Controlling Absorbency in Gelatin Networks: Preparation and Characterization of Alkylated, Crosslinked Gelatin

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Received 23 April 1997; accepted 23 September 1997

ABSTRACT: Gelatin is a highly abundant and relatively inexpensive protein which is used in a variety of gel applications including photography, drug delivery, microencapsulation, and food preparation. Control of network formation in gelatin may therefore provide methods of preparing protein gels tailored for specific applications. In this study, circular dichroism (CD), Raman, and nuclear magnetic resonance (NMR) spectroscopies were used to characterize ordering processes which occur in gelatin and their relationship to absorbency. Ordering of the unmodified protein, studied as a function of concentration, temperature, and time after initial preparation, correlates inversely with absorbency. Chemical modification was used to control the absorbency and solubility of the protein gels. Alkylation of gelatin using glycidyltrimethylammonium chloride causes substantial increases in water absorbency with degrees of substitution as low as 0.5%. Increases in saline swelling were observed only after additional modification of the alkylated gelatin with a nonionic polyoxyalkyleneamine crosslinking agent (Jeffamine®). Limiting the initial degree of substitution prior to crosslinking (to just below 1%) plays a key role in optimizing the absorbency and minimizing the dissolution of the gel in saline. The methods and principles used to manipulate the absorbency of gelatin may also find use for other natural protein systems. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 68: 281–292, 1998

Key words: absorbents; proteins; gelatin; protein modification

INTRODUCTION

Natural gel-forming polymers have the potential to provide the fluid uptake and physical properties needed in a variety of applications and are desirable for these uses because of their safety, biodegradability, and potential low cost. Protein systems are particularly promising in these end uses as they are well hydrated, with water playing an important role in stabilizing their structure. Indeed, some proteins naturally absorb large amounts of water to form gels. The ability of natural proteins to function as highly absorbent materials is influenced by folding and network formation, which control the physical properties of the gels. Control of these interactions in proteins may therefore permit purposeful production of highly absorbent network structures.

Gelatin is an abundant and relatively inexpensive protein derived from collagen, with the capacity to form network structures and gels. It is used in a variety of gel applications including photography, drug delivery, holography, microencapsulation, and food preparation. Under certain condi-

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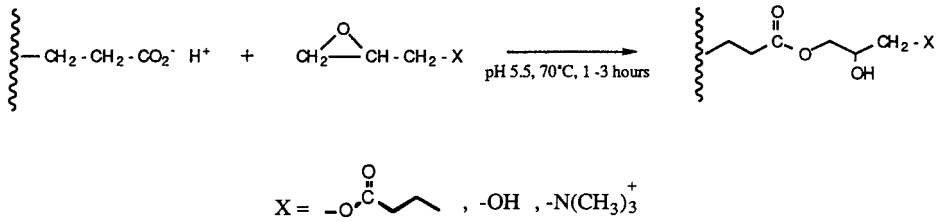
Contract grant sponsors: Kimberly-Clark Corp.; Center for University of Massachusetts–Industry Research on Polymers, University of Massachusetts Amherst.

Journal of Applied Polymer Science, Vol. 68, 281–292 (1998)
 CCC 0021-8995/98/020281-12



Scheme 1 Gelatin alkylation. Although the scheme shows modification of glutamic acid side chains, it is likely that the other nucleophilic sites are alkylated as well.

tions, gelatin absorbs 200 times its mass in water, but forms very weak gels at this high water content. Furthermore, swelling from the collapsed, dry state (xerogel) is severely restricted, with absorption of only 15 g water/g protein.

The ordering of gelatin networks is characteristic of the amino acid sequence, which contains a well-conserved glycine–proline–hydroxyproline triad throughout the protein polymer. These three amino acids comprise over 50% of the overall amino acid composition. The pyrrolidine-rich sequences serve as nucleation sites for partially reforming the collagen triple helix,[1] with interchain hydrogen bonding between gelatin strands.[2] Weaker hydrogen bonding stabilizes the network structure which comprises collagen triple helix crosslinks and single-strand gelatin amorphous regions.[3]

Control of the ordering process in gelatin may allow purposeful formation of absorbent network structures. The swelling of network structures depends on factors such as crystallinity, crosslink density, molecular weight, and hydrophilicity, which can be controlled by chemically modifying the protein. The introduction of sterically bulky and/or charged groups along the protein chain may disrupt ordering without compromising hydrophilicity. Indeed, alkylation of gelatin and collagen by carboxymethylation and epoxide addition has been shown to alter the solubility properties of both proteins.[4–6] Other modifications which have also been shown to alter the physical

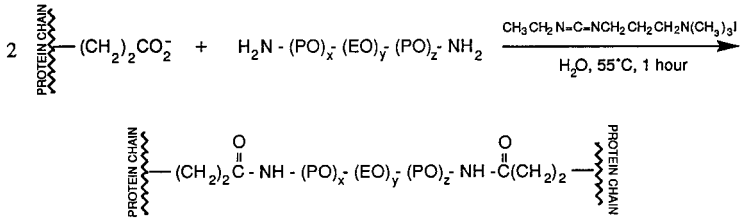
Table I Gelatin Modification: Representative Reactant Masses and Degrees of Modification

Sample	Mass Gelatin (g)	Mass GTAC[a] (g)	Mass Jeffamine® (g)	Mass DEAC[b] (g)	Degree Epoxide Substitution (%)	Degree Jeffamine® Modification (%)
1	10.7	1.5	N/A	N/A	0.51	N/A
2	10.0	6.1	N/A	N/A	1.5	N/A
3	10.0	18.6	N/A	N/A	2.9	N/A
4	5.1	37.2	N/A	N/A	7.6	N/A
5	4.1	N/A	0.74 (ED2001)	0.23	N/A	0.13
6	5.0	37.4	1.15 (ED2001)	0.40	7.4	0.16
7	10.0	15.2	1.99 (ED2001)	0.59	6.7	0.26
8	5.1	50.0	2.50 (ED2001)	0.75	9.5	0.41
9	1.5	0.3	0.25 (ED148)	0.25	0.77	1.6
10	1.5	1.0	0.15 (ED148)	0.20	1.2	0.66
11	5.0	3.1	0.64 (T403)	0.42	1.6	0.34
12	1.5	0.50	0.20 (ED900)	0.05	0.85	0.20
13	1.5	0.22	2.35 (ED900)	0.78	0.70	1.8

Alkylation: Reagents added as above to 20 wt % gelatin solution (pH 5.5) and stirred at 65°C for 1.5 h. Jeffamine® modification: Reagents added to alkylated gelatin (20 wt %, pH 9.0–11.0) and stirred at 55°C for 1 h.

[a] Glycidyltrimethylammonium chloride.

[b] 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide methiodide.



Scheme 2 Crosslinking of gelatin by polyoxyalkyleneamine crosslinkers.

properties of gelatin include acetylation, lauroylation, succinylation, and alkylsulfonation.[4] None of these reactions has been reported to significantly improve the swelling of the protein network. Crosslinking of gelatin has also been extensively studied as a means to improve the material and/or controlled release properties of the protein, but compromises water absorbency[4,7,8] unless modification of the gelatin [e.g., grafting of poly(acrylic acid)] is conducted prior to crosslinking.[9]

In this study we report the (i) characterization of the ordering in gelatin as it relates to absorbency and (ii) the chemical modification of the protein to alter the swelling of the network. Ordering of the noncovalently crosslinked protein network correlates inversely with swelling of the protein gel. Chemical modification of gelatin with glycidyltrimethylammonium chloride causes marked changes in the swelling of the protein in water. Subsequent crosslinking by nonionic polyoxyalkyleneamine crosslinking agents (Jeffamines®) further increases swelling in saline while preventing dissolution.

EXPERIMENTAL

Materials

Porcine skin gelatin (Hormel Foods Corp., 265 Bloom number, Type A) was used in all experiments. Glycidyltrimethylammonium chloride (Aldrich), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide methiodide (Aldrich), and polyoxyalkyleneamines (Jeffamines®, Huntsman Corp., Texas) were used as received in the chemical modification and crosslinking reactions. Deionized water was used as the solvent.

Sample Preparation

The porcine skin gelatin used in the spectroscopic studies and free swell capacity experiments was sieved to particle sizes of 300–600 μm and stored at controlled relative humidity (RH) over a saturated aqueous solution of $MgCl_2 \cdot 6H_2O$ (33% RH).[10] Protein concentrations are reported as weight percent (wt %) and solutions were prepared using distilled water (or 0.15M NaF) to yield final concentrations of 0.005–20 wt % with a resulting pH of approximately 5.5. Initial dissolution was achieved by heating the solution to 40–60°C for 5–10 min; preparation conditions were consistent within sets of samples prepared for spectroscopic analysis. In experiments designed to probe the effect of aging on the secondary structure of the protein, samples were stored at 5°C to minimize hydrolysis and bacterial growth. No

a) $H_2N\text{-}CH(CH_3)\text{-}CH_2\text{-}[O\text{-}CH(CH_3)\text{-}CH_2]_a\text{-}[O\text{-}CH_2\text{-}CH_2]_b\text{-}[O\text{-}CH_2\text{-}CH(CH_3)]_c\text{-}NH_2$

Jeffamine	b	a+c	Approx. Molec. Weight
ED900	15.5	2.5	900
ED2001	40.5	2.5	2000

b) $H_2N\text{-}CH_2CH_2\text{-}OCH_2CH_2\text{-}OCH_2CH_2\text{-}NH_2$

c) A — $[OCH_2CH(CH_3)]_x\text{-}NH_2$, $[OCH_2CH(CH_3)]_y\text{-}NH_2$, $[OCH_2CH(CH_3)]_z\text{-}NH_2$

Figure 1 Jeffamine® polyoxyalkyleneamine crosslinking agents: (a) ED900 and ED2001; (b) EDR148, with a molecular weight of approximately 148; (c) T403 has a molecular weight of approximately 440, 5–6 moles propylene oxide, and trimethylolpropane for A.

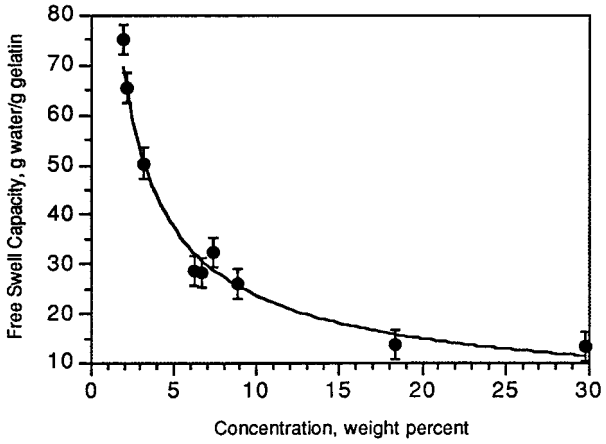


Figure 2 Free swell capacity of gelatin gels as a function of drying to a specific water content. The line represents the equation $y = 107.95\, x^{-0.66}$; $R = 0.99$.

significant mass loss was recorded for gels stored at 5°C for the duration of the experiments.

Swelling Studies

Comparisons of the absorbency of unmodified and chemically modified (see below) gelatins were made on the basis of free swell capacity measurements. Free swell capacity (absorbency) experiments were conducted by adding deionized water or isotonic (0.9% [0.15*M*]) saline (10 mL) to 0.1 g of dry gelatin (300–600-μm particle size, either modified or unmodified) and allowing the gelatin to swell for 1 h. The swollen gel was then retained in a tared, 100-μm sieve and its mass measured. A modified procedure was used in a separate set of experiments designed to determine the dependence of swelling on the concentration of unmodified gelatin gels. In these experiments, gels were prepared (as described above) at varied weight percentages, allowed to set in the refrigerator overnight, and cut into pieces of 3–5-mm dimension. These gel pieces were tested according to the standard protocol and the free swell capacity calculated based on the mass of the dry gelatin. Free swell capacities are reported as g water/g dry gelatin and are calculated by subtracting the dry mass of the protein from the swollen mass and then dividing by the dry mass of the protein.

Spectroscopic Characterization of Ordering

Circular dichroism (CD) spectra were recorded from 190 to 250 nm using an Aviv Model 62DS circular dichroism spectrometer equipped with a Brinkmann RM6 temperature control unit. Samples of 0.005–0.025 wt % concentration were measured in a 1-mm pathlength quartz cell at 25°C unless otherwise noted. Samples with concentrations of approximately 2 and 5 wt % were measured in a 0.01-mm pathlength demountable cell to obtain CD spectra at higher concentrations. Spectra are reported using mean residue ellipticities based on an average amino acid residue molecular weight of 110.[11] Ordering of the unmodified and modified proteins was monitored via changes in the mean residue ellipticity at 198 nm

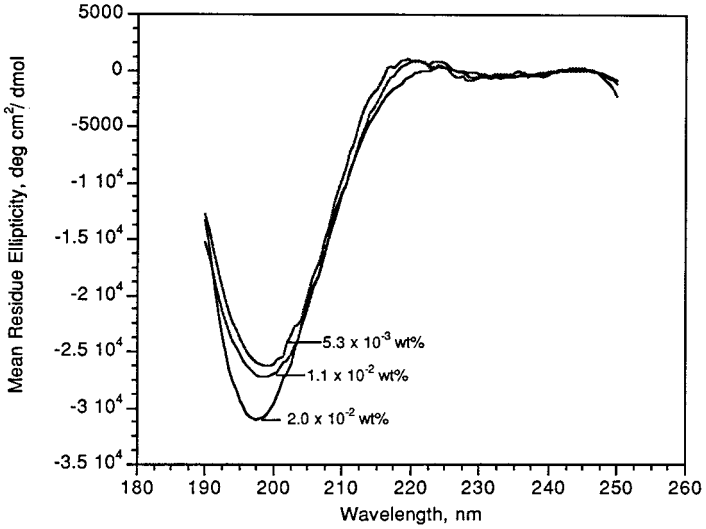


Figure 3 Concentration dependence of the circular dichroism spectrum of gelatin.

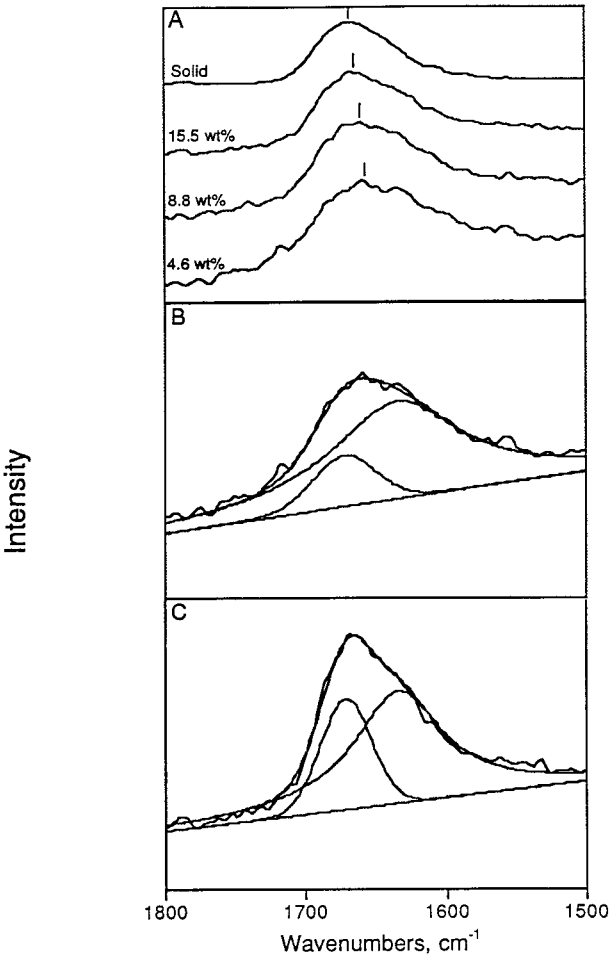


Figure 4 (A) Expanded Raman amide I region of the gelatin spectrum at various gel concentrations. The spectrum of the solid sample is that of the gelatin powder as obtained. (B) Peak contributions to the amide I band for 4.6 wt % gelatin; peaks at 1672 cm^{-1} (18%) and 1637 cm^{-1} (82%). (C) Peak contributions to the amide I band for 15.5 wt % gelatin; peaks at 1672 cm^{-1} (31%) and 1635 cm^{-1} (69%).

as a function of concentration, temperature, time after initial preparation, and chemical modification. Estimates of the triple helix content are based on a mean residue ellipticity of −55,000 deg cm^2/dmol for collagen.[12] Gelatin solutions were prepared by heating the protein in distilled water or 0.15*M* NaF at 40°C for approximately 5 min followed by equilibration to room temperature. Comparisons of ordering in chemically modified samples were conducted after allowing the samples to equilibrate at 5°C overnight. No change in mean residue ellipticity was observed in the samples even after 5 days of storage at 5°C.

Raman spectra (4 cm^{-1} resolution) were recorded using a Bruker FRA 106 spectrometer with a Nd : YAG laser (1064 nm wavelength) as the irradiation source, with a backscattering collection geometry. Intensities of vibrations were calculated using a Lab Calc software package. Amide I bands could be fit using two vibrations, each with a combination of Gaussian and Lorentzian line shapes. Frequency, width at half-height, intensity, and percentage of Gaussian and Lorentzian bands were varied to yield the best fit. Resulting c-square values were on the order of 10^{-4} to 10^{-5}. Gelatin solutions (4.6–15.5 wt %) were prepared by heating the protein in distilled water at 40–60°C for 10 min with equilibration to room temperature for an additional 10 min. Solutions were then allowed to gel in a Raman liquid sample cell at 5°C for 6–8 min, and Raman spectra were subsequently recorded at room temperature. Storage at 5°C for up to 1 week did not result in any significant spectral changes. The addition of sodium azide (0.02%, to prevent bacterial growth) also did not cause any changes in the vibrational spectra.

Proton NMR spectra were recorded using a Bruker AMX 500 NMR spectrometer with proton acquisition at 500 MHz. Twenty-four scans were collected at 22.7°C with a 1-s cycle delay. Presaturation of the water peak eliminated the interfering HDO resonance. Chemical shifts are reported in parts per million (ppm) from 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid (as the sodium salt, TSP), which is also used as an internal peak area reference. Gelatin samples were dissolved in D_2O

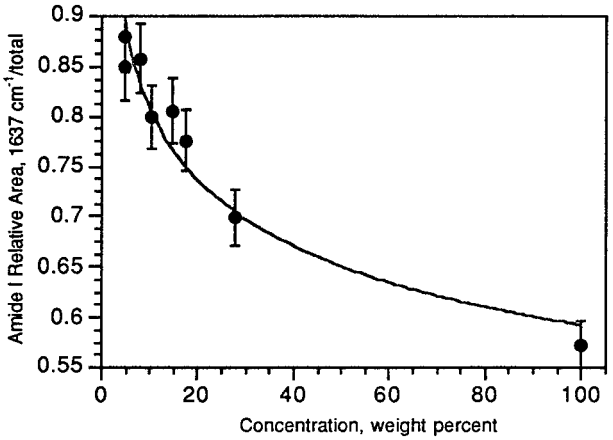


Figure 5 Relative peak area of the 1637 cm^{-1} Raman amide I band of gelatin as a function of concentration. The line represents the fit $y = 1.10\,x^{-0.13}$; $R = 0.96$.

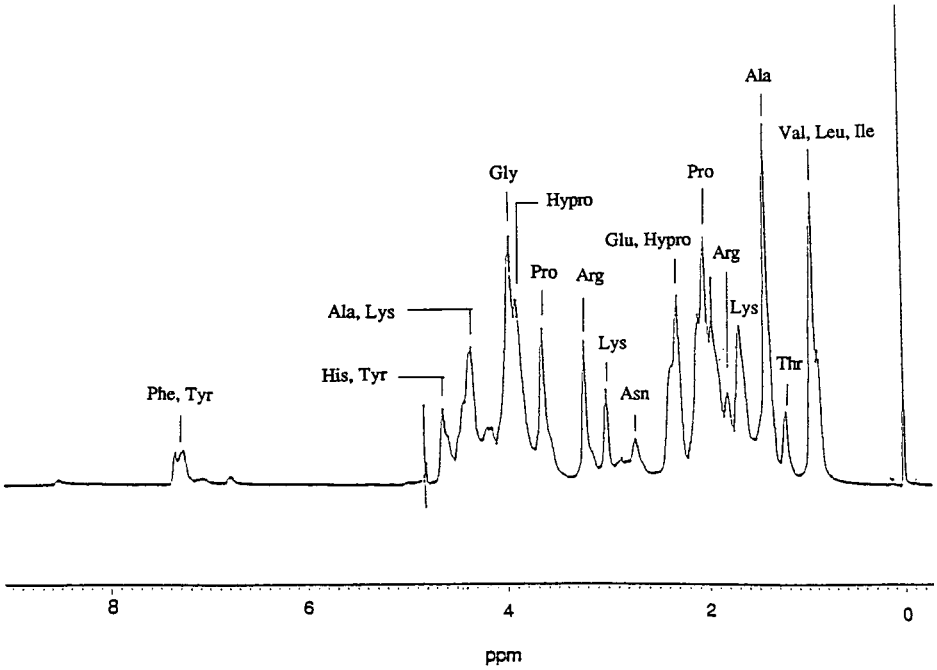


Figure 6 Proton NMR (500 MHz) spectrum of 4.1 wt % gelatin in D_2O.

(0.02% in sodium azide) by gentle heating in a sand bath at 65°C for 1 h to yield final concentrations of 1.8–8.8 wt %. Samples were stored overnight at 5°C prior to spectral acquisition at ambient temperatures. No changes in the spectra were observed with longer storage times. Nuclear magnetic resonance spectroscopy (Bruker AC 200 NMR spectrometer with proton acquisition at 200 MHz) was also used to characterize the chemically modified gelatin samples. Lithium bromide (LiBr) was added to the chemically modified gelatin samples at a ratio of approximately 7 g per g gelatin in D_2O as above to minimize the network formation which decreases gelatin peak intensity. This procedure permitted a more accurate estimate of the degree of substitution and degree of modification (by the crosslinking agent) observed after chemical modification of the gelatin. The spectra were acquired at room temperature immediately after NMR sample preparation.

Chemical Modification of Gelatin

The chemical modification (alkylation) of gelatin using epoxide compounds was conducted using glycidol, glycidyl butyrate, and glycidyltrimethylammonium chloride (Scheme 1 and Table I). Gelatin was dispersed in distilled water at room temperature and then heated to approximately 65°C, yielding a gelatin solution with a concentration of approximately 20 wt % and a pH of 5.5. The epoxide was added dropwise to the stirring solution in varying amounts depending on the desired degree of substitution. (Generally, addition of 1.5 g glycidyltrimethylammonium chloride to 10 g of gelatin yielded a degree of substitution of 0.5%.) The reaction was covered and allowed to stir at 60–70°C

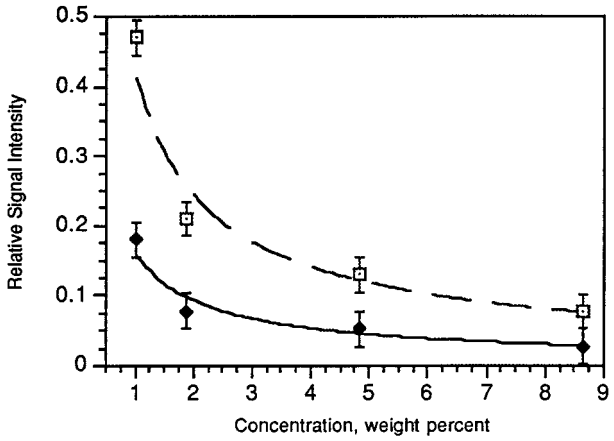


Figure 7 Concentration dependence of NMR signal intensity. The lines are fit according to the following equations: (solid line) Pro, 3.64 ppm, $y = 0.162\,x^{-0.80}$, $R = 0.97$; (dashed line) Gly, 3.96 ppm, $y = 0.423\,x^{-0.79}$, $R = 0.98$.

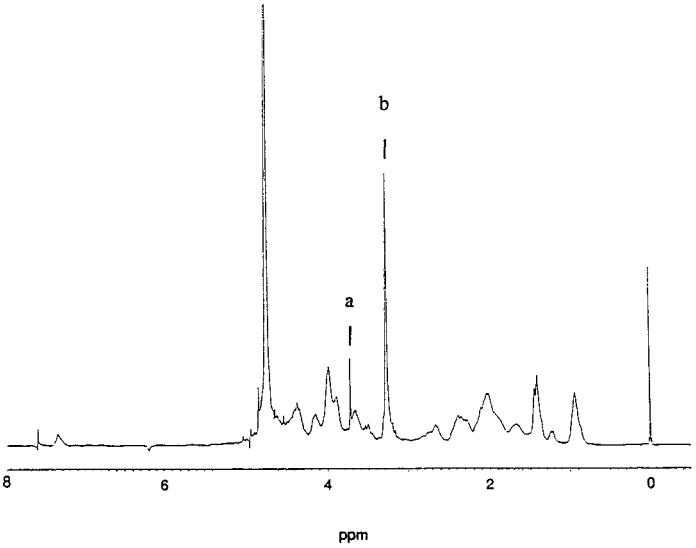


Figure 8 Proton NMR spectrum (200 MHz) of gelatin modified with glycidyltrimethylammonium chloride and Jeffamine® ED900. The resonance at 4.8 ppm corresponds to HDO. Labeled resonances correspond to (a) Jeffamine® ED2001 (CH_2) and (b) glycidyltrimethylammonium chloride (CH_3).

for 1.5 h. During the reaction, the pH of the solution increased from 6.5 to 8.5, consistent with reaction of side-chain carboxylic acid groups. These solutions were either dialyzed as described below and tested directly for changes in ordering and absorbency (CD and NMR) or were reacted with Jeffamine® polyoxyalkyleneamine crosslinking reagents.

A series of Jeffamine® crosslinking reagents (diamines ED2001, ED900, and EDR148; triamine T403; see Fig. 1 for chemical compositions) were added directly to alkylated gelatin prepared as described above (Scheme 2 and Table I). The reactants were stirred at 55°C for approximately 5 min prior to addition of a water-soluble carbodiimide (1-(3-dimethylaminopropyl)-3-ethyl carbodiimide methiodide, Aldrich).[13,14] The Jeffamine® reagents were added first because in most cases addition of the carbodiimide to gelatin solutions prior to the addition of the Jeffamine® nucleophile resulted in immediate gelation of the protein. After carbodiimide addition, the solutions were stirred at 55°C for approximately 1 h. Solution pH values ranged from 9.0 to 11.0. For reactions with over 5 mol % Jeffamine® (based on the total number of amino acid residues), reactions were conducted in 1*M* HEPES buffer with an initial pH of 6.5 (which yielded a reaction pH of 9.0–11.0) in order to minimize hydrolysis of the protein which is accelerated at high pH.[4]

After the reaction, the warm gelatin solution was placed in 12,000–14,000 molecular weight cutoff dialysis tubing (Spectrapor) and dialyzed for at least 48 h against 4–6 300 mL changes of distilled water at 40°C. (Gelatin remains in solution under these conditions.) Dialysis for 48 h was sufficient for removing excess reactant from the

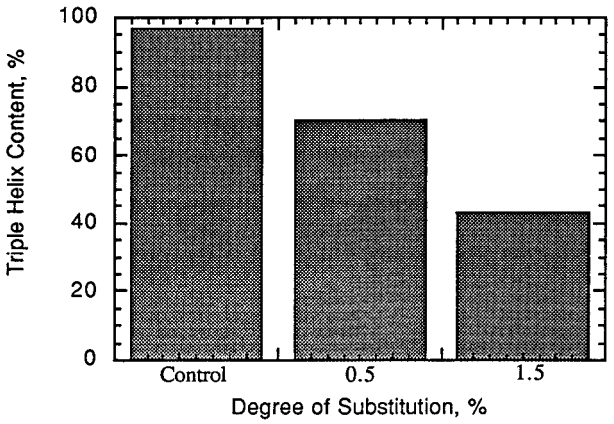


Figure 9 Triple helix content of gelatin (0.02 wt % samples, estimated from mean residue ellipticity at 198 nm) with varying degrees of substitution (Table I, samples 1–4).

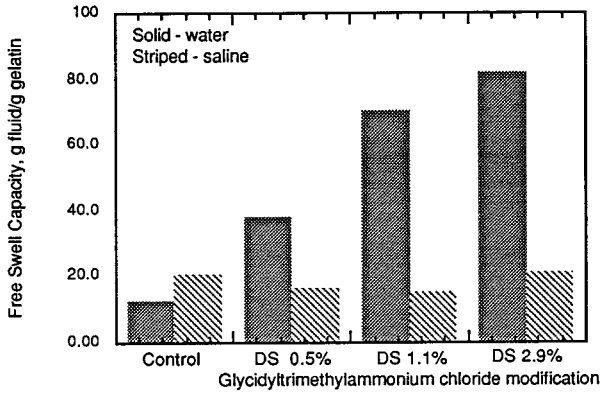


Figure 10 Free swell capacities of control and alkylated gelatins (Table I, samples 1–4) in water and in isotonic saline (0.9% NaCl).

reactant/product mixture as assessed via NMR spectroscopy. The gelatin solutions were stored at 5°C overnight, and the resulting gels were cut into small pieces and dried over P_2O_5 for 12 h under a vacuum or in a fume hood for 48 h. The resulting solids were ground at liquid nitrogen temperatures to a particle size of 300–600 μm and used for free swell capacity and spectroscopic experiments.

NMR spectroscopy allowed estimation of the degree of epoxide substitution and the degree of Jeffamine® modification of the modified gelatin. The methyl protons of glycidyltrimethylammonium chloride (3.28 ppm) and the ethyl protons of the Jeffamine® ethyleneoxide units (3.72 ppm) each have distinct NMR signals which can be used to estimate the degree of substitution and degree of modification, respectively. The glycine/hydroxyproline signal (3.95 ppm) is used to estimate the gelatin content for these calculations, knowing that porcine skin gelatin has 421 glycine and hydroxyproline amino acid residues per 1000 total residues.[4] Degrees of substitution and modification are reported as percentages of the total number of gelatin amino acid residues.

RESULTS AND DISCUSSION

Spectroscopic Characterization of Gelatin

The absorbent capacity of gelatin gels is dependent on the initial gel concentration, with the free swell capacity of the gelatin xerogel an order of magnitude less than that of a 2 wt % gel (approximately 15 versus 200 g/g). It is likely that an increase in ordering during the drying process plays a critical role in this behavior and that the effects of drying might be approximated simply by varying the gelatin concentration. As shown in Figure 2, the free swell capacity varies smoothly as a function of concentration. This concentration dependence is observed regardless of whether the gel is prepared at a specific initial concentration or is dried down to that concentration from a 1.95 wt % gel.

Circular dichroism spectroscopy was used to characterize the conformational ordering as it relates to absorbency. The changes in the CD spectra as a function of concentration are demonstrated in Figure 3 for gelatin at concentrations of 0.005–0.020 wt %. The spectra are consistent with that reported for the collagen triple helix, with a small positive feature at 221 nm and a large negative mean residue ellipticity at 198 nm.[12,15] It is apparent from the spectra that the nature of the protein structure does not change significantly with increasing concentration, but that an increase in order, manifest as an increase in the mean residue ellipticity at 198 nm (which

Table II Triple Helix Content and Swelling of Alkylated, Crosslinked Gelatin with High Degrees of Substitution

Sample	Degree Epoxide Substitution (%)	Degree Jeffamine® Modification[a] (%)	Triple Helix Content (%)	Free Swell, Saline (g/g)
Control	0	0	97	14
6	7.4	0.16	50	41, then dissolves
7	6.7	0.26	44	Dissolves (50 min)
8	9.5	0.41	40	Dissolves (15 min)

[a] Jeffamine® ED2001.

by definition is normalized for differences in concentration[11]), does occur.

Raman spectroscopic studies were also conducted as an alternate means to assess the ordering which occurs in gelatin. The conformation-sensitive amide I band region for gelatin gels of varying concentrations (4.6, 8.8, and 15.5 wt %, and as the obtained powder) is shown in Figure 4 and is consistent with that reported for gelatin,[16] with the amide I band maximum located at approximately 1668 cm^{-1}. As demonstrated in the figure, the amide I band of the protein shifts perceptibly to higher wavenumbers with increasing concentration. This is consistent with the formation of collagen triple helix, as the amide I band of collagen (1670 cm^{-1}) is shifted to slightly higher wavenumbers relative to the gelatin amide I band.[16] Curve fits of the amide I region indicate that the amide I bands of all spectra can be fit to contributions from two peaks: one at approximately 1670 cm^{-1} and another at approximately 1637 cm^{-1} (Fig. 4).

The decrease in the contribution from the band near 1637 cm^{-1} (which arises from hydrogen bonding of water with the amide group) with increasing concentration is shown in Figure 5. The area of this band correlates inversely with the ordering in the protein; a decrease in its area reflects an increase in the ordering of the protein and should correlate with an decrease in absorbency. Indeed, the relative area of this band exhibits a concentration dependence that is qualitatively like that observed for the free swell capacity. The functionally different forms of the data from the swelling and Raman experiments likely result, in part, from differences in experimental conditions; a more detailed comparison between these data sets is therefore not warranted.

NMR spectra of gelatin samples were recorded as functions of concentration to probe for any changes in individual amino acid resonances as the ordering process advances. A 500 MHz proton NMR spectrum of a 4.1 wt % gelatin gel at room temperature is shown in Figure 6; resonances corresponding to individual amino acids are indicated.[17,18] This spectrum is observed for gelatin under all conditions studied (including variations in temperature and time after initial preparation); there is no dependence of chemical shift, line width, or relative intensity on any variable investigated. Plots of signal intensity (relative to TSP) as a function of concentration (1.0, 1.9, 4.8, and 8.7 wt %) for the glycine and proline resonances at 3.96 and 3.64 ppm, respectively (Fig. 7), illustrate the results for all amino acids in the spectrum. The observed concentration dependence matches that observed in absorbency and Raman experiments and further demonstrates the dependence of absorbency on the structure as monitored by these spectroscopic methods. The results also demonstrate that there is no preferential amino acid dependence of the concentration-related loss in resonance intensity. Nevertheless, ordering in gelatin networks (reformation of the collagen triple helix) does limit absorbency. A series of chemical modifications of gelatin was therefore conducted to frustrate the ordering in gelatin and to determine its impact on absorbency.

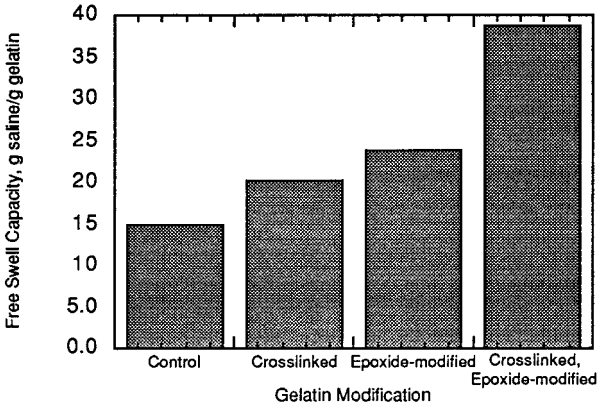


Figure 11 Gelatin absorbency in saline after various chemical modifications (Table I, samples 1–8, 12–13).

Chemical Modification of Gelatin and Resulting Absorbency

Alkylation

Gelatin was covalently modified in order to disrupt folding and network formation and increase the observed free swell capacity of the protein. Initial experiments which varied solution composition (pH, temperature, denaturing salts) and drying conditions (freeze drying, organic precipitation, critical point drying) in an effort to disrupt ordering yielded xerogels with free swell capacities of only 22 g water/g gelatin.[19]

Sterically bulky and/or ionic groups were incorporated into the gelatin chain via the reactions shown in Scheme 1. Modification by each of the epoxides listed in Scheme 1 results in gelatin gels that exhibit greater water retention than does an

Table III Swelling of Alkylated, Crosslinked Gelatins

Sample	Degree Epoxide Substitution (%)	Degree Jeffamine® Modification (%)	Free Swell, Saline (g/g)
9	0.77	1.6[a]	23
10	1.2	0.66[a]	Dissolves
11	1.6	0.34[b]	22

[a] Jeffamine® ED148 crosslinking agent.
[b] Jeffamine® T403 crosslinking agent.

unmodified control, as assessed by the increased time required to dry the modified gelatin under a vacuum. Only glycidyltrimethylammonium chloride causes a measurable change in the absorbency of the protein, however, resulting in water solubility at room temperature. The improvement in water solubility which results from incorporation of quarternary ammonium groups (but not hydroxyl or butyrate groups) may result from increased electrostatic repulsive forces between the added cationic sites and the protonated basic side chains of the protein. The glycidyltrimethylammonium chloride modification was therefore investigated in greater detail.

NMR spectroscopy permitted simple characterization of the product, as the methyl signal from the glycidyltrimethylammonium sites (3.28 ppm) is easily distinguishable from the gelatin resonances (Fig. 8). Degrees of substitution calculated from the NMR spectra as described in the Experimental section are reported as percentages of the total number of gelatin amino acid residues. The NMR spectrum shown in Figure 8 corresponds to a modified gelatin with a calculated degree of substitution of approximately 7%.

Comparison of samples with varying degrees of substitution via CD spectroscopy demonstrates that the ordering in gelatin is significantly reduced by the incorporation of small amounts of glycidyltrimethylammonium chloride and that ordering decreases with increasing degrees of alkylation, as shown in Figure 9. The triple helix content of the modified protein decreases from control values of approximately 97% to values of approximately 75% with degrees of substitution as low as 0.5%. The mean residue ellipticities for the control shown in Figure 9 differ from those shown in Figure 3 due to slight sample preparation and instrumental differences; data are compared only within each set of experimental data.

The observed decrease in ordering does correspond to an increase in absorbency as compared to gelatin controls, as shown in Figure 10. The values reported correspond to a free swell time of 1 h; the alkylated gelatins dissolve in water within 2 h. The maximum free swell observed in water for these modified gelatins was 88 g/g for a gelatin with a degree of substitution of 2.9%. At greater degrees of substitution, samples dissolved within the hour of soaking. The increase in absorbency/solubility is not a result of base hydrolysis of the protein; gelatin heated for 1 h at 70°C at pH 9.0 in the absence of epoxide yields the same free swell values as those of an untreated control gelatin.

As also shown in Figure 10, saline free swell values remain similar to those observed for unmodified gelatin (approximately 20 g/g), independent of the degree of substitution. Even increasing the degree of substitution of the epoxide to 8.6% (which corresponds to alkylation of 85% of the carboxylic acid groups on the protein) does not improve the free swell of the protein in saline. We suggest that the reduction in swelling in saline is due to shielding of the electrostatic repulsion that is likely responsible for the water solubility of the modified protein. The electrostatic screening may, in this case, also limit protein swelling by inducing conformational changes which promote network formation in the protein. In support of this hypothesis, an increase in the triple helix content of alkylated gelatin (from 48 to 67% for a 0.02 wt% solution of gelatin with a degree of substitution of 2.9%) was observed with increasing ionic strength in 0–0.5*M* NaF.

Crosslinking

As a means to improve swelling in saline, a series of nonionic, water-soluble polyoxyalkyleneamine crosslinking agents (Jeffamines®) were added to the alkylated gelatin. Jeffamines® were chosen as modifying agents because of their hydrophil-

Table IV Triple Helix Content and Swelling of Alkylated, Crosslinked Gelatin with Low Degrees of Substitution

Sample	Degree Epoxide Substitution (%)	Degree Jeffamine® Modification (%)	Triple Helix Content (%)	Free Swell, Saline (g/g)
Control	0	0	97	14
1	0.51	0	69	24
5	0	0.13[a]	61	22
12	0.85	0.20[b]	40	32
13	0.70	1.8[b]	42	34

[a] Jeffamine® ED2001 crosslinking agent.
[b] Jeffamine® ED900 crosslinking agent.

icity, nonionic character, steric bulk, and ability to crosslink protein materials via carbodiimide-activated amide bond formation (Fig. 1 and Scheme 2). Jeffamine® crosslinking agents were added to the alkylated gelatins (degrees of substitution ranging from 6.7 to 9.5%) to yield calculated degrees of Jeffamine® modification of 0.14–0.41%, calculated as described in the Experimental section. The modification decreases the triple helix content from the control value of 97% to values of 40–50% (Table II). This reduction in order, as expected, corresponds to an increase in the saline absorbency of gelatin from approximately 22 to 41 g/g in saline, as shown in Table II and Figure 11. The results demonstrate the utility of adding nonionic groups to gelatin to improve the protein absorbency in saline solutions.

However, as shown in Figure 11, modification of gelatin by only nonionic Jeffamine® ED2001 or ED900 (degrees of modification ranging from 0.04 to 0.16%) results in free swell capacities in saline of less than 25 g/g. *Both* alkylation by glycidyltrimethylammonium chloride and modification with Jeffamine® (ED2001 or ED900; results reported are an average of both) are required to improve saline absorbency of gelatin to the approximately 41 g/g maximum observed. It may be that the ionic groups introduced onto the gelatin chain by alkylation disrupt the ordered regions of the protein enough to slightly improve absorbency and that the large, nonionic groups open up the network further after initial hydration. The gelatin modified by both glycidyltrimethylammonium chloride (6.7–9.5%) and Jeffamine® (0.14–0.41%) is soluble, which suggests that no significant crosslinking occurs at these apparent low degrees of modification.

Crosslinking reagents with higher reactivity (EDR148, Fig. 1) or increased number of functional groups (T403, Fig. 1) were also reacted with alkylated gelatin in an effort to prevent saline solubility. As shown in Table III, saline absorbency values of only 23 g/g were observed and dissolution was not prevented. Neither the added reactivity nor the increased functionality appeared to improve the reaction efficiency or the physical properties of the modified protein. Modification with Jeffamine® ED2001 or ED900 yielded similar degrees of modification and free swell values (Table I and Fig. 11). Because the Jeffamine® ED900 is simpler to handle (it is a liquid at room temperature), it was used in experiments designed to test the effect of the degree of epoxide substitution on subsequent crosslinking reaction efficiency and gelatin absorbency.

In an effort to improve absorbency without causing sample dissolution, gelatin was modified with glycidyltrimethylammonium chloride at slightly less than 1% degree of substitution followed by Jeffamine® modification (ED900). Triple helix contents and swelling for gelatin modified in this manner are shown in Table IV. While the triple helix content (40–42%) and degree of substitution (0.18%) in these systems are similar to those observed for the saline soluble, Jeffamine®-modified, alkylated gelatin (Table II), these samples are *not* soluble, which suggests an improved reaction efficiency and successful crosslinking with lower degrees of alkylation. Overall absorbency behavior is improved, with free swell

capacities in saline of 32–34 g/g observed without subsequent dissolution (Table IV). It appears that limiting the amount of glycidyltrimethylammonium chloride used in the first step conserves carboxylic acid sites for the subsequent carbodiimide-activated crosslinking with Jeffamine®. The increased crosslinking plays a key role in improving the absorbency of the crosslinked gelatin; optimization of degrees of alkylation and crosslinking may result in further improvements.

CONCLUSIONS

These studies have demonstrated that absorbency of physically crosslinked gelatin gels correlates inversely with the ordering of the protein, which involves reformation of the collagen triple helix. Furthermore, the absorbency can be readily manipulated through slight modification of the gelatin chain (approximately 0.7% alkylation and 0.18% crosslinking) using very simple reaction schemes. Specifically, alkylation by epoxide ring opening (glycidyltrimethylammonium chloride) followed by modification with polyoxyalkyleneamine (Jeffamines®) has proven successful. Furthermore, while alkylation with the ionic glycidyltrimethylammonium chloride alone causes a significant change in gelatin water absorbency, modification with *both* the glycidyltrimethylammonium chloride and the nonionic Jeffamine® crosslinking agent is required to substantially improve the saline absorbency of the protein. Modification with the bulky nonionic substituent is necessary as electrostatic repulsion is a poor promoter of saline absorbency for gelatin. It is important to alkylate the gelatin at a degree of substitution of less than 1% prior to crosslinking to optimize the observed absorbency and prevent dissolution, indicating the importance of covalent interactions in stabilizing the network for absorbency applications. These basic principles and chemical modification methods may be useful in developing other protein-based absorbent materials.

This work was supported under a collaborative agreement between Kimberly-Clark Corp. and the Center for University of Massachusetts–Industry Research on Polymers at the University of Massachusetts Amherst. NMR spectra were recorded in the University of Massachusetts NMR Facility, which is supported in part by the NSF Materials Research Science and Engineering Center at the University.

REFERENCES

1. R. D. B. Fraser, T. P. MacRae, M. W. K. Chew, and J. M. Squire, in *Fibrous Protein Structure,* J. M. Squire and P. J. Vilbert, Eds., Academic Press, New York, 1987, p. 177.
2. J. Bella, M. Eaton, B. Brodsky, and H. M. Berman, *Science,* **266,** 75 (1994).
3. P. I. Rose, in *Encyclopedia of Polymer Science and Engineering,* H. F. Mark, N. M. Bikales, C. G. Overberger, and G. Menges, Eds., Wiley, New York, 1987, Vol. 7, p. 505.
4. A. G. Ward and A. Courts, Eds., *The Science and Technology of Gelatin,* Academic Press, New York, 1977.
5. R. E. Merrill, U.S. Pat. 3,207,613 (1965).
6. J. W. Gates, W. G. Lovett, and P. E. Miller, U.S. Pat. 3,184,312 (1963).
7. H. Akin and N. Hasirci, *J. Appl. Polym. Sci.,* **58,** 95 (1995).
8. M. M. Welz and C. M. Ofner, *J. Pharm. Sci.,* **81,** 85 (1992).
9. P. R. Chatterji, *J. Appl. Polym. Sci.,* **37,** 2203 (1989).
10. L. Greenspan, *J. Res. Natl. Bur. Stand.,* **81A,** 89 (1977).
11. T. E. Creighton, Ed., *Protein Structure: A Practical Approach,* IRL Press, New York, 1989.
12. T. Hayashi, S. Curran-Patel, and D. J. Prockop, *Biochemistry,* **18,** 4182 (1979).
13. K. L. Carraway and D. E. Koshland, *Methods Enzymol.,* **25,** 616 (1972).
14. J. C. Sheehan and J. J. Hlavka, *J. Am. Chem. Soc.,* **79,** 4528 (1957).
15. R. Wetzel, E. Buder, H. Hermel, and A. Huttner, *Colloid Polym. Sci.,* **265,** 1036 (1987).
16. B. G. Frushour and J. L. Koenig, *Biopolymers,* **14,** 379 (1975).
17. P. Rose and S. Gross, *J. Photogr. Sci.,* **23,** 59 (1975).
18. K. Wuthrich, *NMR of Proteins and Nucleic Acids,* Wiley, New York, 1986.
19. R. T. Shet, personal communication.