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ARTICLE

Alternative Reaction Mechanism for the Cross-Linking of Gelatin with Glutaraldehyde

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FT-IR, in combination with residual amino group determination using a fluorescence technique, has been used to investigate the chemical functional groups involved in the cross-linking reaction between glutaraldehyde and gelatin molecules. The results suggest that, at high pH values (i.e., close to the pK_a of lysine), the cross-linking reaction is mainly governed by the well-known Schiff base formation, whereas at low pH (i.e., when the amino groups of lysine are protonated), the reaction may also involve the −OH groups of hydroxyproline and hydroxylysine, leading to the formation of hemiacetals.

KEYWORDS: Gelatin; glutaraldehyde; cross-linking mechanism; FT-IR; fluorescence titration

INTRODUCTION

There has been a rapid growth of new materials from renewable resources during the past decades, as demonstrated by the large amount of published papers in the field (*1*−*8*). To date, due to their biocompatibility, biodegradability, and low toxicity, a wide range of natural biopolymers have been developed for biomedical (*9*−*11*) and pharmaceutical (*12*−*14*) as well as food-packaging applications (*15*−*17*). Depending upon their specific usages, such biomaterials can be made in many different forms, such as contact lenses, capsules for oral ingestion, coatings, membranes, slabs, and micro- and nanoparticles. Due to their natural origin, biopolymers can be used as green biomass and offer "social" advantages of renewable characteristics with reduced waste disposal management.

Gelatin is a biomacromolecule obtained by the hydrolysis of collagen, the most abundant protein in the skin, connective tissue, bone, and cartilage of animals. To date, gelatin has been used as the wall material for microcapsules and microspheres, as the sealant for vascular prostheses and wound dressing, or as an adsorbent pad for surgical purposes in clinical applications (*18*). More recently, the possibility to fabricate three-dimensional gelatin-based polymer scaffolds is an appealing approach for tissue-engineering research (*19*−*21*). Gelatin has also been used in combination with other molecules of biological, synthetic, and inorganic origin through different techniques. For example, it can be combined with poly(methacrylic acid) to produce interpenetrating polymeric networks (IPN) (*22*), with DNA to form semi-IPN (*23*), with methacrylate to generate graft copolymers (*24*), and with tetraethoxysilane to fabricate nanohybrid composites (*25*).

The use of gelatin for the aforementioned purposes is mainly due to its gel-forming properties at temperatures around 35 °C. Because of the partial recovery of the collagen triple-helix structure, this temperature may vary depending on parameters such as collagen concentration, type, presence of other molecules, and pH (*26*). In addition to the gel-forming property, the gelatin molecule exhibits an excellent versatility due to its amino acid composition. The presence of both positively charged (arginine, lysine, and histidine) and negatively charged (glutamic acid and aspartic acid) amino acids results in its polyampholyte nature, which enables complex formation with oppositely charged polymers at specific pH values. Furthermore, the presence of other amino acids with hydrophobic groups along the gelatin backbone makes hydrophobic interactions with other molecules possible. Biodegradability, biocompatibility, nonimmunogenic properties, and relatively low cost make gelatin an appealing biomacromolecule in the design and development of new functional materials.

Despite numerous attempts to fully exploit gelatin-based biomaterials, some drawbacks still hinder their applications and marketing. Among them, poor mechanical properties and water sensitivity are generally recognized as the most limiting ones (*7*, *27*, *28*). Although different approaches can be pursued to overcome these hurdles, chemical cross-linking is by far the most widely used technique to improve the thermal, mechanical, and water-sensitive properties of gelatin devices intended for long-term usages. For this purpose, a wide variety of reactive molecules have been used to modify gelatin via its amino, carboxyl, or hydroxyl groups. Gelatin has been cross-linked with chemicals such as glyoxal (*29*), epoxides (*30*, *31*), isocyanates (*32*), carbodiimides (*31*, *33*), and formaldehyde (*29*−*31*) or with natural molecules such as tannin and ferulic acids (*34*), glyceraldehyde (*35*), and genepin (*31*, *33*, *36*) or enzymes such as transglutaminase (*37*). However, glutaraldehyde is by far the most widely used cross-linking molecule due to its low cost and excellent efficiency on the stabilization of collagenous materials (*38*), which enables achieving strength and water resistance of the obtained structure (*39*), reducing its cytotoxicity when used at very low concentration (*40*). Many bifunctional aldehydes are able to react

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Table 1. Amino Acid Composition of Pigskin Gelatin

amino acid	pigskin gelatin (mol %)	amino acid	pigskin gelatin (mol %)
alanine	11.05	leucine	2.35
arginine	4.96	lysine	2.65
asparagine	0.60	methionine	0.32
aspartic acid	4.42	phenylalanine	1.38
cysteine	nd	proline	13.10
glutamic acid	7.10	serine	3.40
glycine	32.20	threonine	1.80
histidine	0.45	tryptophan	nd
hydroxyproline	9.80	tyrosine	0.35
hydroxylysine	0.75	valine	1.90
isoleucine	1.02		

with different functional groups, such as carboxyl, hydroxyl, and amide. It is widely accepted that the cross-linking of gelatin is mediated by glutaraldehyde through the unprotonated ε-amino groups of lysine and hydroxylysine and the amino groups of the N-terminal amino acid (*18*). Therefore, the pH value of the medium is a pivotal factor to control the cross-linking reaction. At high pH values, few amino groups are protonated; thus, more free amino groups are available for the cross-linking reaction. In contrast, lowering the pH increases the amount of protonated amino groups; thus, the possibility of cross-linking reactions may be significantly reduced. Otherwise, cross-linking reactions may follow different reaction mechanisms. Nevertheless, the cross-linking of gelatin at pH of approximately 4.5 has been demonstrated, which gives rise to the hypothesis that a different chemical route may be involved in this reaction (*41*).

The aim of this paper is to demonstrate the existence of different gelatin cross-linking mechanisms by glutaraldehyde at different pH values of the medium. In particular, we propose that at acidic pH conditions, the cross-linking reaction involves the formation of hemiacetals, whereas at high pH values, the cross-linking reaction is mainly governed by the formation of Schiff bases. The combination of FTIR and fluorescence techniques has been used to test this hypothesis.

EXPERIMENTAL PROCEDURES

Materials. Pigskin gelatin powder (type A, 250 Bloom, pharmaceutical and food grade) was purchased from Weishardt International, Grauliet Cedex, France. The amino acid composition of gelatin (**Table 1**) was determined after hydrolysis with 6 N HCl at approximately 110 °C for 24 h. The amino acid analysis of the hydrolyzed sample was performed using an Amino-Acid-Analysis System (Waters PICO-TAG System, Milford, MA). Milli-Q water (18.3 MΩ) and a 25% w/w glutaric dialdehyde−water solution (Acros, Morris Plains, NJ) were used to obtain the cross-linked gelatin films.

Film Preparation. A 14% w/w gelatin water solution (native pH ∼4.5) was obtained by heating at 60.0 ± 0.5 °C for 1 h. The temperature was then decreased to 40.0 ± 0.5 °C. At this point, pure gelatin films were obtained by spreading part of the solutions on the bottom of Petri dishes. The remaining part was used to cross-link gelatin with glutaraldehyde (0.3 and 1.0% w/w). Both non-cross-linked and cross-linked films were obtained after water evaporation in a vacuum oven (model 282, Fisher Scientific, Pittsburgh, PA) at 30.0 ± 0.5 °C for 24 h. In addition, the cross-linked samples were repeatedly washed with Milli-Q water and air-dried at room temperature for 1 day. Finally, they were kept in a desiccator for 15 days before analyses. This avoids the undesired influence of residual water during FTIR measurement. The thicknesses of the final films, measured with a micrometer (Dialmatic DDI030M, Bowers Metrology, Bradford, U.K.) to the nearest 0.001 mm at 10 different random locations, were in the range of 100 ± 5 μm.

Attenuated Total Reflectance−Fourier Transform Infrared (ATR-FTIR). Structural characteristics and chemical bonding of both non-cross-linked and cross-linked films at different glutaraldehyde concentrations were investigated by a Perkin-Elmer FT-IR Spectrum 100 series spectrometer (Perkin-Elmer, Waltham, MA) equipped with a universal attenuated total reflectance (UATR) accessory with the single-reflection sampling plate of 1.8 mm round germanium surface. To ensure satisfactory physical contact between samples and the germanium crystal surface, a high-pressure clamping device was used. Spectra were recorded at room temperature within the range of 650−4000 cm^{-1} at 4 cm^{-1} resolution. Spectrum 6.0 software was used for data acquisition and analysis.

Determination of Residual Amino Groups. Free amino groups of gelatin molecules were determined by a fluorescence titration experiment carried out on both non-cross-linked and cross-linked films using NHS-Fluorescein (Pierce, Rockford, IL) as the probe molecule (MW = 473.39; Ex/Em 494/518 nm). NHS-Fluorescein reacts efficiently with the primary amines in the side chain of lysine. In a dark room, 1 mg of NHS-Fluorescein was dissolved in 300 μL of dimethyl sulfoxide (DMSO), and 3 mL of 50 mM sodium bicarbonate buffer (pH ∼8.5) were then added. A constant amount of the solution obtained was properly put on previously prepared gelatin-based strips, which were then stored under no-light conditions at room temperature for 40 min. Strips were first washed extensively by distilled running water, then kept in Milli-Q water at 4 °C for 2 h. Fluorescence emission of NHS-Fluorescein, which reacted with the free amino groups of gelatin molecules, was determined by placing dried film strips into a VersaDoc Image System apparatus (Bio-Rad, Hercules, CA) incorporated with an illumination module for UV excitation (290−365 nm) and coupled to a 520LP-Long Pass filter wheel optimized for single-color detection of fluorescein. It allowed capturing high-resolution digital images, where the light and dark areas indicated free and reacted amino groups, respectively.

RESULTS AND DISCUSSION

Cross-Linking Chemistry. Due to its unique amino acid sequences and numerous functional groups, gelatin is well-suited for producing chemical hydrogels in the form of sheets, films, or membranes by reacting with small molecules containing reactive functional groups, such as an aldehyde group (*42*). As mentioned before, it is generally accepted that the mechanism of gelatin cross-linking mediated by glutaraldehyde can be explained through the reaction of the aldehyde functional groups with free nonprotonated ε-amino groups ($-NH_2$) of lysine or hydroxylysine through a nucleophilic addition-type reaction. Although such reaction normally requires subacid conditions, neutral to slightly alkaline pH values are more favorable for gelatin cross-linking. More specifically, the first step of the reaction involves the nucleophilic addition of the ε-NH_2 groups to the carbonyl groups (C═O) of the aldehyde to form a tetrahedral unstable intermediate called carbinolamine. In a second step, protonation of the −OH group followed by loss of a water molecule yields the conjugated Schiff bases. The scheme of the reaction is reported in **Figure 1**. Such a mechanism results in the formation of new covalent bonds between gelatin molecules at either intramolecular (short-range) or intermolecular scale (long-range). The long-distance bridges form through the polymerization of glutaraldehyde in aqueous solution or aldol condensation reaction (**Figure 2**) (*43*). The final result is a clear improvement of the mechanical, thermal, water resistance, and proteolytic resistance properties of the reconstituted collagen in the form of films, fibers, or membranes. Nevertheless, such properties are strongly affected by the amount of cross-linkers used (*40*,*44*). Not only the cross-linker concentration but also the pH at which the cross-linking reaction occurs may have a significant impact on the final properties of the gelatin films. It is known that the pH can directly influence the charge density and charge distribution of gelatin molecules. More specifically, the pH determines the degree of protonation of ε-amino groups, as well as the presence of negative charges on the carboxylic groups. Therefore, at high pH values, very few amino groups become protonated, and there exists a large amount of free amino groups in gelatin molecules.

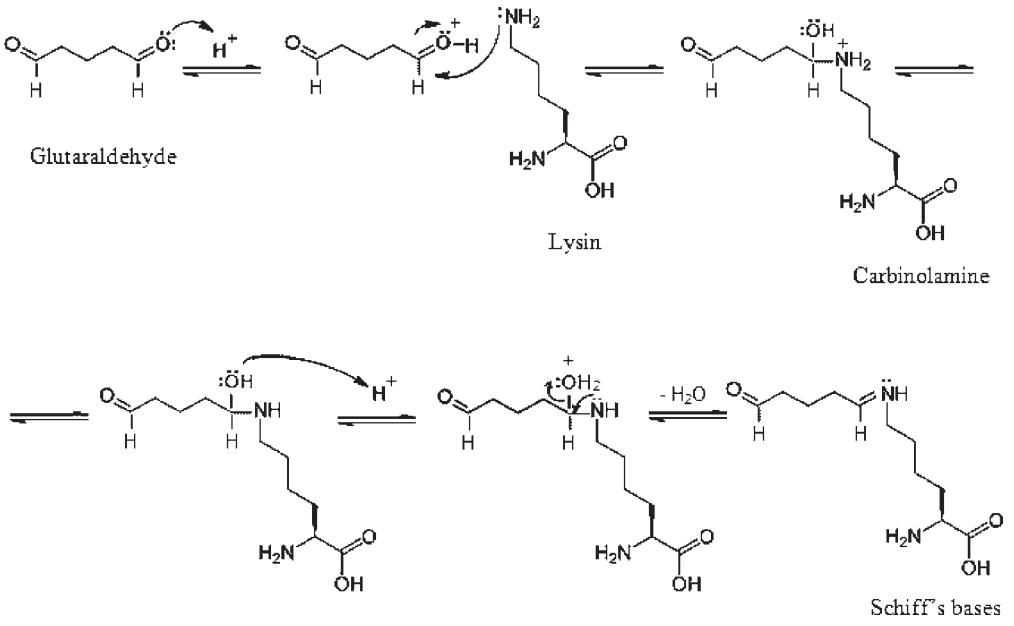

Figure 1. Reaction mechanism between amino groups of lysine and carbonyl groups of glutaraldehyde for the formation of Schiff base.

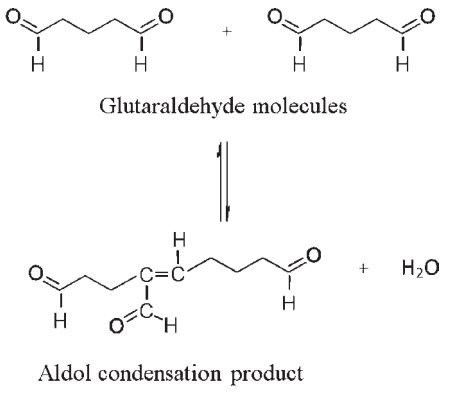

Figure 2. Scheme of the aldol condensation reaction.

Conversely, a decrease in pH leads to an increase in positively charged amino groups, which are unavailable for the cross-linking reaction with glutaraldehyde. In our system, the cross-linking reaction was first carried out at pH 4.5. Because the isoelectric point (p*I*) of type A gelatin is approximately 8.5 and the pK_a value for the ε-amino group of lysine is 10.53 (*45*), at this pH, three basic amino acids in gelatin (i.e., 4% lysine, 1% hydroxylysine, and < 1% histidine) are mostly positively charged. Therefore, it is less likely that the cross-linking reaction took place between these protonated amino acids and glutaraldehyde molecules. Instead, glutaraldehyde molecules may react with other functional groups.

ATR-FTIR Analysis. To prove the previous hypothesis, FTIR spectra were collected from gelatin films prepared with and without cross-linker at pH 4.5. For comparison, the FTIR spectrum of gelatin films cross-linked by glutaraldehyde at pH 11.0 was also provided, as shown in **Figure 3**. One notes that, in the absence of cross-linker, there is a "shoulder-like" peak centered at around 3500 cm^{-1}, which corresponds to the non-bonded −OH stretching band (*46*). At pH 11.0, this wide "bump" remains. However, at pH 4.5, it nearly disappears for the cross-linked gelatin sample, suggesting that the −OH groups may be involved in the reaction with glutaraldehyde. Spectra drawn from the same cross-linker concentration (0.3% w/w glutaraldehyde) exhibit two additional major bands, which are typical of gelatin

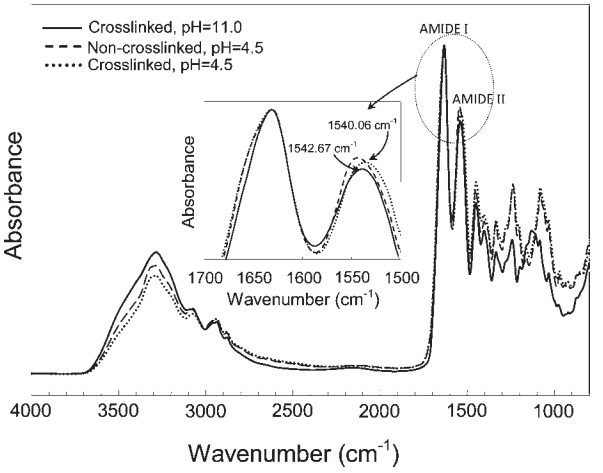

Figure 3. FTIR spectra of non-cross-linked (pH 4.5) and cross-linked gelatin films prepared at pH 4.5 and 11.0.

and, generally speaking, protein matrices. The first one at ∼1630 cm^{-1} is the amide I band, mainly associated with the C=O stretching vibration (70−85%), and is directly related to the backbone conformation (*47*). One notes that, for all three samples mentioned above, this peak is centered at the same wavenumber, suggesting that the carboxyl groups of amino acids, such as glutamic acid and aspartic acid, are likely not involved in the chemical cross-linking with glutaraldheyde. Otherwise, a shift in band position would have been detected. The absorbance at ∼1540 cm^{-1} is due to the amide II band, which originates from the N−H bending vibration (40−60%) and the C−N stretching vibration (18−40%) (*47*). One notes a shift in the amide II band of gelatin films cross-linked at pH 11 (1542.7 cm^{-1}) compared to that cross-linked at pH 4.5 (1540.1 cm^{-1}), indicating that the amide groups may be involved in the cross-linking reaction to form Schiff bases. When the glutaraldehyde concentration was increased to 1.0% w/w (**Figure 4**), it can be noted as gelatin samples cross-linked at pH 4.5 no longer exhibit the previously mentioned shoulder. It can be seen that the left-side shoulder of the graph drops straight away, in contrast to the sample cross-linked at pH 11, for which the bump is still evident. In addition, at

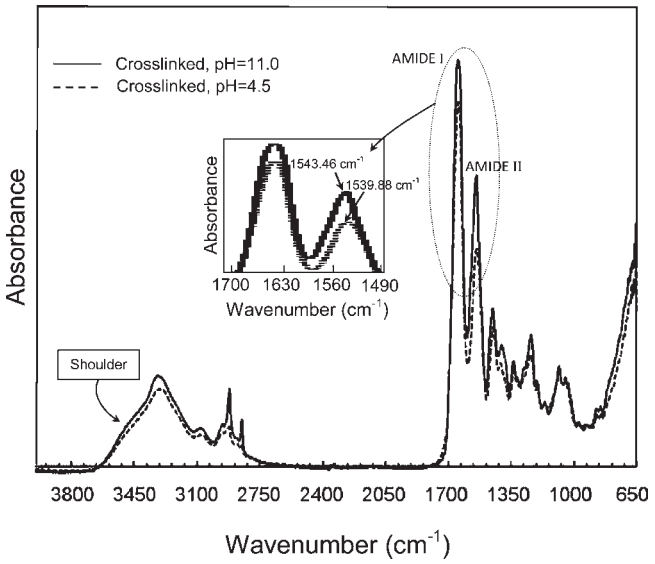

Figure 4. FTIR spectra of cross-linked gelatin films (1 wt % glutaraldehyde) prepared at pH 4.5 and 11.0.

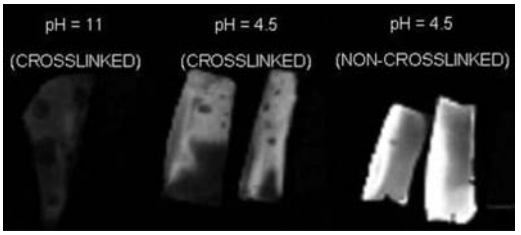

Figure 5. Fluorescence images of non-cross-linked and cross-linked gelatin films (0.3% w/w glutaraldehyde) prepared at pH 4.5 and 11.0.

this cross-linker concentration, the peak position of the amide II band was found to shift to a higher wavenumber for gelatin samples cross-linked at a higher pH value (i.e., pH 11.0), supporting the hypothesis that, at basic pH, $-NH_2$ groups can react with the carbonyl groups of glutaraldehyde to form new covalent C−N bonds.

Fluorescence Titration Experiment. To further understand the effect of pH on the cross-linking reaction between glutaraldehyde and gelatin molecules, fluorescence titration experiments were carried out to determine the free residual amino groups of gelatin films cross-linked at either pH 4.5 or 11.0. **Figure 5** depicts the digital images obtained from the fluorescence experiments performed at 0.3% w/w glutaraldehyde. It is found that the sample cross-linked at pH 11.0 exhibited a darker color than those cross-linked at pH 4.5. Indeed, at pH 4.5, although the majority of ε-amino groups were protonated and unable to react with NHS-Fluorescin, there were still some free noncharged amino groups that interacted with NHS-Fluorescin, as demonstrated by the small darker domains in the middle image of **Figure 5**. When gelatin films were cross-linked at pH 11.0, which is above the pK_a of lysine, almost all of the noncharged amino groups were involved in the cross-linking reaction with glutaraldehyde, as evidenced by the fully dark image observed at the left of **Figure 5**. The existence of a larger amount of unreacted amino groups at pH 4.5 than at pH 11.0 is also consistent with our FTIR results (**Figures 3** and **4**) discussed previously.

Alternative Cross-Linking Mechanism. On the basis of the results from both FTIR and fluorescence titration experiments, we propose that, at pH 4.5, glutaraldehyde may react with other functional groups in gelatin molecules in addition to the few

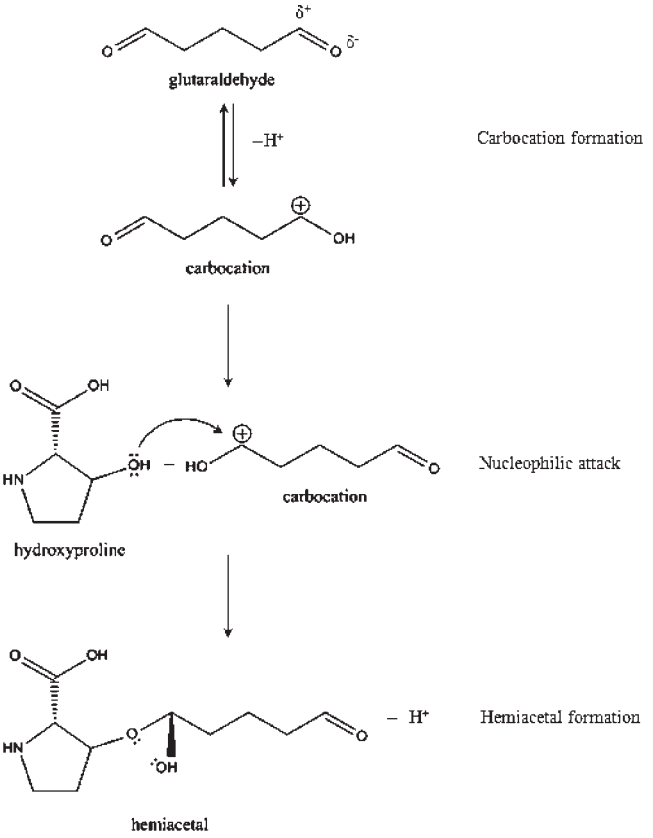

Figure 6. Proposed mechanism for the cross-linking reaction of gelatin by glutaraldehyde at acidic pH values.

unprotonated amino groups that still exist at this pH value. As a consequence, a new mechanism was proposed to explain the cross-linking reaction between gelatin and glutaraldehyde at acidic pH. According to our hypothesis, new covalent bonds are produced through the reaction between the aldehyde groups of glutaraldehyde and the hydroxyl groups of hydroxyproline and hydroxylysine (approximately 98 and 7.5 residues per 1000 residues, respectively, in type A gelatin, as shown in **Table 1**) to form hemiacetals. As proposed in **Figure 6**, the acidic medium makes the α-carbon of the aldehyde a highly reactive carbocation, making the subsequent nucleophilic attack by the −OH groups of hydroxyproline and hydroxylysine possible. As a result, a hemiacetal is formed and H^+ is regenerated in the medium. The low pH as well as the homogeneous charge distribution along the hemiacetal oxygen bridge contributes to the stability of the whole system. To the best of our knowledge, this chemical pathway has never been reported before for gelatin-based matrices cross-linked with glutaraldehyde. However, a similar cross-linking mechanism was also proposed by Reis and co-workers (*46*) for the PVA−glutaraldehyde pair, with the formation of acetals as the final structures.

The formation of different types of covalent bonds during the cross-linking reaction may contribute to the remarkably different properties of the resulting gelatin films. Our preliminary experimental results seem to confirm this (data not shown). In particular, films cross-linked with glutaraldehyde (0.3% w/w) at pH 4.5 exhibited elastic modulus and elongation at break lower and higher than those of non-cross-linked films, respectively. At the same time, it was found that the addition of glutaraldehyde did not result in any significant change in either glass transition temperatures (T_g) or melting temperatures (T_m) of gelatin films, which, however, had a lower helix−coil transition enthalpy (ΔH). These results disagree with the majority of similar data reported

in the literature. Such a discrepancy could be indeed explained by the different cross-linking mechanism herein proposed. The original feature of the chemical route described before lies in the pivotal role of the hydroxyproline, which represents one of the most abundant amino acids in gelatin molecules (**Table 1**). It is well established that the amino acid hydroxyproline, different from hydroxylysine, points out from the gelatin triple-helix (*48*). As a consequence, it cannot form direct hydrogen bonding with any other group within the molecule (i.e., it did not participate in the intramolecular bridges formation). Otherwise, its role in stabilizing the structure of the triple helix should have been elucidated by water-mediated hydrogen bonding between nearby molecules (*49*). Therefore, two results are expected: first, because hydrxyproline is involved in the cross-linking reaction, the possibility for gelatin molecules to recover their original ordered structure becomes fairly low, presumably due to the unavailability of those sites involved in the formation of triple-helix domains. Second, it is more favorable to form covalent intermolecular cross-links than intramolecular ones, which conversely appear predominantly when the cross-linking mechanism is governed by the reaction between ε-amino groups of lysine and aldehyde groups to form Schiff bases. Accordingly, the prevalence of one mechanism to the other will dictate the ultimate properties of the gelatin-based materials produced.

In conclusion, the results obtained by FTIR and fluorescence analyses support our hypothesis that, at acidic pH, another reaction may take place in addition to the well-known Schiff base formation. More specifically, under acidic pH conditions, only partial ε-amino groups are able to react with the aldehyde groups due to their protonation, whereas the cross-linking reaction is predominantly governed by the mechanism involving the hydroxyl groups of hydroxyproline and hydroxylysine amino acids and the carbonyl groups of glutaraldehyde through a typical nucleophilic attack scheme. Such a mechanism may explain why gelatin-based matrices cross-linked at different pH conditions exhibit distinctive mechanical, thermal, and water resistance properties. In addition, we believe that the findings arising from this work may be exploited toward the production of tailored structures, that is, devices in the form of films, sheets, membranes, capsules, etc., that have the necessary properties required for targeted applications. Nevertheless, the use of glutaraldehyde as a cross-linker has here been intended only for nonfood applications, such as tissue engineering and biomaterials fabrication, as well as protecting/insulating materials for the building and electro/electronic industries. Conversely, due to its cytotoxicity, the use of glutaraldehyde in food formulation as well as for all packaging applications that require direct contact with food must be avoided. In these cases, an alternative naturally occurring cross-linking agent is preferred.

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