1. Introduction

Chirality plays an important role in the function of biological processes. The different enantiomers of a chiral drug usually exhibit different pharmacological activities, metabolic effects, metabolic rates, and toxicities due to the high degree of stereoselectivity. [1-3] In some cases, only one of the enantiomers of a chiral drug contributes to its pharmacodynamic behavior, while the other shows no or a much weaker effect as well as side-effects or even toxicity. [4] Besides the use of single enantiomers in the pharmaceutical industry, enantiomerically pure compounds are becoming significant in the production of other chemical products increasingly, such as agrochemicals, fragrances, and foods. [5] Currently, separation of racemic mixtures is typically performed by column chromatography [6], preferential crystallization [7] or kinetic resolution [8]. These methods have various advantages but also disadvantages, including high energy consumption, high cost, low efficiency, and discontinuous operation. Low-cost, continuous, high-efficiency separation technology is clearly needed for commercial-scale preparation of enantiomerically pure substances. Membrane technology, fortunately, fulfills this need very well because of its high efficiency, low energy usage, simplicity, convenience for up- and/or downscaling, and continuous operability. [1-3] Enantioselective membranes include liquid membranes and solid membranes that can achieve enantioseparation by binding the two enantiomers with different affinities. Solid membranes are usually long-term stable because of self-supporting structure or existence of attached base membranes. [9] Among solid membranes, polymer membranes containing amino acid have been widely studied. [10-13] But these membrane systems did not show response to external stimuli. In our previous studies, [14] we have reported a functional molecular membrane composed of peptide which is sensitive to pH conditions.

Poly (N-isopropylacrylamide) (PNIPAm) and its copolymers are the most extensively studied thermo-sensitive polymer, which exhibit a lower critical solution temperature (LCST) in aqueous solution. PNIPAm chains hydrate to form expanded structures in water when the temperature is below its LCST, but become compact structures by dehydration when heated above its LCST. The volume-phase transition brings about drastic changes in the physical properties of the PNIPAm gels.

In this research, according to our previous work we synthesized a membrane consisting of a PNIPAm network with peptide graft chains and porous support membrane. The network membrane has pH and thermo-responsiveness. We investigated the pH and thermo-induced permeability and permselectivity changes through the network membrane.

# corresponding author
2. Experimental section

2.1 Materials

*N*-isopropylamide (NIPAm, TCI, Tokyo, Japan) was purified by recrystallization from hexane. *N,N*-methylenebisacrylamide (BisAAM, Nacalai tesque, Kyoto, Japan) and 2-hydroxy-2-methylpropiophenone (HMPF, Aldrich, USA) were used without purification. Ultrapure water with a conductivity of 18 µS cm\(^{-1}\) was used in all experiments. MF-millipore membrane filter (MF, thickness 180 µm, pore size 0.22 µm, porosity 75%, Millipore, USA) was used as a porous supported membrane.

2.2 Preparation of polymer membrane

The peptide (\(\text{L-Leu-L-Lys}\)_8-vinyl ((LK)_8-vinyl)) was synthesized by the way that we reported. [15] The typical procedure for photopolymerization was used; 10 mg of NIPAm, 20 mg of (LK)_8-vinyl, and 1.5 mg of BisAA as a cross-linker were dissolved in 1 ml of water and stirred in a 10 ml flask at room temperature. The molar ratio of NIPAm : (LK)_8-vinyl: BisAA was 0.988: 0.011: 0.001. Then 100 µL acetone solution of photoinitiator (containing HMPF 10 µL) was added into the monomer solution. Freeze-deaeration was carried out three times to remove the oxygen. The MF membrane was immersed in the reaction mixture to impregnate the monomer solution in the pore of the membrane. After this process, the membrane was took out from the solution and put on a Teflon dish in a glove box. The polymerization was carried out by UV irradiation for 2h. After the polymerization, the membrane was immersed in the pure water to remove the unreacted monomer and the cross-linking agent.

2.3 Characterization of the peptide grafted membrane

In this research, we synthesized the membrane without the porous support membrane at the same condition to characterize the degree of hydration and elongation, composition, pKa and conformation of the peptide graft chains in the membrane. The synthesized polymer without the MF membrane was dried in a vacuum drier for 2 days. From the element analysis we calculated the molar ratio of NIPAM; 0.64, peptide graft chain; 0.05, cross-linker; 0.04. The structure of the membrane is schematically shown in Figure 1. The pKa of the amino group of the lysine residues in the membrane was determined to be 7.5 by the pH titration.

We think that the permeability through the peptide grafted PNIPAm network membrane is influenced by the conformation of the peptide graft chains and the PNIPAm main chain. The degree of hydration and elongation of the membrane are sensitive to the structural changes of the PNIPAm main chain and the peptide graft chains. Here we use the water content of the swollen membrane indicating the degree of hydration of the membrane. The equation to calculate the degree of hydration of the membrane \(H\) is as follows: \(H = (M_s - M_0) / M_s\), where \(M_s\) and \(M_0\) are the weights of swollen and dried membranes, respectively. The elongation of the membrane was measured by an optical microscope (USB Microscope M2, Scalar, Tokyo, Japan). The degree of elongation of the membrane under the several conditions was estimated from the changes in the length of the membrane. The degree of elongation value, \(L\), was calculated as follows: \(L = L_m / L_0\), where \(L_m\) is the length that is measured under a predetermined condition and \(L_0\) is the length of the membrane dipped in aqueous solution at pH 6.5 and 20 °C as a standard value. The membrane was swollen in the aqueous solution under various conditions (pH and temperature), blotted, and weighed and measured repeatedly until constant weight and length were obtained. The pH of the aqueous solution was adjusted with 0.1M HCl or 0.1 M NaOH. The results of hydration and elongation are listed in Table 1.

2.4 FT-IR measurements

Secondary structural changes of the peptide graft chains in the polymer membrane were investigated by the Transmittance Fourier transform infrared (TM-FTIR) spectroscopy. The TM-FTIR spectra were measured with a Perkin-Elmer Spectra 2000 (resolution : 4 cm\(^{-1}\), number of scan : 32). The membranes were dipped in the aqueous solutions under various conditions (pH and temperature) and quickly frozen in liquid nitrogen, and then the frozen samples were lyophilized to obtain the dried membrane powder. Beforehand we checked that the conformational changes did not occur during the lyophilization process.
The pellets for TM-FTIR measurements were prepared by the mixing of the membrane powder and KBr. The fraction of the membrane was fixed at 1 wt%.

2.5 Permeability measurements

The permeability measurements of the phenylalanine through the network membrane were carried out with a Pyrex glass permeation cell. The network membrane was placed between the two parts of the cells pressed by two pieces of Millipore supported membranes to form the sandwich structure. A 5 mM aqueous solution of L-phenylalanine or D-phenylalanine was introduced into one side of the cell, and phenylalanine free water was taken into the other side (the permeated side) of the cell. The pH of the both solution in the cell was adjusted with 0.1 M HCl or 0.1 M NaOH. The changes in the phenylalanine concentration with time on the permeated side were measured with an UV-vis spectrophotometer (UV-3600, Shimadzu, Japan) from the absorbance at 256.5 nm on the basis of the molar extinction coefficient of the phenylalanine. Figure 2 shows a typical example of the time-dependent changes of the L- and D-phenylalanine concentration of the permeation side at 20 °C under the neutral condition (pH 6.5), respectively. From the initial slopes of the curves of Figure 2 (denoted by the dotted lines), the fluxes of L- and D-phenylalanine \([J\text{ (mol} \cdot \text{cm}^{-2} \text{ s}^{-1})]\) through the membrane were calculated. The permeation coefficients \(P\text{ (cm}^2\text{ s}^{-1})\) of L- and D-phenylalanine were calculated by the equation as follow; \(P = J \cdot \delta / \Delta c\), where \(\delta\) is the membrane thickness and \(\Delta c\) is the external concentration difference of L- or D-phenylalanine across the membrane. In this study, the membrane area was the area of the swollen polymer incorporated domain in the MF-membrane for an effective permeation area. The permeability measurements of the L- or D- phenylalanine were performed 3 times under the same condition, and the \(P\) values for the L- and D-phenylalanine were obtained as an averaged value, \(P_L\) and \(P_D\), respectively. The permselectivity, \(\alpha\), of L-phenylalanine to D-phenylalanine through the membrane was obtained as follows; \(\alpha = P_L / P_D\).

3. Result and discussion

3.1 Degree of hydration and elongation of the membrane affected by external conditions

In this research, the main component of the membrane is PNIPAm, which was introduced to give the thermal sensitive property for this polymer membrane. PNIPAm gels have high swellability in water and it is known that the gels shrink above 32 °C in water. [16] Hydration degrees of the membrane under different conditions are shown in Table 1.

<table>
<thead>
<tr>
<th>pH</th>
<th>T / °C</th>
<th>H / %</th>
<th>L / %</th>
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<tr>
<td>3.0</td>
<td>20</td>
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<td>96.3</td>
<td>77.1</td>
</tr>
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</table>

The degree of hydration reached maximum of 98.2% at pH 6.5 and 20 °C. This is mainly because of the hydration of the PNIPAm main chains. At 20 °C, the PNIPAm was hydrated and formed expanded structure. When temperature is increased to 40 °C, which is higher than the LCST temperature of PNIPAm, the PNIPAm is known to adopt a compact structure by dehydration. [17] However, the water content was relatively high even at 40 °C, as shown in Table 1. Probably, the conformational transition of the PNIPAm main chain was disturbed because the cross-linking degree of the membrane was relatively high (BisAA content was 16%) and the membrane trapped the water. At pH 3.0 and pH 9.0, the degree of the hydration slightly decreased compared with that under the neutral pH condition. Under the acidic condition, pH 3.0, the peptide graft chains are protonated. The ionized amino groups of the Lys residues that exist in the vicinity of the PNIPAm main chain should have disturbed the hydration of the polar parts of the PNIPAm by the ionic effect [18, 19]. On the other hand, under the basic condition, pH 9.0, hydrophobic interaction among the deprotonated peptide graft chains may have induced the relatively lower hydration of the PNIPAm main chain.
The degree of the elongation of the membrane showed same tendency (Table 1). The degree of elongation of the membrane clearly decreased under the basic condition (pH 9.0). This membrane shrinkage was remarkable at 40 °C because of the conformational transition of the PNIPAm main chains and the hydrophobic interaction among the deprotonated peptide graft chains.

3.2 pH-induced conformational transition of the peptides graft chain in the membrane

We investigated the conformational changes of the peptide graft chains in the membrane at 20 °C and 40 °C by the TM-FTIR measurements. Figure 3 shows the TM-FTIR absorption spectra of the polymer membranes under the various conditions. In the spectra, characteristic absorptions of the amide I band with α-helix, β-sheet, and random coil conformations were observed at 1650, 1630, and 1675 cm⁻¹, respectively. The ratio of integrated peak intensities assigned to individual secondary structure, which was obtained by peak deconvolution of the amide I band, gave the percentage of conformational changes of the peptide graft chain in the membrane. The results of conformational analysis are summarized in Table 2.

From this table, we could find that temperature had little effect on the conformational changes of the peptide graft chain. The composition of secondary structure was merely not changed when the temperature increased from 20 °C to 40 °C. At pH 6.5, the content of β-sheet structure was slightly higher than that under the acidic and basic conditions. This could be explained as follows; under the acidic condition, pH 3.0, the inter-molecular hydrogen bonding to form the β-sheet structure among

<table>
<thead>
<tr>
<th>pH</th>
<th>T / °C</th>
<th>α-helix / %</th>
<th>β-sheet / %</th>
<th>random coil / %</th>
</tr>
</thead>
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<tr>
<td>3.0</td>
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<td>37.2</td>
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<tr>
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the protonated peptide graft chains was disturbed owing to the electrostatic repulsion. Under the basic condition, pH 9.0, the deprotonated peptide chains aggregated rapidly owing to the hydrophobic interaction. The aggregation disturbed the formation of regular structure, and resulted in the increase of the random coil conformation.

On the other hand, under the neutral condition, pH 6.5, the peptide graft chains were partially protonated. The electrostatic repulsion among the partially protonated peptide chains disturbed the rapid formation of the disordered aggregation and aided in the formation of β-sheet structure owing to the intermolecular hydrogen bonding. However, the degree of the pH induced conformational transition was very little. This is because of the low graft content of the peptide chain and the high degree of cross-linking of the PNIPAm main chain, which

![Fig. 3](image)

**Fig. 3** TM-FTIR spectra of peptide graft chains in the PNIPAm membrane prepared by quick freezing and lyophilization at a) pH 3.0, 20 °C; b) pH 3.0, 40 °C; c) pH 6.5, 20 °C; d) pH 6.5, 40 °C; e) pH 9.0, 20 °C; and f) pH 9.0, 40 °C respectively. Broken lines show the peak deconvolution of the amide I band to 1; α-helix, 2; β-sheet, and 3; random coil conformations.
disturbed the formation of regular arrangement of the peptide graft chains.

3.3 Permselectivity of L- and D-phenylalanine induced by pH and thermal stimuli

In the previous studies, we have reported that the $\beta$-sheet domain in the peptide molecular membrane acted as a binding site of the amino acid, which had same chirality with the peptide [21], and the domain acted as a selective permeation path through the membrane for the amino acid [14]. We investigated the permselectivity of L- and D-phenylalanine through the peptide grafted PNIPAm network membrane, whose peptide chains consisted of L-amino acids. The typical permeation curves of L- and D-phenylalanine at pH 6.5 and 20 °C are shown in Figure 2. The permeability coefficient of L-phenylalanine, $2.86 \times 10^{-7}$ cm$^2$ s$^{-1}$, was very high (self-diffusion constant: $9.0 \times 10^{-6}$ cm$^2$ s$^{-1}$). We think that the deviation of the permeation curve from the expected linearity is due to the cancellation of the concentration difference. On the other hand, the permeation of D-phenylalanine showed the slight delay time owing to the dissolution of D-phenylalanine into the membrane. Figure 4a shows the permeation coefficients of L- and D-phenylalanine through the membrane under the different pH and temperature conditions. The permeation coefficients were calculated as an averaged value of the 3 measurements. Figure 4b shows the permselectivity, $\alpha$, which is the ratio of the permeability coefficient of L-phenylalanine to that of D-phenylalanine. The permeability of both L- and D-phenylalanine through the membrane under the basic and high temperature condition (pH 9.0 and 40 °C) was the largest among the studied conditions. Under this condition, the peptide grafted PNIPAm was shrunk in the MF-supported membrane (Table 1), and the pore was formed in the membrane. This is the reason for the relatively higher permeability and the non-permselectivity of L- and D-phenylalanine ($\alpha=1.0$, Figure 4b). At low temperature, 20 °C, under the basic condition, the membrane did not show the permselectivity ($\alpha=1.0$). The PNIPAm main chains were hydrated, but the peptide graft chain aggregated under this condition. The conformation of the membrane disturbed the formation of permselectivity path for the phenylalanine.

Under the acidic condition, pH 3.0, the permeation coefficients of L- and D-phenylalanine at 20 °C and 40 °C show the same value, and the permselectivity of the phenylalanine did not appear. The peptide grafted chains protonated and had the negative charge (pKa of the peptide graft chain was 7.5), and permeant, phenylalanine, was positively charged at pH 3.0. This electrostatic repulsion between the membrane and permeant disturbed the specific interaction in the binding site composed of $\beta$-sheet domain in the membrane and resulted in the same permeability of the L- and D-phenylalanine through the membrane above and below the LCST.

On the other hand, under the neutral pH condition, pH 6.5, the significant difference was recognized in the permeability of L- and D-phenylalanine at 20 °C. Under this condition, the membrane was most swollen, and the peptide graft chains were partially protonated and formed considerable amount of $\beta$-sheet structure. Furthermore the permeant, phenylalanine, was negatively charged (pI of phenylalanine was 5.48). The attractive force between the negatively charged phenylalanine and the $\beta$-sheet peptide domain which was positively charged induced the increase of the phenylalanine concentration at the interface. The $\beta$-sheet peptide domain composed of L-amino acids acted as effective L-phenylalanine binding site and relatively higher permeability of L-phenylalanine was achieved compared with that of D-isomer ($\alpha=2.6$). With the increase in temperature, the PNIPAm main chains were dehydrated and the polymer was shrunk in the MF-support membrane to form the void. The void acted as the permeable path for both the L- and D-phenylalanine.

![Fig. 4](image_url) Permeation coefficients (a) and the permselectivity of L- and D-phenylalanine (b) through the peptide grafted PNIPAm network membrane.
phenylalanine, which results in the non-permselectivity through this membrane.

4. Conclusion

In this research we prepared a peptide grafted PNIPAm network membrane by the UV photopolymerization. This polymer membrane shows very high permeability, and has a chiral selectivity due to the grafted peptide chains. The permeability of the amino acids through this membrane is sensitive to pH and temperature. At the temperature lower than the LCST of the PNIPAm and under the neutral pH conditions, β-sheet peptide domain composed of L-amino acids in the membrane acted as the effective permeable path for its own optically active amino acid (L-phenylalanine). When temperature is higher than the LCST of the PNIPAm, the shrinking of the PNIPAm main chain formed void structure that acted as permeable paths through the membrane for the both isomer (L- and D-phenylalanine). We think that the permselectivity of the peptide grafted PNIPAm network membrane can be improved for the practical application by decreasing cross-linking density and increasing the relative amount of the peptide graft chains.

References