Synthesis and Properties of Hydrophilic Copolypeptide Membranes Containing L-Aspartic Acid as One Component

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Synopsis

Three-component random copolypeptide consisting of *N*-hydroxypropyl-L-glutamine, L-aspartic acid, and L-lysine, related two component random copolypeptides and homopolypeptides, were prepared by carrying out aminolysis reaction with 3-amino-1-propanol, followed by crosslinking reaction with 1,8-octamethylenediamine on starting polymer membranes consisting of γ-methyl-L-glutamate, L-aspartic acid, and L-lysine. The effective crosslink density was shown to be proportional to the content of the crosslinker in the reaction mixture. The tensile properties of these hydrophilic membranes were highly dependent on the degree of swelling in the pseudo-extracellular fluid, hydrophobicity of the side chains, and the effective charge density of membranes, and their behavior was typical of an elastomer. A higher rate of water permeability was obtained with charged membranes than noncharged and/or compensated charged membranes with the same order of the degree of swelling in the pseudo-extracellular fluid. Biodegradation of the samples *in vitro* by papain indicated that the degradation could be regarded as a bulk rather than a surface phenomenon. The rate of degradation was also highly dependent on the degree of swelling of membranes, as well as on the hydrophobicity and the effective charge density of side chains of sample membranes.

INTRODUCTION

Poly(α-amino acid)s and their copolymers are very useful for biodegradable medical applications such as temporary artificial skin substitutes in burn therapy, temporary barriers to prevent adhesion between natural tissues planes damaged either by accident or surgery between the pericardium, and heart wall during open-heart surgery. Polymer carriers for conjugates coupled to proteins for therapeutic use and drug delivery systems.[1] On the other hand, proteins contain both anionic and cationic groups in their molecules. Thus, it is interesting to investigate confirmations as well as membrane properties of copolypeptides carrying both negative and positive charges in the side chains of the same molecules from the standpoints of basic and applicable considerations.

In this study, we prepared three component random copolypeptides (DMK) consisting of L-aspartic acid (D), *N*-hydroxypropyl-L-glutamine (M), and L-lysine (K), as well as the corresponding two component random copolypep-

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tides, copoly(*N*-hydroxypropyl-L-glutamine/L-aspartic acid) (DM) and copoly(*N*-hydroxypropyl-L-glutamine/L-lysine) (KM), and homopolypeptides, such as poly(L-aspartic acid) (PLAA), poly(L-lysine) (PLLys), and poly(*N*-hydroxypropyl-L-glutamine) (PHPG), and investigated the relation between their bulk structures and membrane properties, such as the degree of swelling in the pseudo-extracellular fluid (PECF),[2] tensile properties in PECF, water vapor permeability, and enzymatic degradation behavior *in vitro* of the hydrophilic membranes in PECF from an applicable point of view for biomedical materials.

EXPERIMENTAL

Materials

Synthesis of Copolypeptides

Three component random copolypeptide, copoly(β-benzyl-L-aspartate/γ-methyl-L-glutamate/ε-*N*-carbobenzyloxy-L-lysine) (DMK), related two component random copolypeptides, copoly(β-benzyl-L-aspartate/γ-methyl-L-glutamate) (DM) and copoly(γ-methyl-L-glutamate/ε-*N*-carbobenzyloxy-L-lysine) (KM), and the corresponding homopolymers, poly(β-benzyl-L-aspartate) (PBLA), poly(γ-methyl-L-glutamate) (PMLG), and poly(ε-*N*-carbobenzyloxy-L-lysine) (PCBL) were synthesized by the *N*-carboxyanhydride (NCA) method. The monomers, D-NCA, M-NCA, and K-NCA, were prepared according to the method reported in a previous paper,[3] and purified by recrystallization from an ethyl acetate solution with the addition of petroleum ether. Recrystallization was repeated more than three times. The polymerization was initiated with triethylamine (TEA) at an NCA-to-TEA molar ratio of 50. All starting polymers were purified and fractionated as described in a previous paper.[4] The results of all the polymerization are summarized in Table I.

Preparation of Hydrophilic Polymer Membranes

The debenzylation of β-benzyl-L-aspartate as well as the decarbobenzylation of ε-*N*-carbobenzyloxy-L-lysine residues in copolymers was performed by anhydrous HBr treatment according to the method of Idelson and Blout.[5] After a membrane of ca. 100 μm in thickness cast from an appropriate solvent

TABLE I
Copolymerization of Random Copolypeptides

Sample code	(mol %) D	M	K	[η] (dL/g) (DCA, 25°C)
PBLA-1	100	0	0	0.79
PMLG-1	0	100	0	0.98
PCBL-1	0	0	100	1.88
DM-1	12	88	0	[illegible]
KM-1	0	87	13	1.55
DMK-1	9	80	11	1.40
DMK-2	6	87	7	1.50

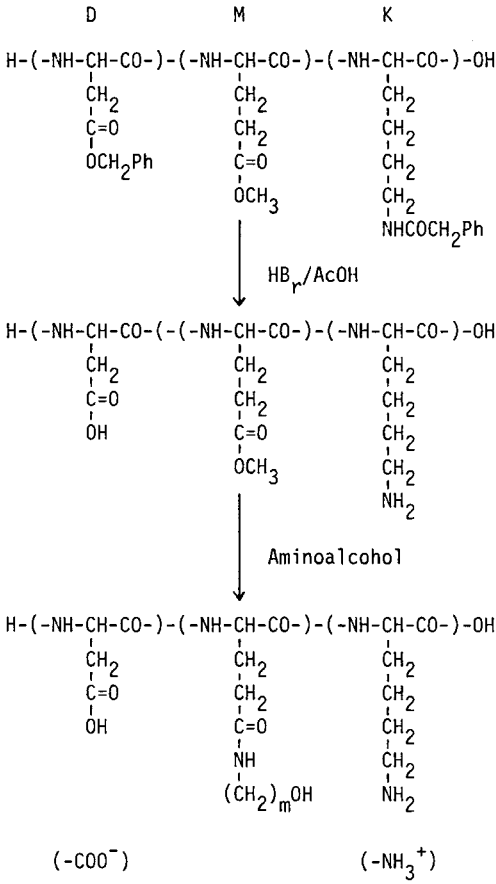

Fig. 1. A schematic diagram of the preparation of hydrophilic copolymer membranes.

was immersed in the mixture of 3-amino-1-propanol and the crosslinker, 1,8-octamethylenediamine (OMDA), at 58°C for 48 h, the membrane was washed with pure water, ethyl ether, and stored in ethanol. Figure 1 denotes a schematic diagram of the preparation of the hydrophilic membranes.

Measurements

Molecular Characterizations of the Starting Copolymers

The intrinsic viscosity $[\eta]$ was measured in a dichloroacetic acid (DCA) solution, using an Ubbelohde type viscometer at 25°C. The copolymer composition was determined by amino acid analysis. These data are listed in Table I.

Physical Properties of Hydrophilic Membranes

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

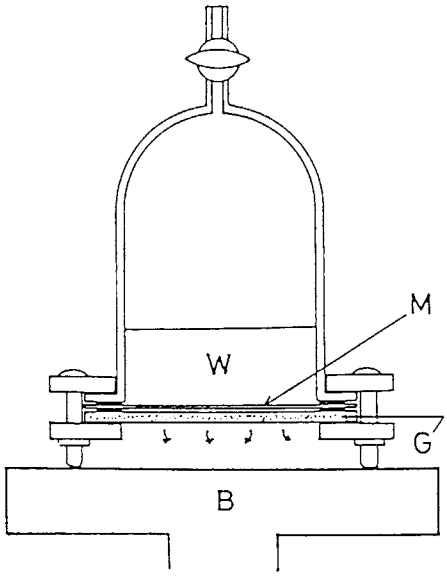

Fig. 2. A schematic model of a cylindrical glass cell for measuring the water vapor permeability: (B) balance; (G) porous glass; (M) sample membrane; (W) water (PECF).

ratio of the amount of PECF to weight of the dried crosslinked hydrophilic membrane.

The tensile properties of hydrophilic membranes were measured by a Tensilon UTM-II-20 (Toyo-Boldwin Co.) using the standard techniques in PECF at 25°C. All these samples were tested at an elongation rate of 100%/min.

Water vapor permeation through the membranes was measured with a cylindrical glass cell[6] designed by us (Fig. 2) at 37.0°C. The exposed membrane area was 12.57 cm^2.

Biodegradation of Hydrophilic Membranes in vitro

Enzymatic degradation studies *in vitro* were carried out by using papain. Papain (3.5 m Anson $\mu m\ mg^{-1}$, No. 7144) was purchased from Nakarai Chem. Co., and used without further recrystallization. The papain was activated in PECF at pH 7.4 with 0.01*M* cystein and 0.04*M* EDTA.[7] Polymer membranes were removed from the papain solution at appropriate time intervals after being exposed in PECF solution of papain, and weighed. Then the membrane was vacuum dried at 50°C to constant weight.

The change of the molecular weight distribution curves with papain digestion of the corresponding water-soluble polymer samples without crosslinker OMDA was investigated by gel permeation chromatograph (GPC) on a Toyo-Soda high-speed liquid chromatography HLC-803D equipped with TSK-Gel Type G4000SW, C-No. SW46A0015 in PECF at 25°C.

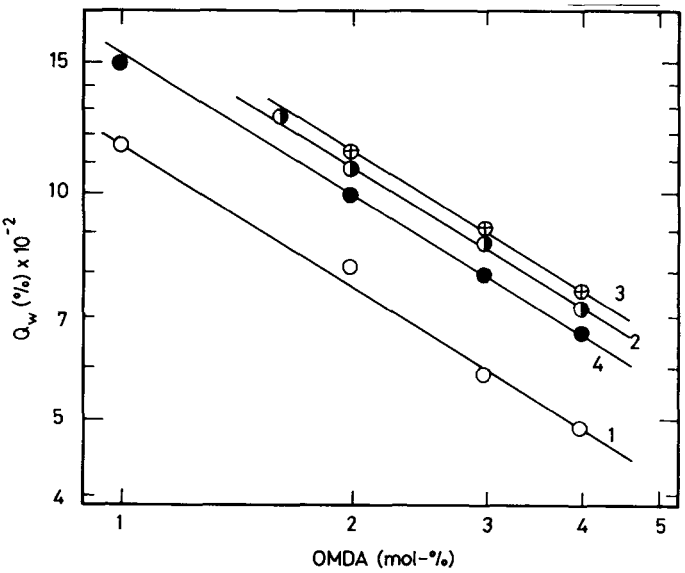

Fig. 3. The degree of swelling Q_w (%) of hydrophilic polypeptide membranes in PECF as a function of OMDA mol % for: (1) PHPG (○); (2) DM(P) (◒); (3) KM(P) (⊕); (4) DMK(P) (●).

RESULTS AND DISCUSSION

Degree of Swelling of Hydrophilic Membranes in PECF

The degree of swelling in a solvent is determined by the interaction energy between the solvent molecules and polymer segments as well as the elastic energy (crosslink density) for a solvent-swollen polymer. On the other hand, the precise crosslink density has not been determined because of the uncertainty in the relative reactivities of 3-amino-1-propanol and OMDA, and also because estimation of the fraction of the reacted diamine molecules which form effective crosslinks is difficult. The effect of crosslinker (OMDA) concentration in the reaction on the degree of swelling of the crosslinked membranes in PECF is shown in Figure 3. The degree of swelling in PECF decreases with increasing OMDA molar concentration in the reaction solution.

When the degree of swelling is quite large, it is given by the following equation according to rubber elasticity theory[8]:

$$Q_w^{5/3} = (\bar{v}M_c)(1 - 2M_c/M)^{-1}(1/2 - \chi_1)/V_1 \quad (1)$$

where M_c is the molecular weight per crosslinked unit, M the primary molecular weight, $\bar{v}$ the specific volume of polymer, V_1 the molar volume of solvent, and χ_1 the interaction parameter. The factor $(1 - 2M_c/M)$ expresses the correction for network imperfections resulting from chain ends. For a quite high molecular weight polymer chain, it reduces to unity. As the effective crosslink density, f_c is proportional to the value of M_0/M_c, where M_0 is the molecular weight of the repeat unit (monomeric unit); eq. (1) may be

TABLE II
Preparative Data of Copolypeptide Hydrogels

Sample code	Starting polymer	OMDA (mol %)	Q_w (%)
PHPG-11	PMLG-1	1.0	1170
PHPG-12	PMLG-1	2.0	810
PHPG-13	PMLG-1	3.0	580
PHPG-14	PMLG-1	4.0	480
DM(P)-11	DM-1	1.5	1200
DM(P)-12	DM-1	2.0	1080
DM(P)-13	DM-1	3.0	880
DM(P)-14	DM-1	4.0	720
DM(P)-12	KM-1	2.0	1130
DM(P)-13	KM-1	3.0	910
KM(P)-14	KM-1	4.0	740
DMK(P)-11	DMK-1	1.0	1480
DMK(P)-12	DMK-1	2.0	1000
DMK(P)-13	DMK-1	3.0	790
DMK(P)-14	DMK-1	4.0	660

simplified as

$$Q_w^{5/3} = (\bar{v}M_0)(1/2 - \chi_1)/V_1 f_c \tag{2}$$

The slope of log–log plots in Figure 3 has the value of $-3/5$ as predicted by eq. (2). The effective crosslink density f_c is proportional to the crosslinker OMDA concentration (mol %) in the reaction solution. The Q_w values obtained with charged membranes, DM(P) and KM(P), were higher than those obtained with the compensated-charged membranes, DMK(P), as well as the noncharged membranes, PHPG, at the same order of OMDA molar concentration, indicating that Q_w value depends on the charge density in membranes.

Tensile Properties of Hydrophilic Membranes in PECF

The tensile properties of hydrophilic membranes are highly dependent on the degree of swelling in PECF. Further, elastomeric membranes are highly suited to biomedical applications, such as membranes for artificial organs, reconstructive prosthesis, and cosmesis.

Figure 4 illustrates the stress–strain curves of the original hydrophilic membranes in PECF at 25°C. While DM(P)-13 and PHPG-12 membranes gave lower strength, DMK(P)-12 and DMK(P)-13 membranes attained higher strength with a moderate modulus. Table III lists the experimental findings of Young's modulus E at an elongation of 1%, the tensile strength σ_B and elongation ϵ_B at the breaking point with Q_w values for the original hydrophilic membranes in PECF. For example, tensile properties of DMK(P)-12 membrane were more than those of PHPG-12 or DM(P)-13 even when the Q_w of DMK(P)-12, a compensated-charged membrane, was more than the corresponding noncharged membrane, PHPG-12, and DM(P)-13, a charged membrane, suggesting charge interactions between cation and anion in the molecular chains to affect the tensile strength of the membrane.

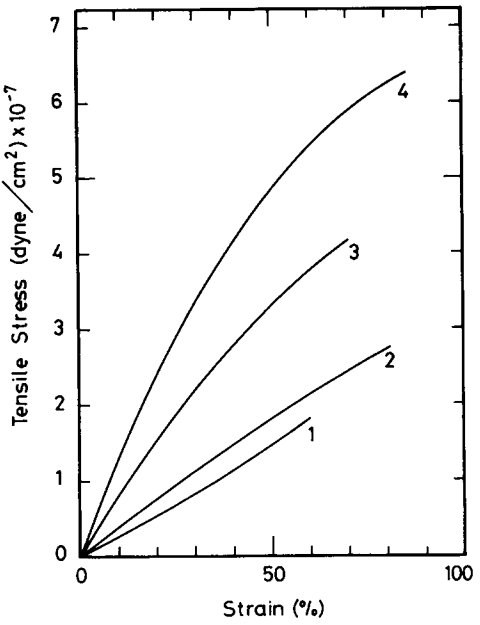

Fig. 4. Stress–strain properties of the original hydrophilic polypeptide membranes in PECF at 25°C for: (1) DM(P)-13; (2) PHPG-12; (3) DMK(P)-12; (4) DMK(P)-13.

TABLE III
Mechanical Properties of Hydrogels in PECF at 25°C

Sample code	Q_w (%)	E (dyn/cm^2)	σ_B (dyn/cm^2)	ϵ_B (%)
PHPG-12	810	4.2×10^7	2.8×10^7	80
PHPG-13	580	8.8×10^7	4.7×10^7	75
DM(P)-13	880	2.7×10^7	1.8×10^7	60
DM(P)-14	720	3.0×10^7	2.1×10^7	65
DMK(P)-12	1000	8.2×10^7	4.2×10^7	70
DMK(P)-13	790	1.3×10^8	6.4×10^7	85
DMK(P)-14	660	1.7×10^8	6.9×10^7	80

One of the most essential points required to the biodegradable membrane is the retention of the proper tensile strength and/or of stability of the material dimension until tissue repair is completed. Thus, it is important to know the change of the remaining tensile strength of the membrane under enzymatic digestion from the applicational point of view. It is pointed out that nonenzymatic degradable materials, such as polyglycolic acid membrane, are apt to lose their mechanical strength almost perfectly while their substantial weight is still remaining without any definite change.[9] On the other hand, the decreasing of the mechanical strength for collagen membrane, which is a typical enzymatic degradable material, was reported to occur parallel with that of the remaining weight of the material with the enzymatic digestion, though the order of the decreasing was practically different.[10]

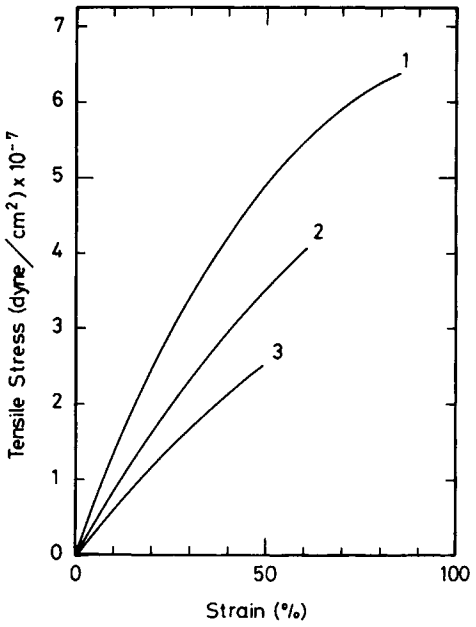

Fig. 5. The change of the stress–strain properties of DMK(P)-13 membrane in PECF at 25°C with papain digestion for: (1) original; (2) 30 min of digestion (W_r/W_0 = 0.87), and (3) 48 min of digestion (W_r/W_0 = 0.74). Papain concentration: 0.15 mg/mL in PECF.

Figure 5 illustrates an example of the change of the stress–strain properties of DMK(P)-13 membrane in PECF with papain digestion. It is clearly shown that the tensile strength decreases drastically with the papain digestion. It means that the action of papain decreases the effective tensile strength of the membrane in the higher order than the decreasing of the weight of the membrane, even though the behavior is characteristic for the enzymatic degradable material.

Water Vapor Permeability of Hydrophilic Membranes

A large variety of synthetic polymer membranes has been investigated in the treatment of burns.[11-13] Among them, for example, the formulation of a crosslinked polymer in the form of hydrogel appears to have added capability for encouraging cellular migration into the graft and vascularization.[14] In designing an effective wound closure or an artificial skin inner-layer substitute, at least two functions of skin are urgently essential for survival. The first is the ability of skin to keep most bacteria out. The second is its ability to control water passage moderately from tissue and organ. Thus, it is important to know the value of the rate of water vapor permeability V_f (g/m^2 day) through the membrane. If the V_f value is excessively low, water accumulates at the interface between the woundbed and impermeable graft, and edema results. The graft–woundbed interfacial contact is thereby undermined. As a result, maintain the ability to wet the woundbed and thereby maintain an air-free interface, an inner skin substitute membrane should have a higher V_f value than that of the human physiological level of about V_f = 500 (g/m^2 day).

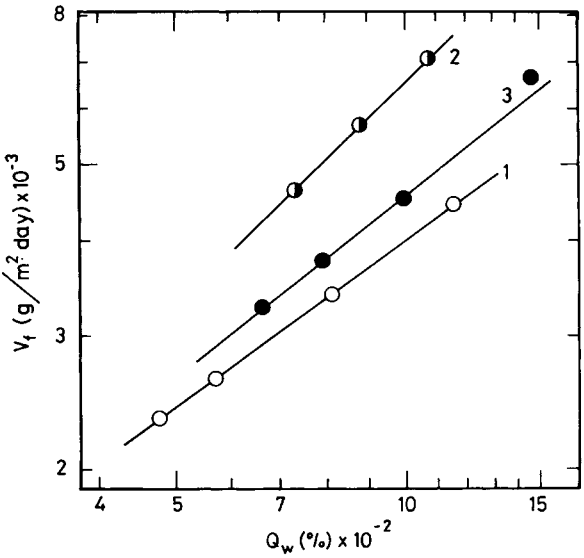

Fig. 6. Rate of water vapor permeability V_f (g/m^2 day) as a function of Q_w for membranes: (1) PHPG (○); (2) DM(P) (◒); (3) DMK(P) (●).

Figure 6 illustrates the relation between the rate of water vapor permeability V_f of PECF and the degree of swelling Q_w for membranes at 37.0°C. It may be also shown that the V_f value is highly dependent on the Q_w value in PECF. Further, it may be judged in Figure 6 that the V_f value of the charged membranes, DM(P), is more than those of the compensated charged or noncharged membranes, DMK(P) or PHPG, at the same value of Q_w, indicating that the charge density in membranes affects the state of water in membranes.

Biodegradation of Hydrophilic Membranes in vitro

Numerous proteases may be present at a wound site.[15] These proteases are divided into some classes, depending on the structure of active sites. Enzymes of inflammatory response that are likely to degrade poly(α-amino acid)s include the endopeptidase Cathepsin B and the exopeptidases Carboxypeptidase and Leucine amino peptidase.[16] In the present study, the plant thiol endopeptidase papain was selected as a commercially available analog of Cathepsin B released during the acute and chronic stages of the inflammatory response. Although papain is a general plant 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.[17]

First, to check whether random degradation of the main chain of a polypeptide is dominated by the reaction with papain, GPC analyses of partially degraded polypeptides were carried out by using water-soluble polypeptide samples prepared without crosslinker OMDA. Figure 7 illustrates a typical example of GPC curves for DMK(P)-10 sample. From Figure 7, it may concluded that DMK(P)-10 is dominantly degraded by a random main-chain fraction as in the case of the degradation of PLGA by endopeptidases such as

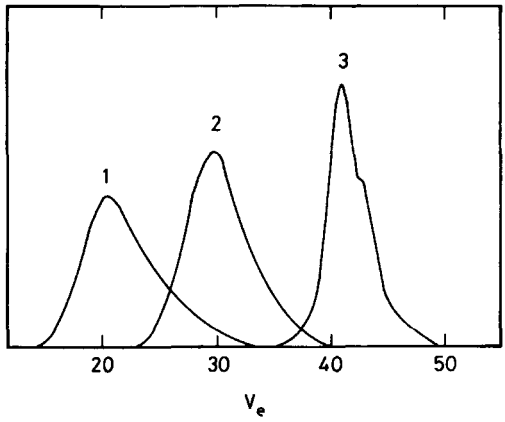

Fig. 7. GPC elution curves for reaction products of DMK(P)-10 in PECF solution of papain for: (1) original; (2) 30 min of digestion; (3) 300 min of digestion. Papain concentration: 0.15 mL/mg.

chymotrypsin, elastase, papain, ficin, or subtilisin, previously reported by Miller.[18] Dickinson and Hiltner[19] reported that the main products of PHPG by papain digestion are oligomers of polymerization degree 4–9, rather than monomers.

Preweighed DMK(P)-13 membranes were exposed to papain and the results are illustrated in Figure 8. The dry weights of membranes began to decrease

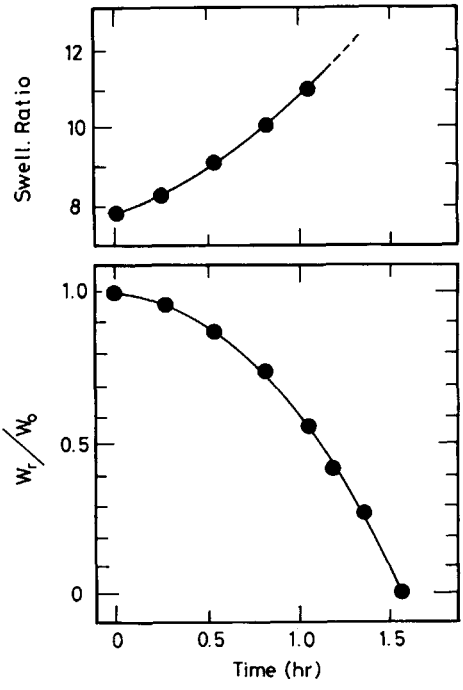

Fig. 8. Dry weight ratio (W_r/W_0) and the swelling ratio of PECF for DMK(P)-13 membrane as a function of papain digestion time at 37°C and pH 7.4.

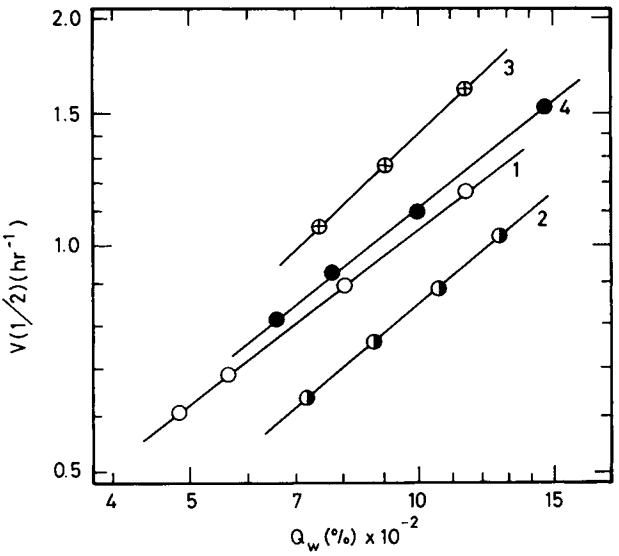

Fig. 9. Rate of papain digestion $V(1/2)$ (h^{-1}) as a function of Q_w (%) for: (1) PHPG (○); (2) DM(P) (◒); (3) KM(P) (⊕); (4) DMK(P) (●).

with papain digestion, but a lag time with respect to weight loss was observed so that when the experiment was half over, i.e., at half the time needed to dissolve the specimen, about 75% of the dry weight remained. The increase in swelling ratio was also observed with papain digestion. Judging from the immediate increase in the swelling ratio, the degradation of the DMK(P)-13 membrane should be bulk rather than a surface phenomenon. Papain, 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 swelling ratios of membranes. Thus, the initial effect of papain is therefore to decrease the effective crosslink density without producing soluble materials.

Figure 9 summarizes the rates of papain digestion $V(1/2)$ (h^{-1}) as a function of the degree of swelling Q_w in PECF for hydrophilic polymer membranes. $V(1/2)$ is defined as the reciprocal of the time required for the sample weight to be reduced to one-half its initial value. It is clearly shown that the order of degradation rates among these hydrophilic membranes is as follows: KM(P) > DMK(P) > PHPG > DM(P) at the same order of Q_w for each membrane.

In conclusion, the effective crosslink density was shown to be proportional to the content of the crosslinker OMDA in the reaction mixture. Q_w values were highly dependent on the charge density in membranes. The tensile properties of these hydrophilic membranes depended on Q_w in PECF, charged interactions between cations and anions in the molecular chain, and their behavior was typical of an elastomer. Biodegradation of these hydrophilic membranes by papain *in vitro* indicated that the degradation could be regarded as a bulk rather than a surface phenomenon. The rate of degradation of sample was also highly dependent on Q_w of membranes, as well as on charged density of the sample side chains.

References

1. 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.
2. C. A. Homsey, *J. Biomed. Mater. Res.*, **4**, 341 (1971).
3. T. Hayashi, E. Nakanishi, and A. Nakajima, *Kobunshi-Ronbunshu*, **43**, 633 (1986).
4. T. Hayashi, E. Nakanishi, and A. Nakajima, *Polym. J.*, **19**, 1025 (1987).
5. M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 4631 (1958).
6. T. Hayashi, K. Takeshima, E. Kobatake, and A. Nakajima, *Kobunshi-Ronbunshu*, **42**, 777 (1985).
7. J. Lowbridge and J. S. Fruton, *J. Biol. Chem.*, **249**, 6754 (1974).
8. P. J. Flory, in *Principle of Polymer Chemistry*, Cornell Univ. Press, Ithaca, NY and London, 1953, p. 576.
9. K. Jamshidi, S. H. Hyon, T. Nakamura, Y. Ikada, Y. Shimizu, and T. Teramatsu, in *Biological and Biomechanical Performance of Biomaterials*, Elsevier, Amsterdam, 1986, p. 227.
10. H. R. Dickinson, A. Hiltner, D. F. Gibbons, and J. M. Anderson, *J. Biomed. Mater. Res.*, **15**, 577 (1981).
11. J. Hubacek, K. Kliment, J. Dusek, and J. Hubacek, *J. Biomed. Mater. Res.*, **1**, 387 (1967).
12. I. R. Schmolka, *J. Biomed. Mater. Res.*, **6**, 571 (1972).
13. J. Guldalian, C. Jelenko, III, D. Calloway, and J. T. McNight, *J. Trauma*, **13**, 32 (1973).
14. I. V. Yannas and J. F. Burke, *J. Biomed. Mater. Res.*, **14**, 65 (1980).
15. D. F. Williams, *J. Bioeng.*, **1**, 279 (1977).
16. T. N. Salthouse, *J. Biomed. Mater. Res.*, **10**, 197 (1976).
17. J. Lowbridge and J. S. Fruton, *J. Biol. Chem.*, **249**, 6754 (1974).
18. W. G. Miller, *J. Am. Chem. Soc.*, **86**, 3913 (1964).
19. H. R. Dickinson and A. Hiltner, *J. Biomed. Mater. Res.*, **15**, 591 (1981).

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