary method to obtain pure enantiomers in industry [2-4].

Molecularly imprinted polymers (MIPs) are a novel class of selective sorbents due to their manufacturing procedure [5, 6]. They are usually made for every particular substrate that should be selectively bound and separated. A heavily crosslinked rigid polymer is usually synthesized in the presence of the template molecule. After completion of polymerization, template molecules are removed by washing the polymer with a suitable solvent. When exposed next to a solution of mixture containing template molecules, the polymer will adsorb the template molecule with notable selectivity over other substances like enantiomers [7, 8]. There are two main types of imprinting: covalent and noncovalent [9-12]. Molecularly Imprinted Membranes (MIMs), which are viewed as very promising materials for practical applications with the advantages of low energy consuming and easy to scale up, have also attracted much research interest [13-18].

One of the main issues is the selection of appropriate polymeric membrane material. Acrylic acid (AA) and acrylonitrile (AN) are most commonly available and have been considered as the most promising materials in a wet phase inversion method [19-25]. AA contains only one hydroxyl group capable of assembling with template molecules such as amino acids through forming hydrogen bonds. The AN residues work as solidified parts for membrane formation while AA residues interact with the template molecules to fix the template molecules in the polymeric membrane [26].

Many researchers have used poly(AA-co-AN) imprinted membrane for the separation of target molecule (template). Trotta *et al.* imprinted poly(AA-co-AN) membrane with tetracycline hydrochloride [24] and naringin [25] for the separation of tetracycline hydrochloride from chloramphenicol; and to separate naringin from orange juice, respectively. Kobayashi *et al.* [19-21] employed Theophylline (THO) as template molecule to imprint poly(AA-co-AN) membrane for the separation of THO and Caffeine (CAF). Cristallini *et al.* [23] prepared Uric acid (UA) and THO imprinted poly(AA-co-AN) membranes for separation of UA and THO.

Several groups have devoted their efforts for the optical resolution of amino acids. But very few researcher have employed MIMs for the optical

resolution of Phenylalanine. Takeda *et.al.* [27] used L-Phe imprinted nylon 6, nylon 6,6, and terephthalic phenylene polyamide (TPPP) membranes for optical resolution of phenylalanine (Phe) in batch binding using ultrafiltration cell. The apparent partition coefficients of L – and D – forms by the imprinted membranes were 6.8, 4.2, and 1.7 for nylon 6, nylon 6,6, and TPPP, respectively. Takeda *et.al.* might have got much better results if they would have considered the rejection of the solute by membranes. Jiang *et.al.* [28] imprinted chitosan (CS)/ γ -glycidoxypropyltrimethoxysilane (GPTMS) hybrid membrane with L-Phe for chiral resolution of Phe by diffusion cells, improving significantly selectivity and achieved a separation factor of the order of 4.5 was achieved in 24 hrs.

First, the Kobayashi group [19-22, 26, 27] had introduced imprinting via phase separation starting with a solution containing the copolymer and the template; same approach has also been used, for instance, by the Drioli group [24,25]. Later, Kobayashi introduced the method via copolymerization of (mono) functional monomers in the presence of a template and subsequent film casting and non-solvent induced phase separation. In this study we have prepared poly(AA-co-AN) MIM by wet phase inversion method, in which the in situ implantation of template (L-Phe or D-Phe) was done by non-covalent interactions for the optical resolution of Phenylalanine. Ultrafiltration experiments were performed so as to develop better understanding of sorption ability, binding and permeation selectivity of membranes, for chiral separation of the underivatized Phe aqueous mixtures. Significantly high selectivities were achieved using ultrafiltration technique in a very short time. Selective rejection of solute was also observed. The morphological structure of membrane was characterized by SEM. Chemical structure of membrane was studied with FT-IR spectroscopy.

Results and Discussions

Structure Analysis and Morphology of Membranes

Hydrogen bonding plays an important role in molecular imprinting [29]. It is interesting to mention that a single hydrogen bond interaction is sufficient for imprinting, or recognition, in the presence of water. It is an established fact that water has a weakening effect on the noncovalent interactions, that is why other polar interactions in aqueous system are limited. In this study we have chosen AA as functional monomer with one

carboxylic group which was quite sufficient for the recognition of target molecule in aqueous medium. The molecular cavities with distinct size, shape and chemical functionality remained in the membrane matrix and the specific recognition sites were formed after removal of the template molecules.

The spectra of L-Phe imprinted poly(AA-co-AN) and D-Phe imprinted poly(AA-co-AN) membrane were analyzed by FT-IR. The interpretation of FT-IR spectra is summarized in Table-1. The OH dimer and free OH stretching can be realized at 3466 cm^{-1} and 3242 cm^{-1} respectively, in L-Phe imprinted membrane, and in D-Phe imprinted membrane OH dimer and free OH stretching appeared at 3461 cm^{-1} and 3243 cm^{-1} , respectively (Fig. 1). These free OH groups are might be due to the presence of COOH in imprinted poly(AA-co-AN) membranes, and are responsible for the formation of hydrogen bond with the template. SEM studies revealed that the average thickness of membrane was $25\ \mu\text{m}$ and average thickness of dense top layer was $6\ \mu\text{m}$. The measured pore sizes of membrane were less than 25 nm .

Table-1: Assignment of FT-IR spectra L-Phe and D-Phe imprinted P(AA/AN) membranes.

Peak Assignment	Segment	L-Phe Imprinted Membrane	D-Phe Imprinted Membrane
OH Stretching	AA	3466	3461
Free COOH Group			
OH Stretching	AA	3360	3360
Dimerized COOH group			
OH Stretching	AA	3242	3243
Free COOH group			
CH Stretching	AA,AN	2939	2939
CN Stretching	AN	2244	2244
C=O Stretching	AA	1734	1734
NH Stretching	AN	1634	1634

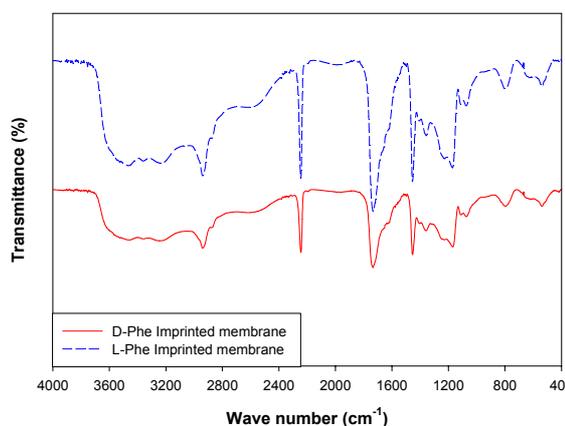


Fig. 1: FT-IR spectra of L-Phe & D-Phe imprinted P(AA/AN) membrane.

Effect on Swelling Separation Ability of Membrane

The polymer swelling changes the three-dimensional configuration of functional groups which are taking part in the recognition process [30]. The swelling rate of L-Phe imprinted membrane was 73% and that of D-Phe imprinted membrane was 75%. It was observed that elasticity and swelling ability of membrane increased in the aqueous medium. According to the theory of “induced fit effect”, Piletsky et. al. [31] concluded that solvation of the functional monomer binding ligands are the cause of swelling. Most of functional ligands (from the functional monomers) after the removal of template are probably produced inside the selective cavities. After selective rebinding, the volume of the polymer reduced nearly to the original volume. While Ulbricht observed that the increase in permeability is due to the membrane swelling as a consequence of template binding to imprinting sites [32].

Template Effect on Selective Solute Rejection

During ultrafiltration process it was observed that solute not only adsorbed on membrane but also rejected by membrane. Fig. 2 shows that in L-Phe imprinted membranes, the rejection of D-Phe was higher than the rejection of L-Phe. In case of filtration from D-Phe membranes, the rejection of L-Phe was higher than that of D-Phe. The substrates (L-Phe or D-Phe used as template during the synthesis of membrane) after removal left imprinting cavities and channels (corresponding to the size and shape of L-Phe or D-Phe). The recognition of template took place by imprinted cavities and channels with in the membrane matrix worked as gate between pores [33]. When L-Phe imprinted membrane was used, these gates allowed L-Phe pass through it and rejected D-Phe. Similarly, when D-Phe imprinted membrane was used; L-Phe was rejected and D-Phe was allowed to pass through membrane. When L-Phe imprinted membranes were used; the rejection of D-Phe after 16 ml of filtration increased by 3.17-folds than rejection of L-Phe, and D-Phe imprinted membranes resulted rejection of L-Phe of the order of 3.53-times higher than the rejection of D-Phe. The rejection selectivity for L-Phe imprinted membrane was 0.32 and that of D-Phe imprinted membrane was 0.28. The nano pores and rough surface of membrane can also be the reason of rejection [34]. T. Gotoh *et.al.* have reported that the amino acid rejection decreased with increasing the amino acid concentrations [35]. NTR-7450

nanofiltration membrane was used for the separation of glutathione and its related amino acids (L-glutamate, L-cysteine, glycine, and L-glutamine). We used D-Phe and L-Phe imprinted membranes for chiral separation of Phe. We observed that the concentration of solute in retentate increased gradually with filtration time while rejection decreased. So we can assume that decrease in rejection with filtration time is due to the increase in concentration of solute in retentate. From above results we can also conclude that selective rejection is the combine effect of selective adsorption and selective permeation.

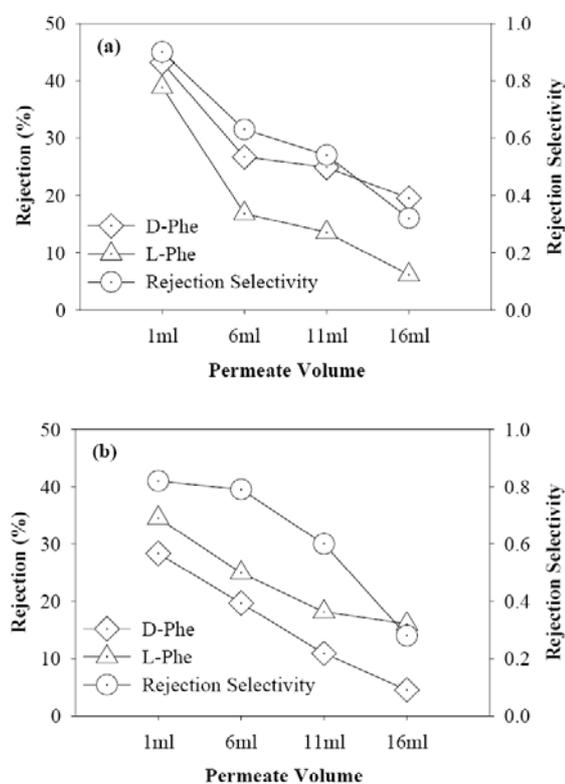


Fig. 2: Rejection profile of (a) L-Phe and (b) D-Phe imprinted AA/AN membranes after 16 ml filtration of 100-ppm racemate mixture of Phenylalanine.

Template Effect on Selective Solute Adsorption

The adsorbed amounts of D-Phe and L-Phe were 0.0647 mg/g of membrane and 0.1685 mg/g of membrane respectively, and adsorption selectivity $[\alpha_{Ads}]_L$ of 2.6 was achieved using L-Phe imprinted membranes. While D-Phe imprinted membrane

showed adsorption selectivity $[\alpha_{Ads}]_D$ of 2.40 and adsorbed amount of D-Phe was 0.1674 mg/g of membrane while that of L-Phe was 0.0698 mg/g of membrane. The enantioselectivity of imprinted membranes was attributed to the selective recognition and binding ability of imprinting sites with “memory” created after the removal of templates [28]. L-Phe and D-Phe imprinted membranes showed both strong binding ability and higher adsorption selectivity (Table-2). Fig. 3. shows the chiral recognition ability of L-Phe and D-Phe imprinted membranes. There was preferential adsorption of L-Phe over D-Phe using L-Phe imprinted membrane and when D-Phe imprinted membrane was used the adsorbed amount of D-Phe was much more than the amount of L-Phe. The selective recognition of L-Phe imprinted membrane is much better than that of D-Phe imprinted membrane which is confirmed by FT-IR spectra. While the adsorption capacity of D-Phe was more than that of L-Phe it might be due to swelling effect [31, 32] We can say that selective recognition of template by imprinted cavities in the membrane matrix directly effect on selective permeation and selective rejection.

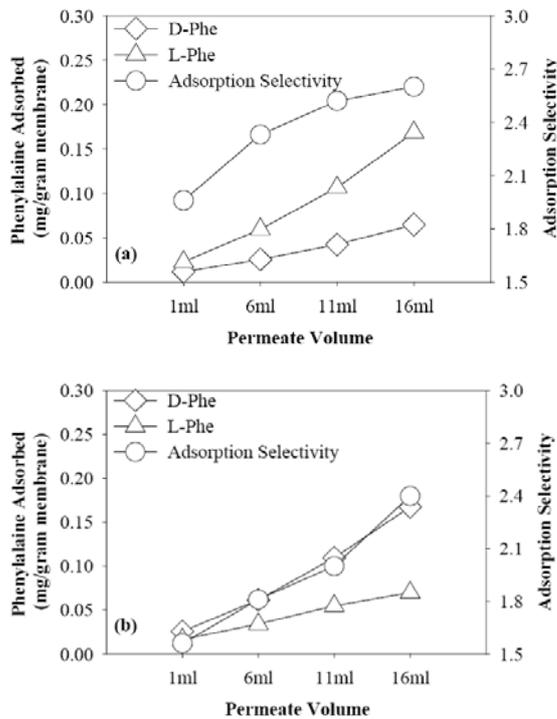


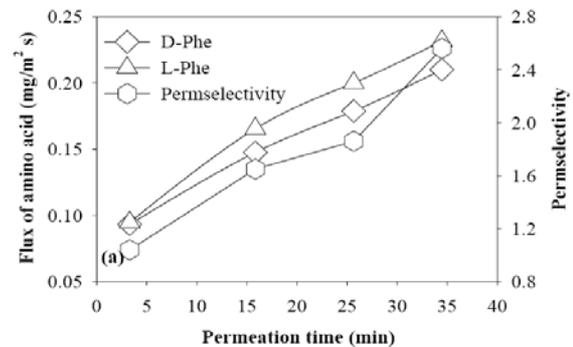
Fig. 3: Adsorption profile of (a) L-Phe and (b) D-Phe imprinted AA/AN membranes after 16 ml filtration of 100-ppm racemate mixture of Phenylalanine.

Template Effect on Selective Permeation

Fig. 4 shows typical permeation curves for concentration and flux versus time for separation of Phe isomers mixture obtained by permeation experiments using imprinted poly(AA-co-AN) membranes fixed in ultrafiltration kit by applying a pressure of 1 atm. The concentration of permeate increased gradually with time. The fluxes of the isomers in permeate also increased with time, increase in permeability is due to membrane swelling [31, 32]. Both L-Phe and D-Phe showed similar trend. The concentration and flux of the two isomers were different and chiral resolution of D, L-Phe was thus realized. The maximum separation factor (permselectivity) achieved in this study were about 2.56 and 2.03 for L-Phe imprinted poly(AA-co-AN) membranes and D-Phe imprinted poly(AA-co-AN) membranes, respectively (Table-2) and the permeability coefficient P was in $9 \times 10^{-9} \text{ m}^2/\text{s}$. Fig. 4 illustrates that the permselectivity of L-Phe imprinted and D-Phe imprinted membrane increased with ultrafiltration. The permselectivity of L-Phe was found to be better than that of D-Phe imprinted membrane. It is concluded that the template plays an important role on the performance of imprinting membrane used for chiral resolution, facilitated permeation through imprinted gates in the membrane and directly influence on selective rejection and selective adsorption.

Table-2: Separation factor, adsorption selectivity and transport selectivity of L-Phe and D-Phe imprinted membranes after 16 ml filtration of 100-ppm racemate mixture of Phenylalanine.

Characterizations	L-Phe imprinted membrane	D-Phe imprinted membrane
S_{Ratio}	73%	75%
α_{Rej}	0.32	0.28
α_{Ads}	2.60	2.40
α_{Perm}	2.56	2.03
α_{Trans}	0.98	0.84



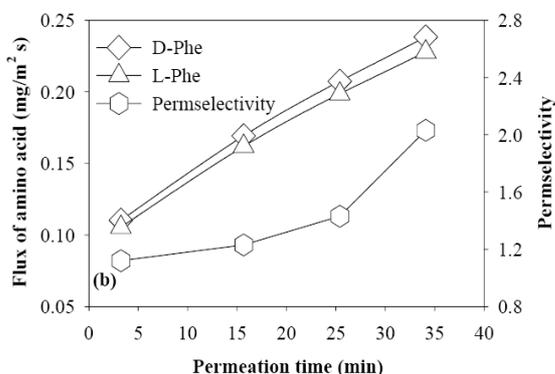


Fig. 4: Phe flux and permselectivity of (a) L-Phe and (b) D-Phe imprinted AA/AN membrane after 16 ml filtration of 100-ppm racemate mixture of Phenylalanine.

Template Effect on Solute Transport Mechanism

In MIMs transport of solute can be considered by (1) facilitated permeation and/or (2) retarded permeation [33]. The facilitated permeation is driven by preferential sorption of the template due to affinity binding. In facilitated permeation transport of solute depend on the structure of membrane, concentration and distribution of MIP sites coupled with transport phenomenon [32]. Separation selectivity can only be achieved for relatively small diameters of transmembrane pores due to the coupling with non-selective transport. Most of synthetic carrier membranes based on facilitated transport are liquid membranes, i.e. they have a non-porous barrier structure. In retarded permeation other solute transports faster due to affinity binding, until a saturation of MIP sites with template is reached. MIP binding capacity helps to evaluate separation efficiency due to the saturation behavior. Those MIM can be solute adsorbers as selectivity is caused by specific adsorption [36]. Separation efficiency is determined by MIM binding capacity due to the saturation behavior. Based on the α_{Sep} data obtained by permeation of substrate and α_{Ads} data obtained by uptake values of membrane, transport selectivity α_{Trans} was calculated according to the solution-transport mechanism model using equation (8) and listed in Table-2. The transport selectivity of L-Phe imprinted membranes $[\alpha_{Trans}]_L$ was 0.98 and transport selectivity of L-Phe imprinted membranes $[\alpha_{Trans}]_D$ was 0.84. From these data we conclude that after 16 ml of permeation the permselectivity was

higher than adsorption selectivity for both L-Phe and D-Phe imprinted membranes. From these three selectivity factors, it could be deduced that the L-Phe imprinting membranes lead to an improved preferential adsorption for L-Phe, the transport of D-Phe molecules were retarded presumably due to the strong trapping effect of the imprinting cavities (i.e. gate effect [33]) and facilitated transport of L-Phe. The D-Phe imprinted membrane showed similar behavior. D-Phe imprinted membrane rejected L-Phe and retarded transport of L-Phe while D-Phe was successfully recognized by membrane as adsorbed amount of D-Phe was much higher than that of L-Phe and permeation curves show facilitated permeation of L-Phe (template). Therefore, the separation mechanism of L-Phe and D-Phe imprinted poly(AA-co-AN) membranes for D,L-Phe isomer separation agreed well with the above mechanism (1). Thus we can concluded that the template recognition and increase in facilitated permeation are also functions of membrane swelling along with imprinted gates and cavities in the membrane matrix.

Experimental

Materials

The chemicals purchased from Sigma-Aldrich (USA) were 2,2-Azobisisobutyronitrile (AIBN), D-Phenylalanine (D-Phe), L-Phenylalanine (L-Phe), underivatized mixture of D,L-Phenylalanine (Phe) and Trifluoroacetic acid (TFA). Dimethyl sulfoxide (DMSO) was product of Kanto (Japan). Acrylic acid (AA) and copper sulfate (CuSO₄) were obtained from Junsei (Japan). Acrylonitrile (AN) was purchased from Yakuri (Japan). The HPLC solvents acetonitrile and methanol were obtained from Scharlau (Spain). All reagents were of analytical grade and used without further purification.

Preparation of Molecularly Imprinted Membrane

To prepare molecularly imprinted poly(AA-co-AN) membranes by wet phase inversion method, imprinted polymer was prepared by radical polymerization. In 50 ml DMSO 7.19 ml AA, 0.5 g template (L-Phe or D-Phe) and 2 ml TFA were dissolved at 50 °C for 2 h in a polymerization reactor. To the above solution 37.72 g AN and a solution 50 ml DMSO and 0.22 g AIBN were added to above solution and nitrogen gas was purged for 5~10 minutes. The polymerization was done at 60 °C for 6 h under nitrogen atmosphere. The solution was

stirred at uniform rotation speed of 200 rpm. 100 ml DMSO was added to the polymer and stirred for 20 h with a uniform rotation speed of 200 rpm at 25 °C. Then the polymer solution was placed in vacuum oven for 24 h, at 0.8 atm and 25 °C. With the help of gardener knife polymer solution was cast on glass plate and coagulated in deionized water at 25 °C to get polymeric membrane. DMSO was removed from membrane by extensive washing. 5 % (V/V) acetic acid solution was used for the removal of template.

Characterization of poly(AA-co-AN) Membranes

FT-IR spectra of dried poly(AA-co-AN) samples (grounded with KBr pellets at room temperature) were recorded using a Mattson Galaxy 7020A FT-IR spectrophotometer (with a resolution of 0.025 cm⁻¹ and wavelength range from 4000 cm⁻¹ to 400 cm⁻¹) and a DTGS detector. The surface and cross-section morphology of poly(AA-co-AN) membranes were observed with Hitachi S-4300 Field Emission Scanning Electron Microscope (FE-SEM). Freeze dryer was used to dry samples of membrane, then samples were sputtered with gold and observed at 15 and 20 kV Energy Dispersive X-ray Spectrophotometer Image Processing System was used.

Separation Experiment

A 30 ml aqueous solution containing 100 mg Phe/l (50 mg for each enantiomer) with pH value of 2 was filtered through 5 sheets of membranes fixed in Millipore Ultrafiltration kit to determine the separation ability driven by a pressure of 1 atm. The amounts of L-Phe and D-Phe in samples were measured by HPLC consist of M 930 solvent delivery pump & M 720 UV Absorbance detector made of Young-Lin Instruments (Korea). The column TSKgel Enantio L2 made of Tosoh (Japan) with dimensions 4.6 mm id. X 250 mm was used. To check the reproducibility of results the experiments were repeated three times.

Rejection Selectivity of Membrane

The equation of rejection R used by other researchers [31] was modified using mass balance equation considering feed solution volume and concentration; permeate volume and concentration; volume and concentration of retentate; and amount of Phe adsorbed on membrane. The rejection R was calculated by following equations.

$$R_L = \frac{V_R [C_R - C_O]_L}{V_P [C_O]_L} \times 100 \quad (1)$$

where R_L is rejection of L-Phe, subscript L represents L-Phe, V_R and V_P represents volume (ml) of retentate and permeate respectively; C_R and C_O are concentrations of Phe (mg/l) in retentate and in feed solution respectively. The rejection selectivity α_{Rej} , is defined as

$$[\alpha_{Rej}]_L = \frac{R_L}{R_D} \quad (2)$$

where $[\alpha_{Rej}]_L$ represents rejection selectivity when L-Phe imprinted membrane was used and R_D is rejection of D-Phe. If $[\alpha_{Rej}] < 1$, then it shows that the rejection of template was more than the counter enantiomer but, if $[\alpha_{Rej}] > 1$, this indicates that the rejection of counter enantiomer was more than template molecule.

Adsorption Selectivity of Membrane

The adsorption of L-Phe, Q_L (mg/g of membrane) on membrane was calculated by

$$Q_L = \frac{[M_O - (M_P + M_R)]_L}{W_D} \quad (3)$$

where M_O , M_P and M_R are amounts of Phe (mg) in feed solution, in permeate and in retentate respectively; and W_D is dry weight of membrane. The adsorption selectivity α_{Ads} was calculated by using following equation [19-21].

$$[\alpha_{Ads}]_L = \frac{Q_L}{Q_D} \quad (4)$$

where $[\alpha_{Ads}]_L$ represents adsorption selectivity when L-Phe imprinted membrane was used and Q_D is adsorption of D-Phe (mg/g of membrane). When $[\alpha_{Ads}] < 1$, then it shows that the adsorption of template was more than the counter enantiomer and $[\alpha_{Ads}] > 1$ show that adsorption of counter enantiomer was more than template enantiomer.

Solute Transportation Across Membrane

The L-Phe flux J_L (mg/m²s) was calculated by the following equations: [37].

$$J_L = \frac{[M_p]_L}{AT} \quad (5)$$

where, A is the effective area (m²) of membrane and T represents time (sec) required by solution to pass through membrane. The permeability coefficient P_L (m²/s) of L-Phe is defined as:

$$P_L = \frac{J_L \delta}{[C_o - C_p]_L} \quad (6)$$

where δ is the membrane thickness (m) and C_p is concentration of Phe (mg/l) in permeate. The permselectivity $[\alpha_{perm}]_L$ using L-Phe imprinted membrane was calculated by:

$$[\alpha_{perm}]_L = \frac{P_L}{P_D} \quad (7)$$

where, P_D is permeability coefficient (m²/s) of D-Phe. The $[\alpha_{perm}] < 1$ illustrate that membrane showed facilitated permeation and $[\alpha_{perm}] > 1$ shows that membrane retarded permeation of template.

The diffusion selectivity of the membranes was calculated by Jiang *et.al.* [28] method, for the chiral separation of amino acid. We calculated solute selectivity of the membrane using ultrafiltration technique considering solution transport mechanism by equ (8) after certain modifications.

$$[\alpha_{Trans}]_L = \frac{[\alpha_{perm}]_L}{[\alpha_{Ads}]_L} \quad (8)$$

where $[\alpha_{Trans}]_L$ represents transport selectivity when L-Phe imprinted membrane was used. When $\alpha_{Trans} > 1$ then permselectivity is higher than adsorption selectivity and when $1 > \alpha_{Trans}$ then adsorption selectivity is higher than permselectivity.

Swelling Study of Imprinted Membranes

The Phe extracted membranes were soaked in distilled water for 72 h to ensure swelling equilibrium. Then the swollen membranes were taken out and water on the surface of membrane was blotted carefully with filter paper and weighed immediately. Then the membrane was dried under vacuum with a flat bottomed weighty object placed on the filter paper to avoid the shrinking. The following equation was used to determine swelling ratio (S_{Ratio}) of the membrane [38]:

$$S_{Ratio} = \frac{W_w - W_D}{W_D} \quad (9)$$

where, W_w is wet weight of membrane.

Conclusions

The L-Phe and D-Phe imprinted membranes prepared by AA and AN, successfully recognize template, facilitate permeation of template and reject other enantiomer. It was observed that one carboxylic molecule is sufficient for imprinting and recognition. The interacting imprinting sites in membrane matrix successfully bind template resulting in significantly improved chiral separation followed by ultrafiltration. Both L- and D-Phe imprinted membranes show similar trends. The results of L-Phe imprinted membranes were found to be remarkable. The L-Phe imprinted membrane was much better than D-Phe imprinted membrane, in terms of permselectivity, adsorption selectivity, rejection selectivity and transport selectivity.

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