Chemical and Swelling Evaluations of Amino Group Crosslinking in Gelatin and Modified Gelatin Matrices

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Purpose. To determine the extent of amino group crosslinking in gelatin matrices by chemical assay, and to compare these results to crosslinking evaluations from swelling measurements.

Methods. Matrices crosslinked with a water soluble carbodiimide (EDC/G), glutaraldehyde (GTA/G), as well as a GTA crosslinked matrix prepared from gelatin modified to contain 230% greater crosslinking sites (GTA/Mod) were evaluated. Crosslinking extent, X_c, was determined by a UV assay of uncrosslinked amino groups before and after crosslinking, and was used to obtain crosslinking densities. Equilibrium swelling ratios, Q_m, at 37°C in isotonic pH 7.4 were used to calculate crosslinking degree from the Flory equation for swelling of ionic polymers for comparison to the chemically determined crosslinking densities.

Results. Of the original 33×10^{-5} moles ϵ-amino groups/g gelatin, 91 to 95% were crosslinked in EDC/G and GTA/G. GTA/Mod lost 95% of the original 108×10^{-5} moles amino groups/g gelatin. Crosslinking densities were 4.1×10^{-4} and 4.2×10^{-4} moles/mL for EDC/G and GTA/G, respectively. The value for GTA/Mod increased to 14.2×10^{-4} moles/mL. Values of Q_m followed the same trend. The Flory crosslinking degrees for both gelatin matrices were 12×10^{-4} and 13×10^{-4} moles/mL, respectively. The value for the more extensively crosslinked GTA/Mod was 280×10^{-4} moles/mL.

Conclusions. The swelling and chemical evaluations of crosslinking are in general agreement for matrices with the lower of two crosslinking levels. The chemical determination appears suitable for evaluating amino group crosslinking in gelatin and it may be suitable for other proteinaceous materials.

KEY WORDS: gelatin crosslinking; crosslinking determination; crosslinking densities; gelatin swelling; equilibrium swelling ratios; Flory equation for swelling of ionic polymers; Flory crosslinking degree.

INTRODUCTION

Proteinaceous materials such as albumin (1), collagen (2), and gelatin (3), have been investigated as biodegradable carriers for implantable drug delivery. Covalent crosslinking is important in these systems because it is usually required during preparation, it changes important properties such as solubility and density, and because it has the potential to control release of macromolecular therapeutic agents such as peptides, proteins, and oligonucleotides. In spite of its broad use, there are few direct, accurate and reliable measures of crosslinking available. Most evaluations of covalent crosslinking in macromolecules are based on the amount, or duration of exposure to the crosslinking agent, or on indirect physical measurements of the crosslinked material. An approach that is more direct and has the potential for quantitative determination is to follow loss of the functional group participating in the crosslinking process.

Gilbert and Kim (2,4) were among the first to follow loss of amino groups as a measure of crosslinking in collagen matrices for drug delivery. Several modifications of a basic procedure have been used to determine amino groups in proteins (5–7). A recent modification (8) is used in the current investigation to assay uncrosslinked amino groups and subsequently to determine crosslinking extent in gelatin matrices. Matrices crosslinked with two different crosslinking agents are evaluated. A third matrix prepared from gelatin modified to contain substantially higher levels of crosslinking is also evaluated.

Swelling measurements have been used to evaluate crosslinked polymers since the introduction of the Flory-Rehner model of equilibrium swelling (9). The Flory swelling equation for ionic polymers (10) has been modified to account for covalent crosslinking in solution (11,12). Additional modifications to this equation have been reported to describe ionic polymer swelling, but they are based on specific assumptions and involve cumbersome mathematics (13). The swelling equation accounting for solution crosslinking is expected to provide reasonable accuracy for the swelling conditions in this investigation. Crosslinking degrees from the Flory swelling equation for ionic polymers can be calculated for an independent comparison to chemically determined crosslinking densities.

MATERIALS AND METHODS

Type B USP granular gelatin from Rousselot (Paris, France; Lot 10287) was used without further purification. This gelatin contains 33×10^{-5} moles ϵ-amino groups/g (8) on the lysine and hydroxylysine residues, and contains approximately 126×10^{-5} moles carboxylic acid groups (14) on aspartic and glutamic acid residues. The moisture content of the granules was approximately 9.5% w/w. The pH of a 1% (w/v) solution was 5.8, and the isoionic point (pI) was 5.2 (3). Trinitrobenzenesulfonic acid (TNBS; solid, ≥98% pure) was purchased from Pierce (Rockford, IL). Ethylenediamine (ED; liquid free base, ≥98% pure) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC; solid, ≥99% pure) were purchased from Sigma (St. Louis, MO). Glutaraldehyde (GTA; 25% in water, certified) was purchased from Fisher Scientific (Fairlawn, NJ). All other reagents were ACS reagent grade. Water was purified by reverse osmosis.

Preparation of Crosslinked Gelatin Matrices

Five grams of gelatin granules, or modified gelatin, were dissolved in warm water and crosslinked with an amount of GTA to produce a 10:1 molar ratio of crosslinking agent to gelatin ϵ-amino groups or to this amount plus new amino groups in modified gelatin (see below). Gelatin concentration at this point was 10% w/v. The resulting gels were allowed to cool and crosslink for 4 hours. The gels were cut into disc matrices (14 mm diameter, 5 mm height) then washed with 4°C water for 7 hours by which time GTA was negligible (15). Matrices were dried at 7% relative humidity for 7 days at room temperature, and stored in closed vials inside a desiccator until used.

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EDC crosslinked matrices were prepared as above with the following differences: 8.0 g of gelatin was used, and one hour of crosslinking time was used. Also, the crosslinked gel was cut into small pieces, washed for 72 hours, dried at ambient conditions for 48 hours, and stored in a desiccator for later use.

Preparation of Modified Gelatin

Gelatin carboxylic acid groups were reacted with ED in the presence of EDC to produce amino groups by modifying previously published methods (16,17) as shown in Figure 1. Briefly, 6.0 g of gelatin were dissolved in warm water and mixed with a 100:1 molar excess of ED to the amount of EDC to be used. The mixture was adjusted to pH 5 with 6 N HCl. An 18:1 molar excess of EDC to gelatin carboxylic acid groups, dissolved in 15 mL of water, was added and the reaction was continued for 24 hours while pH 5 was maintained with HCl. The resultant dark orange modified gelatin solution was dialyzed (Spectar/Por molecular porous dialysis membrane; MW cutoff 1000) for 48 hours at 4°C. The dialysate was lyophilized to a white fibrous amorphous solid (Virtis Unitop 200 freeze dryer) and stored in a desiccator until used.

Determination of Amino Groups in Gelatin and Modified Gelatin Matrices

A previously reported assay was used to determine the number of uncrosslinked ϵ-amino groups in the crosslinked gelatin and modified gelatin matrices (8). Briefly, 11 mg of gelatin was mixed with 1 mL of 4% $NaHCO_3$ and 1 mL of 0.5% TNBS, and heated at 40°C for 4 hours. Three mL of 6 N HCl were added and the mixture was autoclaved for 1 hour at 120°C and 15–17 psi. The hydrolysate was diluted with 5 mL of water, then extracted with ethyl ether. A 5 mL aliquot of the aqueous phase was removed from each sample and heated for 15 minutes in a hot water bath, cooled to room temperature, and diluted again with 15 mL of water. The absorbance was measured at 346 nm in a Perkin-Elmer model 552A double beam spectrophotometer against a blank. Four or five replicates were used in each determination. Blanks were prepared in triplicate by the same procedure as above except that HCl was added before TNBS to prohibit reaction of the gelatin amino groups with TNBS.

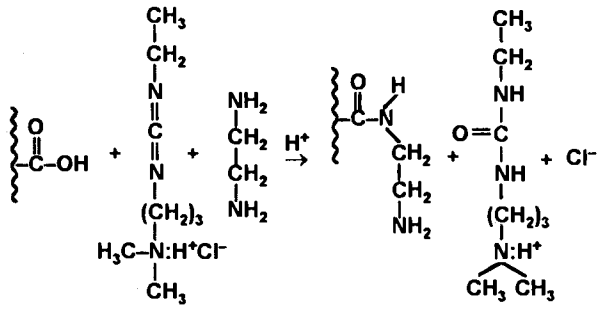

Fig. 1. Formation of modified gelatin containing enhanced number of amino group crosslinking sites by reacting gelatin carboxylic acid groups with ethylenediamine using the water soluble carbodiimide, EDC.

Results were obtained by converting absorbance values to moles ϵ-amino groups per gram gelatin using Eq. 1:

$$\frac{\text{moles } \epsilon\text{-amino groups}}{g \text{ gelatin}} = \frac{2(\text{Absorbance})(0.020L)}{(1.46 \times 10^4 \text{ L/mole·cm})(b)(x)} \quad (1)$$

where 1.46×10^4 L/mole·cm is the molar absorptivity of TNP-lys, b is the cell path length in cm, and x is the sample weight in grams. The accuracy and precision of this assay were previously evaluated on insulin, bovine albumin, and granular gelatin; the determinations differed from literature values by ≤3.3%. The coefficient of variation for these determinations was ≤5.3% (8).

The above procedure was modified to determine the extent of carboxylic acid modification to amino groups because the original procedure led to precipitation of the trinitrophenyl labeled material. Smaller sample concentrations than previously evaluated, ranging from 0.0249 to 0.0512 mg/mL of modified gelatin, were assayed in 1 mL of 4% $NaHCO_3$. Plots of these sample absorbances as a function of concentration were linear but yielded a substantial non-zero intercept. The intercept was subtracted from absorbance in Eq. 1 to calculate the number of residue amino groups per gram of modified gelatin for each sample. The accuracy of this procedure was verified using insulin, bovine albumin, and gelatin; the assay values were within 1% of the literature values (18).

Physical Measurements

Density

Densities of matrix polymers and crosslinked matrices were evaluated using a Quanta Chrome micropycnometer (model MPY-1). Moisture contents ranging from 7.85 to 11.4% (w/w) were used to calculate anhydrous density. Densities of the matrix polymers, ρ_p, were 1.369 and 1.374 g/mL for gelatin and modified gelatin, respectively.

Swelling

Swelling of the crosslinked matrices was evaluated in 0.069 M phosphate bufferred saline (PBS), pH 7.4, at 37°C, with shaking at 100 rpm in a rotary shaker bath. Size changes (height, diameter) were measured manually until maximum swelling was reached by about five hours. Equilibrium swelling volumes were determined after 72 hours swelling with a liquid pycnometer at 37°C by the displacement of toluene using Eq. 2:

$$V_S = \frac{W_{mt} - W_t}{\rho_t} \quad (2)$$

where V_s is volume of the swollen matrix, W_{mt} is weight of the swollen matrix and toluene, W_t is weight of toluene, and ρ_t is density of toluene. A check for dissolved gelatin using a BCA protein assay verified that no gelatin was extracted out of the swollen matrices. The equilibrium swelling ratio, Q_m, was calculated from:

$$Q_m = \frac{V_S}{V_p} = \frac{V_S \rho_p}{W_p} = \frac{1}{v_{2m}} \quad (3)$$

where V_p is the unswollen matrix polymer volume (gelatin or

modified gelatin), W_p and ρ_p are the anhydrous weight and density of the matrix polymer, and v_{2m} is the volume fraction of the matrix polymer at maximum or equilibrium swelling.

Moisture Content

The loss of sample weight after heating at 105°C for 72 hours was used to calculate moisture content.

RESULTS AND DISCUSSION

Feasibility of Chemical Crosslinking Determination

The reaction of TNBS with primary amino groups of gelatin is used to determine uncrosslinked groups in the crosslinked matrices. The number of groups lost to crosslinking is the difference between the chemically determined number of uncrosslinked groups before and after crosslinking. An accurate determination will depend on virtually all uncrosslinked groups participating in the TNBS reaction. While crosslinked gelatin usually does not dissolve in aqueous solutions at room temperature, it swells to produce a large volume fraction of solvent which would favor complete reaction of the uncrosslinked amino groups. Two preliminary experiments, however, were conducted to corroborate this expectation, and demonstrate feasibility of the chemical determination procedure. EDC, a water soluble carbodiimide, was used as the crosslinking agent because it specifically crosslinks the amino and carboxylic acid groups of proteins into an amide (or peptide) bond as shown in Figure 2. GTA, by comparison, has some reactivity with groups besides amino groups, adds some distance as a spacer between participating functional groups, and has an incompletely understood mechanism (19).

In the first preliminary experiment, TNBS reaction time of the amino group assay was evaluated on EDC crosslinked gelatin. Figure 3 shows assay values after TNBS reaction times of 3–6 hours. The latter values from 4–6 hours have no statistically significant difference and indicate that the standard 4 hour reaction time is sufficient for completion of the TNBS reaction. In addition, the assay values during this time indicate that the amide crosslinks do not degrade under the TNBS reaction conditions. The dashed horizontal line represents the number of amino groups in gelatin before EDC crosslinking.

For the second feasibility experiment, the EDC crosslinked gelatin was exposed to 105°C at a reduced pressure of 10 μm Hg for 5 days to produce a more insoluble material that may further reduce availability of the uncrosslinked groups for reaction with TNBS. The assay values of uncrosslinked ϵ-amino groups per g gelatin before and after oven heating were 3.1×10^{-5} and 3.4×10^{-5} moles/g gelatin, respectively. The SD of these values were 0.4×10^{-5} and 0.2×10^{-5}, respectively. These assay values are virtually identical which indicates the same availability of the uncrosslinked amino groups. These preliminary experiments support the assumption of near or complete determination of uncrosslinked amino groups in these crosslinked matrices.

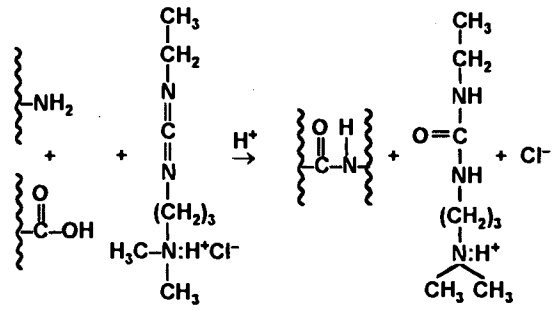

Fig. 2. EDC crosslinking in gelatin to form an amide bond between carboxylic acid and amino groups.

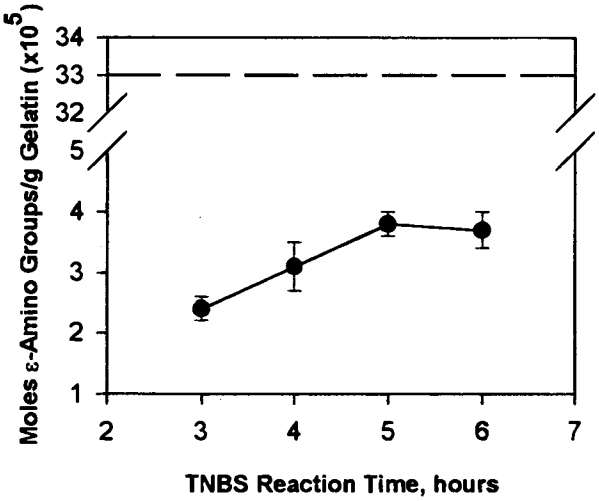

Fig. 3. Determination of time for complete reaction of TNBS with uncrosslinked amino groups in poorly soluble EDC crosslinked gelatin. Dashed line represents the experimentally determined ϵ-amino groups before crosslinking. Each point is the mean of 4–5 determinations; bars represent ± SD.

Crosslinking Evaluations from Chemical Determinations

Table I lists the determinations of uncrosslinked amino groups for the evaluation of EDC and GTA crosslinked gelatin and modified gelatin matrices. The tabulated values have units of moles amino groups/g crosslinked gelatin or modified gelatin ($\times 10^5$) and correspond to the number of groups on one molecule of 1000 amino acid residues for an ideal gelatin molecular weight of 100,000. Values for the modified gelatin represent the original ϵ-amino groups plus the amino groups from the ED converted carboxylic acids (see Figure 1). The determinations in all three crosslinked matrices indicate a loss of 91 to 95% amino groups after crosslinking. The lost amino groups in these determinations represents the extent of crosslinking. Determination of EDC crosslinking is believed to be quantitative because each lost amino group participates in a crosslink. Evaluations of GTA crosslinking, however, are semi-quantitative because each crosslink might contain one or more GTA molecules (19), GTA might react with only one amino group and not complete the crosslink, and GTA might react with non-amino groups.

Table II lists the chemically determined and related crosslinking parameters of the three crosslinked matrices. The number of amino groups lost to crosslinking, or crosslinking extent, is designated X_c, and is listed for each of the matrices. It represents the moles of crosslinks per g of matrix gelatin or modified gelatin. Under these conditions of excess crosslinking agent, crosslinking extent is similar and near the theoretical maximum

Table I. Determination of Uncrosslinked Amino Groups Before and After Covalent Crosslinking in Gelatin and Modified Gelatin Matrices

Before or After Crosslinking	Gelatin Crosslinked with EDC[b]	Gelatin Crosslinked with Glutaraldehyde	Modified Gelatin[a] Crosslinked with Glutaraldehyde
Before	33.0 ± 0.8 (4)[c]	33.0 ± 0.8 (4)	108 ± 3 (4)[d]
After	3.09 ± 0.44 (5)	1.6 ± 0.0 (5)	5.35 ± 0.46 (5)

[a] Increased number of amino groups by modifying carboxylic acid groups to amino groups using ethylenediamine in the presence of EDC; see text.
[b] 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.
[c] Value ± SD (n), units are moles amino groups/g material ($\times 10^5$). Values represent number of amino groups per molecule of 1000 amino acid residues; ϵ-amino groups determined in gelatin.
[d] Value is total of original ϵ-amino groups plus new amino groups.

for EDC and GTA crosslinked gelatin matrices. The crosslinking extent in modified gelatin is also near maximum, but this value is more than three-fold greater than the values for EDC and GTA crosslinked gelatin. The molecular weight between crosslinks, M_c, has been used as a crosslinking parameter; it varies inversely with the extent of crosslinking. Estimates of this parameter are listed in Table II from calculations using X_c and M, the average gelatin molecular weight (M cancels out when units of X_c are converted to moles gelatin using M).

$$M_c = \frac{M}{X_c} \approx \frac{1}{X_c} \tag{4}$$

At low degrees of crosslinking this value can be 10,000 or more. The values for EDC and GTA crosslinked gelatin matrices are both approximately 3300. The M_c value for the extensively crosslinked modified gelatin matrix is approximately 1000. Density measurements of the crosslinked matrices follow the same trend as the other chemically determined parameters in Table II.

A crosslinking density of the polymer in the unswollen matrix can be obtained using crosslinking extent and matrix polymer density, ρ_p. Crosslinking density, ρ_c, is calculated from the product $(X_c)(\rho_p)$. It has units of moles crosslink/mL of unswollen matrix polymer. As expected from the similar crosslinking extents, the crosslinking densities in Table II are virtually the same for the EDC and GTA crosslinked matrices. The value for the extensively crosslinked modified matrix is more than three-fold greater.

Crosslinking Evaluations from Swelling Measurements

Table III contains swelling measurements and related parameters as an independent evaluation of crosslinking. The equilibrium swelling ratios, Q_m for each of the crosslinked matrices are listed and corroborate the chemically determined values of X_c. Both parameters have similar values for the EDC and GTA crosslinked gelatin matrices, and both have a substantially different value for the more extensively crosslinked modified gelatin matrices.

The Flory swelling equation for ionic polymers was derived for isotropic equilibrium swelling of an amorphous, ionic, tetrafunctionally crosslinked polymer (10). Adjusting this equation for crosslinking in solution (11) produces:

$$V_1\Delta c_j = [\ln(1 - v_{2m}) + v_{2m} + \chi_1 v_{2m}^2] + V_1(\nu_e/V_o)v_{2o}\left[\left(\frac{v_{2m}}{v_{2o}}\right)^{1/3} - \left(\frac{v_{2m}}{2v_{2o}}\right)\right] \tag{5}$$

where Δc_j is the concentration difference of mobile ions inside and outside the swollen gel, V_1 is the molar volume of the solvent, v_{2m} is the volume fraction of polymer in the swollen network at equilibrium or maximum swelling, χ_1 is the Flory-

Table II. Measured and Calculated Parameters Used in Chemical Evaluation of Covalent Crosslinking in Gelatin and Modified Gelatin

Parameter	Gelatin Crosslinked with EDC[b]	Gelatin Crosslinked with Glutaraldehyde	Modified Gelatin[a] Crosslinked with Glutaraldehyde
Crosslinking Extent[c], X_c, ($\times 10^5$) moles/g	30	31	103
Molecular Weight Between Crosslinks[d], M_c ($\times 10^{-3}$)	3.3	3.2	0.97
Matrix Density, g/mL	1.32	1.29	1.39
Crosslinking Density[e], ρ_c, ($\times 10^4$) moles/mL	4.1	4.2	14.2

[a] Number of amino groups increased by modifying carboxylic acid groups to amino groups using ethylenediamine in the presence of EDC; see text.
[b] 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.
[c] Amino groups lost to crosslinking; see Table I.
[d] From Equation 4.
[e] Product of crosslinking extent and matrix polymer density; see text.

Table III. Measured and Calculated Parameters Used in Swelling Evaluation of Covalent Crosslinking in Gelatin and Modified Gelatin

Parameter	Gelatin Crosslinked with EDC[b]	Gelatin Crosslinked with Glutaraldehyde	Modified Gelatin[a] Crosslinked with Glutaraldehyde
Equilibrium Swell Ratio, Q_m	7.54 + 0.32 (5)[c]	8.01 ± 0.33 (4)	4.10 ± 0.17 (5)
Polymer Volume Fraction at Equilibrium Swell, v_{2m}	0.133	0.125	0.244
Matrix Polymer Net Charge[d], ($\times 10^5$), moles/g	−23	−54	19
Matrix Polymer Charge Concentration, c_p, ($\times 10^5$), moles/mL	4.2	8.8	6.5
Swelling Medium Mobile Ion Concentration, c_e, ($\times 10^4$) moles/mL	1.5	1.5	1.5
Mobile Ion Concentration Difference[e], Δc_j, ($\times 10^5$) moles/mL	0.29	1.3	0.70
Flory Crosslinking Degree[f], v_e/V_o, ($\times 10^4$), moles/mL	12	13	280

[a] Number of amino groups increased by modifying carboxylic acid groups to amino groups using ethylenediamine in the presence of EDC; see text.
[b] 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.
[c] Value ± SD (n).
[d] From Ref. 21 and adjusted for loss of crosslinked ionic groups; see text.
[e] From Equation 10.
[f] From Equation 6.

Huggins polymer-solvent interaction parameter (dimensionless), v_e is the moles of elastically effective crosslinked chains in the network, V_o is the volume of the unswollen network, and v_{2o} is the polymer volume fraction at network formation, i.e. at crosslinking in solution. The term v_e/V_o represents the concentration of crosslinked active chains which Flory designated as crosslinking degree. Flory pointed out that this parameter will vary somewhat from a true crosslinking degree because it will be influenced by entanglements and dangling terminal chains.

Equation 5 was derived from a phase equilibrium model which at equilibrium swelling equates the reduction of the chemical potential of the solvent inside the gel with the reduced chemical potential of the solvent outside the gel. The reduced chemical potential of the solvent inside the swollen gel is divided into three terms which represent the changes due to the mixing of polymer and solvent, the elastic deformation of the polymer network, and the mixing of mobile ions and solvent. The left-hand side of Eq. 5 represents the ionic contribution and it is absent for nonionic polymers. Eq. 5 is solved for crosslinking degree, v_e/V_o, as:

$$v_e/V_o = \frac{V_1\Delta c_j - [\ln(1 - v_{2m}) + v_{2m} + \chi_1 v_{2m}^2]}{V_1 v_{2o}[(v_{2m}/v_{2o})^{1/3} - (v_{2m}/2v_{2o})]} \quad (6)$$

Crosslinking degree then can be calculated given the additional values of $\chi_1 = 0.495$ for isoelectric gelatin (22), 18 mL/mole of water for V_1, the experimentally determined values of v_{2m} listed in Table III, an estimate of 0.1 for v_{2o}, and the appropriate values of Δc_j for each of the swollen gels.

The concentration difference of mobile ions, Δc_j, is driven by Donnan equilibria (20), and is dependent on the polyampholyte net charge and the mobile ion concentration in the swelling medium. At equilibrium swelling the mobile ion distribution produces an internal excess of mobile ions, or Δc_j. An expression to calculate Δc_j can be derived in which c_e represents the initial mobile ion concentration in the swelling medium, c_p represents the polyampholyte net charge concentration, and x represents the mobile ions that have migrated from the swelling medium into the gel at equilibrium (concentration in the swelling medium changes negligibly). This equilibrium state can be represented below using NaCl as the swelling medium ions and P as an anionic polyampholyte:

Inside the Gel			Outside the Gel	
P^-	Na^+	Cl^-	Na^+	Cl^-
c_p	$c_p + x$	x	c_e	c_e

The ion activity product for the mobile ions is constant inside and outside the gel (20):

$$(a_{Na_+})_i(a_{Cl\_})_i = (a_{Na_+})_o(a_{Cl\_})_o \quad (7)$$

The above concentration terms can be substituted for their respective activities to produce a quadratic equation of x describing the concentration of mobile ions migrating into the gel:

$$x^2 + c_p x - c_e^2 \quad (8)$$

The difference between the mobile ion concentrations inside the gel and outside the gel at equilibrium (from the schematic above) is:

$$\Delta c_i = [(c_p + x) + x] - [c_e + c_e] \quad (9)$$

Combining equations 8, 9, and simplifying produces an expression for the concentration difference of mobile ions inside and outside the swollen gel:

$$\Delta c_j = (c_p^2 + 4c_e^2)^{1/2} - 2c_e \quad (10)$$

The value of c_p for the crosslinked gelatin or modified gelatin matrix is calculated by (net charge/g polymer)(g polymer in matrix)/equilibrium swelling volume of matrix. Table III lists the net charges and the values used to calculate Δc_j in Eq. 10 for the respective matrices. The net charges are from titration of a similar gelatin (21) and adjustment for the appropriate loss of ionic groups from crosslinking. The isotonic concentration of NaCl can be used in place of the ion concentrations in PBS for the external mobile ions without significant loss of accuracy.

The values of the Flory crosslinking degree for each of the matrices are listed in Table III. The values for EDC and GTA crosslinked gelatin are nearly equal. A previously reported Flory crosslinking degree for gelatin was calculated without adjustment for solution crosslinking (22). Calculating the current values in the same manner produces results nearly identical to the earlier value. The crosslinking degree for the more extensively crosslinked modified gelatin is ~22 times greater than the unmodified gelatin matrices. These swelling determined crosslinking degrees offer an inexact, but useful, comparison to the chemically determined crosslinking densities. The swelling determined Flory crosslinking degrees follow the same trend as the chemically determined crosslinking densities in Table II. The Flory crosslinking degree values for EDC and GTA crosslinked gelatin are 3-fold greater than the corresponding values of the chemically determined crosslinking densities. The swelling determined value for the extensively crosslinked modified gelatin, however, is 20 times greater than the chemically determined value. Possible explanations for the greater swelling determined values include entanglements which act as crosslinks, and errors in the estimate of the polymer volume fraction during solution crosslinking.

It should be noted that the more convenient nonionic form of Equation 6 can be used in some conditions of ionic polymer swelling. The calculated value of the Flory crosslinking degrees without the ionic contributions are 5.6%, 23%, and 2.1% lower than the values for EDC, GTA, and the modified crosslinked matrices, respectively, listed in Table III. The error introduced by using the nonionic form increases as the absolute value of the matrix net charge increases.

Insight into crosslinking mechanisms might be gained using both chemical and swelling evaluations. One GTA molecule reacting between two amino groups in the gelatin matrix would produce an average 15.5 crosslinks per molecule from the 31 lost amino groups per molecule. EDC crosslinking, by comparison, produces an average 30 crosslinks per molecule from its 30 lost amino groups per molecule. This crosslinking difference would produce substantially different swelling, but both EDC and GTA crosslinked matrices swelled to similar extents producing similar calculated crosslinking degrees. Therefore, the single GTA molecule crosslinking mechanism is not supported by the swelling results. A GTA crosslinking mechanism which produces one crosslinking for each lost amino group, however, is supported by the swelling results. Such a mechanism is plausible considering the potential of GTA to react with other functional groups, the 10-fold molar excess of GTA, and the incompletely understood mechanism of GTA crosslinking in proteins (19).

CONCLUSIONS

Amino group crosslinking in gelatin matrices has been measured by chemical and swelling methods. The chemically determined crosslinking extent was 95% of the theoretical maximum for gelatin matrices crosslinked with a water soluble carbodiimide and with glutaraldehyde. Gelatin matrices modified to substantially increase the number of crosslinking sites were also measured at 95% crosslinking, but the absolute crosslinking extent was more than three-fold greater than the lesser crosslinked matrices. Molecular weight between crosslinks and crosslinking densities were also calculated. Equilibrium swelling ratios, as an independent evaluation, followed the same trends as the chemically determined crosslinking parameters. Values for a crosslinking degree term from the Flory equation for swelling of ionic polymers were obtained that are in general agreement with the chemically determined values of crosslinking density for the lessor of the two crosslinking levels. The chemical determination appears suitable for evaluating amino group crosslinking in gelatin. Since the chemical assay has been used on albumin and insulin, this procedure may also be suitable for evaluations in other proteinaceous materials. Using both chemical and swelling evaluations may provide insight into crosslinking mechanisms.

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REFERENCES

1. O. P. Rubino, R. Kowalsky, and J. Swarbrick. *Pharm. Res.* **10**:1059–1065 (1993).
2. D. L. Gilbert, T. Okano, T. Miyata, and S. W. Kim. *Int. J. Pharm.* **47**:79–88 (1988).
3. M. M. Welz and C. M. Ofner III. *J. Pharm. Sci.* **81**:85–90 (1992).
4. D. L. Gilbert and S. W. Kim. *J. Biomed. Mat. Res.* **24**:1221–1239 (1990).
5. A. F. Habeeb. *Anal. Biochem.* **14**:328–336 (1966).
6. R. Fields. In C. H. W. Hirs and S. N. Timasheff (eds.), *Methods of Enzymology*, Academic Press, New York, 1972, Vol. 25, pp. 464–468.
7. A. K. Hazra, S. P. Chock, and R. W. Albers. *Anal. Biochem.* **137**:437–443 (1984).
8. W. A. Bubnis and C. M. Ofner III. *Anal. Biochem.* **207**:129–133 (1992).
9. P. J. Flory and J. Rehner, Jr. *J. Chem. Phys.* **11**:521–526 (1943).
10. P. J. Flory. *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, p. 591 (1953).
11. J. C. Bray and E. W. Merrill. *J. Appl. Polym. Sci.* **17**:3781–3796 (1973).
12. L. Brannon-Peppas and N. A. Peppas. *J. Contr. Rel.* **16**:319–330 (1991).
13. W. Oppermann. In R. S. Harland and R. K. Prud'homme (eds.), *Polyelectrolyte Gels-Properties, Preparation, and Applications*,

American Chemical Society, Washington DC, pp. 159–170 (1992).
14. A. Veis. *The Macromolecular Chemistry of Gelatin*, Academic Press, New York, 1964, chapter 1.
15. J. Boratynski and T. Zal. *Anal. Biochem.* **184**:259–262 (1990).
16. D. G. Hoare and D. E. Koshland. *J. Biol. Chem.* **242**:2447–2453 (1967).
17. M. R. Mejillano and R. H. Himes. *J. Biol. Chem.* **266**:657–664 (1991).
18. W. A. Bubnis. Ph.D. Dissertation, Philadelphia College of Pharmacy and Science, 1994.
19. D. T. Cheung and M. E. Nimni. *Conn. Tiss. Res.* **10**:187–199 (1982).
20. P. C. Hiemenz. *Principles of Colloid and Surface Chemistry*, Marcel Dekker, New York, p. 154 (1986).
21. C. M. Ofner III and H. Schott. *J. Pharm. Sci.* **74**: 1317–1321 (1985).
22. I. D. Robinson. *Phot. Sci. Eng.* **8**:220–224 (1964).