Characterization of the Network Structure of Carbodiimide Cross-Linked Gelatin Gels

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ABSTRACT: The network structure of native and carbodiimide cross-linked gelatin A and B gels was studied based on their rheological behavior. Gelatin A and B contain different numbers of carboxylic acid groups caused by different preparation conditions and had previously shown different characteristics in controlled release applications. It was evaluated to which extent chemical cross-linking densified the network structure of physical gelatin gels. After normalization of the equilibrium shear modulus (G_e) with respect to swelling (Q), it was observed that the normalized G_e values largely depend on the way gelatin is prepared from collagen. At an equal number of chemical junctions, chemically cross-linked gelatin B gels had a lower elasticity modulus than chemically cross-linked gelatin A gels. This seemed contradictory as gelatin B contains more carboxylic acid groups, available for cross-linking, but is related to a higher probability for intramolecular cross-linking, as was validated quantitatively by chemical and rheological analysis of the number of cross-links. Assuming an ideal network, the average molecular weight of the elastic network chains (M_c) was calculated for physical and chemical gelatin A and B networks, and on the basis of M_c the mesh sizes of the gels were estimated. The calculated mesh sizes were experimentally confirmed by lysozyme and albumin diffusion. Chemical cross-linking increased the resistance of the gels toward thermal degradation, resulting in a more gradual disintegration of physical cross-links upon heating. Moreover, chemical cross-linking prevented recombination of these cross-links upon cooling.

Introduction

Gelatin is a protein that is obtained by breaking the triple-helix structure of collagen into single-strain molecules.[1] It is biodegradable, biocompatible, and nonimmunogenic, which makes it a suitable compound for biomedical applications, such as sealant for vascular prostheses,[2,3] and in drug delivery, e.g., as hard and soft capsules, hydrogel,[4] or microspheres.[5,6]

There are two types of gelatin: gelatin A and gelatin B, which differ in the way of preparation. Gelatin A is processed by an acidic pretreatment before thermal denaturation, while gelatin B is processed by an alkaline pretreatment. The alkaline pretreatment is supposed to convert amide residues of glutamine and asparagine into glutamic and aspartic acid, which leads to a 25% higher carboxylic acid content for gelatin B than for gelatin A.[7]

In aqueous solution, gelatin forms physical thermoreversible gels upon lowering the temperature below 35 °C as the chains undergo a conformational coil-to-helix transition during which they tend to recover the collagen triple-helix structure.[8] The melting temperature of physical gelatin gels is determined by several parameters, e.g., aging time, gelation temperature, pH,[9] and concentration of the gel.[10]

As the physical network breaks down at higher temperatures, for long-term biomedical applications at 37 °C the thermal and mechanical stability of gelatin hydrogels has to be improved for instance by chemical cross-linking. In a previous study the application of gelatin gels for controlled delivery purposes was described.[11] These gelatin gels were chemically cross-linked with *N*,*N*-(3-(dimethylamino)propyl)-*N*′-ethyl carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS). The chemical cross-linking of gelatin gels resulted in chemically cross-linked physical gelatin networks, so-called "chemical gelatin gels" which probably show a network structure as illustrated in Figure 1. It was shown that the properties of these chemical gelatin A and gelatin B gels concerning lysozyme release differed with respect to initial lysozyme uptake from solution by the gels and the total release time. These differences are caused either by differences in lysozyme interaction with gelatin A or B or by differences in the network structures of both gelatin types.

This paper aims to elucidate the effect of chemical cross-linking on the network structure of gelatin gels and to evaluate to which extent the network structure of chemical gelatin A differs from chemical gelatin B. Physical measurements of the rheological, swelling, and thermal properties of the gels and chemical analysis of the number of junctions introduced by chemical cross-linking were used to elucidate the structure of the chemical gelatin networks. A second aim was to determine the effect of chemical cross-linking on the thermal

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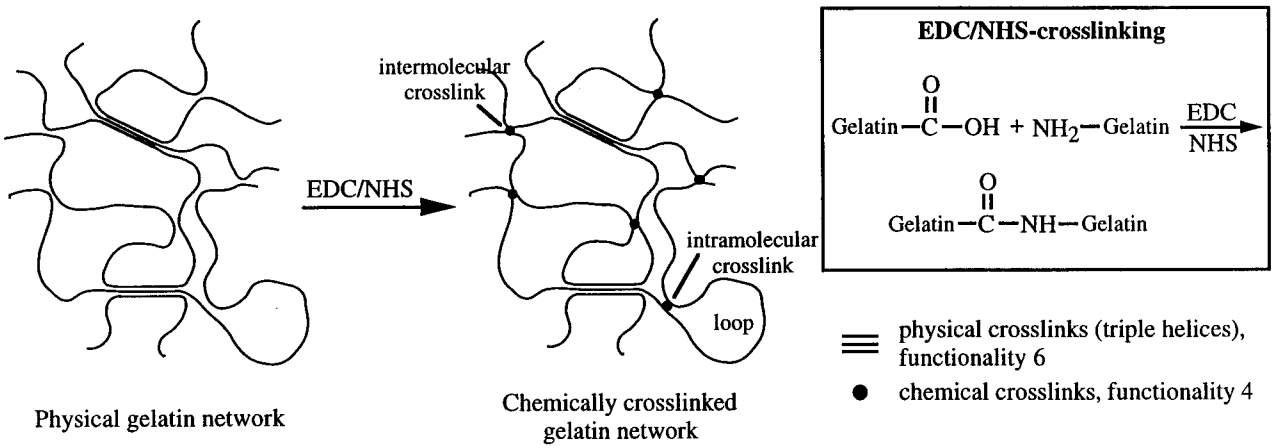

Figure 1. Schematic representation of a physical and a chemical gelatin network. A physical gelatin network contains triple-helix junctions (≡) with a functionality of 6. By chemical cross-linking of this physical network with EDC and NHS, junctions are introduced (●) with a functionality of 4.

behavior of gelatin gels and to correlate these results to their network structure. It should be realized that the gelatin gels studied, either chemically cross-linked or not, do all consist of a physically cross-linked network.

An important characteristic of a polymer network is the degree of cross-linking, i.e., the number density of junctions or cross-links connecting the chains in a network structure.[12,13] In general, the number of moles of elastic junctions per unit volume of the network (μ) and the number of moles of elastic network chains per unit volume of the network (ν) are used to describe the density of cross-links and network chains of the polymer network. Consequently, ν and μ determine the average molecular weight between the junctions. The functionality of the junctions (f), being the number of chains leaving from one junction, determines the relation between ν and μ by the following equation:[12]

$$\mu = 2\nu/f \tag{1}$$

In physical gelatin gels the functionality of the junctions (f_{ph}) formed by collagen-like triple helices is 6, whereas the functionality of the chemical junctions introduced by EDC/NHS cross-linking (f_{ch}) in chemical gelatin gels is 4 (Figure 1). Although the length of the physical cross-links might be considerable due to aging of the gels after preparation,[14] in the continuation of this study variations in the length of the physical cross-links were not considered for reasons of simplification.

Generally, the equilibrium shear modulus of a polymer network (G_e) or its swelling behavior is used to estimate μ, ν, and M_c. In this study, G_e was used to obtain information on the network structure, based on the constrained junction model of Flory and Erman which predicts that G_e is given by[13]

$$G_e = (\nu - h\mu)RT \tag{2}$$

where R is the gas constant and T is the absolute temperature. In eq 2, h ranges between 0 for an affine network and 1 for a phantom network.[15] In an affine network, it is assumed that the junctions of the network do not fluctuate and transform affinely (linearly) with the macroscopic deformation. For a phantom network it is assumed that the junctions do fluctuate over time. Real networks are expected to show characteristics somewhere between the properties of affine and phantom models.[13] Considering a "bimodal distribution" of the network structure,[16] for chemical gelatin gels, G_e is assumed to be based on independent contributions from $G_{e,ph}$, the equilibrium modulus of the physical gelatin gel before EDC/NHS cross-linking, and $G_{e,ch}$, being the contribution to G_e of the chemical cross-links:

$$G_e = G_{e,ph} + G_{e,ch} = (\nu_{ph} - h\mu_{ph})RT + (\nu_{ch} - h\mu_{ch})RT \tag{3}$$

where ν_{ph} is the molar concentration of elastic network chains in the physical gelatin gels before EDC/NHS cross-linking and ν_{ch} is the molar concentration of elastic network chains which are additionally created by chemical cross-linking; μ_{ph} and μ_{ch} are the molar concentrations of respectively the physical and the chemical elastic junctions.

Experimental Section

Materials. Gelatin A (porcine skin, 300 bloom, lot 054H0724) and gelatin B (bovine skin, 225 bloom, lot 56H0658), lysozyme, and albumin were purchased from Sigma Chemical Inc., St. Louis, MO. 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) (1 M) and *N*-hydroxysuccinimide (NHS) were purchased from Fluka, Buchs, Switzerland. Coomassie Plus Protein Assay Reagent was obtained from Pierce, Rockford, IL. Deionized water was obtained from a Milli-Q plus apparatus from Millipore (Molsheim, France). The phosphate buffer used in swelling experiments was an aqueous solution of sodium dihydrogen phosphate and disodium hydrogen phosphate (pH 7.1, 66 mM phosphate). Phosphate buffered saline (PBS) (pH 7.4, [NaCl] = 0.140 M) was purchased from NPBI, Emmer Compascuum, The Netherlands. All other reagents were obtained from Merck, Darmstadt, Germany.

Preparation of Physical Gelatin Gels. Gelatin A and gelatin B were dissolved in deionized water (10 wt %, 540 mL) at 50 °C. After 1 h of stirring the solution was sonicated to remove air bubbles. The solution was poured onto a silanated glass plate (28 × 38 cm) and dried on a flat surface overnight at room temperature. Smaller gels (5 cm diameter) were cut from these gels. Silanation of the glass plate was carried out with 50 mL of a mixture of a saturated (dimethylamino)-pyridine solution in toluene and chlorotrimethylsilane (7:3 v/v). After incubation of the glass plate under nitrogen for 5 h, it was washed with ethanol (100 mL), petroleum ether 40−60 (100 mL), and acetone (100 mL) and dried under nitrogen.

Chemical Cross-Linking of Gelatin Gels. Before cross-linking, the gels were dried in vacuo for 1 day. Cross-linking of gelatin gels with EDC and NHS was carried out in 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.3, 0.05

M) at 4 °C during 16 h. In all experiments the amount of gelatin film in buffer was 1 g in 50 mL of solution. The molar ratio of EDC to carboxylic acid groups of gelatin (EDC/$COOH_{gelatin}$) varied from 0.2 to 3.0 for both gelatin A and B. As gelatin A contains 77 COOH per 1000 amino acids while gelatin B contains 118 COOH per 1000 amino acids,[1] the EDC concentration varied from 3 to 60 mM for gelatin A and from 4 to 80 mM for gelatin B while the molar ratio of NHS to EDC was kept constant at 0.2. The reaction mixture was quenched with a solution containing 0.1 M disodium hydrogen phosphate and 2 M sodium chloride for 2 h (pH 8.5, 100 mL) and subsequently washed four times for 0.5 h with deionized water (100 mL) to remove salt from the gels. The washing water was checked by measuring the conductivity. All washings were performed at 4 °C. The chemical gelatin gels were dried and punched into samples with a diameter of 4 cm.

Swelling Measurements. The physical and chemical gelatin gels were dried at a reduced pressure for 1 day and weighed. The gels were swollen in phosphate buffer for 2 h at 22 °C (after which equilibrium was reached), blotted with a tissue, and weighed again. Experiments were carried out in triplicate. The volume degree of swelling (Q) was calculated:

$$Q = \frac{V_{gel}}{V_{dry}} \tag{4}$$

where V_{gel} is the volume of buffer taken up by the gel and V_{dry} is the dry volume of the gelatin film. The volumes were calculated from the weight of the dry and wet gelatin gels and the densities of gelatin (1.3×10^3 kg/m^3) and phosphate buffer.

Chemical Determination of Free Amine Groups in Gelatin Gels. Freeze-dried gelatin gels (2−4 mg) were incubated in 2 mL of a solution of TNBS (0.01 M) in sodium hydrogen carbonate (pH 8.2, 4 w/v %) for 2 h at 40 °C. Then hydrochloric acid (6 M, 3 mL) was added to the solution to hydrolyze the gelatin gels in 1.5 h at 60 °C. After cooling to room temperature, deionized water (5 mL) was added to the solution, and the absorbance at λ 345 nm was measured against a TNBS solution without gelatin, which had been treated in exactly the same way as the cross-linked gelatin samples. Using the absorption coefficient of 2,4,6-trinitroaniline derivatized (hydroxy)lysine residues ($\epsilon = 14\,600$ L/(mol cm)),[17] the amount of free amine groups per 1000 amino acids was calculated, assuming a molecular weight for a gelatin chain of 1000 amino acids of 10^5 g/mol. The experiments were performed in triplicate.

Rheological Characterization of Physical and Chemical Gelatin Gels. The rheological measurements were performed on an AR1000-N controlled stress rheometer from TA Instruments, Ghent, Belgium. The plate/plate geometry of the rheometer was adapted for measurements on hydrogels by sticking sandpaper on the plate to avoid slippage of the gels between the plates. All measurements were performed with an acrylic top plate (diameter 4 cm). Unless described otherwise, all experiments were done in oscillation mode at 1 Hz in the linear viscoelastic range by applying a constant strain of 5×10^{-4} rad. Before the measurement, the gelatin gels were swollen in phosphate buffer outside the rheometer. While the chemical gelatin gels were swollen to equilibrium, the physical gelatin gels tend to dissolve in solvent and therefore never reach an equilibrium swelling value. Therefore, the swelling of these gels was limited in order to obtain a series of physical gelatin gels with different degrees of swelling. The swollen gels, having a thickness between 1.2 and 3.2 mm, were applied between the plates of the rheometer. G_e was measured following the methodology for elasticity modulus measurements on gel slabs as recently developed by Meyvis et al.[18] This method defines an optimal gap setting between the plates of the rheometer for measuring hydrogel slabs prepared outside the rheometer. By gradually decreasing the gap between the rheometer plates (in steps of e.g. 25 μm) and measuring G' at each position, it allows to find to which extent the hydrogel slab has to be compressed between the plates of the geometry in order to perform reliable G_e measurements. The normal force sensor of the rheometer was used to measure the first contact between the hydrogel slab and the geometry. The normal force measured in this study arose from the resistance of the hydrogels to compression and was by no means related to the normal stress components infor example polymeric liquids under flow. At optimum compression of the hydrogels G' was independent of the applied frequency (G' equaled G_e), indicating the existence of a real rubbery network. To be certain that a linear behavior occurred and that slippage did not influence the measurements, a creep-recovery test was introduced after each change of gap.

Diffusion Measurements. Chemical gelatin B gels (EDC/$COOH_{gelatin}$ molar ratio of 0.8) were swollen in PBS to equilibrium. The diffusion of lysozyme and albumin through the gels was measured in time using a 2×8.3 mL two-chamber diffusion cell at 37 °C. The membrane surface was 2.27 cm^2. Lysozyme and albumin were used in initial donor concentrations of 4 mg/mL in PBS. After certain times samples (300 μL) were taken from the acceptor compartment, and the protein concentration was measured with Coomassie Plus Protein Assay Reagent, using calibration curves for lysozyme and albumin.

Temperature-Dependent Rheological Measurements. Since preliminary experiments on chemical gelatin gels showed a large increase in normal force when the temperature was raised, the compression of the gels between the plates was limited. Although a firm contact between the film and the rheometer plates was established, the gels were not ideally compressed to reach G_e. Starting from 20 °C, the temperature was increased with fixed intervals of 5 °C, and an equilibrium time of 5 min was respected before oscillation experiments were performed for 3 min. Non-cross-linked gels were heated to 45 °C while cross-linked samples were heated to 70 °C, unless the normal force became too high and the temperature increase had to be stopped. The gels were quickly cooled to 20 °C, and oscillation experiments were performed continuously up to 30 min.

Results and Discussion

Chemical Cross-Linking of Gelatin Gels. Gelatin A and B gels, which were obtained by solution casting, were cross-linked with EDC and NHS. The carboxylic acid residues of glutamic and aspartic acid on gelatin chains are activated with EDC. The activated carboxylic acid groups can react with free amine groups, hydrolyze, or rearrange to *O*-acylisourea residues. However, by adding NHS to the reaction mixture, NHS will react with the activated carboxylic acid groups and form NHS-activated carboxylic acid groups which are less susceptible to hydrolysis and rearrangement of the carboxylic acid groups.[19] The reaction of activated carboxylic acid residues with the free amine residues of lysine and hydroxylysine residues on (other) gelatin molecules results in the formation of amide bonds as chemical cross-links between the gelatin chains and an urea derivative as leaving molecule (Figure 1).

Figure 2 shows the swelling behavior of the chemical gelatin hydrogels thus obtained and the amount of free amine groups still present after cross-linking. At an EDC/$COOH_{gelatin}$ molar ratio of 0, physical gelatin gels were considered. When the amount of EDC cross-linker was increased, less free amine groups were present after cross-linking; thus the cross-link density increased, and the hydrogels showed a lower degree of swelling at equilibrium. The reduction in free amine groups with increasing EDC/$COOH_{gelatin}$ molar ratio is larger for gelatin B than for gelatin A. The carboxylic acid content for gelatin B is higher than for gelatin A, so that at equal molar ratio of EDC more carboxylic acid groups are activated. Assuming that every "lost" amine group was consumed in a chemical junction, the number of

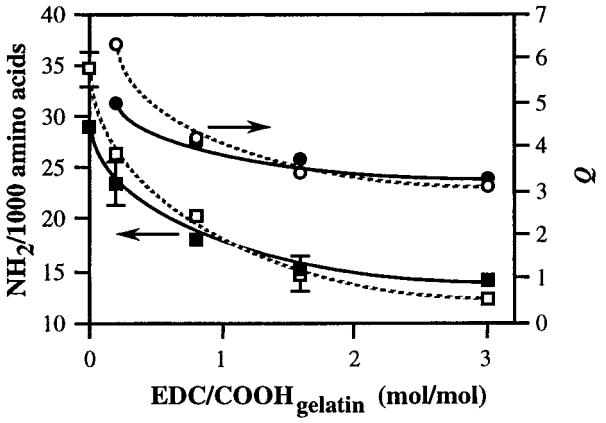

Figure 2. Swelling Q of the gelatin gels and the number of free amine groups per 1000 amino acids by EDC/NHS reaction for gelatin A and B as a function of $EDC/COOH_{gelatin}$ molar ratio during cross-linking: Q_A (●), Q_B (○), NH_{2A} (■), NH_{2B} (□). $n = 3$.

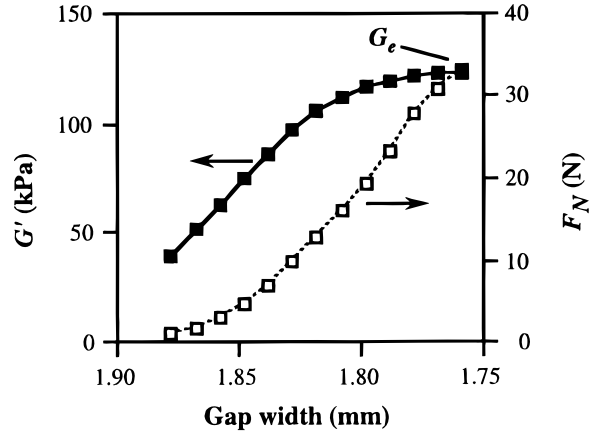

Figure 3. G' (■) and F_N (□) as a function of the gap (mm) between the upper and the lower plate for a gelatin A gel cross-linked at an $EDC/COOH_{gelatin}$ molar ratio of 0.2.

chemical junctions is 15 for chemical gelatin A and 22 for gelatin B gels at the highest $EDC/COOH_{gelatin}$ molar ratio used for cross-linking.

Rheological Characterization of Physical and Chemical Gelatin Gels. The physical and chemical gels were swollen outside the rheometer, before application on the plate of the rheometer. Although apparently a good contact was obtained between the swollen gels and the plates of the rheometer at low compression, it was likely that this was not so, due to the inhomogeneity of the surface of the hydrogels. The structure of the sandpaper had to be pushed into the hydrogel to establish perfect contact between the sandpaper and the hydrogel. Upon compression of the gelatin gels the normal force kept increasing. As outlined in the methodological section, G' was measured after each compression step. G' increased until it leveled off, and only changes of a few percent were observed upon further compression (Figure 3). The systematic compression of the gelatin gels resulted into very reproducible G' profiles.

G_e was measured for gelatin A and B gels both before and after chemical cross-linking. As G_e depends on μ and ν, which are the concentrations of respectively elastic junctions and network chains in the gels, it is influenced by the degree of swelling of the gels. As clearly observed in Figure 4, at equal degree of swelling, G_e of physical gelatin A gels is consistently higher than

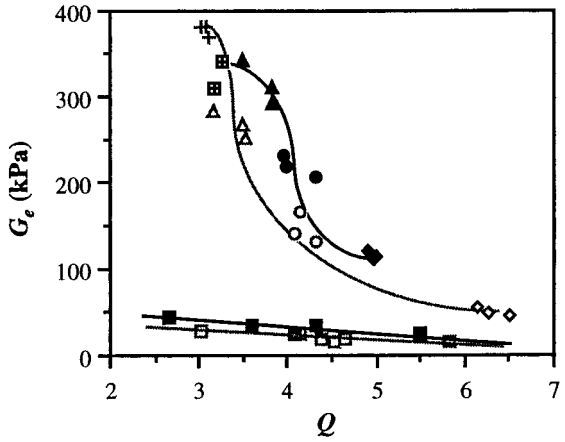

Figure 4. G_e as a function of Q for physical and chemical gelatin A and B gels with different $EDC/COOH_{gelatin}$ molar ratios: 0 (■), 0.2 (◆), 0.8 (●), 1.6 (▲), and 3.0 (crossed □), with the filled symbols for gelatin A and the open symbols for gelatin B.

G_e of physical gelatin B gels. This shows that the "rigidity" or gelling power of gelatin is determined by the way it is prepared from collagen, as has previously been reported[20] and is also reflected in the bloom value (see Materials section).[1] This probably arises from differences in the gelatin molecular weight and in the number and the organization of physical cross-links.[21] Gelatin A and B contain different numbers of amide and carboxylic acid residues, which play an important role in hydrogen-bond formation and thus also in the formation of triple helices. Figure 4 also shows that the introduction of chemical cross-links in gelatin hydrogels causes an increase in G_e. In the continuation of this paper it is explained why G_e of chemical gelatin A gels is larger than G_e of chemical gelatin B gels although there are more free carboxylic acid groups on gelatin B chains than on gelatin A chains.

Structural Rheological and Chemical Analysis of Gelatin Networks. As explained in the Introduction, ν and μ provide information on the structure of the polymer network in gels. The swelling degree influences G_e, which is related to ν and μ (eqs 2 and 3). To compare ν and μ of physical and chemical gelatin gels, it is necessary to know G_e of the gels at an equal degree of swelling. Experimentally it was impossible to obtain an equal swelling for all the gelatin gels during rheological analysis. Therefore, an alternative approach was followed. The experimentally measured G_e values were normalized for the swelling of the gels by multiplying G_e with Q. To verify whether this approach is valid, the normalized G_e values of the series of identical physical gelatin gels that only differed in their degree of swelling were compared. It was expected that the normalization of G_e of these physical gels would result in one single normalized G_e value for all the physical gelatin A gels and one single value for the physical gelatin B gels. As Figure 5 shows, this was observed taking into account the experimental error of G_e. The average normalized G_e value for physical gelatin A gels was 125 ± 19 kPa while it was only 87 ± 8 kPa for physical gelatin B gels.

Figure 6 shows the normalized G_e values of gelatin gels as a function of the EDC/COOH molar ratio. It appears that chemical cross-linking of gelatin A gels results in a higher modulus (and consequently a higher concentration of elastic network chains) than chemical

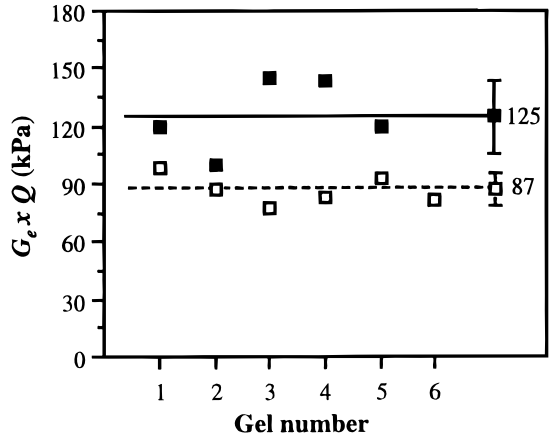

Figure 5. G_e values normalized to Q for a series of physical gelatin A (■) and gelatin B (□) gels.

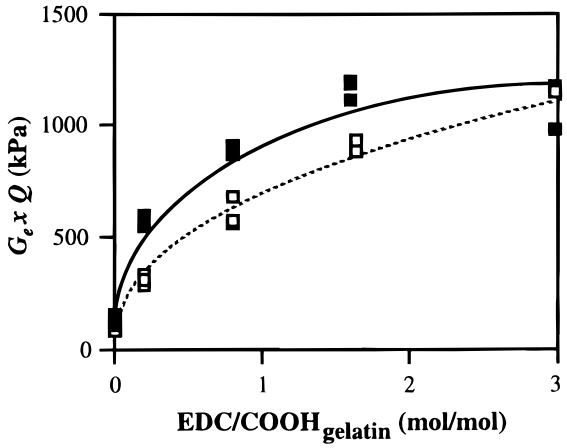

Figure 6. G_e values normalized to Q for chemical gelatin A (■) and B (□) gels as a function of $EDC/COOH_{gelatin}$ molar ratio during cross-linking.

cross-linking of gelatin B gels. On first impression this seems contradictory as the number of free carboxylic acid groups on gelatin B chains is higher than on gelatin A chains, meaning that more possible cross-link sites are available. It is generally known that, upon increasing the number of reactive groups on a polymer chain, the chance for intramolecular cross-linking increases.[22−24] This results into intramolecular cross-links and loops (see Figure 1) instead of intermolecular cross-links between the gelatin chains. Only intermolecular cross-links contribute to G_e; loops do not as they are nonelastic chains. It may therefore be postulated that the higher probability for intramolecular cross-linking accounts for the lower elasticity modulus of gelatin B gels. Figure 6 also shows that for gelatin A gels, above an EDC/COOH molar ratio of 1.6, the normalized G_e value seems to level off, which may indicate a saturation of the cross-linking between carboxyl and amine groups. As the number of carboxylic acid residues on gelatin B chains is higher than on gelatin A chains, this saturation was not observed for gelatin B and may only occur at EDC/COOH molar ratios higher than 3. Besides the higher chance for the formation of intramolecular cross-links in the case of gelatin B gels, the lower elasticity modulus of the *physical* gelatin B gels in comparison to the physical gelatin A gels (Figure 5) may be a second reason the normalized G_e values of *chemical* gelatin B gels are lower compared to those of chemical gelatin A gels.

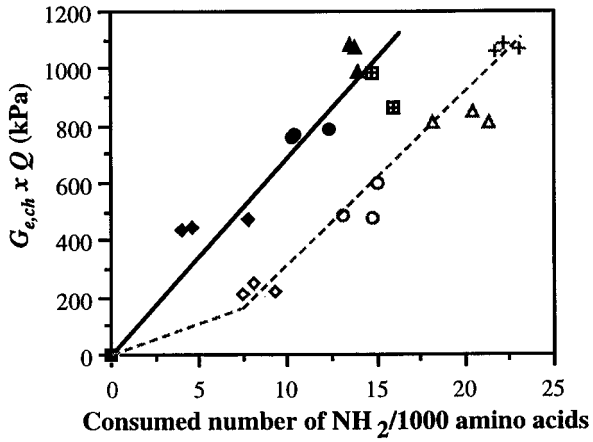

Figure 7. $G_{e,ch}$ values normalized to Q for chemical gelatin gels as a function of the amount of amine groups per 1000 amino acids consumed and $EDC/COOH_{gelatin}$ molar ratio: 0 (■), 0.2 (◆), 0.8 (●), 1.6 (▲), and 3.0 (crossed □), with the filled symbols for gelatin A and the open symbols for gelatin B.

To confirm the hypothesis of intramolecular cross-linking as described above, the contribution of chemical cross-linking to G_e ($G_{e,ch}$) and the number of chemical cross-links (as analytically quantified with TNBS) were compared. Figure 7 clearly shows that an equal number of chemical cross-links resulted in chemical gelatin A gels, which have a higher elasticity modulus than chemical gelatin B gels. This can only be explained by the formation of a higher number of nonelastic intramolecular chemical cross-links in the case of gelatin B. The lower slope for gelatin B at low $EDC/COOH_{gelatin}$ molar ratios is attributed to the preferential formation of intramolecular cross-links. At higher EDC concentration also intermolecular cross-links are formed, and the slope of $G_{e,ch}$ to the number of chemical cross-links approaches the slope observed for gelatin A. Figure 7 also confirms that in case of gelatin A, when the $EDC/COOH_{gelatin}$ molar ratio is higher than 1.6, a saturation of the cross-linking between carboxyl and amine groups seems to occur as only a minor increase in the consumption of amine groups is observed.

To know to which extent the degree of cross-linking in gelatin gels increases by the chemical EDC/NHS reaction, the molar concentration of elastic network chains in the physical gels (ν_{ph}) was compared with the molar concentration of elastic network chains originating from the chemical cross-links (ν_{ch}). The calculation of ν_{ch} and ν_{ph} was based on eq 3 using the normalized G_e values of the physical and chemical gelatin gels. Although it can be expected that real networks such as gelatin gels show a deformation behavior somewhere between the deformation properties of affine and phantom models, an affinelike behavior of the gelatin gels was assumed ($h = 0$) in the calculation of ν_{ch} and ν_{ph} as it is generally difficult to find out precisely the deformation behavior of a polymer network.[25] At low deformations and limited extensions of the network chains due to a low degree of swelling, the behavior becomes closer to that of affine networks.[13] Figure 8 shows the ratio $(\nu_{ch} + \nu_{ph})/\nu_{ph}$ as a function of the $EDC/COOH_{gelatin}$ molar ratio. $(\nu_{ch} + \nu_{ph})/\nu_{ph}$ indicates the number of chemical elastic junctions per physical elastic network chain. It also shows how the average molecular weight of the elastic chains (M_c) decreases upon chemical cross-linking. The average molecular weight of the elastic

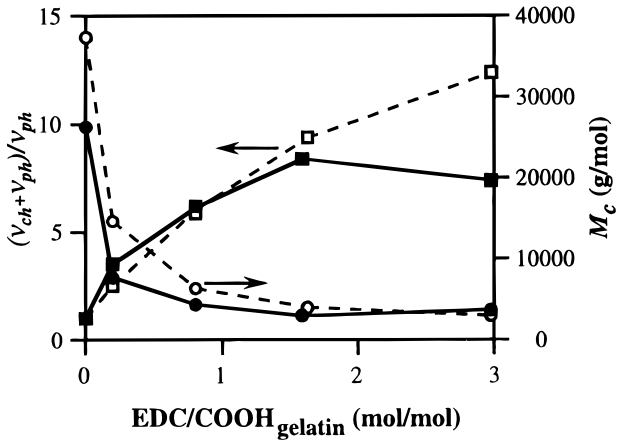

Figure 8. Number of elastic chemical junctions per physical elastic network chain ($(\nu_{ch} + \nu_{ph})/\nu_{ph}$) and M_c as a function of EDC/COOH$_{gelatin}$ molar ratio: $(\nu_{ch} + \nu_{ph})/\nu_{phA}$ (■), $(\nu_{ch} + \nu_{ph})/\nu_{phB}$ (□), M_{cA} (●), and M_{cB} (○).

chains in physical gelatin gels ($M_{c,ph}$) was calculated from the following equation:

$$M_{c,ph} = \frac{c}{\nu_{ph}} \tag{5}$$

where c is the concentration of gelatin involved in elastic chains. As no quantitative information on the concentration of gelatin involved in nonelastic chains such as dangling ends, loops, and sol chains was available, an ideal network without nonelastic chains was assumed in the calculation of $M_{c,ph}$. $M_{c,ph}$ equaled 26 000 ± 4000 g/mol for physical gelatin A and 37 000 ± 3000 g/mol for physical gelatin B gels. M_c was estimated from

$$M_c = \frac{M_{c,ph}}{(\nu_{ch} + \nu_{ph})/\nu_{ph}} \tag{6}$$

M_c decreased gradually to 3500 g/mol for chemical gelatin A and to 3000 g/mol for chemical gelatin B, both cross-linked with an EDC/COOH$_{gelatin}$ molar ratio of 3 (Figure 8).

The number of *elastic* chemical cross-links per 1000 amino acids was calculated from the M_c values assuming that a single-strain gelatin molecule with a molecular weight of 10^5 g/mol consists of 1000 amino acids. These values were compared to the number of *chemical* junctions per 1000 amino acids as determined with TNBS (Figure 9). For gelatin A the number of chemical cross-links is equal to the number of elastic network junctions determined from rheological measurements. At the highest EDC/COOH$_{gelatin}$ molar ratio a saturation of the chemical cross-linking of gelatin A is observed. Gelatin B has more chemical cross-links than elastically active cross-links, which is caused by intramolecular cross-linking.

As explained above, all G_e values were normalized with respect to swelling to compare the network structure of physical and chemical gelatin A and B gels. However, to use these results in studies on the diffusion of proteins in gelatin gels, it was interesting to calculate the mesh size of the chemical gels. The average distance between the junctions (ξ) was estimated from M_c based on an "equivalent network model".[26] The network is represented as a collection of "blobs", whose diameters represent ξ of the entangled structure.[27] As it is impossible to describe the real network in detail, we can replace it by an idealized network for which it is possible to calculate ξ.

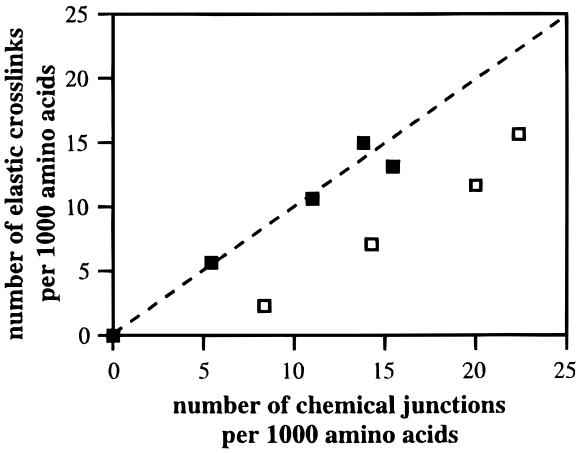

Figure 9. Comparison between the number of chemical junctions (as determined with TNBS) and the number of elastic cross-links (from rheology) for gelatin A (■) and gelatin B (□).

Table 1. Concentration, M_c, and ξ for Chemical Gelatin Gels

	EDC/COOH$_{gelatin}$	c (g/L)	M_c (g/mol)	ξ (nm)
gelatin A	0.2	3.27×10^2	7500	4.2
	0.8	4.46×10^2	4300	3.1
	1.6	4.78×10^2	3200	2.8
	3.0	5.71×10^2	3600	2.7
gelatin B	0.2	2.44×10^2	14700	5.8
	0.8	4.22×10^2	6200	3.6
	1.6	5.26×10^2	4000	2.9
	3.0	6.29×10^2	3000	2.5

$$\xi = \sqrt[3]{\frac{6M_c}{\pi c N_{AV}}} \tag{7}$$

where c is the polymer concentration (calculated from Q) and N_{AV} is Avogadro's number. Table 1 shows that the mesh size of the chemical gelatin gels decreases with cross-link density and is on the order of a few nanometers. It is expected that random coil molecules with molecular weights up to a few thousand g/mol may diffuse through these chemical gelatin gels without any sterical hindrance.

To verify the calculated mesh sizes experimentally, lysozyme and albumin diffusion through chemical gelatin B gels was studied. The gels used for the diffusion measurements were 0.5 mm thick (in swollen state) and cross-linked under the same conditions as the gelatin gels used in rheology. The gels had an equal degree of swelling and a slightly lower number of free amine groups after cross-linking. The chemical gelatin gels cross-linked with an EDC/COOH$_{gelatin}$ molar ratio of 0.8 were permeable for lysozyme but not for albumin (Figure 10). The hydrodynamic diameter of lysozyme has been reported to be at least 3 nm,[28,29] and the hydrodynamic diameter of albumin is 7.2 nm.[29] Furthermore, it has been observed that chemical gelatin gels with a higher cross-link density (EDC/COOH$_{gelatin}$ molar ratio of 1.6 and 3.0) became impermeable for lysozyme.[30] The diffusion experiments confirmed the mesh size calculations from rheological data.

Thermal Behavior of Chemical Gelatin Hydrogels. Figure 11 shows the thermorheological behavior of physical gelatin gels during heating. The physical cross-links in the gelatin A network start to break at

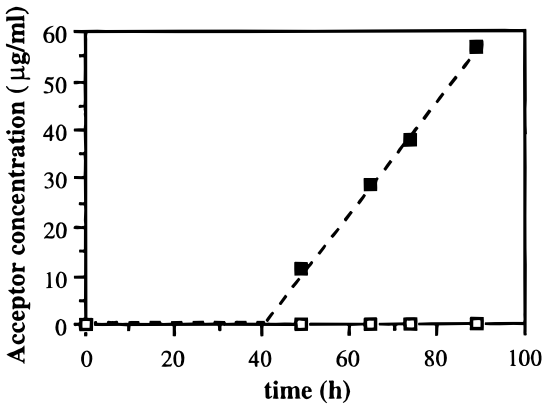

Figure 10. Concentration of lysozyme (■) and albumin (□) in the acceptor compartment of the diffusion cell (μg/mL) in time (h) during diffusion through a chemical gelatin B gel cross-linked with an EDC/$COOH_{gelatin}$ molar ratio of 0.8.

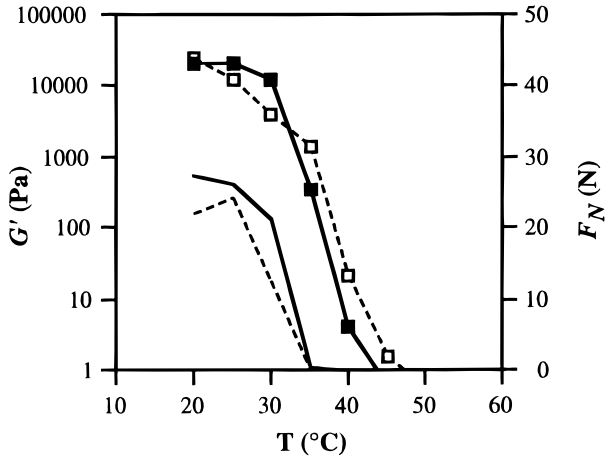

Figure 11. G' and F_N as a function of temperature for physical gelatin A and B: G'_A (■), G'_B (□), F_{NA} (—), and F_{NB} (---).

approximately 30 °C (decrease of G_e). The unwinding of triple helices occurs in a narrow temperature window since the network is completely disintegrated at 40 °C. As expected, a drop of the normal force is clearly observed. This is associated with the liquefaction of the gelatin film as the liquid cannot be compressed between the plates of the geometry. The degradation of physical gelatin B gels seems to start already at 20 °C and occurs over a broader temperature range. The slightly higher melting temperature of physical gelatin A compared to that of gelatin B is possibly explained by the higher number of physical network junctions for gelatin A. Gel preparation parameters such as gelation temperature, aging time, concentration, etc., do also influence the gel melting temperature.[9]

When chemical cross-links are introduced, the thermal response clearly changes. As illustrated in Figures 12 and 13, the degradation of the network upon heating clearly diminishes and extends throughout a large temperature window. For the highest cross-linked gelatin gels (Figure 13) the G' profile no longer shows a drop but a continuous decrease. As the unwinding of helical junctions by thermal heating needs mobile chains, the gradual thermal degradation of the chemical gels may be attributed to a restriction of the mobility of the chains by the chemical cross-links.[31] The polymer network in chemical gelatin A gels seems to be more thermoresistant than the one in chemical gelatin B gels. This may indicate that especially elastic cross-links hinder the

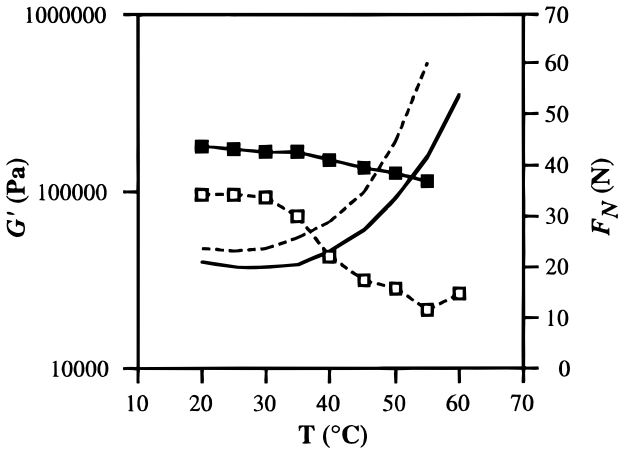

Figure 12. G' and F_N as a function of temperature for chemical gelatin A and B (cross-linked at an EDC/$COOH_{gelatin}$ molar ratio of 0.8): G'_A (■), G'_B (□), F_{NA} (—), and F_{NB} (---).

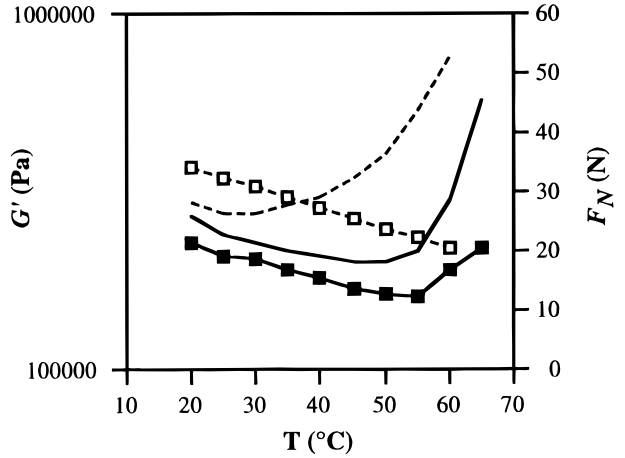

Figure 13. G' and F_N as a function of temperature for chemical gelatin A and B (cross-linked at an EDC/$COOH_{gelatin}$ molar ratio of 3.0): G'_A (■), G'_B (□), F_{NA} (—), and F_{NB} (---).

mobility of the chains, since the amount of elastic cross-links is higher in gelatin A, while the amount of total chemical cross-links is higher in gelatin B (Figure 9). As the contribution of physical cross-links to G_e is only minor compared to the contribution of chemical cross-links, which are assumed to be thermoresistant, it is obvious that the reduction in G_e upon heating is less pronounced than for physical gelatin gels. Even at higher temperatures the chemical cross-links keep the film in a gel state as was observed by a dominance of G' over G'' (data not shown).

Another remarkable observation from Figures 12 and 13 was the drastic increase of the normal force during heating of chemical gelatin gels. This was explained by a reorientation of gelatin chains in the gel which could also be observed visually as the diameter of the gels became smaller. An interesting observation was also that this reorientation of the gelatin gels always occurred in the same direction: the diameter of the gels diminished, while the thickness increased. This supports the hypothesis that there is an orientation of gelatin molecules in the gels, caused by unidirectional drying.[32] The observed reorientation is probably caused by shrinkage phenomena. Shrinkage of chemical gelatin gels occurs as a result of melting of the ordered crystalline structure of the native gels.[33]

As the distance between the plates of the rheometer is fixed and consequently expansion of the gels in height

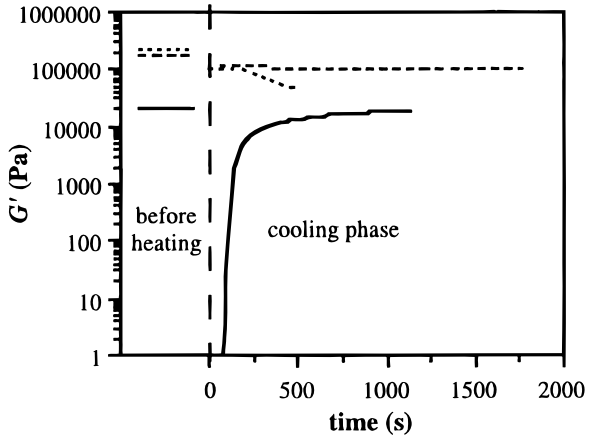

Figure 14. G' of physical and chemical gelatin A gels before heating and during cooling at 20 °C (after heating the gel to 50−65 °C, depending on the cross-link density): physical gel (—); EDC/$COOH_{gelatin}$= 0.8 (---); EDC/$COOH_{gelatin}$= 3.0 (· · ·).

is prevented, shrinkage will increase the cross-link concentration, which should result in an increase of G_e. This may explain why an increase in normal force coincides with an increase in G' (Figure 13). It should be noted that because of the drastic increase in normal force upon heating, the initial compression of the samples was limited to about 20 N. For this reason G' values measured in the thermorheological measurements cannot be compared directly to the G_e values as represented in Figure 4. For the chemical gelatin gels prepared with an EDC/$COOH_{gelatin}$ molar ratio of 0.8 (Figure 12) it seems that, although shrinkage was clearly observed, breaking of physical cross-links during heating of the gels dominates the G' profile. For the chemical gelatin A gels prepared with an EDC/$COOH_{gelatin}$ molar ratio of 3.0 (Figure 13), the sharp G' increase at higher temperatures probably indicates that no further degradation of the physical network occurs while the shrinkage of the gels starts to dominate the G' profile. The onset of the increase of the normal force and consequently the onset of the shrinkage of the gels is different for both gelatins. For gelatin B it is almost independent of the amount of chemical cross-link density while for gelatin A there is a clear shift toward higher temperatures as the cross-link density increases.

Figure 14 shows G' during cooling for physical and chemical gelatin A gels. The physical gelatin A gels show a clear recovery of the network structure. This did not occur for the chemical gels, which suggests that compatible sites present on gelatin chains that were originally joined in physical cross-links are prevented to rejoin due to the presence of chemical cross-links which may alter the spatial organization of the gelatin chains.

Conclusions

The network structure of physical and chemical gelatin A and B (Figure 1) gels was studied based on their rheological behavior. Although the gelatin gels were produced in large quantities and small differences in gel preparation conditions may influence the gel properties, the rheological measurements led to very reproducible values for G_e. By calculating the concentration of elastic network chains from G_e, it was evaluated to which extent chemical cross-linking densifies the network structure of physical gelatin gels. The degree of swelling of the gels influences G_e, and it was experimentally impossible to obtain equal swelling degrees for all gelatin gels during rheological analysis. So to compare the network characteristics calculated from G_e, the measured G_e values were normalized for the degree of swelling of the gels by multiplying G_e with Q. This approach was valid, as the normalized G_e values of a series of identical physical gelatin gels, which only differed in their swelling degree, were equal within experimental error (Figure 5). The normalized G_e values for physical gelatin A gels were significantly higher than for physical gelatin B gels, which showed that the way of preparing gelatin from collagen affects the formation of physical cross-links.

Chemical cross-linking of gelatin gels caused a marked increase in G_e (Figure 6). It appeared that chemical cross-linking of gelatin B gels resulted in gels with a lower elasticity modulus compared to chemically cross-linked gelatin A. On first impression this seemed contradictory as the number of free carboxylic acid groups (i.e., potential sites for cross-linking) on gelatin B chains is higher than on gelatin A chains. However, this also increased the probability for intramolecular cross-linking, leading to a higher amount of nonelastic cross-links. The hypothesis on intramolecular cross-linking was confirmed by quantitative chemical analysis of the number of chemical cross-links. Figure 7 showed that at equal number of chemical cross-links chemical gelatin A gels have a higher elasticity modulus than chemical gelatin B gels, which can only be explained by the formation of a higher number of nonelastic intramolecular chemical cross-links in chemical gelatin B gels. This was also illustrated in Figure 9.

Assuming an ideal network structure, the average molecular weight of the network chains between the elastic junctions was estimated from the elasticity moduli of the gels. For physical gelatin gels $M_{c,ph}$ equaled 26 000 ± 4000 g/mol for gelatin A and 37 000 ± 3000 g/mol for gelatin B. Chemical cross-linking of these physical gelatin gels led to M_c values of 3500 g/mol for chemical gelatin A and 3000 g/mol for chemical gelatin B, cross-linked with an EDC/$COOH_{gelatin}$ molar ratio of 3. Consequently, the average mesh sizes of the chemical gelatin gels were estimated from the M_c values and were between 2 and 5 nm (Table 1). These values were experimentally confirmed as lysozyme (3 nm) did diffuse and albumin (7.2 nm) did not diffuse through the gelatin gels.

The thermorheological measurements showed that the introduction of chemical cross-links in gelatin gels increased the resistance of the gels toward thermal degradation by breaking physical cross-links (Figures 11−13). Moreover, compared to the physical gelatin gels, breaking of the physical cross-links in chemical gelatin gels occurred more gradually over a broader temperature range. While the network structure of thermally degraded physical gelatin gels was completely recovered upon cooling to room temperature, the chemical cross-links in chemical gelatin gels prevented a recombination of physical cross-links.

Finally, the results of this study will hopefully contribute to a better understanding of the structure and the mechanical behavior of chemically cross-linked physical gelatin networks. These fundamental physicochemical insights may promote the development of these gels for medical applications.

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