

# Elastomeric Polypentapeptides Cross-Linked into Matrixes and Fibers

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Received September 11, 2000; Revised Manuscript Received December 15, 2000

Microbially prepared polypentapeptides were cross-linked by two chemical methods. In one chemical approach, (GVGIP)<sub>260</sub> where G = glycine, V = valine, I = isoleucine, and P = proline with no functional groups in its side chains, was cross-linked using dicumyl peroxide, and reaction conditions were systematically examined. Successful cross-linking was obtained even under severe conditions for proteins, i.e., 3 h at 120 °C, without having significant side reactions. In the second chemical approach, two separate polymers, (GVGVPGVGVPGXGVPGVGVPGVGVPGVGVPG)<sub>n</sub> where X is either E (the carboxylic acid containing glutamic acid residue) or K (the lysine residue with an  $\epsilon$ -amino function), were mixed and cross-linked using a carbodiimide reagent. The reaction temperature was found to affect the equilibrium swelling behavior of the resulting cross-linked hydrogels. In hydrogels cross-linked at a temperature above their hydrophobic folding and assembling transition temperature, 3D continuous filamentous microstructures were observed. Chemically cross-linked hydrogel fibers were also prepared and their anisotropy in swelling was confirmed. Uniaxial tensile moduli and equilibrium weight swelling ratios of the chemically cross-linked samples were compared to those of (GVGVPG)<sub>251</sub> and (GVGIP)<sub>260</sub>,  $\gamma$ -irradiation cross-linked at different Mrad doses.

## Introduction

Recently, interest in protein-based polymers has been growing. This new class of materials has many advantages over the conventional petroleum-based polymers. They can be prepared by chemical synthesis and by recombinant DNA technology involving gene construction, *E. coli* transformation and expression by means of fermentation.<sup>1</sup> The former can enable the efficient screening of numerous combinations of amino acids for a desired property. The latter is good for large-scale production, and its cost is continually decreasing. Using recombinant DNA technology, there can be precise control of not only the sequence and stereochemistry of amino acids but also the molecular weight and its distribution. It is another advantage that the production and disposal of protein-based polymers can be done in an environmentally friendly manner.

In addition to the advantages, protein-based polymers can be designed for a specific function using the biologically available 20 amino acids and subsequent chemical modification of side chains with functional groups. Mimicking the functions of natural proteins<sup>2</sup> is also possible. Poly(GVGVP) and its modified polymers have been studied based on this idea.<sup>3</sup> The amino acid sequence has been found in tropoelastin, the precursor protein of mammalian elastin,<sup>4–7</sup> which is responsible for the resilience of tissues. Interestingly, on cross-linking, elastic matrices were formed that are dominantly entropic elastomers.<sup>8–10</sup> Furthermore, when the cross-linked polymer has thermal energy input, it can produce

mechanical energy output.<sup>11</sup> This energy conversion ability has been demonstrated for various types of energy including mechanical, electromagnetic, electrical, thermal, chemical, and pressure energy.<sup>3</sup> Related energy converting functions occur in natural proteins.

The energy conversion ability is possible, because of the hydrophobic folding and assembling transition of protein-based polymers in water. This transition has been found to be very sensitive to various stimuli, resulting in the remarkable ability. The polymers have a significant amount of hydrophobic hydration around their hydrophobic moiety. Water of hydrophobic hydration is believed to be in a more ordered state,<sup>12–15</sup> and its ordered state has been variously referred to as a “clathrate,” “cathedral,” or “caged” structure.<sup>16</sup> The numbers of waters of hydrophobic hydration (bound water) molecules must be reduced upon thermal energy input, because they are not entropically favorable. Thus, the polypeptide chains collapse and increase inter or intramolecular contacts. This contraction produces mechanical energy output in the energy conversion experiments. In the contracted state of poly(GVGVP), the polymer chains were found to have a supramolecular secondary structure, i.e., twisted filament structure of  $\beta$ -spirals, which have type II  $\beta$ -turns.<sup>3,17–19</sup>

The hydrophobic folding and assembling transition and its temperature will be designated as the  $T_t$ -transition and  $T_i$ , respectively. These terminologies have been proposed and used in the previous studies on poly(GVGVP).<sup>3</sup>

The ultimate goal of our study is to build our knowledge base for developing this interesting class of materials. In this study, focus is on the preparation of cross-linked materials having mechanical strength. Our work begins with the task

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**Table 1.** Designations, Amino Acid Sequences and Molecular Weights of Polymers

designations; amino acid sequence; cross-linking reactions			mol wt before cross-linking (kg/mol)
$\gamma$ -V; (GVGV <sub>251</sub> ); $\gamma$ -irradiation			102
dose of $\gamma$ -irradiation (Mrad)	20	30	
mol wt between cross-links (kg/mol)	13.8	12.3	
$\gamma$ -I; (GVGIP) <sub>260</sub> ; $\gamma$ -irradiation			109
dose of $\gamma$ -irradiation (Mrad)	22	30	
mol wt between cross-links (kg/mol)	21.6	13.4	
c-V(EK); 50 mol % of (GVGV <sub>251</sub> GGV <sub>251</sub> GEGVP GGV <sub>251</sub> GGV <sub>251</sub> GGV <sub>251</sub> ) <sub>n</sub> GGV <sub>251</sub> and 50 mol % of (GVGV <sub>251</sub> GGV <sub>251</sub> GKGVP GGV <sub>251</sub> GGV <sub>251</sub> GGV <sub>251</sub> ) <sub>n</sub> GGV <sub>251</sub> ; condensation			88 (V(E)) and 87 (V(K))
p-I; (GVGIP) <sub>260</sub> ; free radical reaction using DCP			109

of chemically cross-linking two protein-based polymers, poly(GVGVP) and poly(GVGIP), which lack functional side chains (designations = V and I, respectively). Various chemical cross-linking methods were first tried, and the success of cross-linking was checked by solubility in dimethyl sulfoxide (DMSO) and water. Cross-linking using  $\gamma$ -irradiation has already been developed,<sup>10</sup> but this method may not be suitable for many applications.

Dimethylaniline/benzyl chloride/acetic acid initiator system had been reported to successfully generate radicals in polyamides.<sup>20</sup> Because of its mild reaction condition, the cross-linking of poly(GVGIP) solution was tried following the same methods as described in ref 20. Reaction temperature, time, and polymer concentrations were varied in a wide range. In various trials, however, an insoluble cross-linked polymer was never obtained.

Cross-linking using Cr(VI)/HClO<sub>4</sub> redox initiator system,<sup>21</sup> diisocyanates and their catalysis in DMSO (N-substitution reactions), and radical initiators such as benzoyl peroxide (BPO) were also tried to cross-link poly(GVGIP). Among the trials, successful cross-linking was found when dicumyl peroxide (DCP) was used as an initiator at 120 °C in DMSO. DCP seems to have a stronger tendency toward cross-linking than chain scission for our polymers. In fact, recent electron spin resonance (ESR) spectrometer studies<sup>22–24</sup> on the cross-linking of polyamides showed the higher cross-linking tendency of DCP.

In this study, the DCP cross-linking of protein-based polymer is examined in detail. Additionally, cross-linking using a carbodiimide reagent is studied also. In this approach, one polymer having a glutamic acid (E) every 30th residue of the poly(GVGVP) host polymer, designated as V(E), and another polymer having a lysine (K) residue recurring every 30 residues, V(K), are used (Table 1). According to turbidity measurements, the two polymers have their own  $T_i$ 's above 60 °C, but a 1:1 mixture solution shows  $T_i$  around 35–37 °C, near body temperature. This is because ion pairing between carboxylic acids and amines lowers the value of  $T_i$ . After cross-linking, the resulting hydrogels will have a similar structure to poly(GVGVP). The condensation cross-linking method using a carbodiimide reagent does not involve chain scission reactions, unlike the radical cross-linking methods.<sup>25</sup> Furthermore, this reaction is conveniently used to prepare hydrogel fibers, which show interesting swelling behavior. The swelling and mechanical properties of the

hydrogels prepared by the two cross-linking methods are compared with those of  $\gamma$ -irradiation cross-linked hydrogels.

## Experimental Section

**Materials.** Protein-based polymers were expressed and purified using the biosynthesis technique as previously reported.<sup>26–28</sup> After the purification, polymers were lyophilized. The amino acid sequences of polypeptides are listed in Table 1. Deionized ultra-filtered water from the Fisher Scientific was used in this experiment. DCP, DMSO, D<sub>2</sub>O, 1-ethyl-3-[3-(dimethylamino)propyl] maleimide (EDC), and 1-hydroxybenzotriazole hydrate (HOBt) were purchased from the Aldrich Co. For fluorescence tagging on the unreacted amines of carbodiimide cross-linked polymers (c-V(EK) in Table 1), the ATTO-TAG CBQCA detection reagent, 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde, was purchased from Molecular Probes Co.

**Preparation of Protein-Based Hydrogels.** Protein-based hydrogels prepared in this study are listed in Table 1 with their designations. Reaction conditions, which specify the types of samples, will follow the designations, e.g.,  $\gamma$ -V-20 Mrad.

Cross-linking using  $\gamma$ -irradiation was done following the same experimental methods described elsewhere.<sup>29</sup> First, polymer solution in a mold was heated above  $T_i$  and dense polymer phase was separated from water-rich phase by centrifuging. Then,  $\gamma$ -irradiation was applied to the polymer phase (concentration  $\approx$  500 mg/mL) in the mold at 23 °C. The intensity of  $\gamma$ -irradiation was ca. 0.3 Mrad/h. Before any characterization, hydrogels were cleaned in a shaking water bath for more than 2 weeks, changing water every 24 h.

The preparation of peroxide cross-linked hydrogels (p-I) was done using DCP. The dried poly(GVGIP) was mixed with DCP in DMSO (DMSO/pentamer = 36.11 mol ratio) and then the mixture was poured into a mold placed in an oven (chamber volume = ca. (25 cm)<sup>3</sup> of a certain temperature. The oven was filled with N<sub>2</sub>(g). Cross-linking reaction occurred from 1 h 40 min to 7 h. After cross-linking, the resulting hydrogels were cleaned in a shaking DMSO bath for more than a week with DMSO changed every 24 h and then in a shaking methanol bath for more than 3 days with the same solvent changing. As the final cleaning step, the hydrogels were cleaned in a shaking water bath for more than a week. The water was also changed every 24 h. Detailed reaction conditions can be found in Table 2.

For the carbodiimide cross-linking of "slab" specimens, two polymers, one having carboxylic acids (V(E)) and the other having amines (V(K)) recurring with a periodicity of every 30 residues (Table 1), were used. The two polymers were first dissolved in water at room temperature. The polymer solution was then mixed with an aqueous solution of EDC and HOBt. After thorough mixing

**Table 2.** Equilibrium Weight Swelling Ratio of p-I in DMSO at 22 °C (Batches 1–15) and in Water at 5 °C (p-I-0.20 to p-I-0.47)<sup>a</sup>

batch	reacn conditions		mole ratio DCP/ pentamer	equilib wt swelling ratio, $Q_{we}$	transparency at 27 °C in water
	temp, °C	time			
1	100	3 h	0.16	no gel	opaque
2	110	3 h	0.16	24.7	opaque
3	120	3 h	0.16	13.6	opaque
4	130	3 h	0.16	10.8	opaque
5	140	3 h	0.16	8.3	opaque
6	120	1 h 40 min	0.16	17.1	opaque
7	120	2 h 10 min	0.16	14.1	opaque
8	120	3 h	0.16	13.6	opaque
9	120	5 h	0.16	13.6	opaque
10	120	7 h	0.16	38.0	opaque
11	120	3 h	0.02	no gel	opaque
12	120	3 h	0.14	18.9	opaque
13	120	3 h	0.30	10.4	opaque
14	120	3 h	0.83	3.8	transparent
15	120	3 h	1.46	2.0	transparent

designation	reacn temp, °C	reacn time	DCP/ pentamer	equilib wt swelling ratio, $Q_{we}$	mol wt between cross-links, kg/mol
p-I-0.20	120	3 h	0.20	5.93	21.9
p-I-0.31	120	3 h	0.31	5.81	19.0
p-I-0.39	120	3 h	0.39	5.15	15.5
p-I-0.47	120	3 h	0.47	4.17	13.2

<sup>a</sup> The molecular weight between cross-links was obtained from uniaxial tensile modulus data. The transparency is based on unaided eye observation. Transparency difference between transparent and opaque hydrogels is similar to that found in the  $T_1$  transition of poly(GVGVVP) solutions.<sup>3</sup>

for 2–3 min, the mixture was poured into a Teflon mold placed in a temperature-controlled oven and cross-linked for 24 h at a temperature between 5 and 55 °C after sealing the mold. After cross-linking, the hydrogels were cleaned following the same method as in the preparation of  $\gamma$ -irradiation cross-linked hydrogels. Their reaction conditions can be found in Table 3.

The same EDC reaction was used to cross-link fibers. The two polymers, V(E) and V(K), were first dissolved in water. The concentrations of both polymers were 20 wt %. A small drop of polymer solution (ca. 200  $\mu$ m diameter) was placed at the tip of a glass fiber of ca. 100  $\mu$ m diameter. Then, the tip of another glass fiber of the same diameter was placed at the polymer solution drop. The drop placed between the two tips of glass fibers was then stretched into a fiber by pulling one of glass fiber at a speed of 200  $\mu$ m/s using the motor of a syringe injector (Havard Apparatus Pump II). Solvent drying in air during this operation was enough to make the drop spinnable. The drawn fibers were dried in a vacuum for more than 24 h and cross-linked at room temperature in the aqueous solution of EDC, HOBt, NaCl (7.2, 1.4, and 18.8 wt %, respectively) for 12 h. NaCl was used to decrease the solubility of dried fibers in the reactant mixture, and to keep the preformed fiber structure during cross-linking. The cross-linked fibers were then cleaned in an excess amount of water (>1 L/0.1 mg) for more than 3 days, changing water every day.

**Characterizations: Equilibrium Weight Swelling Ratio and Dimension of Fibers.** Thin disk specimens of ca. 100 mm<sup>2</sup>  $\times$  0.7 mm (above  $T_1$ ) were used for the measurements of equilibrium weight swelling ratio ( $Q_{we}$ ), which was the ratio of the weight of fully swollen hydrogel ( $W_s$ ) to that of dried gel ( $W_d$ ). They were immersed in DMSO or water for more than 24 h at 22 °C and their

**Table 3.** Equilibrium Weight Swelling Ratio of c-V(EK) in Water at 22 °C<sup>a</sup>

samples	reacn temp, °C	mole ratio				equilib wt swelling ratio
		E/K	H <sub>2</sub> O/K	EDC/K	HOBt/EDC	
1	22	0.98	1356	10.20	0.28	7.2
2	22	0.98	2034	10.20	0.28	9.9
3	22	0.98	2712	10.20	0.28	12.0
4	22	0.98	3390	10.20	0.28	17.9
5	22	0.98	4068	10.20	0.28	22.8
6	22	0.98	2712	4.33	0.28	14.1
7	22	0.98	2712	10.20	0.28	12.0
8	22	0.98	2712	12.74	0.28	14.9
9	22	0.98	2712	17.08	0.28	16.4
10	22	0.98	2712	10.20	0.28	12.0
11	22	0.98	2712	10.20	0.85	49.5
12	22	0.98	2712	10.20	1.42	46.0
13	22	0.98	2712	10.20	2.84	50.1
14	22	0.98	2712	10.20	0.28	12.0
15	22	1.38	2712	10.20	0.28	9.3
16	22	1.97	2712	10.20	0.28	5.3
17	22	2.95	2712	10.20	0.28	4.1

designation	reacn temp, °C	equilib wt swelling ratio	mol wt between cross-links, kg/mol
c-V(EK)-5	5	7.9	20.6
c-V(EK)-11	11	7.2	20.4
c-V(EK)-22	22	7.2	20.5
c-V(EK)-29	29	6.9	21.2
c-V(EK)-40	40	6.6	20.2
c-V(EK)-55	55	6.9	18.9

<sup>a</sup> For the preparation of c-V(EK)-5 to c-V(EK)-55, E/K = 0.98, H<sub>2</sub>O/K = 1356, EDC/K = 10.2, and HOBt/EDC = 0.28 (mol ratios).

$W_s$  value was measured using a Sartorius B120S balance.  $W_d$  was measured after drying the sample for more than 1 week under vacuum at room temperature.

$Q_{we}$  was also measured with changing temperature. A specimen was first immersed in a 40 mL water bottle and the bottle was preconditioned in a temperature controlling water bath (Neslab endocal refrigerated circulating bath, RTE-5DD) at 1 or 2 °C for more than 2 days. Then, the specimen was heated at 2 °C steps per 12 h and its weight was measured after every 12 h of equilibration time. This weight measurement was usually done in 15 s and water loss during this 15 s was found to be insignificant (diffusion coefficient of our hydrogels  $\approx 10^{-8}$  cm<sup>2</sup>/s<sup>30</sup>).

Optical measurement was used to study the swelling of fibers as a function of temperature. A fiber was first mounted between a concave glass slide and a cover slide with water, and then the slide was placed in a thermal stage, Mettler FP800HT. While the fiber was being heated in the stage, its dimensions were measured using an Olympus BH2–UMA microscope with a Sony DXC-750 CCD camera. Cooling of the thermal stage was done using N<sub>2</sub>(g) flow directly produced from N<sub>2</sub>(l).

**Attenuated Total Reflectance Fourier Transform Infrared Spectrometer (ATR–FTIR) Analysis.** For FTIR analysis, dried polymers were swollen in D<sub>2</sub>O (>0.1 L/1 mg) for more than 2 days. Then, the D<sub>2</sub>O was exchanged and more than 24 h was given as an equilibration time before measurements. For un-cross-linked polymers, D<sub>2</sub>O was kept at  $T_1 + 10$  °C to prevent the formation of homogeneous solution. A Nicolet Magna 750 ATR–FTIR was used. The scan resolution was 1 cm<sup>-1</sup>, and the number of scans was 1024.



**Uniaxial Tensile Tests.** Because of the limited amount of polypeptides, small rectangular strips (gauge section =  $5 \times 10$  mm, thickness  $\approx 0.7$  mm) were prepared from cross-linked polypeptides for uniaxial tensile tests [ASTM-D412-98a]. They were uniaxially loaded in an MTS MicroBionix instrument with a 4 N load cell at a crosshead speed of 3 mm/min. The whole sample and gripping sections were immersed in a water bath during the tests. The temperature of the water bath was controlled using ethylene glycol circulation around it. Tests of more than 4 specimens gave a Young's modulus value.

**LSCFM (Laser Scanning Confocal Fluorescence Microscopy).** Tagging by a fluorescent dye was performed following the information obtained from the Molecular Probes Co and ref 31. Hydrogel specimens (thinner than 0.5 mm and ca.  $5 \times 5$  mm<sup>2</sup>) were first immersed in the mixture of ATTO-TAG CBQCA (5 mg), potassium cyanide (22 mg), DMSO, and water for 24 h with shaking,<sup>32</sup> then the hydrogels were cleaned in an excess amount of water ( $>0.5$  L/1 mg of hydrogel) for 24 h, changing water at every 12 h. Cleaned specimens were mounted in concave glass slides with water. Visible light illumination of the specimens was prevented by means of aluminum foil. A Bio-Rad MRC 1024 ES confocal system was used in a fluorescence/transmission mode using 488 nm blue laser light. To examine the 3-D structure of hydrogels, microscopic thin sectioning was performed at every 3  $\mu$ m z-step.

## Results and Discussion

**Optimization of Cross-Linking Reactions.** As a test of cross-linking,  $Q_{we}$  was measured on hydrogels prepared from various reaction conditions. The  $Q_{we}$  of hydrogels can be converted into cross-link density, since the two quantities are inversely proportional to each other.<sup>33</sup> Table 2 shows  $Q_{we}$  of p-I in DMSO at 22 °C. In general, the reaction window where the polypeptide can be successfully cross-linked without having serious side reactions such as oxidation is narrow. Always, there is the competition between chain scission and cross-linking reactions, and determining the proper window, where cross-linking is promoted and chain scission is suppressed, is one of the important components of this current work.

As can be seen in Table 2, the effect of reaction temperature on cross-linking is significant. At 100 °C, cross-linking is not enough, but as temperature increases, successful cross-linking can be achieved.  $Q_{we}$  decreases with an increase in reaction temperature. As described above, this reflects an increase in cross-link density. This result is consistent with the known fact that DCP starts to decompose into acetophenone and dimethyl carbinol at ca. 120 °C (information from the manufacturer). From the trend in Table 2, it can be expected that higher cross-link density may be achieved as temperature increases above 140 °C. However, above 140 °C, cross-linking induced yellow color became obvious in resulting hydrogels, which indicates oxidation. Thus, samples prepared above 140 °C were not compared in Table 2.

Oxidation, which can be noticed from color change, could also be detected after more than 12 h of reaction time. Thus, in Table 2, only reaction times between 1 and 7 h were compared. Reaction times of 3–5 h gave the lowest  $Q_{we}$ , and both longer or shorter reaction times produce less cross-linked hydrogels. In previous reports,<sup>22–24</sup> DCP radicals were found to be effective for cross-linking in an early stage and

for chain scission in a later stage of decomposition. Thus, our results appear consistent with those reports.

The concentration of DCP was found to be a very important parameter for controlling cross-link density (Table 2). Generally, as the concentration increases,  $Q_{we}$  decreases. As DCP/pentamer mole ratio increases above 0.83, the hydrogels became yellowish brown, and above 1.46, the color was quite distinct. Thus, like the other reaction conditions, DCP concentration seems to have an optimal range as well. The samples of batches 14 and 15 were brittle and glassy rather than rubbery, even in swollen states (in water or DMSO). Furthermore, they did not show any turbidity change due to  $T_g$ -transition, which all the other hydrogels did. As a consequence, the cross-link density of these samples seems to be too high, resulting in a loss of rubbery nature and  $T_g$ -transition behavior. Table 2 shows that controlling DCP content may be the easiest way to control the cross-link density of p-I.

By control of the DCP content, a series of hydrogels having different cross-link density could be prepared for the detailed study of swelling and mechanical properties. They are p-I-0.20 to p-I-0.47, and their  $Q_{we}$  values are given in Table 2. The  $Q_{we}$  shows a systematic decrease with the increase of DCP concentration. It can be noticed in the table that  $Q_{we}$  of ca. 5 in water may correspond to  $Q_{we}$  of ca. 10 in DMSO.

Table 3 shows the effect of reaction conditions on the cross-linking of c-V(EK). Mild reaction conditions and no chain scission involved are the advantages of this reaction over radical cross-linking methods. Water content is definitely one of the important factors determining  $Q_{we}$ . As the content increases,  $Q_{we}$  increases as well, which indicates a decrease in cross-link density. This result can be easily understood, but to increase cross-link density, reducing water content is not always easy. It is because of the increase of viscosity of polymer solution and difficulty in homogeneous mixing.

EDC content is not as effective as water content in controlling the  $Q_{we}$  of hydrogels, as can be seen in Table 3. However, the variation in  $Q_{we}$  here is significant. The optimum content seems to be around the mole ratio of 10:2. This is far more than the equivalent amount of EDC to the numbers of amines and carboxylic acids.  $Q_{we}$  is also found to be a function of HOBt content. HOBt is known to be able to reduce acylurea rearrangement side reactions and prolong the lifetime of reactive ester intermediate for increasing reaction efficiency. As the content over the amount of EDC increases,  $Q_{we}$  increases significantly, indicating that the content should be limited less than ca. 0.8 mol ratio. A similar result was reported in the study on the cross-linking of alginate hydrogels<sup>34</sup>

One finding difficult to understand is the effect of the mole ratio of glutamic acids (E) to lysine (K). Intuitively, 1:1 mixture is expected to produce lowest  $Q_{we}$ , because it can give highest cross-link density. However, the results in Table 3 show that  $Q_{we}$  decreases as the content of E increases more than its equivalent amount. As the content of a polymer increases, the chemical nature of cross-linked network changes, and so the relationship between  $Q_{we}$  and cross-link

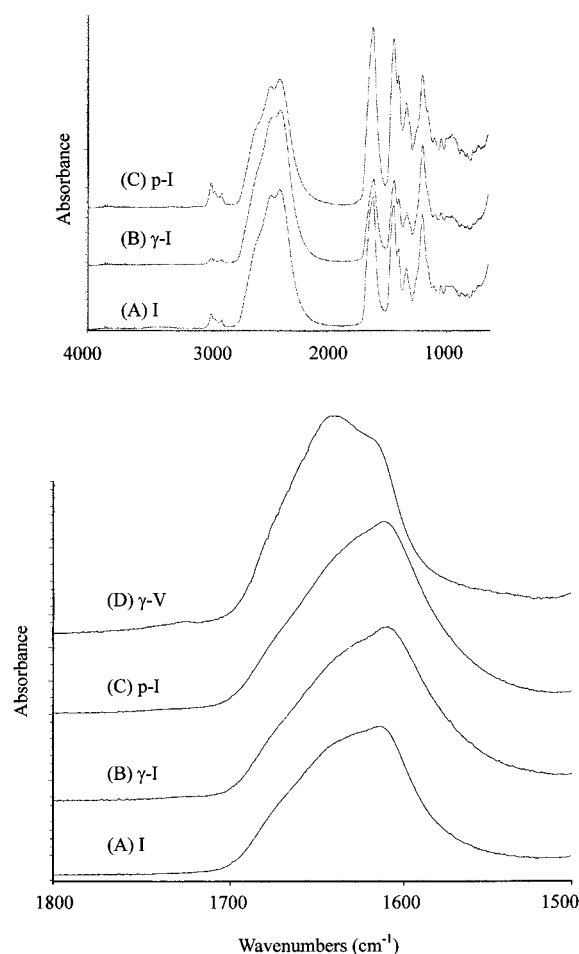
density changes too. The increase of  $E$  in Table 3 will increase the amount of unreacted carboxylic functional groups, which reduces the hydrophobicity of hydrogels. Thus, an increase in  $Q_{we}$  can be expected, instead of a decrease. Situations in reality could be different. EDC can first react with carboxylic acids and produce *O*-acylisourea derivatives. This can further react with amine or result in acylurea by rearrangement.<sup>35</sup> Because of the side reactions, an excess amount of carboxylic acids may be necessary. The optimum EDC content may also indicate the effect of the side reaction.

The effect of cross-linking temperature is particularly worthy of note, because of their hydrophobic folding and assembly transition. Hydrogels were cross-linked at five different temperatures and their  $Q_{we}$  was measured (Table 3). Unexpectedly, the variation in reaction temperature does not appear to significantly affect  $Q_{we}$ . There is only a very small difference in  $Q_{we}$  between the hydrogels prepared below and above  $T_i$ .

After cross-linking, c-V(EK)-29, c-V(EK)-40, and c-V(EK)-55 were cloudy, while all the others were transparent. Among the three hydrogels, cloudiness was found to increase as reaction temperature increases. If the transparent c-V(EK)-5 was heated above  $T_i$ , it became cloudy too. However, hydrogels cross-linked above  $T_i$  were cloudier at a temperature above  $T_i$ . By the same token, at a temperature below  $T_i$ , hydrogels cross-linked below  $T_i$  were more transparent. Among c-V(EK)-5, c-V(EK)-11, and c-V(EK)-22, no difference in transparency was noticed. From these observations, it can be conjectured that cross-linking can partially immobilize the microstructure (or nanostructure) of un-cross-linked polymer/water mixture at cross-linking conditions. However, Table 3 shows no significant dependence of  $Q_{we}$  on the reaction temperature. Later, in the discussion of the temperature dependence of  $Q_{we}$ , it will be revealed that  $Q_{we}$  in fact significantly depends on reaction temperature.

It has been reported<sup>36</sup> that the reactivity of carbodiimide reagents depends on the pH of medium. In our experiments, the pH of reaction mixture before cross-linking was found to be 5.2 and that of water-rich phase after cross-linking was 8.2. Since EDC is known to lose its reactivity above 7.5,<sup>36</sup> the use of a buffer solution can be beneficial. However, the use of a buffer was found to reduce  $T_i$ , resulting in difficulty in increasing polymer concentration and mixing.<sup>3,37</sup> Thus, no buffer was used in this experiment.

**ATR-FTIR Analysis.** In our FTIR study, using  $D_2O$  removes overlapping problems between solvent and target polymer peaks but possibly induces the shift of peaks, because of different hydrogen bonding strength.  $T_i$  was reported to be shifted, too.<sup>38</sup> However, the main purpose of this FTIR study is to check whether any side reactions significantly occur during cross-linking. Thus, comparisons between cross-linked and un-cross-linked polymers were made. In the cross-linking of poly(GVGIP), free radicals could be generated in many different sites and follow complicated reaction routes.<sup>22</sup> Among them, the preferred route is cross-linking via radicals in tertiary carbons. Other routes could be acceptable too. The most undesirable route is the generation of radicals in amide bonds and possible subsequent chemical changes of amide backbone including



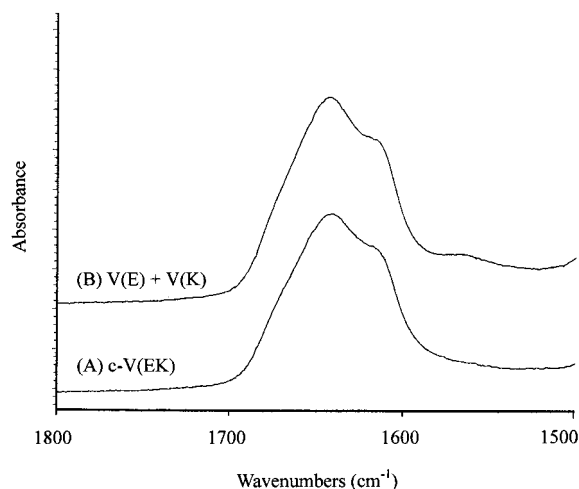
**Figure 1.** ATR-FTIR analysis on un-cross-linked and cross-linked protein-based polymers: (A) (GVGIP)<sub>260</sub>; (B)  $\gamma$ -I-30 Mrad; (C) p-I-0.47; (D)  $\gamma$ -V-30 Mrad.

chain scission. As explained before, the chemical nature of amide bonds should remain intact. To check the side reactions, the amide I bands were mainly examined in this analysis.

As can be seen in Figure 1, the cross-linking reactions ( $\gamma$ -irradiation and DCP radical reactions) do not produce any obvious change in the overall vibrational spectra of cross-linked and un-cross-linked poly(GVGIP). The amide I bands in 1600–1700  $cm^{-1}$  do not show any significant change either. Thus, the side reactions appear to be insignificant, even in the DCP cross-linking at 120 °C.

While the p-I,  $\gamma$ -I, and I (poly(GVGIP)) peaks are almost the same, the  $\gamma$ -V peak is different from the others. Thus, the known secondary structure of poly(GVGVP),  $\beta$ -spirals having type II  $\beta$ -turns,<sup>19,39–41</sup> might become modified in poly(GVGIP). The peak of  $\gamma$ -V in Figure 1 was found to agree with that of un-cross-linked poly(GVGVP). They are also consistent with those in the previous Raman spectrum study.<sup>42</sup> In this study on the amide bands of poly(GVGVP), it was found that the peak in 1600–1700  $cm^{-1}$  is a composite of two amide I bands of 1660 and 1640, and a proline band of 1620  $cm^{-1}$ . However, as argued in ref 42, the structural peak assignment using deconvolution<sup>43–45</sup> does not seem to be reliable for this polymer.

In c-V(EK), a significant number of amide bonds will be generated from the cross-linking reaction, if the yield of



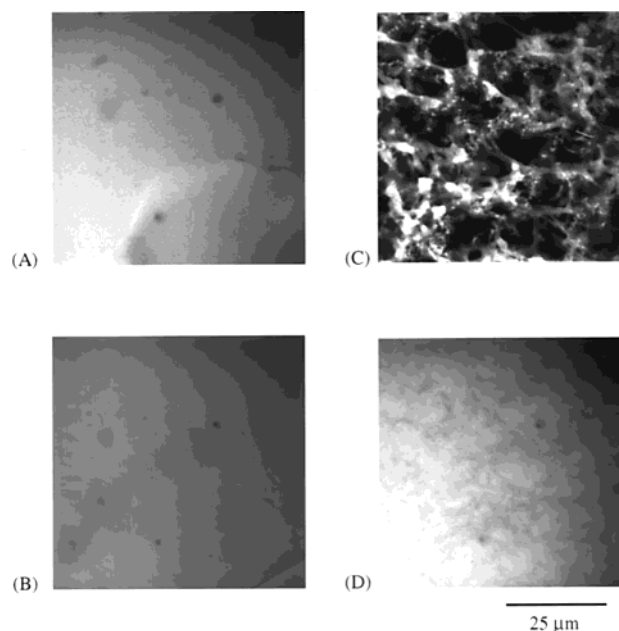
**Figure 2.** ATR-FTIR analysis on cross-linked c-V(EK)-22 Mrad (A) and un-cross-linked 1:1 mixture of V(E) + V(K) (B).

cross-linking reaction is 100%. The existence of these amide bonds can produce differences in FTIR spectra. However, Figure 2 shows no significant difference between the spectra of cross-linked and un-cross-linked polymers. Thus, it seems to be the case that the cross-linking does not produce significant changes in the chemical nature of polymers. Since the molecular weight between cross-links,  $M_c$ , in Table 3 shows that the yield of cross-linking reaction is about 15%, this result is reasonable.

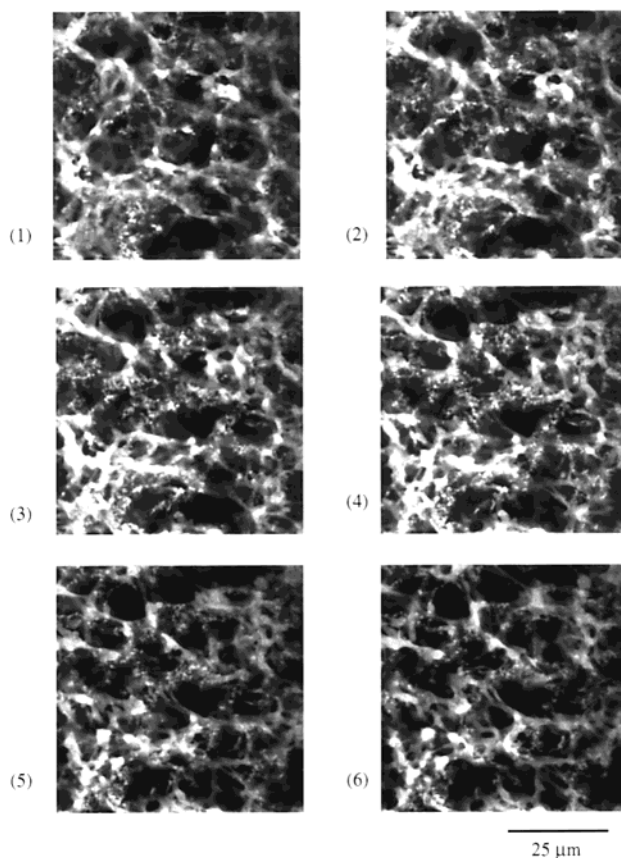
The FTIR results can be supported by  $T_i$  data. As mentioned above,  $T_i$  is known to be very sensitive to the changes of chemical nature of polymers.<sup>3</sup> For example, as mentioned above, the incorporation of 3.4 mol % of glutamic acid residues into poly(GVGVP) can increase  $T_i$  more than 30 °C.<sup>46</sup> It will be found later that the cross-linking reactions do not significantly change the  $T_i$  of polymers. Thus, the reactions do not seem to produce any significant number of functional groups. This result agrees with the FTIR results.

**LSCFM (Laser Scanning Confocal Fluorescence Microscopy).** Figures 3 and 4 show the LSCFM micrographs of two c-V(EK) hydrogels cross-linked at 5 and 55 °C. The two hydrogels can be distinguished by the unaided eye, since the 5 °C hydrogel is transparent and the other is cloudy. This suggests differences in microstructure, which can be directly identified in Figure 3. While the 5 °C hydrogel does not show any inhomogeneity in both fluorescence and transmission micrographs (Figure 3, parts A and B), the 55 °C hydrogel shows distinct continuous filamentous structures in the fluorescence micrograph (C). The light transmission micrograph (D) taken at the same region as in (C) also shows filamentous structures, but they are relatively vague. This filamentous structure was also found in c-V(EK)-40. Thus, cross-linking above  $T_i$  can produce the filamentous structure. Now, the effect of reaction temperature becomes important, which was not in Table 3.

The structure is likely to result from density fluctuation caused by the  $T_i$  transition<sup>47,48</sup> and fixed by the cross-linking reaction, since similar structures were reported in the studies on un-cross-linked poly(GVGVP).<sup>39,49</sup> This structure is quite different from the aggregated particle structure sometimes found in natural protein folding,<sup>50,51</sup> but similar to the “beaded



**Figure 3.** Laser scanning confocal fluorescence micrographs of c-V(EK) cross-linked at 5 °C (A and B) and 55 °C (C and D) taken at 21 °C: (A and C) fluorescence microscopy; (B and D) light transmission microscopy.



**Figure 4.** Series of laser scanning confocal fluorescence micrographs of c-V(EK) cross-linked at 55 °C taken at every 3  $\mu$ m z-step at 21 °C.

filamentous structure” found in coacervation of tropoelastin in vitro.<sup>52</sup> When the cross-linking reaction finished at 55 °C, the separation between filaments should be smaller than that in Figure 3C. After cross-linking, the hydrogels were cooled to room temperature with absorption of more water. During this swelling, less cross-linked regions will expand more,



resulting in more obvious and separated filamentous structure like in part C.

The 3-dimensional continuity of the filamentous structure can be examined by microscopic thin sectioning in  $z$ -direction.<sup>53</sup> Figure 4 shows a series of LSCFM micrographs taken at every 3  $\mu\text{m}$   $z$ -step. The continuity can be noticed in the  $z$ -direction too. Thus, the filamentous structure is continuous in 3 dimensions, which is similar to the structures found in poly(*N*-isopropylacrylamide) (PNIPAM) hydrogels.<sup>54,55</sup> The filamentous structure in Figure 4 seems to be macroscopically isotropic.

Although the hydrogels cross-linked below  $T_i$  did not show any significant inhomogeneity in our LSCFM study, this result does not exclude the possible existence of submicron level inhomogeneity. In fact, it has been reported<sup>48</sup> that there is inhomogeneity of submicron scale in other hydrogels caused by cross-linking, i.e., spatial inhomogeneities other than liquidlike thermal fluctuations. However, this spatial inhomogeneity fixed by cross-linking can hardly be in the micron scale, as our LSCFM data suggest.

**Uniaxial Tensile Modulus.** According to the rubber elasticity theory of random Gaussian chain networks, uniaxial tensile test data can be used to estimate cross-link density<sup>33,56,57</sup>

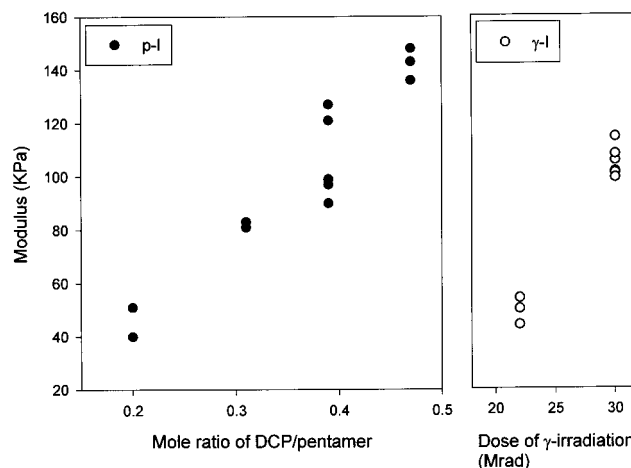
$$\tau = \varphi^{1/3} RT\nu_e \left( \alpha - \frac{1}{\alpha^2} \right), \quad \nu_e = \nu - \mu h \quad (1)$$

where  $\tau$  is the tensile stress,  $\varphi$  the volume fraction of polymers,  $R$  the gas constant,  $T$  temperature,  $\alpha$  the extension ratio, i.e., the current length divided by the initial length, and  $\nu_e$  the effective cross-link density,  $\nu$  and  $\mu$  are the concentrations of elastically active strands and junctions, and  $h$  is an empirical constant which is 0 for affine ( $\nu_e = \nu$ ) and 1 for phantom networks ( $\nu_e = 0.5\nu$ ). The molecular weight between cross-links,  $M_c$ , can have a direct relationship with cross-link density as follows

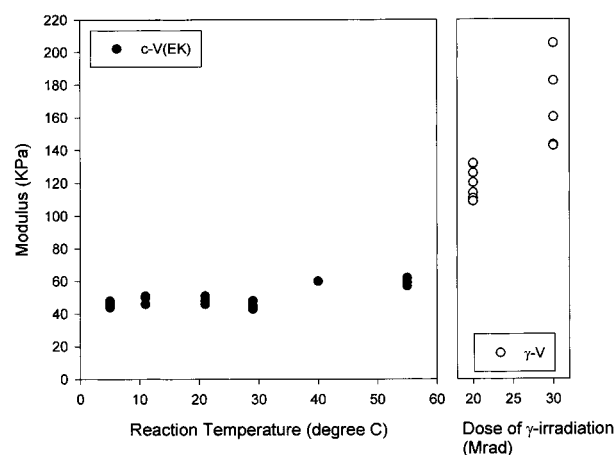
$$\nu = \frac{\rho}{M_c} \left( 1 - \frac{2\bar{M}_c}{M_n} \right) \quad (2)$$

where  $\rho$  is the density of hydrogels,  $\bar{M}_n$  is the molecular weight of linear polymer. These equations are derived under the mean field approximation,<sup>33,58</sup> and elastically ineffective chains such as loops and non-Gaussian behavior are not considered.

Since the rubber elasticity theory can provide us a rough but direct estimation of an important characteristic of hydrogels, i.e., cross-link density, uniaxial tensile tests were performed and their data were used to obtain  $\nu_e$ , which is given in Tables 1–3 ( $h = 1$ ). Figures 5 and 6 show tensile modulus data. All the data were taken below  $T_i$ . It is because our polypeptides have more regular secondary structures such as twisted filaments of  $\beta$ -spirals<sup>3,17–19</sup> above  $T_i$ , as mentioned before. Thus, it seems inappropriate to describe them as random chain networks. There is one more phenomenological reason. Above  $T_i$ , our hydrogels were found to be more viscoelastic, showing significant hysteresis in tensile tests.<sup>30</sup> Thus, the measurement of rubbery tensile modulus (or the ratio between  $\tau$  and  $\alpha - 1/\alpha^2$ ) is more dependent on testing conditions.



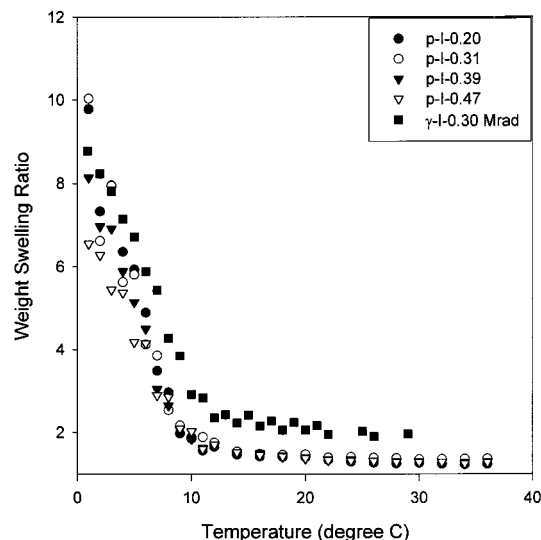
**Figure 5.** Uniaxial tensile moduli of p-I and  $\gamma$ -I measured in water at 7 °C.



**Figure 6.** Uniaxial tensile moduli of c-V(EK) and  $\gamma$ -V measured in water at 21 °C.

Figure 5 shows the tensile moduli of p-I and  $\gamma$ -I. The DCP reaction produces the similar tensile moduli as  $\gamma$ -irradiation of 20–30 Mrad. As DCP content increases, tensile modulus increases, indicating an increase in cross-link density. This trend can also be seen in the average  $M_c$  values of Table 2. The  $M_c$  values show that there is a cross-link junction at every ca. 50–30 pentamers. Thus, the hydrogels seem to be relatively lightly cross-linked.

The minimum  $M_c$  value obtainable from the cross-linking of c-V(EK) is ca. 2.5 kg/mol, because the glutamic acid and lysine residues were introduced at every 30 residues. The tensile modulus of c-V(EK) in Figure 6 corresponds to higher  $M_c$  than the minimum value, which is given in Table 3. The modulus values are even lower than those of  $\gamma$ -V's of 20 and 30 Mrad. An interesting thing is that there is no significant effect of cross-linking temperature on the modulus of c-V(EK). As seen in the LSCFM study, there is a significant difference in microstructure between the hydrogels cross-linked below and above  $T_i$ . However, tensile modulus measured at 21 °C does not reflect this difference. In Figure 6, there is a very small jump in modulus between 29 and 40 °C, but this is not large enough to be related with the difference in microstructure. In fact, a similar result, i.e., no influence of microstructural differences on mechanical properties, can be found in the studies of other hydrogels.<sup>50</sup>



**Figure 7.** Equilibrium weight swelling ratio of p-I as a function of temperature.

This subject will be reexamined in the next section, where the significant effect of cross-linking temperature on  $Q_{we}$  will be found.

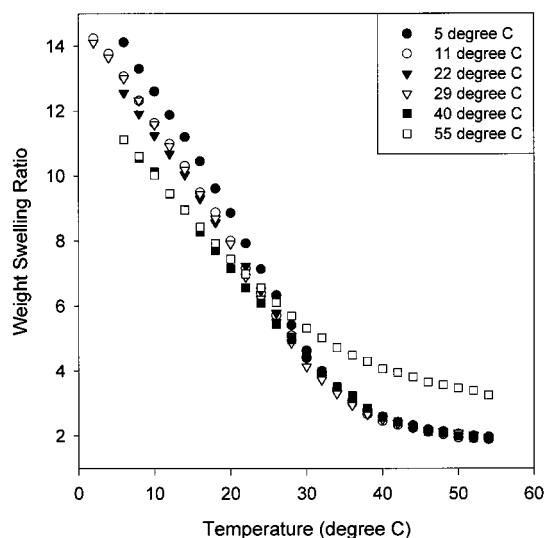
**Equilibrium Weight Swelling Ratio ( $Q_{we}$ ).** The  $Q_{we}$  of p-I is compared with that of  $\gamma$ -I-30 Mrad in Figure 7 as a function of temperature.  $T_t$  seems to be the same in both types of hydrogels, which is around 10 °C. Above  $T_t$ ,  $Q_{we}$  does not significantly depend on temperature, while it does almost linearly below  $T_t$ . The  $Q_{we}$  data at a temperature of even number were taken during heating and the others were taken during cooling. The two sets of data appear to be generally consistent with each other, although there are small differences between them. These results show that the  $T_t$ -transition is continuous and reversible. It looks like a second-order phase transition.

Among the p-I data, there are small differences in  $Q_{we}$  below  $T_t$ , but not above  $T_t$  caused by differences in DCP content. The same result was also found in the swelling study of  $\gamma$ -I,<sup>30</sup> where differences in  $\gamma$ -irradiation dose made differences in  $Q_{we}$  only below  $T_t$ . The differences below  $T_t$  are rather small compared to the scattering of data but are expected to reflect the differences in cross-link density found in Table 2.

The  $Q_{we}$  of our hydrogels is determined by two free energy terms, free energy of mixing ( $\Delta G_m$ ) and rubber elasticity ( $(\Delta G_r)$ ).<sup>33</sup>

$$\Delta G = \Delta G_m + \Delta G_r \quad (3)$$

At a temperature, water diffusion into a polymer network driven by the free energy of mixing term will be counterbalanced by the rubber elasticity term. Above  $T_t$ , the  $\Delta G_m$  term becomes smaller, and so significant differences in  $Q_{we}$  caused by different cross-link densities can hardly be noticed. Thus, above  $T_t$ , no significant variation in  $Q_{we}$  can be found. The collapsed state of macromolecular chains at  $T_t$  is thought to be closer to those in  $\Theta$  solvents, and can be used as the reference state ( $\Delta G_r = 0$ ) for volume expansion due to swelling.<sup>59</sup> However, our protein-based hydrogels have regular structures above  $T_t$ , which is making the situation more complicated.



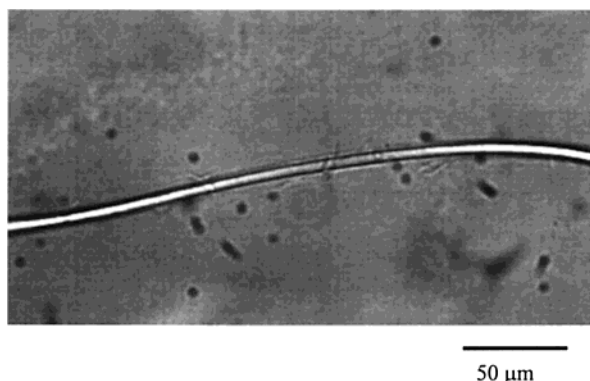
**Figure 8.** Equilibrium weight swelling ratio of c-V(EK) hydrogels as a function of temperature, which were cross-linked at different temperatures.

In all the temperature range of Figure 7,  $Q_{we}$  is smaller in p-I than in  $\gamma$ -I. It might be simply because of residual DMSO or (decomposed) DCP. However, there was no evidence of any residual DMSO or other chemicals in the FTIR analysis, and the purification procedure here is rather thorough. Furthermore,  $T_t$  is likely to be changed, if any residual low molecular weight species remain.<sup>46</sup> The difference may be caused by different chain conformations during cross-linking. Cross-linking can (partially) immobilize the different chain states. While  $\gamma$ -I was cross-linked in water at 23 °C, p-I was cross-linked in DMSO at 120 °C. Figure 7 suggests that the polypeptide chains might be in more collapsed state in DMSO at 120 °C than in water at 23 °C. This can cause differences in the reference state.

A more complicated situation can be found in Figure 8, which shows the  $Q_{we}$  of c-V(EK). The hydrogels cross-linked at 5 to 40 °C have the same  $Q_{we}$  above  $T_t$  of ca. 37 °C (collapsed state), but one cross-linked at 55 °C has different  $Q_{we}$  from all the others. To understand the 55 °C hydrogel, the cross-linking procedure of this experiment needs to be considered. The polymer solution was mixed with the EDC reagent solution at room temperature and then heated to 55 °C. Because the cross-linking reaction starts after the mixing, the macromolecules cannot fully have attained the equilibrium state of 55 °C. Thus, instead of the hydrophobically folded and assembled state of 55 °C, a nonequilibrium intermediate state will result and be fixed by cross-linking. Later, the fixed nonequilibrium (unrelaxed) state may interfere with the collapsing of hydrogels above  $T_t$ , resulting in a higher  $Q_{we}$ , as can be seen in Figure 8. In other words, the 55 °C hydrogel may have a different reference state from others. The 40 °C hydrogel seems to have too small temperature difference between  $T_t$  and cross-linking temperature to observe the same kinetic effect.

In Figure 8, the  $T_t$ -transition occurs in the same temperature range in the cases of 5 to 40 °C hydrogels. It does not depend on reaction temperature. Comparison between these and the 55 °C hydrogel is difficult, because of the broadness of curves. The  $Q_{we}$  data below  $T_t$  show the significant effect





**Figure 9.** Optical micrograph of a cross-linked c-V(EK) fiber in water at room temperature.

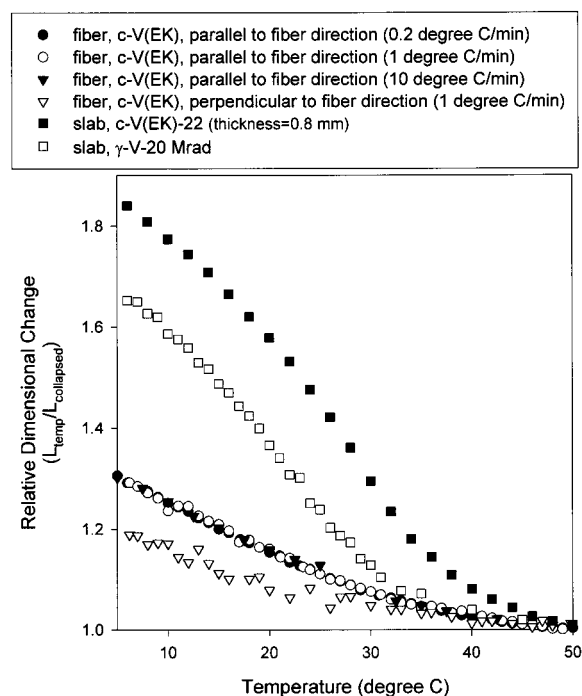
of reaction temperature. Generally, as reaction temperature increases,  $Q_{we}$  decreases. In Table 3 and Figure 6, no significant effect of reaction temperature on  $Q_{we}$  at 22 °C and modulus at 21 °C was demonstrated. However, in a range of temperature below  $T_i$ , the effect on  $Q_{we}$  becomes significant.

Cross-linking at various conditions, which can partially fix microstructures at the conditions, is interesting and useful for the future processing of a desired product. However, understanding the behavior of resulting hydrogels is difficult, mainly because of difficulty in determining their reference states. Thus, how to determine the reference state will be a critical issue for the future development of protein-based hydrogels.

One more challenge for processing is fibrilization. Figure 9 shows the optical micrograph of a fiber of ca. 5  $\mu\text{m}$  diameter prepared in this experiment. The method used is a kind of solution spinning with air-drying. After the drying fixed stretched structures, the fibers were swollen again in EDC reagent solution. During this reswelling, the stretched structures were partially conserved by cross-linking and also partially destroyed by swelling. Depending on the kinetics of the two processes, a different degree of anisotropy will result.

A method to test the anisotropy of a fiber is measuring its dimensional change as a function of temperature. Figure 10 shows a typical result of c-V(EK) fibers. It shows that anisotropy is successfully fixed by cross-linking. Dimensional change due to swelling is much larger in the direction parallel to fiber than perpendicular to fiber. Anisotropy simply introduced by stretching and cross-linking in this experiment is comparable to the anisotropy fixed in the interpenetrating networks of *N*-isopropylacrylamide and acrylamide by two-step cross-linking in ref 60. In conventional synthetic fibers, the coefficient of thermal expansion in the fiber direction is near zero or even negative. This is because stretched chains become relaxed upon heating. The more negative longitudinal dimensional change in Figure 10 might be caused by the same reason. This anisotropy can be expected to increase the modulus of hydrogels in fiber direction.

Interestingly, different heating rates from 0.2 to 10 °C/min do not cause any significant difference in dimensional change. In fact, it was found that the response of fiber dimension was faster than these heating rates. The response



**Figure 10.** Dimensional change of c-V(EK) fiber (diameter = 42  $\mu\text{m}$  at 5 °C) and slab hydrogels as a function of temperature. The dimension of hydrogels at 50 °C is taken as the  $L_{\text{collapsed}}$ . The data of slabs are taken from equilibrium weight swelling ratio measurements.

time of a hydrogel depends on its diffusion coefficient ( $D$ ) and dimension ( $L$ ), if a diffusion-controlled process is assumed.<sup>61</sup>

$$\text{time} \approx L^2/D \quad (4)$$

The diffusion coefficient of our hydrogels was found to be about  $10^{-8} \text{ cm}^2/\text{s}$ .<sup>30</sup> The thickness change of the fiber in Figure 10 is ca. 7  $\mu\text{m}$  on going from 5 to 50 °C. Thus, the response time is ca. 50 s, which corresponds to a heating rate of 54 °C/min. Consequently, unless heating rate is faster than this, the rate dependence of dimensional change could not be noticed. Although this is a rough estimation, the heating rates tried in Figure 10 still seem to be too low. The fast response time is one of the interesting features of protein-based hydrogel fibers, in addition to the built-in anisotropy.

Dimensional increase upon cooling to 5 °C is more than twice in slabs than in fibers (c-V(EK) in Figure 10). This may be simply because of different cross-link densities. Stretching can help the alignment of macromolecules and also increase ion pairing between carboxylic acids and amines. Thus, a higher degree of cross-linking may result. Furthermore, the cross-linking of fibers was done during reswelling. Thus, the water content of fibers during cross-linking may be smaller than that of films. Because of the significant dependence of cross-link density on water content (Table 3), the different water contents will result in different cross-link densities. For a comparison with c-V(EK) data, the dimensional change of  $\gamma$ -V is plotted in Figure 10. In all the samples, the  $T_i$ -transition is continuous.

## Conclusions

In the radical cross-linking of poly(GVGIP) using DCP, both reaction temperature and time were found to be

important variables. Proper temperature and time ranges were ca. 110–140 °C and 2–5 h, which could produce successfully cross-linked hydrogels ( $Q_{we} < 14$  in DMSO). As the amount of DCP increased, the cross-link density and tensile modulus of hydrogels were shown to increase. When it increased above 0.8 mole ratio (DCP/pentamer), hydrogels became glassy. In the cross-linking of c-V(EK) using EDC, the concentrations of water and HOBt were critical. A reaction temperature change from 5 to 55 °C did not produce a significant change in tensile modulus at 21 °C. However, the equilibrium weight swelling ratio below  $T_g$  was demonstrated to be a function of reaction temperature. Three-dimensionally continuous filamentous structures were found in hydrogels cross-linked above  $T_g$ . Cross-linked hydrogel fibers were successfully prepared by the same carbodiimide reaction. They showed reversible anisotropic swelling behavior and faster response time upon temperature changes. During these cross-linking reactions, no significant chemical changes were detected in amide backbone.

**Acknowledgment.** We wish to thank the Bioelastic Research Laboratory in Alabama, M. Neidert, BPTI, and F. Prochazka. This work was supported by the Office of Naval Research (ONR), Contract No. N0001498-1-0656.

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BM0000900