Effects of Side Chain Length on Membrane Properties of Copoly- (*N*-hydroxyalkyl L-glutamine) Hydrogels

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ABSTRACT: Random copolymers, poly(*N*-hydroxyethyl L-glutamine-*co*-*N*-hydroxypentyl L-glutamine) (PHPeEG) and poly(*N*-hydroxypropyl L-glutamine-*co*-*N*-hydroxypentyl L-glutamine) (PHPePG) as well as the corresponding homopolymers, poly(*N*-hydroxyethyl L-glutamine) (PHEG), poly(*N*-hydroxypropyl L-glutamine) (PHPG), and poly(*N*-hydroxypentyl L-glutamine) (PHPeG), were prepared by aminolysis in the presence of the crosslinking agent, octamethylenediamine (OMDA), of a membrane of poly(γ -benzyl-L-glutamate). Membrane properties, such as the degree of swelling Q_w , conformational property, tensile property, water vapor permeability and enzymatic degradation behavior were studied in the pseudo-extracellular fluid (PECF). Q_w and molecular conformation are dependent on the composition of random copolymers. The mechanical strength of PHEG and PHPG hydrogels has been improved largely by introducing HPeG residues. Suitable mechanical property and water vapor permeability were obtained in some copolymers, which were compatible as skin substitutes. When compared at the same Q_w , PHPePG copolymers were shown to have higher helix content and mechanical strength, lower water vapor permeability and faster papain digestion than PHPeEG copolymers. This can be explained by differences in hydrophobicity between PHEG and PHPG.

KEY WORDS Poly(*N*-hydroxyethyl L-glutamine-*co*-*N*-hydroxypentyl L-glutamine) / Poly(*N*-hydroxypropyl L-glutamine-*co*-*N*-hydroxypentyl L-glutamine) / Hydrogel / Degree of Swelling / Molecular Conformation / Tensile Property / Water Vapor Permeability / Enzymatic Degradation /

Hydrogels are water-swollen hydrophilic materials. Several useful applications of hydrogels require not only suitable degree of swelling, but also favourable elastic and mechanical properties in the swollen state.^{1,2} The molecular design for useful hydrogel which is compatible with high water content and moderate mechanical strength is not so easy. Hydrogels of poly(α -aminoacid)s will be useful for medical applications such as temporary artificial skin substitutes, temporary barriers to prevent adhesion, and so on.³

In our previous paper,⁴ mechanical properties of random copolymer PHPeEG consisting

of PHEG and PHPeG were studied in connection with their molecular conformation. The difference in tensile behavior between PHEG and PHPeG was interpreted. Tensile property similar to that of human skin was shown in some PHPeEG hydrogels with low Young's modulus and high tensile strength. Polypeptides in PHEG hydrogel exist in random coil conformation while those in PHPG hydrogel exist in interrupted α -helix conformation. In this study, random copolymers, PHPeEG and PHPePG, were prepared. The membrane properties of both copolymers, such as the degree of swelling Q_w , molecular conformation,

tensile property, water vapor permeability and enzymatic degradation behavior were investigated in pseudo-extracellular fluid (PECF)⁵ (115 mM NaCl, 30 mM NaHCO_3 , 3 mM KCl, and 2 mM K_2HPO_4) at pH = 7.4. The object of this study is to examine membrane properties in connection with molecular conformation, from the viewpoint of possible skin substitutes.

EXPERIMENTAL

Materials

The mother polymer, poly(γ -benzyl L-glutamate) (PBLG) was synthesized by the *N*-carboxyanhydride (NCA) method as described in our previous paper.⁶ Trichloromethyl-chloroformate was supplied by Hodo-gaya Chemical Co. The molecular weight of PBLG was determined as 335,000 by viscosity measurement⁷ in dichloroacetic acid (DCA). All the solvents used in the synthesis were distilled twice.

Hydrophilic copolymer membranes, PHPeEG and PHPePG, were prepared by the aminolysis⁸ of PBLG membranes cast from chloroform solution. PBLG membranes of ca. 60 μm and 1 μm in thickness were immersed in a mixture at various compositions of 2-amino-1-ethanol (EA) and 5-amino-1-pentanol (PeA) and of 3-amino-1-propanol (PA) and PeA, with 3 mol% of the crosslinking agent, 1,8-octamethylenediamine (OMDA), at $58 \pm 0.2^\circ\text{C}$. The aminolytic reaction was complete after 7 to 14 days. Debenzylation of γ -BLG was confirmed by the disappearance of the absorption at 250–260 nm in UV spectra, and of the absorptions at 700 and 750 cm^{-1} in IR spectra. The absence of the γ -benzyl ester group was guaranteed by the disappearance of the absorption due to ester groups at 1730 cm^{-1} in IR spectra. Compositions of all the copolymers were determined by elementary analysis.

Measurements

Degree of Swelling Q_w (%) of Hydrogels.

Q_w (%) was determined by equilibrating the membrane in PECF solution at 37.0°C . The membrane was then blotted to remove surface PECF and weighed until constant weight was achieved. The membrane was then dried in a vacuum oven. Q_w (%) was defined as the ratio of the amount of PECF to weight of the dried crosslinked hydrophilic membranes.

Molecular Conformation of Hydrogels. The molecular conformation of linear PHPeEG and PHPePG in PECF solution was examined by circular dichroism (CD) (Jasco J-40 CD/ORD) measurement. The helix content X_H was evaluated from the residue ellipticity (θ) at 222 nm. The conformations of membranes in xerogel and in hydrogel were examined by IR (Hitachi Model 285) spectra. The Amide V band⁹ in IR spectra was used for identification of molecular conformation in hydrogel.

Tensile Properties of Hydrogels. The tensile properties of hydrogels were measured in PECF at 25°C , using a Tensilon UTM-4LH (Orientec Co.) equipped with a 1 kg load cell. A wrapping film was used at grabbing parts to prevent the hydrogels from breaking or from slippage at the grab. All the hydrogels were tested at a strain rate of 40% per minute. Mechanical parameters such as Young's modulus (E), tensile strength (σ_B), and strain at break point (ϵ_B) were measured.

Water Vapor Permeability of Hydrogels. Water vapor permeation through the membranes was measured with a cylindrical glass cell¹⁰ at 37°C . The rate of water vapor permeability V_f ($\text{gm}^{-2}\text{ day}$) was determined by weighing the cell at constant time intervals. The exposed membrane area was 12.57 cm^2 .

Biodegradation of Hydrogels in Vitro. Enzymatic degradation studies *in vitro* were carried out using papain purchased from Nakarai Chem. Co. Enzyme solution was prepared by standard techniques¹¹ at 37°C . A series of the crosslinked hydrogels was exposed to PECF solution with activator, 0.01 M cysteine and 0.04 M EDTA,¹² at pH 7.4. Polymer membranes were removed from the

enzyme solution at appropriate time intervals, weighed and then vacuum dried at 60°C to constant weight.

RESULTS AND DISCUSSION

Relation of Degree of Swelling to Molecular Conformation on Hydrogels

Linear PHEG, PHPG and PHPeG without crosslinking are water soluble polymers. Molecular conformation of PHEG, PHPG and PHPeG in water are random coil, interrupted α -helix (30% helicity) and complete α -helix, respectively.¹³⁻¹⁵ The helix content X_H of linear PHPeEG and PHPePG copolymers in PECF solution at 25°C is shown as a function of the mole fraction of HPeG in Figure 1. It is suggested that α -helix conformation of PHPeG is disrupted by the introduction of HEG units and is strengthened by HPG units. This can be explained by differences in hydrophobicity of the side chains. The molecular conformation of crosslinked membranes is identified by IR absorption spectra and some spectra in the Amide V region are shown in Figure 2. The number X in sample code PHPeEG-X denotes the molar percent of HPeG in copolymer. Although all the membranes showed α -helix conformation in xerogel, the absorption of 650 cm⁻¹ which corresponds to random coil conformation increases with decreasing HPeG content in hydrogel. PHPePG-21 hydrogel seems to have higher helix content X_H than PHPeEG-61 hydrogel, which is similar to the result of linear copolymers in Figure 1. Thus, the molecular conformations of linear copolymers in PECF solution are considered to reflect those of crosslinked hydrogels. The results from CD measurement of hydrogels also support this viewpoint. The degree of swelling Q_w of crosslinked membranes in PECF solution is shown in Figure 3. The degree of swelling decreases with increasing HPeG content in both copolymer membranes. Q_w values of PHPeEG membranes are higher than those of PHPePG membranes,

Figure 1. The helix content X_H of linear PHPeEG (○) and PHPePG (●) copolymers as a function of HPeG content (mol%) in PECF solution at 25°C.

Figure 2. IR absorption spectra in Amide V region of copolymer membrane in the swollen state.

Figure 3. The degree of swelling Q_w of PHPeEG (○) and PHPePG (●) membranes in PECF solution at 25°C.

which indicates that Q_w also depends on the hydrophobicity of the side chains. However, Q_w of PHPePG-46 membrane is higher than that of PHPeEG-82 membrane which shows

helix content X_H similar to that of PH-PePG-46 membrane in PECF. This may be due to the less content of hydrophobic HPeG in the PHPePG-46 membrane.

Mechanical Properties of Hydrogels

The tensile property of human skin shows characteristic behavior with low Young's modulus and high tensile strength. For hydrogels as skin substitutes, it is necessary to have the above characteristic behavior to prevent exfoliation from a wound site. Hydrogels, which can use water as a plasticizer, are considered promising materials as skin substitutes with respect to the tensile property.

The stress-strain (S - S) curves of PHPeEG and of PHPePG hydrogels in PECF are shown in Figure 4 together with the S - S curve of human skin at lower abdomen in a longitudinal direction.¹⁶ The mechanical parameters obtained from S - S curves, such as Young's modulus (E), tensile strength (σ_B) and strain at break point (ϵ_B) are listed in Table I. In PHPeEG and PHPePG copolymers σ_B and ϵ_B increase with increasing molar fraction of HPeG while low Young's modulus is unchanged. Mechanical properties similar to that of human skin are shown in some copolymers. The S - S curves of hydrogels were classified into an elastomer-type having transition point in the low strain region and a skin-type without this point, in our previous paper.⁴ The skin-type behavior was regarded as entropy elasticity without stress relaxation, creeping and hysteresis, while elastomer-type behavior was considered to include the contribution of energy elasticity with stress relaxation, creeping and hysteresis, but without unrecoverable strain. As shown in Figure 4, this transition from skin-type to elastomer-type in S - S curve occurs in the region between 82 and 92 mole fraction of HPeG, corresponding to 78–92% of the helix content X_H and 110–160% of the degree of swelling Q_w in PHPeE-G copolymers and occurs in the region between 46 and

Figure 4. Stress-strain curves of copolymer membranes, (a) PHPeEG and (b) PHPePG, in PECF solution at 25°C.

Table I. Mechanical Parameters obtained from S-S Curves of PHPeEG Membranes in PECF solution at 25°C

Sample code	E	σ_B	ϵ_B
	MPa	MPa	%
PHEG	0.039	0.12	130
PHPeEG-10	0.042	0.16	150
PHPeEG-32	0.045	0.45	150
PHPeEG-61	0.055	2.1	220
PHPeEG-75	0.11	3.9	220
PHPeEG-80	0.17	5.8	210
PHPeEG-82	0.29	8.0	210
PHPeEG-92	6.8	18	180
PHPeG	38	21	120
PHPG	0.061	1.6	180
PHPePG-21	0.097	3.7	230
PHPePG-46	0.15	4.9	240
PHPePG-70	4.3	12	200
PHPePG-72	5.8	12	170
PHPePG-80	6.9	13	160
PHPePG-88	8.6	14	160
PHPePG-95	12	15	150

Figure 5. Comparison of PHPeEG copolymers (○) with PHPePG copolymers (●) in the relation between Young's modulus E and degree of swelling Q_w of PECF.

70 mole fraction of PeG, corresponding to 76–95% of X_H and 200–510% of Q_w in PHPePG copolymers. It was found that PHPeEG copolymers have the advantage of increasing the mechanical properties while maintaining skin-type behavior compared to PHPePG copolymers. Young's modulus E obtained from initial slope of S-S curves are plotted against the degree of swelling Q_w in Figure 5 and against helix content X_H in Figure 6. In Figure 5, E in PHPePG copolymers is shown to be higher than that of PHPeEG copolymers at the same Q_w owing to higher X_H of PHPePG copolymers. The plot of E against X_H for both copolymers fall on a single curve as can be seen in Figure 6. We proposed in previous paper⁴ that E depends on X_H for PHPeEG copolymers and is related to X_H by

$$E(X_H) = \{X_H/E_{\text{HPeG}} + (1 - X_H)/E_{\text{HEG}}\}^{-1}$$

where E_{HPeG} and E_{HEG} are Young's modulus of PHPeG and PHEG hydrogels, respectively. It was confirmed that E , i.e., the shape of S-S curve is governed by X_H in both copolymers.

Figure 6. Comparison of PHPeEG copolymers (○) with PHPePG copolymers (●) in the relation between Young's modulus E and helix content X_H .

The transition point of S-S curve is considered to correspond to a point $X_H \approx 90\%$. A small part of random coil residues can provide considerable flexibility to the whole chain segments by interrupting the helix parts.

Water Vapor Permeability of Hydrogels

A large variety of synthetic polymer membranes has been tested in the treatment of burns.¹⁷⁻¹⁹ Among them, for example, a crosslinked polymer in the form of hydrogel appears to have added capability for encouraging cellular migration into the graft and vascularization.¹⁷ In designing a material for effective wound closure or artificial skin inner-layer substitute, at least two functions of skin should be maintained. The first is the ability of skin to keep most bacteria out. The second is its ability to control water passage moderately from tissues and organ. Thus, it is important to know the rate of water vapor permeability V_f ($\text{gm}^{-2} \text{ day}$) through the membranes. If V_f through the membrane is extremely low, water accumulates at the interface between woundbed and impermeable graft, and edema results. The graft-woundbed interfacial contact is thereby undermined. Consequently, a skin substitute membrane should have higher V_f , than that of the human physiological level of about $V_f = 500$ ($\text{gm}^{-2} \text{ day}$)²⁰ in order to maintain the ability to wet the woundbed and maintain an air-free interface. Figure 7 shows the relation between water vapor permeability V_f ($\text{gm}^{-2} \text{ day}$) of PECF and degree of swelling Q_w for PHPeEG and PHPePG copolymer membranes at 37°C. It is shown that V_f is highly dependent on the Q_w in PECF and meets the requirement of being a skin substitute; *i.e.*, V_f is higher than human physiological level in all the copolymers. When V_f of PHPeEG and PHPePG membranes are compared at the same Q_w , V_f of PHPeEG is higher than that of PHPePG membranes, indicating that the molecular conformation affects the state of water in membranes.

Figure 7. Comparison of PHPeEG copolymers (O) with PHPePG copolymers (●) in the relation between the rate of water vapor permeability V_f ($\text{g/m}^2 \text{ day}$) and degree of Q_w of PECF at 37°C.

Biodegradation of Hydrogels in Vitro

Numerous proteases may be present at a wound site.²¹ These proteases are divided into some classes depending on the structure of active site. Enzymes of inflammatory response that are likely to degrade poly(α -amino acid)s include the endopeptidase Cathepsin B, exopeptidases Carboxypeptidase and Leucine amino peptidase.²² In the present investigation, the plant thiol endopeptidase Papain was selected as a commercially available analogue of Cathepsin B. Papain is an ellipsoidal protein molecule with approximate dimensions $5.0 \times 3.7 \times 3.7 \text{ nm}$.²³ Cathepsin B is a thiol endopeptidase isolated from mammalian spleen, liver, kidney and lung, and released by the cells of inflamed tissue to cathepsin B. Although Papain is a general thiol endopeptidase, it has preference for peptide bonds where the amino acid residue of the carbonyl group is arginine, lysine or glutamine and where this amino acid is joined on either side by amino acids with hydrophobic side chains.²⁴ Pre-weighed crosslinked copolymer membranes were exposed to 0.1 mg of Papain in 1 ml of PECF with the activator at 37°C and pH 7.4, and the results obtained with PHPG membrane are illustrated in Figure 8. Although Papain is a macromolecule whose molecular weight is about 21,000, it is apparent that the polymer network does not exclude the Papain molecule. Degradation of PHPG was also measured by changes in the degree of

Figure 8. Dry weight ratio (%) and the degree of swelling Q_w of PECE for PHPG membrane as a function of papain digestion time (h) at 37°C and pH = 7.4.

swelling Q_w . Since an immediate increase in Q_w was observed, the degradation must be a bulk rather than a surface phenomenon. Geometrical considerations are also consistent with easy access of the enzyme to the bulk of the membrane. As shown in Figure 8, with Papain digestion an immediate increase in Q_w of PHPG membrane was observed, while weight loss occurred a little bit more slowly. An endopeptidase must make two incisions in a chain segment to produce a soluble fragment, but a single cleavage will decrease the effective crosslink density, resulting in increased Q_w of membranes. Thus, the initial effect of Papain is to decrease the effective crosslink density without producing soluble materials. Figure 9 summarizes the rate of Papain digestion $V_{1/2}$ (h^{-1}) as a function of Q_w in PECE for PHPeEG and PHPePG copolymers. $V_{1/2}$ is defined as the reciprocal of time required for sample weight to be reduced to one-half its initial value. It is also clearly shown that a common relation the same as in the previous paper,²⁵ is obtained between $V_{1/2}$ and Q_w in both copolymers. The

Figure 9. Comparison of PHPeEG copolymers (○) with PHPePG copolymers (●) in the relation between the rate of papain digestion $V_{1/2}$ (h^{-1}) and degree of swelling Q_w of PECE at 37°C and pH = 7.4.

order of the rate of Papain digestion in two copolymers was $\text{PHPePG} > \text{PHPeEG}$ at the same Q_w . Hydrophobic effects of the helix region are considered to be important in explaining the faster digestion of higher helix content X_H in comparing two copolymers.

The major conclusions are as follows.

- (1) PHPeEG and PHPePG copolymers are considered to be promising materials as skin substitutes, showing high degree of swelling, skin-type tensile behavior, high water vapor permeability and enzymatic degradation behavior *in vitro*.
- (2) PHPePG copolymers show higher helix content X_H than PHPeEG copolymers at the same degree of swelling Q_w , due to different hydrophobicities.
- (3) Young's modulus E and tensile behavior are shown to be governed by molecular conformation of copolymers.
- (4) PHPeEG copolymers show higher water vapor permeability and slower enzymatic digestion than PHPePG copolymers at the same Q_w , which is considered due to differences in helix content.

REFERENCES

1. S. D. Bruck, *Biomater. Med. Dev. Artif. Organs*, **1**, 79 (1973).
2. B. D. Ratner and A. S. Hoffman, in "Hydrogels for

- Medical and Related Applications," J. D. Andrade, Ed., ACS Symp. No. 31, ACS, Washington D. C., 1976, Chapter 1.
3. J. M. Anderson, K. L. Spilizewski, and A. Hiltner, in "Biocompatibility of Tissue Analogs," D. F. Williams, Ed., CRC Press, Boca Raton, FL, 1985, p 68.
4. E. Nakanishi, E. Sugiyama, Y. Shimizu, S. Hibi, and T. Hayashi, *Polym. J.*, **23**, 983 (1991).
5. C. A. Homsey, *J. Biomed. Mater. Res.*, **4**, 341 (1971).
6. T. Hayashi, E. Nakanishi, and A. Nakajima, *Kobunshi Ronbunshu*, **43**, 633 (1986).
7. H. E. Auer and P. Doty, *Biochemistry*, **5**, 1708 (1966).
8. T. Sugie and P. A. Hiltner, *J. Macromol. Sci. -Phys.*, **B17**, 769 (1980).
9. T. Miyazawa, J. Masuda, and K. Fukushima, *J. Polym. Sci.*, **62**, 62, (1966).
10. T. Hayashi, K. Takeshima, E. Kobatake, and A. Nakajima, *Kobunshi Ronbunshu*, **42**, 777 (1985).
11. H. B. Bensusan, *Biochemistry*, **8**, 4716 (1969).
12. J. Lowbridge and J. S. Fruton, *J. Biol. Chem.*, **249**, 6754 (1974).
13. N. Lotan, A. Yaron, A. Berger, and M. Sela, *Biopolymers*, **3**, 625 (1965).
14. N. Lotan, A. S. Yaron, and A. Berger, *Biopolymers*, **4**, 365 (1966).
15. N. Lotan, N. Bixon, and A. Berger, *Biopolymers*, **8**, 247 (1969).
16. H. Yamada, in "Strength of Biological Materials," F. G. Evans, Ed., WWC Press, 1970, p 226.
17. J. Hubacek, K. Kliment, J. Dusek, and J. Hubacek, *J. Biomed. Mater. Res.*, **1**, 387 (1967).
18. I. R. Schmolka, *J. Biomed. Mater. Res.*, **6**, 571 (1972).
19. J. Guldalian, C. Jelenko, III, D. Calloway, and J. T. McKnight, *J. Trauma.*, **13**, 32 (1973).
20. I. V. Yannas and J. F. Burke, *J. Biomed. Mater. Res.*, **14**, 65 (1980).
21. D. F. Williams, *J. Bioeng.*, **1**, 279 (1977).
22. T. N. Salthouse, *J. Biomed. Mater. Res.*, **10**, 197 (1976).
23. J. Drenth, J. N. Jansonius, R. Koekoek, and B. G. Solthens, "Papain, X-Ray Structure, in the Enzymes," Vol. III, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971, p 485.
24. J. Lowbridge and J. S. Fruton, *J. Biol. Chem.*, **249**, 6754 (1974).
25. T. Hayashi, E. Nakanishi, and A. Nakajima, *Polym. J.*, **19**, 1025 (1987).