Gelatin with Hydrophilic / Hydrophobic Grafts and Glutaraldehyde Crosslinks*

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Synopsis

Acrylic monomers were graft copolymerized onto gelatin chains using potassium persulfate in aqueous medium. The graft copolymers were then treated with glutaraldehyde to establish crosslinks between gelatin macromolecules. This yielded a rigid water insoluble protein network sporting grafted chains at random. The chemistry of the acrylic monomer decided the hydrophilic/hydrophobic properties of the ultimate product. This paper describes the preparation and properties of such grafted, crosslinked gelatins.

INTRODUCTION

Gelatin is the water-soluble degradation product of the water-insoluble structural protein collagen.[1] A fibrous protein with a molecular weight of the order of 285 kdaltons, collagen is unique in its molecular architecture. Three strands of polypeptide chains are braided into a triple helix stabilized through interchain hydrogen bonds and covalent crosslinks.

The number and nature of these H bonds and crosslinks impart the necessary biological stability and physical strength to the collagen fiber. The collagen to gelatin exhaustive degradative transformation involves the concurrent rupture of the main chain peptide bonds and the interchain covalent crosslinks. Thus while collagen is triple-stranded, tough, insoluble in water and remarkably resistant to degradation, gelatin is low molecular weight, single stranded soft, soluble in water, and rapidly biodegradable. Even aqueous solutions of gelatin do not keep for long. Gelatin retains none of the long range structural orders that distinguish collagen, except perhaps the ability to form inter/intrachain hydrogen bonds which manifests itself in the case with which gelatin solutions undergo sol–gel transition.[1]

If one adequately compensates for the structural deficiencies of gelatin, can it prove to be a versatile biomaterial? Many investigators have tried to answer this through graft copolymerization techniques.[2-8] We sought the answer in two steps. We chose graft copolymerization not only to enhance the effective molecular weight, but also to manipulate the hydrophilic/hydrophobic properties of the system. Next we mimicked collagenlike covalent crosslinks between the gelatin chains using the bifunctional reagent, glutaraldehyde. This paper presents the details of our investigations and the results.

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EXPERIMENTAL

Bacteriological gelatin and 25% aqueous glutaraldehyde solutions were supplied by Loba Chemicals, Bombay. The acrylic monomers (BDH/E Merck) were purified according to the methods of Riddick and Bunger.[9] Potassium persulfate (BDH) and the AR grade solvents were used as such.

Throughout the text the following abbreviations will be used for the various samples:

Sample	Abbreviation
Native gelatin	Gel
Crosslinked gelatin	Gelx
Poly(acrylic acid)-grafted crosslinked gelatin	Gelx-*g*-PAA
Poly(methyl methacrylate)-grafted crosslinked gelatin.	Gelx-*g*-PMMA
Poly(methyl acrylate)-grafted crosslinked gelatin.	Gelx-*g*-PMA
Potassium persulfate	KPS

Preparation of the Glutaraldehyde Crosslinked Gelatin *Gelx*

The method reported by Quiocho and Richards[10] was adopted. One gram of gelatin was immersed in 4% aqueous glutaraldehyde solution (25 mL) overnight. The crisp granules were then washed several times with distilled water, rinsed a couple of times with acetone and dried under vacuum.

Grafting Acrylic Polymers onto Gelatin and Subsequent Crosslinking with Glutaraldehyde

Gelx-g-PAA

Gelatin and 60% aqueous acrylic acid (1:0:5 w/v) containing potassium persulfate (0.005 mol/100 mL) were thoroughly blended. The sticky mass, loosely rolled into shape, was incubated at room temperature overnight. This was then immersed in solution of acrylic acid containing KPS (0.005 mol KPS/100 mL 60% acrylic acid) and the temperature was quickly raised to 70°C to facilitate the decomposition of KPS. After 30 min, the softened mass was transferred to 4% glutaraldehyde containing 0.01% hydroquinone and ripened for 24 h. The product was extensively dialyzed with water at 50°C, filtered, and dried under vacuum. The dry polymer was pulverized and subjected to further washings. Final product was rinsed with acetone several times and dried under vacuum at room temperature.

Gelx-g-PMA and Gelx-g-PMMA

Methyl acrylate and methyl methacrylate monomers were graft-copolymerized into gelatin chain and characterized according to the known methods.[8,11] The graft copolymers were then treated with 4% glutaraldehyde for 24 h to

introduce the crosslinks. The product was washed thoroughly with water, rinsed several times with acetone, and vacuum-dried.

Amino Acid Analysis

Amino acid analysis of the native and modified gelatin samples, hydrolyzed by heating in 6*N* HCl at 110°C for 24 h, was carried out in an LKB alpha plus 4151 Model amino acid analyzer, using a citrate buffer system.

Swelling Studies

The response of the unmodified and modified protein samples towards different solvents was followed gravimetrically as reported by Yeo.[12] Samples were hot pressed into pellets of 0.5 cm radius and 0.5 cm thickness. The weighed pellets were then equilibrated in various solvents for 48 h at room temperature. The pellets were then lifted, patted dry, and weighed. The percentage swelling was calculated as

$$\text{percentage swelling} = \frac{w_f - w_i}{w_i} \times 100$$

where w_i = initial weight of the pellet and w_f = final weight.

Differential Thermal Analysis

Leeds Northrup (USA) DTA unit was used to study the thermal behavior of the samples. Alumina was used both as reference and diluent. Heating rate was maintained at 5°C min.

Enzymatic Degradation Studies

Enzymatic degradation of weighed pellets of different samples was carried out at 44°C in trypsin buffered to pH 8.5.[13] At the end of a fixed time interval (2–3 h), the test pellets were washed thoroughly, dried, and weighed. The control experiments were a parallel set of pellets in distilled water. The weight loss suffered by the distilled-water-treated pellets was used for calibration.

RESULTS AND DISCUSSION

Crosslinking studies form an integral part of the systematic structural investigations of proteins. Glutaraldehyde is one of the most popularly used crosslinking reagents because it reacts easily at room temperature with obvious color change characteristics of the Schiff base linkages.[14] It is well established that the aldehyde group of the reagent interacts with the amino group of the lysine residues of the protein chain forming a Schiff base (Fig. 1). Collagen crosslinks are also mostly lysine based (Fig. 2).

Characterization of the Crosslinked Copolymers

A visible color change accompanies the reaction between glutaraldehyde and gelatin. The pale yellow granules of gelatin turn deep orange within

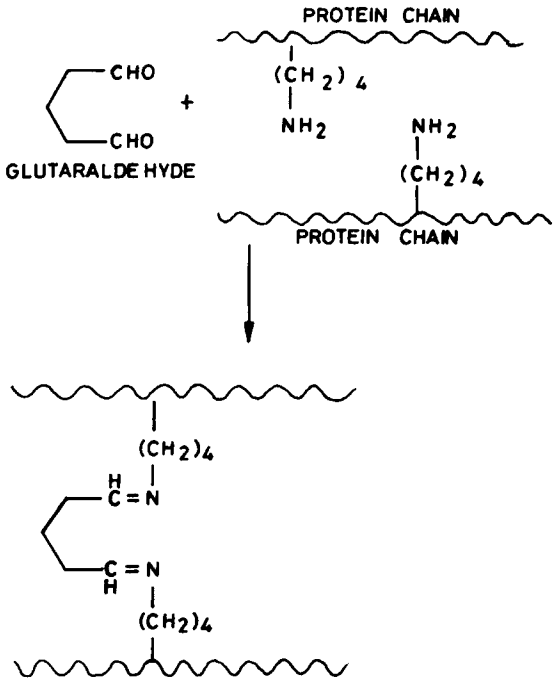

Fig. 1. Scheme of reaction between glutaraldehyde and the E-amino group of lysine residue of the protein chain.

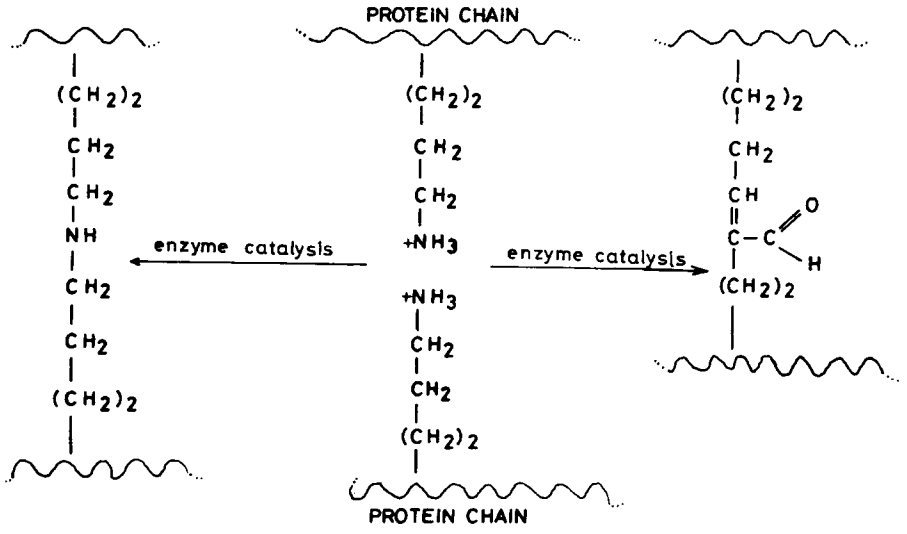

Fig. 2. Crosslinks in collagen.

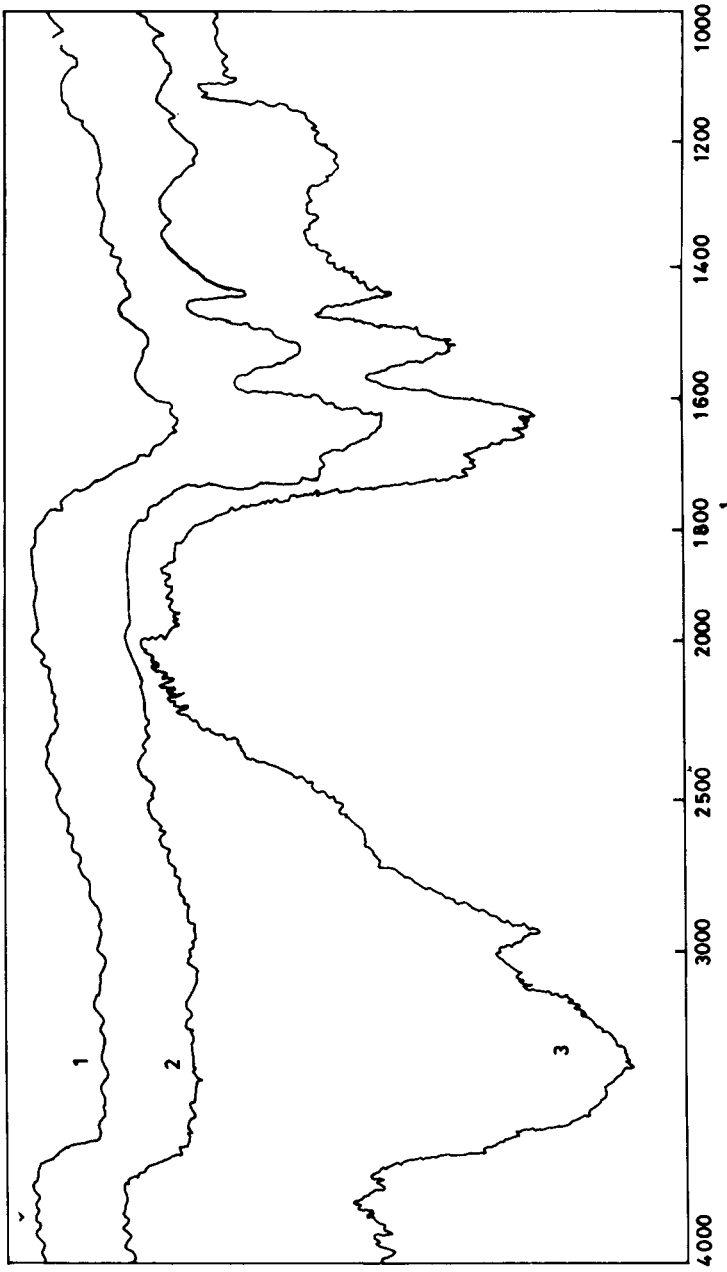

Fig. 3. IR spectra of native and modified gelatin samples: (1) gelatin; (2) Gelx; (3) Gelx-*g*-PAA.

minutes on treatment with glutaraldehyde, while the graft copolymers take on lighter shades. The color is due to the establishment of aldimine (Schiff) linkage (=CHN=), between the free amino groups of the protein and glutaraldehyde. Upon reduction with sodium borohydride, the colour was lost, thus confirming the aldimine linkage.[4]

Further proof for grafting and crosslinking came from the IR spectra (Fig. 3). The characteristic absorptions of the backbone peptide bond occurring at 1540 and 1650 cm^{-1} are the only distinguishing features of the gelatin. The crosslinked gelatin (Gelx) shows, in addition, the aldimine absorption at 1450 cm^{-1}. The spectrum of Gelx-*g*-PAA has absorption due to backbone peptide bonds and the aldimine linkage besides the broad overlapping absorptions due to carboxyl and methylene groups in the region 2500–3700 cm^{-1}.

The amino acid analysis data in Table I categorically establish that when native gelatin is treated with glutaraldehyde, the reaction exclusively involves the lysines, that to almost 100%. The gelatin used in these investigations has a molecular weight of ~ 35,000. This makes it roughly 12 lysines per gelatin chain. It follows naturally that 12 crosslinks per chain if all linkages are intermolecular, a little less if there are a few intramolecular tie-ups (Fig. 4). The amino acid analysis cannot throw much light on this aspect, but it reveals considerable information regarding the extent and sites of grafting. Comparative evaluations of the amino acid compositions of the Gelx-*g*-PAA samples against the Gel or Gelx (except lysine) put the percentage grafting to be 40–50 and the most affected sites to be in the Gly > Glu > Ser = Thr = Asp. X-ray diffraction data show that qualitatively there is a slight decrease in the crystallite size upon crosslinking (data not shown).

TABLE I
Amino Acid Analysis of Native and Modified Gelatin Samples
Residues Expressed as nmol/μg Sample[a]

			Gelx-*g*-PAA		
	Gel	Gelx	I	II	III
Asp	8.92	8.97	5.12	4.6	4.0
Thr	3.39	3.32	2.27	1.90	1.4
Ser	6.22	6.2	3.48	3.4	2.7
Glu	16.02	16.34	9.09	8.6	6.5
Gly	49.46	50.75	23.14	22.2	30.5
Ala	19.35	20.06	11.89	10.8	9.52
Val	3.26	3.92	2.23	2.15	1.83
Ileu + Leu	5.80	5.90	4.39	4.7	3.84
Phe	2.32	1.86	1.61	1.7	1.24
Hy.Lys	1.22	0.28	0.08	—	—
Lys	6.81	0.89	0.40	0.39	0.42
ARG[b]	8.17	7.15	7.4	6.0	6.33
HY.Pro	—	—	5.0	4.3	3.6
Pro	20.46	21.42	14.7	10.0	7.0

[a] *Note*: For Gel and Gelx, 20 μg samples were used. The same sample weight was inadequate for the Gelx-*g*-PAA samples; hence the analysis was carried out on 60 μg samples. The gelatin used in the present investigation did not contain either histidine or tyrosine.

[b] Arginine values were slightly erratic.

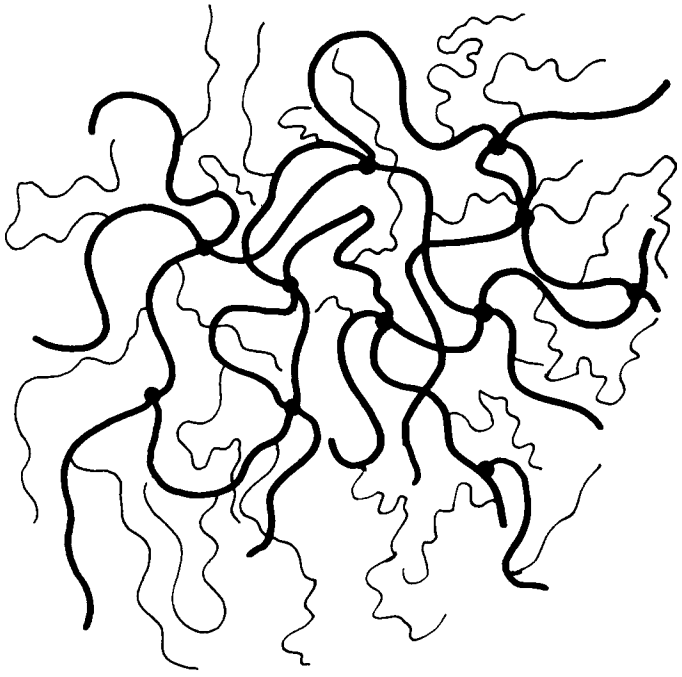

Fig. 4. Schematic representation of graft copolymers of Gelx: (—) gelatin chain; (●) glutaraldehyde crosslings; (⌒) grafted chains of PAA/PMA/PMMA.

Swelling Behavior

The most characteristic property that differentiates the crosslinked copolymers from one another is their response towards various solvents (Table II). The Gelx-*g*-PAA swells extensively in water, taking up 170% water by weight. The Gelx-*g*-PMMA and Gelx-*g*-PMA show hardly any response to water, but they take up solvents like benzene and tetrahydrofuran to the extent of 120–150% by weight. As expected, the swelling behavior is entirely determined by the chemical nature of the grafted chain. Figure 5 represents the optical micrographs of Gelx-*g*-PAA in water and benzene.

Figure 6 and Table III bring out the salient features of the thermal behavior of these samples. The DTA curve of Gelx-*g*-PMMA is not shown in Figure 6 because it overlaps with that of the Gelx-*g*-PAA. The data are

TABLE II
Swelling Behavior of Native and Modified Gelatins

Sample	Water	Methanol	Benzene/THF
1. Gelatin	The pellet dissolves	No uptake	No uptake
2. Gelx	No uptake	No uptake	No uptake
3. Gelx-*g*-PAA	Extensive swelling	Swelling	No uptake
4. Gelx-*g*-PMMA	No uptake	No uptake	Extensive swelling
5. Gelx-*g*-PMA	No uptake	No uptake	Extensive swelling

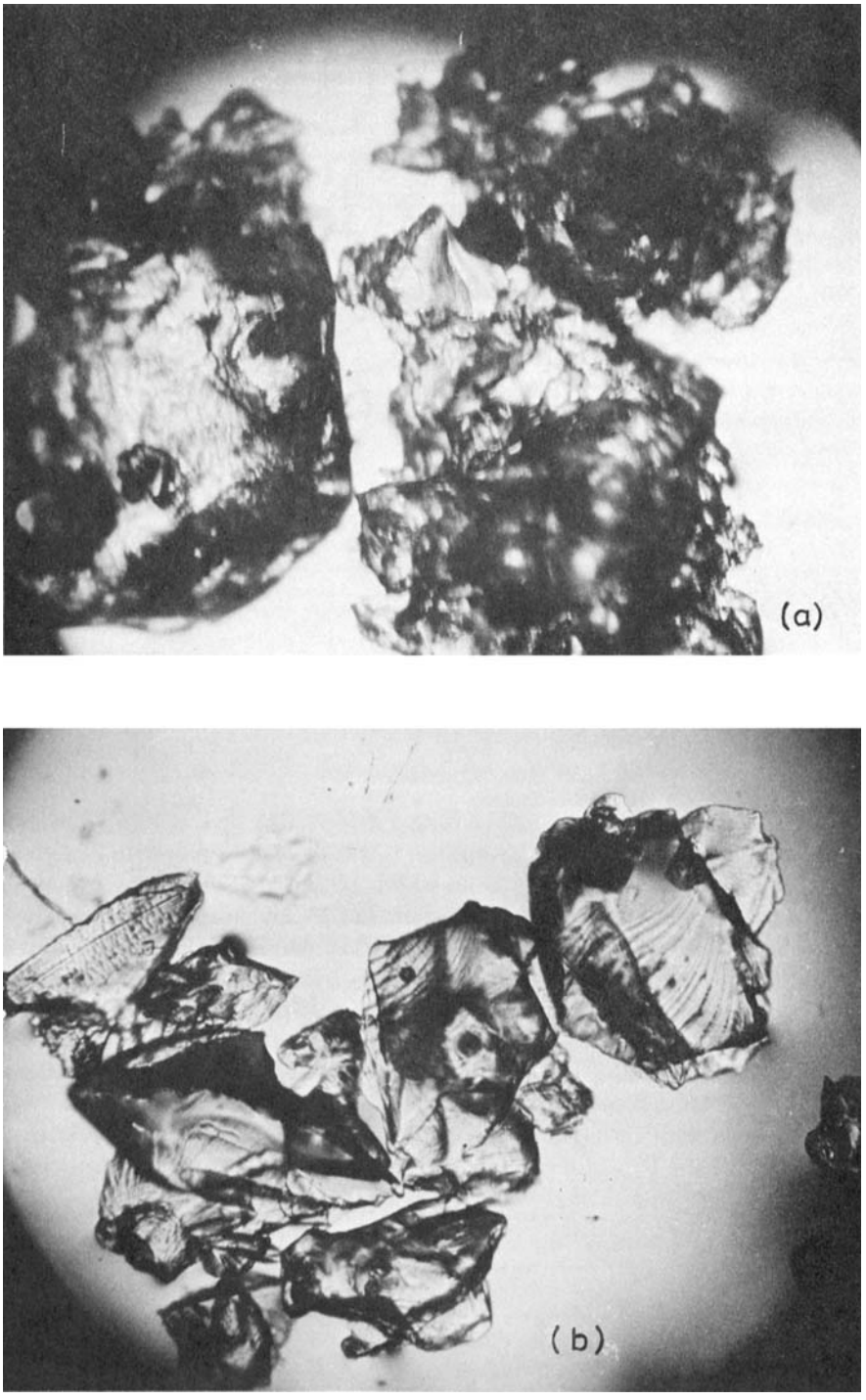

Fig. 5. Optical micrographs of Gelx-*g*-PAA granules in water (a) and benzene (b).

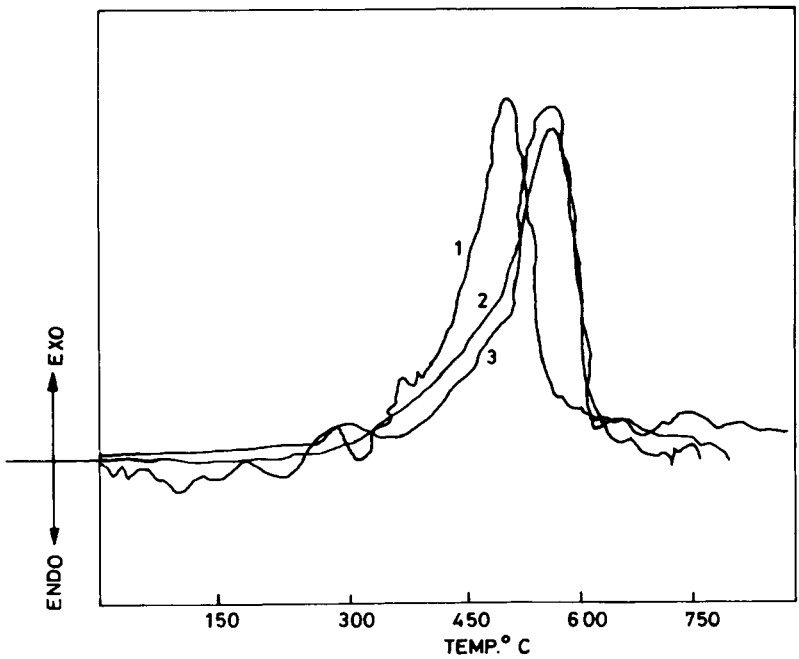

Fig. 6. DTA curves of native and modified gelatins: (1) Gel; (2) Gelx; (3) Gelx-*g*-PAA.

suggestive of the following thermal stability order:

$$\text{Gelx-}g\text{-PMMA} \approx \text{Gelx-}g\text{-PAA} > \text{Gelx} > \text{Gel}$$

Results of the enzymatic degradation studies using trypsin[13] showed an altered trend. The resistivity is in the order

$$\text{Gelx-}g\text{-PMMA} > \text{Gelx} > \text{Gelx-}g\text{-PAA} > \text{Gel}$$

This variation in behavior towards thermal enzymatic degration could be due to the possibility that on heating adjacent carboxyl side chains of poly(acrylic acid) might interact with the elimination of water leading to thermally stable intra- or interchain anhydride linkages.[15] In aqueous medium, abundance and

TABLE II
Peak Characteristics of Gel, Gelx, and Gelx-*g*-PAA/PMMA

Sample	IDT[a] (°C)	Peak temp (°C)	FDT[b] (°C)
Gelatin	330	525	570
Gelx	350	570	630
Gelx-*g*-PAA	390	580	660
Gelx-*g*-PMMA	410	580	660

[a] IDT = initial decomposition temperature.
[b] FDT = final decomposition temperature.

proximity of the carboxyl groups could facilitate the rupture of the aldimine linkage, thus enhancing enzymatic degradation rates.

CONCLUSIONS

Crosslinking gelatin chains provides stable rigid matrix for anchoring a variety of hydrophilic/hydrophobic grafts. The crosslinked, grafted macromolecule exhibits better stability towards thermal and enzymatic degradation. These systems are being studied in detail.

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