Reaction of Gelatin and Chitosan with Water Soluble Carbodiimides*

by
M.M. Taylor, L.P. Bumanlag, N. Latona, E.M. Brown and C.-K. Liu†
United States Department of Agriculture, Agricultural Research Service
Eastern Regional Research Center
600 E. Mermaid Lane, Wyndmoor, PA, 19038

Abstract

Earlier research from this laboratory has demonstrated the feasibility of using chemical and enzymatic treatments on protein and carbohydrate waste products for the purpose of making fillers to enhance the properties of leather. In our ongoing studies, we examined the reactivity of various concentrations of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with gelatin, chitosan and combinations of both, and found that both gelatin and chitosan had reactivity with EDC. Moreover, when the gelatin and chitosan were reacted together in the presence of the carbodiimide, the physical properties improved significantly over those of the protein and carbohydrate when reacted separately. In this continuing study, less expensive, commercially available, water-soluble and environmentally safe multifunctional carbodiimides were reacted with gelatin, chitosan and mixtures of gelatin and chitosan for the purpose of making biopolymers. The physical properties, molecular weight distribution of gel products are reported, as well as epi-fluorescent imaging. Initial results indicate reactivity similar to EDC. These studies should lead to a better understanding of the reactivity of carbodiimide and optimal conditions for developing appropriate products.

Introduction

The extensively reported water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) can be used as a cross-linking agent for collagen-based biomaterials. Cross-linking using EDC involves the activation of carboxylic acid groups to give O-acylisourea groups, which form cross-links after reaction with free amine groups.[1] The authors demonstrated that treatment of dermal sheep collagen with EDC resulted in an increased shrinkage temperature (Ts) and decreased free amine group content, showing that cross-linking occurred.[1] The authors also showed that the addition of N-hydroxysuccinimide (NHS) to the EDC-containing cross-linking solution improved the rate of cross-linking.[1]

Tropini *et al.* have found that wheat gluten films could be cross-linked with EDC and NHS.[2] Recent successful applications of EDC to fish gelatins have been reported.[3-5] Several authors have reported on reaction of EDC with fish gelatin and chitosan and the subsequent effects on properties (e.g., vapor permeability, mechanical properties and solubility) of resultant films [6-10] EDC's ability to crosslink primary amines to carboxylic acid groups enables peptides and proteins to be easily conjugated to one another or to other compounds that contain either carboxyl or amino groups.[11] Thus treatment of waste proteins, perhaps in conjunction with chitosans or other carbohydrates, with EDC, could possibly lead to products with interesting functional properties that could be used in leather processing.

In a recent study,[12] we investigated the EDC reaction with proteins and carbohydrates, for purpose of making products that may be useful to the leather industry. This study examined the reactivity of various concentrations of EDC with gelatin, chitosan and combinations of both, in the presence and absence of NHS at optimal times, and temperatures, and the effect these parameters have on physical properties, molecular weight distribution and free amine content. Both gelatin and chitosan had reactivity with EDC and the physical properties reflected the concentration of both the carbodiimide and gelatin. However when the gelatin and chitosan were reacted together in the presence of the carbodiimide, the physical properties improved significantly over the protein and carbohydrate when reacted

†Corresponding author e-mail: ChengKung.Liu@ars.usda.gov
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separately, resulting in unique products. This was a model study for future investigations in which cost-effective water soluble functional carbodiimides will be examined.

EDC is expensive and potentially toxic. Hesselman *et al.* [13-14] and Levy [15] reported on environmentally-friendly water-soluble multifunctional carbodiimides in which an additional inter-polymer network is formed by the added functional groups. They found that the film properties, such as the film strength, water and chemical resistance, abrasion properties, were enhanced. This present study will examine the reactivity of water soluble carbodiimides with gelatin, chitosan and combinations of both. We will report the optimal pH, temperatures of reactions, time, and concentrations of reactants and look at effects these parameters have on physical properties and molecular weight distribution. We will also be examining resulting products for epi-fluorescent properties, a further indicator of extent of the reaction. This will be a model study for future investigations in which these cost-effective water soluble multi-functional carbodiimides could be reacted with waste proteins and carbohydrates for the purpose of making products for leather processing (films, coatings, fillers, etc.)

Experimental

Materials

Commercial Type B gelatin from bovine skin, characterized in this laboratory as 123g Bloom, EDC, NHS, and chitosan (low molecular weight and 75-85% acetylated) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Picassian® XL702 and XL725 were generously supplied by Stahl USA (Peabody, MA). All other chemicals were analytical grade and used as received.

Methods

Preparation of Carbodiimide-modified Gelatin

Gelatin (123g Bloom) (5g) was suspended in 40 ml of water (Figure 1), held for 2h at RT (room temperature, 22-25°C) and then heated at 45°C for 10 min. The solution was allowed to sit at RT for 2 h and then stored o/n at 4°C.

The solution was then heated at 40°C for 15 min, and when cool (RT), the pH was adjusted to 5.5. EDC (calculated to be 0-10 mM final solution) or Picassian® XL 702 or Picassian® XL 725 (0-25% based on wt of gelatin) was prepared in 10 mL of water and added immediately to gelatin solution. After addition to the solution, the mixture was stirred well. Control samples to which no carbodiimides were added, were run to monitor changes in physical properties. Aliquots (10 mL) of all the reaction mixtures were added to test tubes for melting point determination and 30-mL aliquots were poured into appropriate containers (39-mm diameter jar) for determining gel strength. The samples were warmed to 35°C in a shaker bath and the reaction was carried out for 1h. The samples were cooled to RT and then chilled for 17h at 10°C in a constant temperature bath. Physical analyses (gel strength, melting point and viscosity) were run on these samples. Aliquots of the samples were lyophilized and molecular weight distribution was determined. Sodium azide (70 µL of 1% solution) was added to the remaining treatment solutions as a preservative; the samples were stored at 4°C.

Preparation of Carbodiimide-modified Chitosan

Chitosan (0.35g) was dissolved in 20 ml of 0.15M acetic acid (Figure 1). Water (20 ml) was added and the samples were sonicated. The samples sat at RT for 2h and then o/n at 4°C. The samples were warmed in 40°C bath, cooled and the pH was slowly adjusted to 5.5; precipitation of chitosan was continually monitored. EDC (calculated to be 0-30 mM final solution), or Picassian® XL 702 or Picassian® XL 725 (0-25% based on wt chitosan) were prepared in 10 mL of water and added immediately to chitosan solution. The samples were then treated as described above for carbodiimide-modified gelatin samples. Control samples, with no carbodiimide additions were also run.

Preparation of Carbodiimide-modified Gelatin/chitosan Biopolymer Products

Chitosan (0.35g) was dissolved in 20 ml of 0.15M acetic acid (Figure 1). Gelatin (123g Bloom) (5g) was suspended in 20 ml of water, held for 2h at RT and then heated at 45°C for 10 min. When gelatin went into solution, the chitosan solution was added and mixed well. The solution was allowed to sit at RT for 2h and then stored o/n at 4°C. The solution was then heated at 40°C for 15 min, and the pH slowly adjusted to 5.5; precipitation of chitosan was continually monitored. EDC (calculated to be 0-10 mM final solution), or Picassian® XL 702 or Picassian® XL 725 (0-25% based on combined wt. of gelatin and chitosan) were prepared in 10 mL of water and added immediately to gelatin/chitosan solution. The samples were then treated as described above for carbodiimide-modified gelatin samples. Control samples, with no carbodiimide additions were also run.

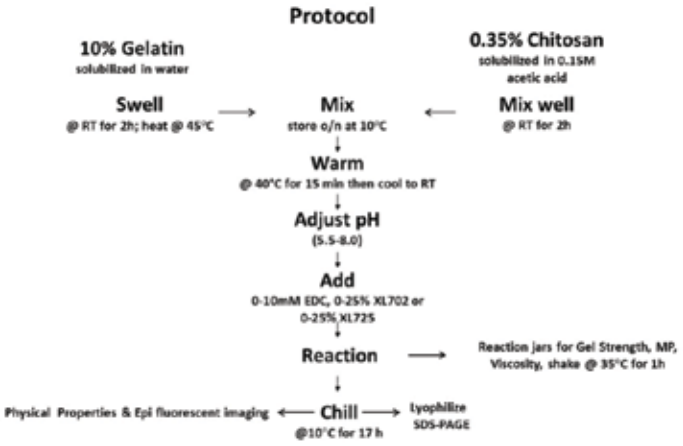

Figure 1. Flow diagram for preparation of carbodiimide-modified gelatin, chitosan and gelatin/chitosan biopolymer.

Analyses

Physical Properties and Molecular Weight Distribution

Gel strength, melting point, and viscosity of the gelatin/chitosan/carbodiimide-treated solutions were determined as described in a previous publication.[16] Protein molecular weights were estimated as described previously.[17] In summary, SDS-PAGE (polyacrylamide gel electrophoresis in sodium dodecyl sulfate) was run using precast 4-15 percent gradient gels. A broad range (BRS) calibration standard (Bio-Rad, Hercules, CA), which contains a mixture of nine proteins ranging in size from 6,500 to 200,000 Daltons, was used. Samples of lyophilized protein were dissolved in sample buffer (10 mM Tris-HCl at pH 8.0 containing 1 mM EDTA, 2.5% SDS, 5% β-mercaptoethanol and 0.01% bromophenol blue) and were then heated at 40°C for 4 h. Separation was achieved using a Phast-Gel System (Pharmacia Biotech Inc., Piscataway, NJ). Gels were stained with Coomassie Blue (Pharmacia).

Optical Microscopy (with Epi-fluorescent Attachment)

Samples of unmodified gelatin, chitosan, and gelatin/chitosan and gelatin, chitosan, and gelatin/chitosan modified with EDC, Picassian® XL702 or XL725, were prepared and then checked for fluorescence using an epi-fluorescent microscope. They were examined using an Eclipse E600 Polarizing Microscope (Nikon Instruments Company, Melville, NY), at 4X magnification, operating in optical mode. The instrument was equipped with a X-Cite™ 120 Fluorescence Illuminator System which was fitted with a metal halide lamp (EXFO Photonic Solutions, Inc., Mississauga, ON, Canada), with two filter cubes or optical blocks, containing epi-fluorescence interference and absorption filter combinations including an excitation filter, dichromatic beamsplitter (often referred to as a mirror), and a barrier (or emission) filter (515-555 nm or 600-660 nm), and with a digital camera (DS-Fi1).[18]

Results and Discussion

Reaction with Carbodiimides

In a previously published paper,[12] the widely reported EDC reaction with proteins and carbohydrates, for purpose of making biomaterials, was investigated. We examined the reactivity of various concentrations of EDC with gelatin, chitosan and combinations of both, in the presence and absence of NHS (an activator of carboxylic acid groups) at optimal times, and temperatures, and the effect these parameters would have on physical properties (Figures 2 a, b, c). In the reaction with chitosan alone, the viscosity decreased with increasing EDC concentration (Figure 2d). Furthermore the physical properties reflected the concentration of both the carbodiimide and gelatin (Figures 3 a, b, c). However when the gelatin and chitosan were reacted together in the presence of the carbodiimide, the physical properties improved significantly over the protein and carbohydrate when reacted separately, possibly resulting in products with unique properties (Figures 3 a,b,c.)

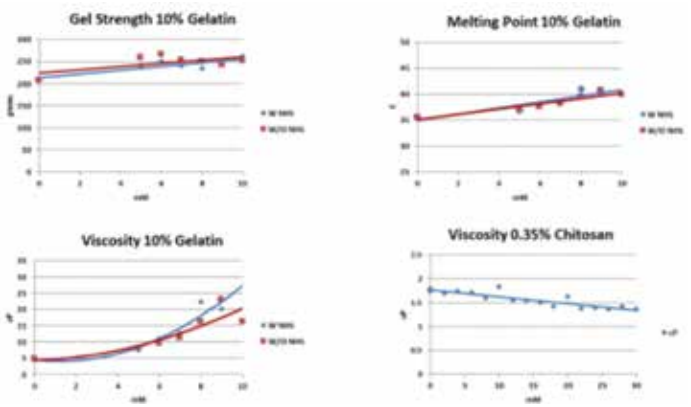

Figure 2. Physical properties of 10% gelatin treated with EDC (w and w/o NHS) and viscosity of 0.35% chitosan treated with 0-30 mM of EDC, both at 35°C, pH 5.5, for 1h.

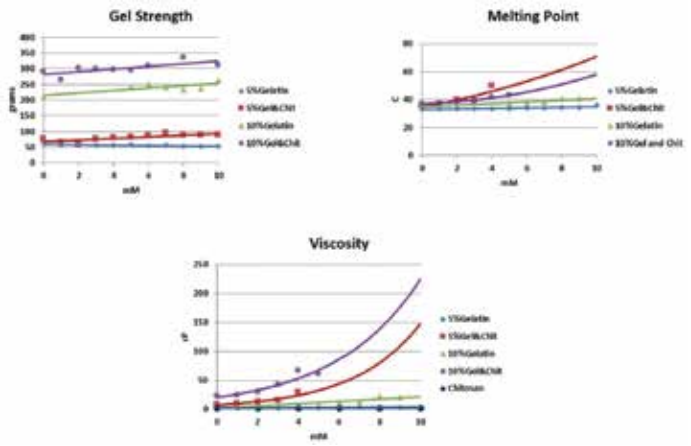

Figure 3. Physical properties of 123g Bloom gelatin (5% and 10% w/v), 0.35% chitosan, and biopolymer with 5% and 10% gelatin and 0.35% chitosan, and viscosity (at 60°C) of 0.35% chitosan, all treated with 0-10 mM EDC at pH 5.5, 35°C for 1 h.

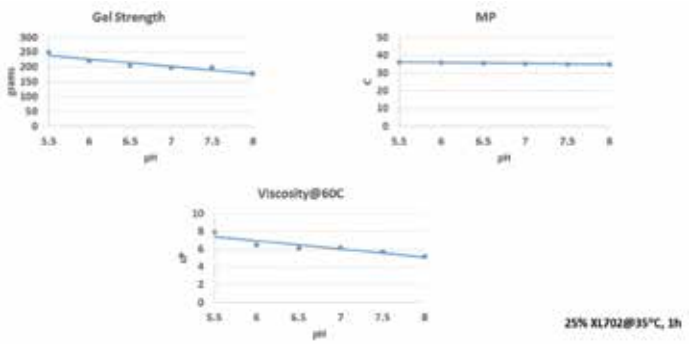

Figure 4. Effect of pH (5.5-8.0) on physical properties of 10% (w/v) 123g Bloom gelatin treated with 25% XL702 at 35°C for 1 h

Since EDC is expensive and potentially toxic, we decided to evaluate the more environmentally friendly, inexpensive, water soluble and commercially available carbodiimides. Picassian® XL702 and XL725 have properties which when reacted with protein and carbohydrates, may demonstrate reactivity similar to EDC. Furthermore, from the supplier's literature, they are also not toxic and are commonly used in the leather industry to make coatings.[19] They report that among the benefits: these carbodiimides are effective due to unique multifunctionality, long pot life, and low/no VOC. With respect to use in leather industry these polycarbodiimides are a suitable alternative to isocyanate and aziridine crosslinkers.

Gelatin, chitosan and gelatin/chitosan were reacted with XL702. Literature has reported and we demonstrated that the EDC reaction took place at a pH of 5.5 [1]. However it is unknown what the optimum pH for reaction with XL702 would be. In our initial experiment we ran the reaction with 25% XL702 at 35°C for 1h with varying pH's and the resulting physical properties are shown in Figure 4.

As the pH increased from 5.5 to 8.0, the gel strength and viscosity decreased significantly while the melting point decreased slightly. We initially used concentrations of XL702 similar to those in our EDC study (0-1.6% EDC based on weight of gelatin), but found little indication of a reaction. In the next set of experiments gelatin, chitosan and gelatin/chitosan were reacted with 0-25% of XL702 (based on weight of gelatin or gelatin and chitosan) at pH 5.5 for 1h at 35°C and the physical properties can be seen in Figure 5. Increasing the XL702 concentration did not have a significant effect on gel strength and melting point, but the combination of gelatin and chitosan modified with increasing amounts of XL702 significantly increased the viscosity.

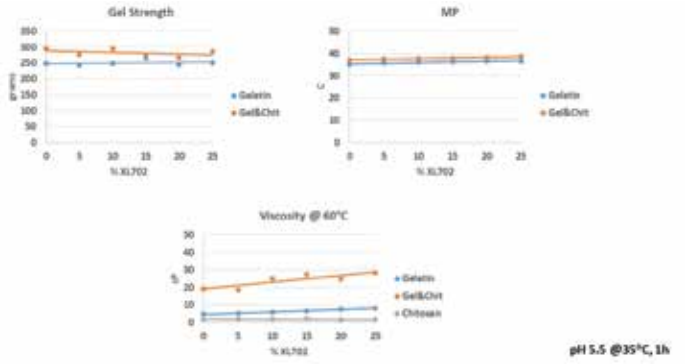

Figure 5. Effect of XL702 concentration on physical properties of treatment of 10% (w/v) gelatin, 10% gelatin/0.35% chitosan biopolymer and 0.35% chitosan with 0-25% XL702, at pH 5.5, 35°C for 1h.

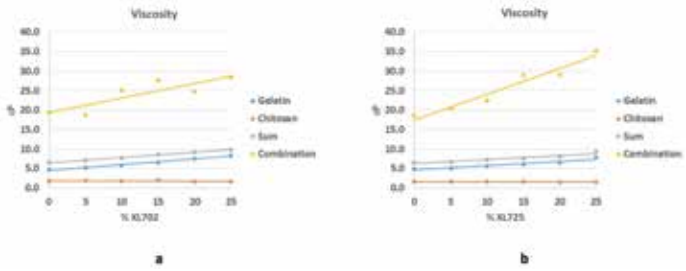

Figure 6. Difference in viscosity between mathematically combined viscosity values (of gelatin and of chitosan) and the viscosity values when gelatin/chitosan are combined in solution and the XL702 (0-25%) (a) and XL725 (b) concentrations are increased.

The viscosities of gelatin alone, chitosan alone and mixtures of both, modified with increasing amounts of XL702 are shown in Figure 6a. Presented are data when the viscosities values of gelatin and chitosan are mathematically added and the values when the two compounds are mixed together in solution. The graph demonstrates that the mathematically added viscosities are quite lower than those when the gelatin and chitosan are mixed together in solution and modified. Furthermore as increasing amounts of carbodiimide are added these latter values become increasingly higher, indicating a synergistic effect between the two, giving physical properties superior to those of the individual substrates.

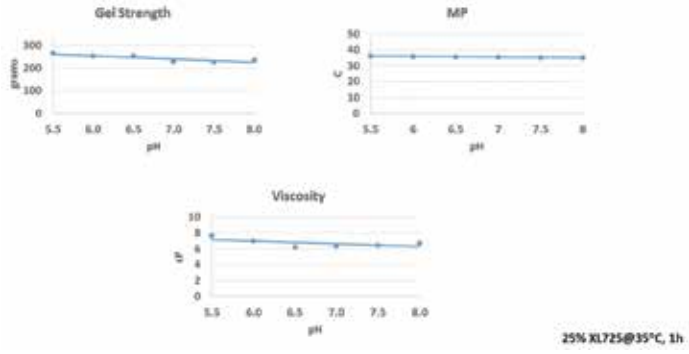

Figure 7. Effect of pH (5.5-8.0) on physical properties of 10% (w/v) 123g Bloom gelatin treated with 25% XL725 at 35°C for 1h.

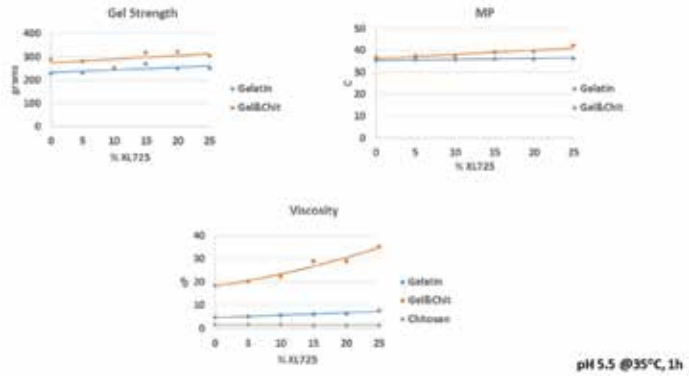

Figure 8. Effect of XL725 concentration on physical properties of treatment of 10% (w/v) gelatin, 10% gelatin/0.35% chitosan biopolymer and 0.35% chitosan with 0-25% XL702, at pH 5.5, 35°C for 1h.

Gelatin, chitosan and gelatin/chitosan were next reacted with XL725. The only difference in handling of the two Picassian® samples was that the XL725 was stored under a nitrogen atmosphere. Like XL702, it is unknown what the optimum pH for reaction would be. In our initial experiment we ran the reaction with 25% XL725 at 35°C for 1 h with varying pH's and the resulting physical properties are shown in Figure 7.

As the pH increased from 5.5 to 8.0, the gel strength, melting point and viscosity all decreased slightly, and we determined that optimal pH was between 5.5. In the next set of experiments gelatin, chitosan and gelatin/chitosan were run at pH of 5.5 for 1h at 35°C and the results can be seen in Figure 8.

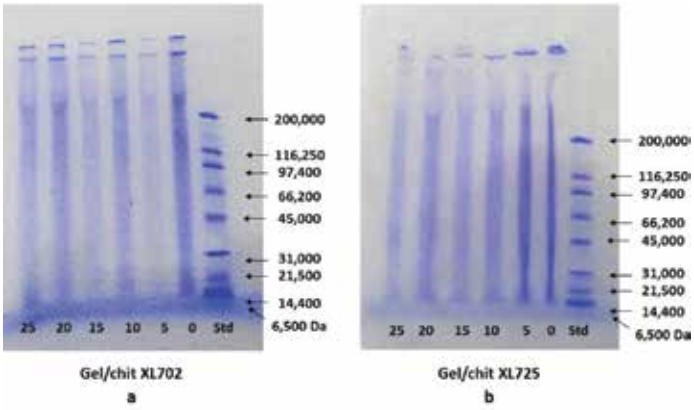

Figure 9. SDS-PAGE of 10% gelatin/0.35% chitosan (biopolymer) reacted with 0-25% XL702 (a) and with 0-25% XL725 (b) (reacted at 35°C, pH 5.5, for 1h); molecular weights are shown in Da.

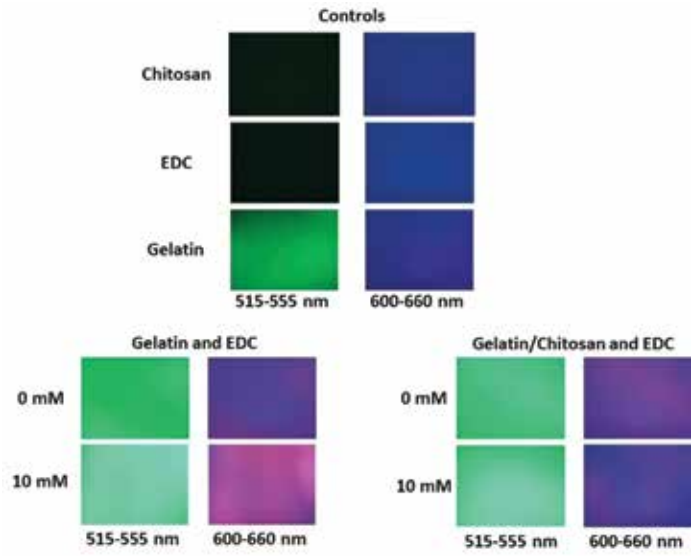

Figure 10. Epi-fluorescent images of gelatin, chitosan and EDC alone and EDC-modified gelatin and gelatin/chitosan gels (reacted at 35°C pH 5.5, for 1h); emission (barrier) filters, 515-555 and 600-660 nm were used.

As the XL725 concentration increased (0-25%), there was a slight increase in the gel strength and melting point, with the most dramatic significant increase in viscosity of the gelatin/chitosan biopolymer as was seen in the reaction with XL702. As seen in Figure 6b, the same synergistic effect was occurring in the viscosity values when one examines the mathematical additions as opposed to the dramatic increase when the gelatin and chitosan were mixed in solution.

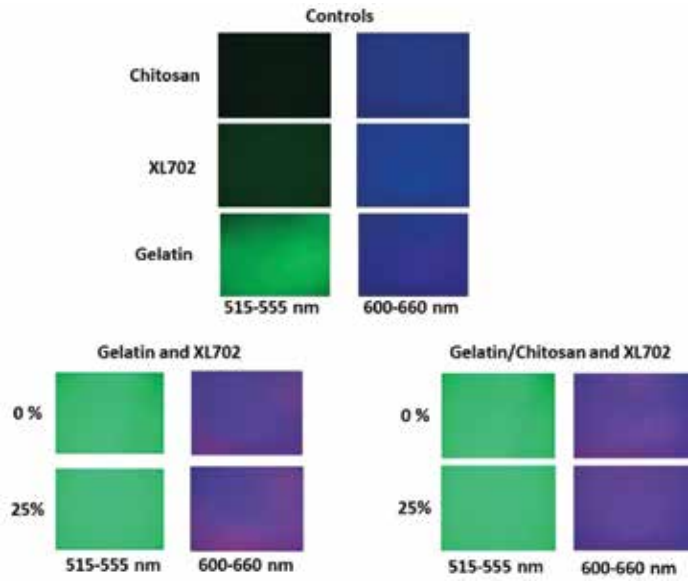

Figure 11. Epi-fluorescent images of gelatin, chitosan and XL702 alone and XL702-modified gelatin and gelatin/chitosan gels (reacted at 35°C pH 5.5, for 1h); emission (barrier) filters 515-555 and 600-660 nm were used.

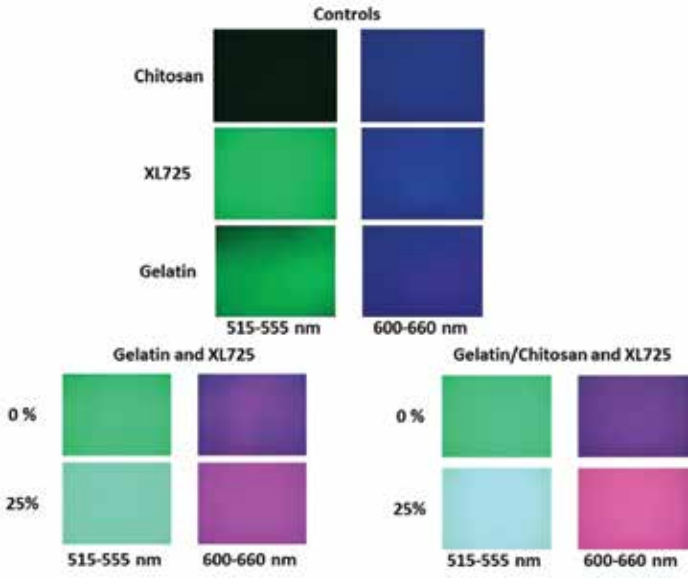

Figure 12. Epi-fluorescent images of gelatin, chitosan and XL725 alone and XL725-modified gelatin and gelatin/chitosan gels (reacted at 35°C pH 5.5, for 1h); emission (barrier) filters 515-555 and 600-660 nm were used.

Finally the gelatin/chitosan products from reaction with XL702 and XL725 were examined by SDS-PAGE and the images shown in Figures 9a and 9b. With respect to XL702, (Figure 9a) the bands indicating gelatin show a slight lightening as the XL702 concentration increases, correlating with physical properties as does the reaction products resulting from increasing XL725 concentration.

Epi Fluorescent Imaging

Epi-fluorescent studies were carried out on products from the carbodiimide-gelatin/chitosan study. As controls, chitosan, gelatin, EDC, XL702, and XL725 alone were examined at 515-555 nm and 600-660 nm emissions. As seen in Figure 10, the chitosan and EDC control samples which were examined at the 515-555 nm and 600-660 nm range exhibited no fluorescence, however for gelatin, the control sample has intense fluorescence in the 515-555 nm range, with very slight fluorescence in the 600-660 range. At the 0 mM offering for gelatin alone, no difference could be distinguished between the control and test samples. In the 600-660 nm range (Figure 10), only the gelatin control samples exhibited a very pale pink fluorescence. At the 10 mM offering, the fluorescence for gelatin alone intensified in both ranges. With respect to EDC and gelatin/chitosan samples that were treated with 0 mM, and 10 mM offerings, the 515-555 nm emission was strong for 0 mM gelatin/chitosan and more intense for 10 mM offering. In the 600-660 range, the emission was subdued.

When gelatin and gelatin/chitosan were treated with the water soluble carbodiimides, XL702 and XL725, and the samples' epi-fluorescence were examined the following was found. The images shown in Figure 11 show basically with respect to XL702 modification, in the 515-555 range, the gelatin and gelatin/chitosan were not indicating any enhanced epi-fluorescence. In the 600-660 range there is minimal enhancement.

With respect to XL725 (Figure 12), the differences in gelatin alone between the 0% and 25% are significant in both ranges, and the gelatin/chitosan samples are showing dramatic changes in epi-fluorescence in both ranges between the unmodified and modified samples.

These results are demonstrating that the individual components alone have little or no fluorescence (at these emissions) but when the concentration of XL725 is increased, the corresponding increase in fluorescence, particularly in the 515-555 for both gelatin and gelatin/chitosan is significant; in the 600-660 nm range, the increase in fluorescence is dramatic. These results suggest a synergistic effect of combining gelatin and chitosan, modifying with EDC and XL 725. These results correlate with physical properties and molecular weight distribution, with less effect seen in XL 702 at least with respect to epi fluorescence. However, if one examines these results from the fluorescence study, and correlates with the physical properties, molecular weight distribution, they are all indicating that a reaction is taking place with the gelatin, chitosan and carbodiimide and this reaction is being driven by the concentration of the carbodiimide.

Conclusions

Water soluble multifunctional carbodiimides have been shown to crosslink gelatin, chitosan, and in preparation of gelatin/chitosan biopolymers. These products are non-toxic, inexpensive and commercially available. They behave similar to EDC in that the biopolymer formed has superior physical properties to those of individually modified protein and carbohydrates. Molecular weight distribution studies indicate that modification has taken place. This is further substantiated by epi-fluorescent imaging, in that the compounds alone have little or no fluorescent but when reacted with the carbodiimide the fluorescence increases significantly, particularly with respect to XL725 reaction, and these fluorescent properties correlate with physical properties and molecular weight distribution. These products have potential to make interesting products such as films.

Acknowledgements

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