Reaction of Protein and Carbohydrates with EDC for Making Unique Biomaterials*

by
M.M. Taylor,† L.P. Bumanlag, 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

Prior 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. These treatments (microbial transglutaminase, genipin, and polyphenols in the form of vegetable tannins), were effective in reacting with gelatins, whey protein concentrate (WPC), and/or chitosan, alone or in combinations, to give products with interesting functional properties. All crosslinkers were either natural products and/or sustainable materials. In our continuing studies of chemoenzymatic methods to crosslink collagen and collagen by-products, we investigated the extensively reported 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), which has been used to crosslink proteins for purpose of making biomaterials. This present study examined the reactivity of various concentrations of EDC with gelatin, chitosan and combinations of both, in the presence and absence of N-hydroxysuccinimide (NHS) at optimal times, and temperatures, and the effect these parameters had on physical properties, molecular weight distribution and free amine content. It was found that both gelatin and chitosan had reactivity with EDC and the physical properties reflected the concentration of both the carbodiimide and gelatin. It was found however that 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, resulting in unique products. This study provides a better understanding of the reactivity of carbodiimide and optimal conditions for developing appropriate products.

Introduction

Gelatin and collagen hydrolysate, extracted from the solid byproducts of leather manufacture, had been chemically crosslinked and grafted with polymerizable monomers to add functionality.[1-4] In earlier research from this laboratory, the use of glutaraldehyde-modified gelatin as a filling agent was described.[5] In our prior research, we examined whether the extensively reported [6-8] enzymatic polymerization of gelatin using environmentally benign and relatively inexpensive microbial transglutaminase would effectively produce filled products similar to those described in chemical modification. Research was directed toward optimizing desirable functional properties such as gel strength and thermal stability, to produce materials with the potential to serve as components of filling or finishing agents in leather manufacturing or for other industrial applications.[9-11] Biopolymers, formed by the enzymatic crosslinking of dissimilar proteins, have the potential for generating novel products.[12-16] Based on this research, we enzymatically reacted gelatin with casein[17-18] or whey proteins[19-21] and after characterization, found that unique, highly polymerized products were obtained. These products were applied to blue stock,[22,23] and we demonstrated that the fillers did not affect the mechanical properties of the treated leather[24] and most importantly, when applied to low quality hides[25] it was determined that these products significantly improved the subjective properties of the leather and presented more cutting area.

*Presented in part at the 111th annual American Leather Chemists Association Meeting, June 10-13, 2015, Pinehurst, NC

†Corresponding author e-mail: taylormaryannm@gmail.com

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

Vegetable tanning, using polyphenols extracted from plant materials, is used primarily for the production of heavy leathers used in saddles, belts and shoe soles.[26-27] Polyphenols involved in this vegetable tannage have recently been investigated for their ability to crosslink gelatin.[28-35] Polyphenols are known to react under oxidizing conditions with side chain amino groups of peptides, leading to formation of cross-links in protein.[28] It was also demonstrated[28,36] that biopolymer type products could be made by reaction of polyphenols with gelatin and pectins as well as with starch and chitosan, respectively.

Vegetable tanning agents, e.g. quebracho and tara, contain polyphenols and when these less expensive tannins were applied to gelatin and other waste agricultural products, it was demonstrated that modification took place similar to using the polyphenolic acids, resulting in products amenable to be used in leather processing.[37-39] The vegetable tannin-modified gelatin and whey products had improved physicochemical properties and resulted in products that could be used as fillers in leather processing.[40,41] The quebracho- and tara-modified gelatin products were applied to chrome tanned hides and demonstrated that quality of resulting leather was far superior to untreated controls. Wet white or chrome free leather has a tendency to be flat and tinny and of a lesser quality than wet blue but after treatment with tara-modified gelatin, the quality was enhanced with special emphasis on handle and break of leather. Also, by improving the quality of wet white, a better economic return should be realized. Thus, inexpensive renewable resources, tannins and agricultural waste products, are being utilized to improve quality of leather.

In our continuing investigation of chemoenzymatic methods for modification of protein and carbohydrate waste products, we investigated a cross-linking method for collagen-based biomaterials that has been extensively reported using the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). Olde Damink *et al*. stated that 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.[42] Treatment of dermal sheep collagen with EDC resulted in materials with an increased shrinkage temperature (Ts) and decreased free amine group content, showing that cross-linking occurred. Addition of N-hydroxysuccinimide (NHS) to the EDC-containing cross-linking solution increased the rate of cross-linking. Liang *et al*. has speculated that carbodiimide could form intramolecular crosslinks within a gelatin molecule or short-range intermolecular crosslinks between two adjacent gelatin molecules.[43] Liang *et al*. has also suggested that carbodiimide-crosslinked gelatin hydrogels could be used as bioadhesives to overcome the cytotoxicity problem associated with formaldehyde-crosslinked gelatin hydrogels. The researchers found that crosslinking a gelatin hydrogel with carbodiimide was rapid and the gelation time was short. Tropini *et al*. have found that wheat gluten films could be cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide.[44] Wheat gluten films were cast from aqueous dispersions containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as cross-linking reagents and glycerol as a plasticizer, with subsequent characterization of the films by measurement of protein and water content, amount of amino groups, swelling of the films in water, and mechanical properties such as tensile strength and strain at maximum stress. Recent successful applications of EDC to fish gelatins have been reported.[45-47] 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[48-52] Finally, Wang *et al*. suggest that EDC modified gelatin/chitosan could be a candidate for implantable bioartificial livers.[53]

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.[54] Thus treatment of waste proteins, perhaps in conjunction with chitosans or other waste proteins, with EDC, could possibly lead to products with interesting functional properties that could be used in leather processing.

This present study will examine the reactivity of EDC with gelatin, chitosan and combinations of both, in the presence and absence of NHS. We will determine optimal time, temperatures, and concentrations of reactants and look at effect on physical properties, molecular weight distribution and free amine content. Furthermore, this will be a model study for future investigations in which cost-effective water soluble functional carbodiimides[55] will be reacted with waste products for the purpose of making products for leather processing (films, coatings, fillers, etc.)

EXPERIMENTAL

Materials

Two commercial Type B gelatins from bovine skin, characterized in this laboratory as 123g and 175g Bloom, EDC, NHS, Ninhydrin reagent, and chitosan (low molecular weight and 75-85% acetylated) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). All other chemicals were analytical grade and used as received.

Methods

Preparation of EDC-modified Gelatin

Gelatin (123g or 175g Bloom) (10g) was suspended in 40 ml of water (Figure 1), held for 2 h 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 hr 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 from 5 to 6. EDC (calculated to be 0-50 mM final solution), and NHS (calculated to be 1:15 ratio to EDC), were 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 EDC/NHS 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 1 h. The samples were cooled to RT and then chilled for 17 h at 10ºC 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 EDC-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 2 h and then o/n at 4°C. The samples were warmed in 40°C bath, cooled and the pH was slowly adjusted from 5-6; precipitation of chitosan was continually monitored. EDC (calculated to be 0-30 mM final solution), and NHS (calculated to be 1:15 ratio to EDC), were prepared in 10 mL of water and added immediately to chitosan solution. The samples were then treated as described above for EDC-modified gelatin samples. Control samples, with no EDC/NHS additions were also run.

Preparation of EDC-modified Gelatin/chitosan Biopolymer Products

Chitosan (0.35g) was dissolved in 20 ml of 0.15M acetic acid (Figure 2). Gelatin (123g Bloom) (10g) was suspended in 20 ml of water, held for 2 h 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 2 hr and then stored o/n at 4°C. The solution was then heated at 40°C for 15 min, and the pH slowly adjusted from 5-6; precipitation of chitosan was continually monitored. EDC (calculated to be 0-10 mM final solution), and NHS (calculated to be 1:15 ratio to EDC), were prepared in 10 mL of water and added immediately to gelatin/chitosan solution. The samples were then treated as described above for EDC-modified gelatin samples. Control samples, with no EDC/NHS additions were also run.

Analyses

Physical Properties and Molecular Weight Distribution

Gel strength, melting point, and viscosity of the gelatin/chitosan/EDC-treated solutions were determined as described in a previous publication [9]. Protein molecular weights were estimated as described previously.[56] 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%

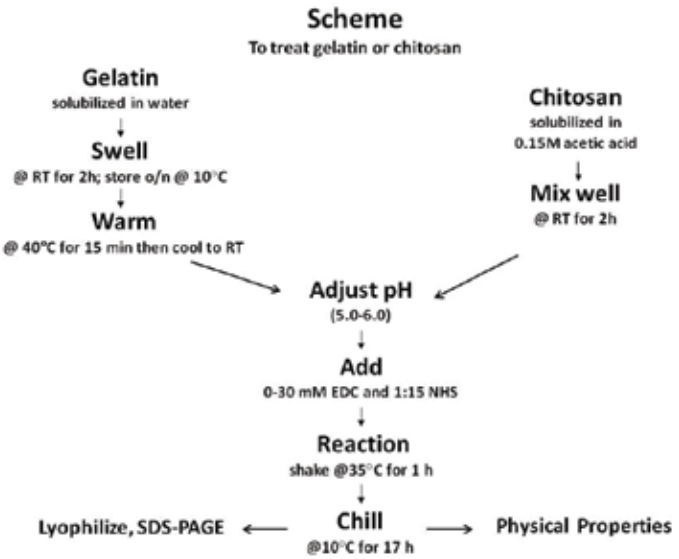

Figure 1. Flow diagram for preparation of EDC-modified gelatin or chitosan.

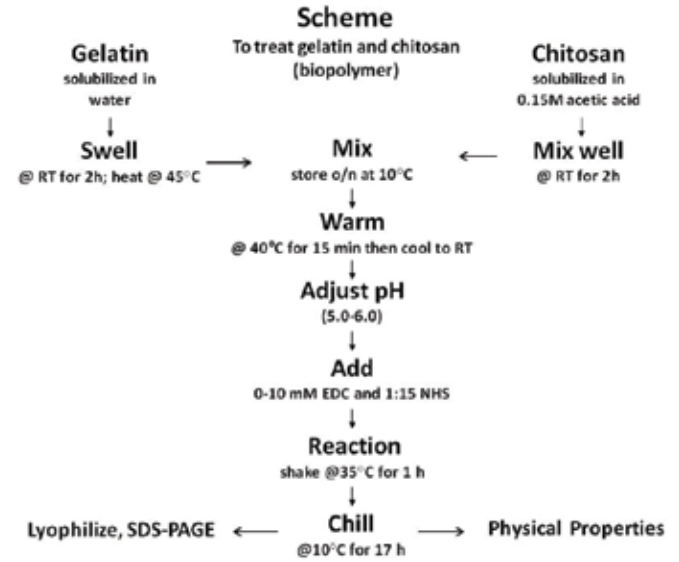

Figure 2. Flow diagram for preparation of EDC-modified gelatin/chitosan biopolymer.

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).

Ninhydrin Test for Primary and Secondary Amines

Determination of primary and secondary amines remaining in protein after reaction with EDC gives an indication of the extent of crosslinking, and this was determined by the

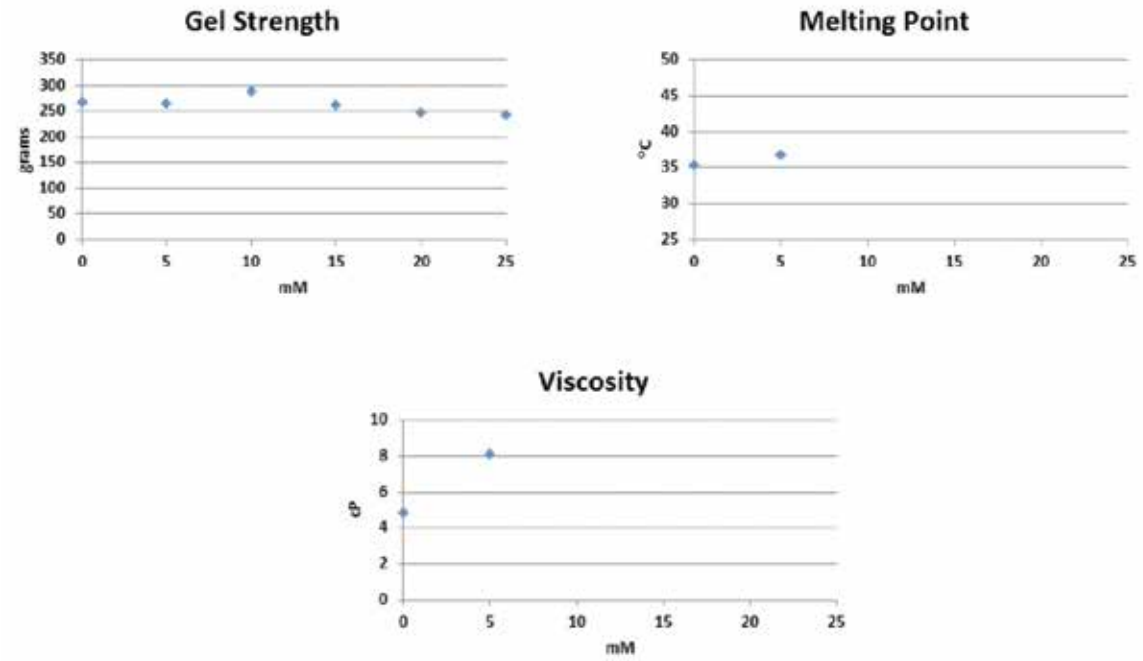

Figure 3. Gel strength, melting point, and viscosity (at 60°C) of 123g Bloom gelatin (10% w/v) treated with 0-25 mM EDC at pH 5.0-6.0, 35°C for 1 h.

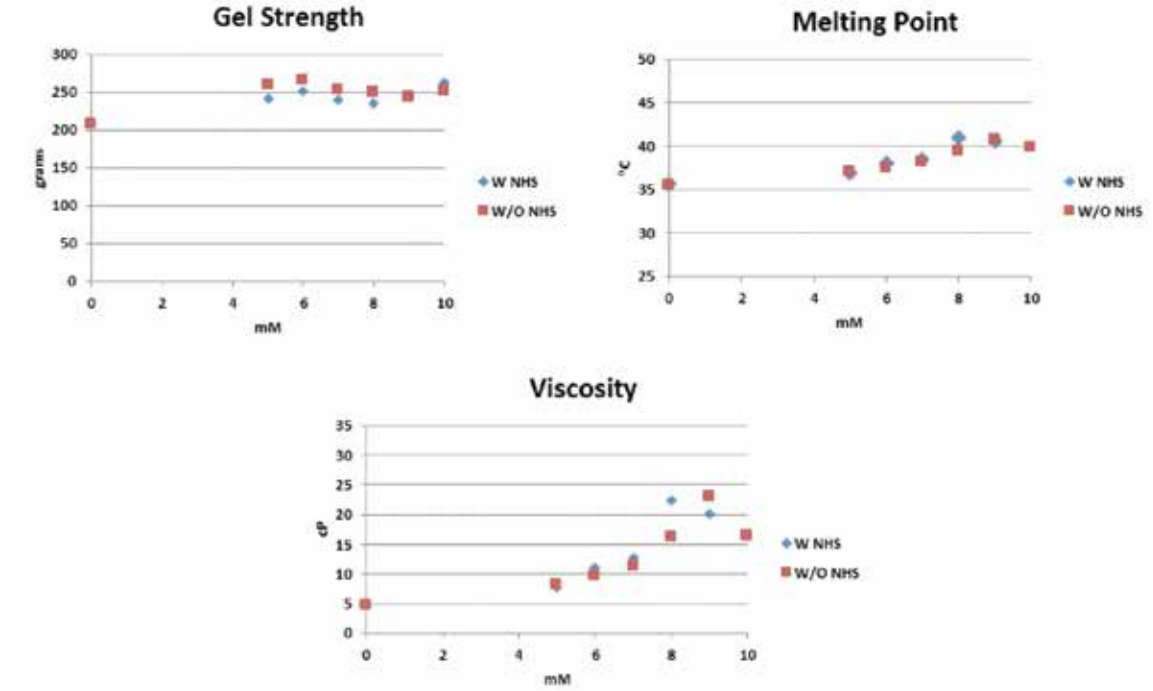

Figure 4. Gel strength, melting point, and viscosity (at 60°C) of 123g Bloom gelatin (10% w/v) treated with 0-10 mM EDC at pH 5.0-6.0, 35°C for 1 h.

Ninhydrin Test.[57] The procedure was carried out as follows. The standard (Leucine) and test samples were prepared as outlined in Tables I and II. The samples were mixed well using a vortex. A marble was added to top of each tube to cover, and then all tubes were heated at 100°C for 10 min using a heater block. The tubes were transferred to ice bath for 10 min. Ethanol (2 ml) was added to each tube and the tubes were mixed using a vortex. The absorbance was read at 570.0 nm; standard and test samples were read vs. a blank. A standard curve was drawn and free amino group concentration was read from the standard curve.

RESULTS AND DISCUSSION

Reaction of 10% Gelatin with EDC (w/wo NHS)

In these initial experiments, conditions outlined in the literature were applied to 175 g Bloom gelatin. Accordingly, a 10% solution of gelatin was prepared and to it was added 30 mM EDC with a 1:15 ratio of NHS. The solution gelled immediately upon addition of the EDC/NHS, and formed an irreversible chemical gel. This reaction was so rapid, that in next experiment, a lower bloom gelatin (123 g) was tried to see the effect that these conditions would have on the reaction. In this set of experiments, 30 mM of EDC with 1:15 NHS was added to a 10% w/v solution of gelatin. This reaction proceeded slightly slower and we were able to transfer the reaction mixture to the appropriate testing vessels (for Bloom and melting point). These samples also formed an irreversible chemical gel. In the next set of experiments, continuing to use the 123 Bloom gelatin, we decreased the concentration of EDC; as a control, 0 mM was added and then the concentration was increased from 5 to 25 mM.

These lower concentrations (from 10 mM to 25 mM, increasingly became more viscous as they were prepared for the 35°C bath. After 1 h only the control (0 mM) and the 5 mM could be analyzed for viscosity. The 10 mM to 25 mM had formed an irreversible chemical gel and melting point and viscosity could not be determined. The gel strengths of the 0-25 mM samples and melting point, and viscosity of the 0-5 mM samples are shown in Figure 3.

TABLE I

Standard

Tube	1	2	3	4	5	6
Standard (uL)	0	100	200	300	400	500
Water (uL)	1000	900	800	700	600	500
Ninhydrin (ml)	0.5	0.5	0.5	0.5	0.5	0.5

TABLE II

Samples

Tube	7	8	9	10	11	12
Sample weight (mg)	4	4	4	4	4	4
Water (uL)	1000	1000	1000	1000	1000	1000
Ninhydrin (ml)	0.5	0.5	0.5	0.5	0.5	0.5

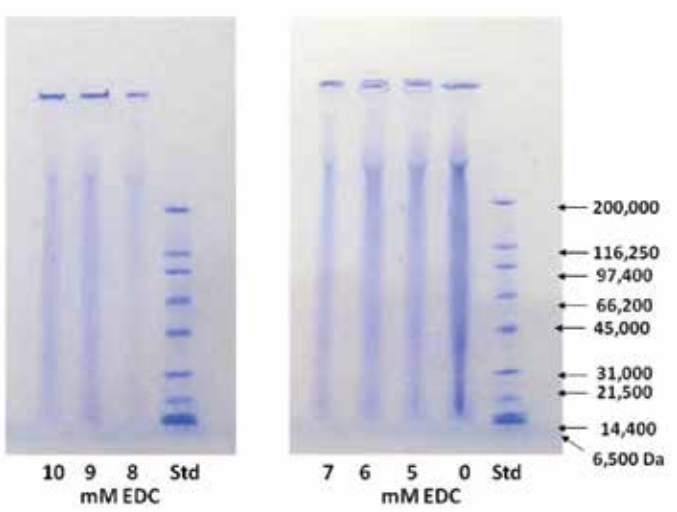

Figure 5. SDS-PAGE of 123g Bloom gelatin (10% w/v) treated with 0-10 mM EDC at pH 5.0-6, 35°C for 1 h; molecular weights are shown in Da.

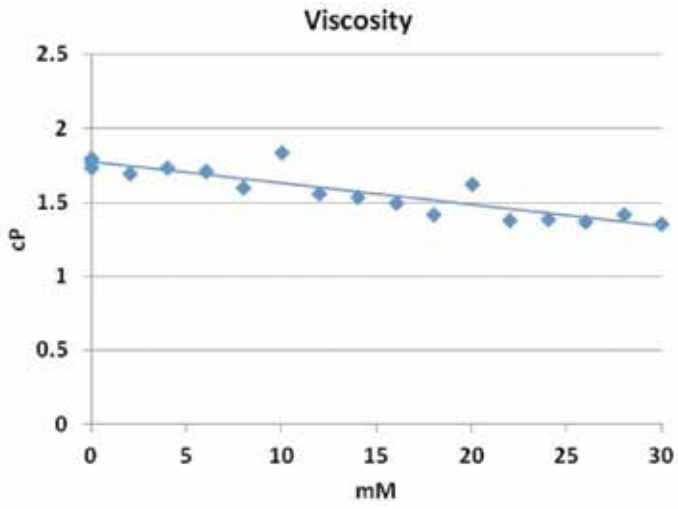

Figure 6. Viscosity (at 60°C) of 0.35% chitosan treated with 0-30 mM EDC at pH 5.0-6.0, 35°C for 1 h.

In the next set of experiments, from 5 to 10 mM of EDC with and without NHS was used to treat 10% gelatin solution. The physical properties, melting point and viscosity (Figure 4) indicate that from 5 to 8 mM, an irreversible gel had not formed. Gel strengths of these samples are also shown in the figure. It appears that in the range from 5-8 mM, the gel strength, melting point and viscosity results are similar with and without NHS. However in 9-10 mM range the melting point and viscosity values with and without the NHS differ, in that the 10 mM range with NHS the samples did not melt and viscosity could not be run. The presence of NHS appears to increase the efficiency of the reaction in the higher EDC concentrations. Finally, as seen in Figure 5, the molecular weight distribution studies are showing that as the EDC concentration increases the density of the gelatin bands decreases. These results correlate with the physical properties, particularly in the results for viscosity.

Reaction of 0.35% Chitosan with EDC

Literature has suggested that chitosan can take part in the EDC reaction.[48-53] In preparation for making biopolymers utilizing gelatin and chitosan treated with EDC, we ran a series of experiments in which we observed physical properties of EDC-treated chitosan. Chitosan (0.35% w/v) was treated for 1h, at pH 5-6 and 35°C, with varying amounts of EDC (0-30 mM). The samples did not gel so no melting point could be run; as seen in Figure 6, the viscosity of the chitosan decreased as the EDC concentration increased (perhaps due to the slight change in the linear oligomer as influenced by the EDC reaction). The viscosity of the control sample, which contained 0 mM of EDC, was 1.80 cP with STD of 0.05 (n=3), was significantly higher than the samples treated with varying amounts of EDC.

Reaction of 10% Gelatin and 0.35% Chitosan with EDC

Several authors have reported on reaction of EDC with fish gelatin and chitosan and the subsequent effects on properties of resultant films.[48-52] In the following set of experiments, 10% gelatin and 0.35% chitosan were treated with varying amounts of EDC (0-10 mM) at pH 5-6, 35°C. The effect on physical properties of the products and molecular weight distribution were examined. In the initial experiment, in which 0-10 mM of EDC was reacted with gelatin and chitosan, the gel strength was higher than when gelatin alone was treated. With respect to melting point and viscosity the results are more dramatic (Figure 7) in that after 5 mM offering, the samples did not melt and viscosity could not be run. Even the samples that did melt had much higher values than the gelatin (viscosity) and chitosan (viscosity) alone at the same concentration of EDC. The molecular weight distribution of the samples was run (Figure 8) and the disappearance of the gelatin bands at the higher EDC concentration correlates with the changes in the physical properties.

Reaction of 5% Gelatin and 0.35% Chitosan with EDC

Having achieved a significant effect on physical properties using 10% gelatin, 0.35% chitosan and varying mM of EDC, we then examined the effect that a lesser amount of gelatin,

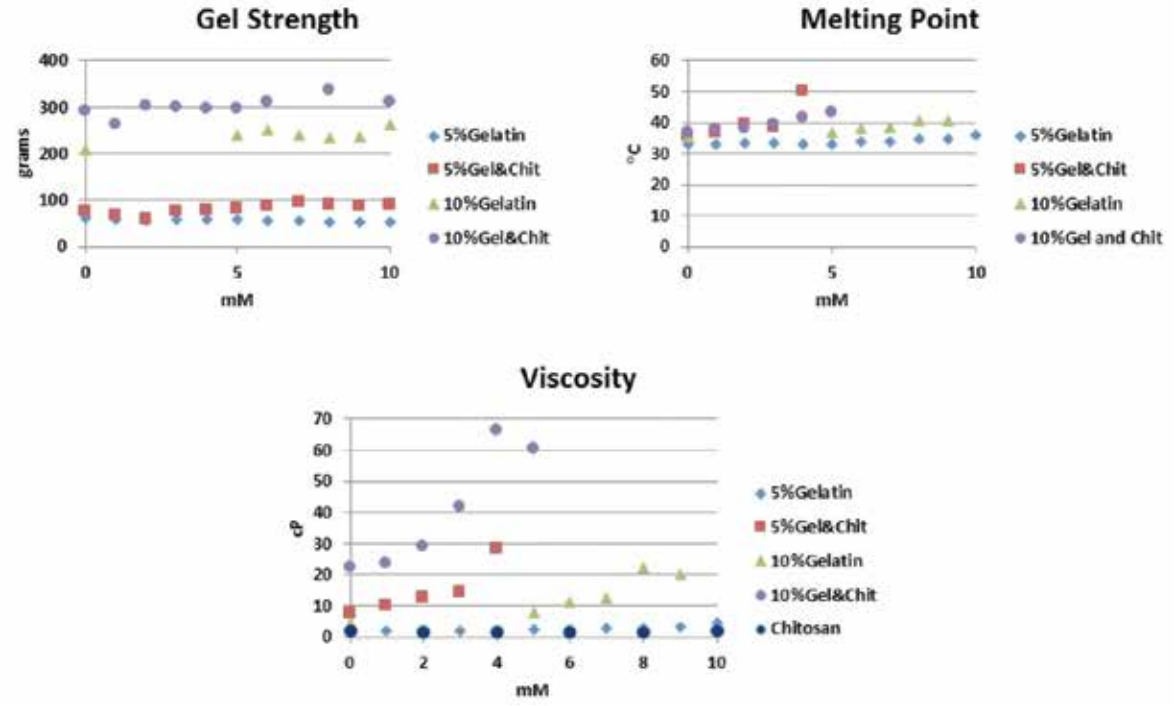

Figure 7. Gel strength, melting point, and viscosity (at 60°C) of 123g Bloom gelatin (5% and 10% w/v), 0.35% chitosan, biopolymer with 5% and 10% gelatin and 0.35% chitosan, and viscosity (at 60°C) of 0.35% chitosan, all treated with 0-10 mM EDC at pH 5.0-6.0, 35°C for 1 h.

while keeping the chitosan at 0.35% and using similar amounts of mM of EDC, would have on these properties. We first ran a series in which 5% gelatin (w/w) was reacted with varying mM concentrations of EDC with NHS. The results of these experiments can be seen in Figure 7. Gelatin and chitosan were then reacted with EDC (0-10 mM) and as was shown in reaction of 10% gelatin, the physical properties of the resulting products were significantly different than the modified individual gelatin and chitosan (Figure 7). After the 5 mM EDC concentration, a chemical gel was formed and the melting point and viscosity could not be determined. Finally SDS PAGE was run on these samples (Figure 9) and the results are corroborating the data from the physical properties.

When the 5 and 10% loadings of gelatin biopolymer products were compared (Figure 7), the data indicate that the samples behaved similarly when increasing amounts of EDC were added, but as one would expect, the physical property values were reflecting the concentration (5% or 10%) of the added gelatin.

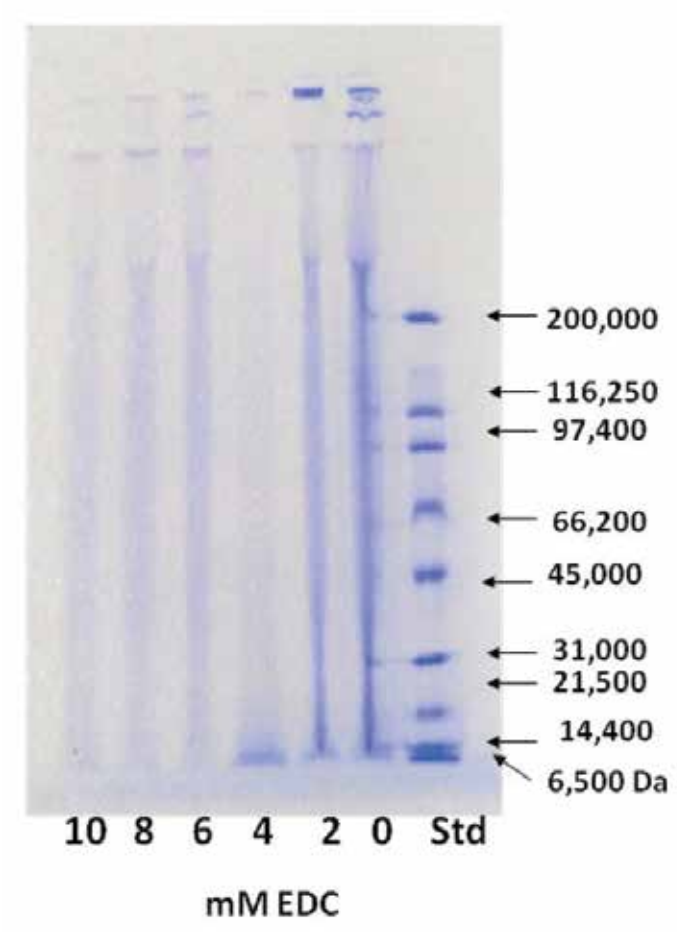

Figure 8. SDS-PAGE of 123g Bloom gelatin (10% w/v) and 0.35% chitosan (biopolymer) treated with 0-10 mM EDC at pH 5.0-6, 35°C for 1 h; molecular weights are shown in Da.

Free Amine Determination

Primary and secondary amines remaining after reaction with EDC will give an estimation of extent of crosslinking. The ninhydrin test[57] was used for this analysis. The products resulting from reaction of 10% gelatin alone with 0-10 mM

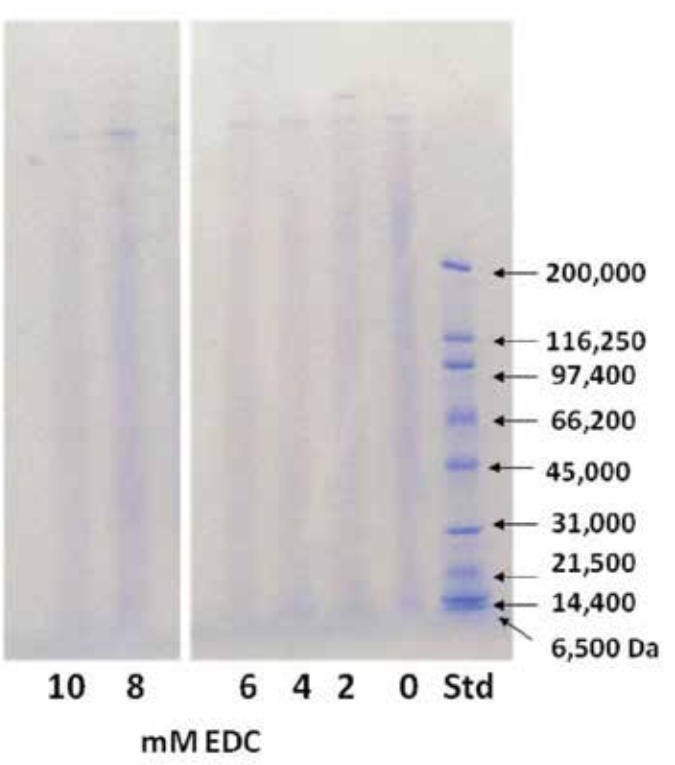

Figure 9. SDS-PAGE of 123g Bloom gelatin (5% w/v) and 0.35% chitosan (biopolymer) treated with 0-10 mM EDC at pH 5.0-6.0, 35°C for 1 h; molecular weights are shown in Da.

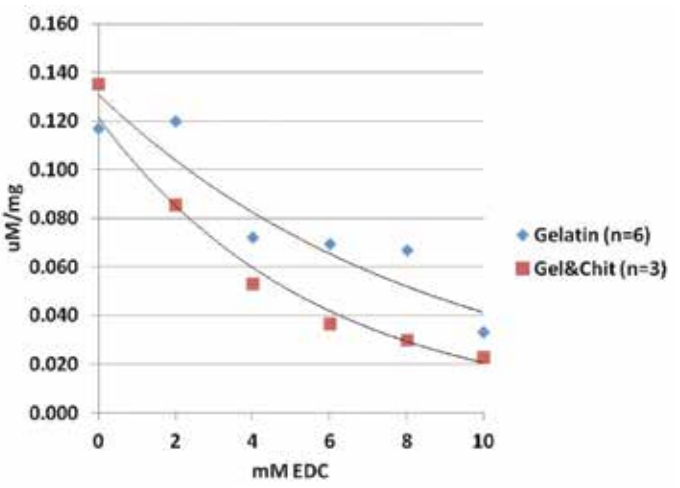

Figure 10. Primary and secondary amino groups (uM/mg) remaining after treatment of 10% (w/v) 123g Bloom gelatin alone and 10% (w/v) 123g Bloom gelatin and 0.35% chitosan (biopolymer), with 0-10 mM EDC at pH 5.0-6.0, 35°C, for 1 h.

EDC and 10% gelatin and 0.35% chitosan with 0-10 mM EDC were analyzed and the results can be seen in Figure 10. The data are indicating that the increasing concentration of EDC has an effect on the free primary and secondary amines remaining in these samples. The gelatin and chitosan reacted together with EDC show a slightly higher amount of free amine to begin with and its curve is showing a steeper decline than that for gelatin alone with EDC. These data correlate with physical properties (melting point and viscosity).

CONCLUSION

The extensively reported 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) reaction with proteins and carbohydrates, for purpose of making biomaterials, was investigated. The 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. It was found that both gelatin and chitosan had reactivity with EDC and the physical properties reflected the concentration of both the carbodiimide and gelatin. It was found however that 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, resulting in unique products. This is a model study for future investigations in which cost-effective water soluble functional carbodiimides[55] will be reacted with waste products for the purpose of making products for leather processing (films, coatings, fillers, etc.)

REFERENCES

1. Chatterji, P.R.; Gelatin with hydrophilic/hydrophobic grafts and glutaraldehyde crosslinks. *J. Appl. Polym. Sci.* **37**, 2203-2212, 1989.
2. Chatterji, P.R.; Glutaraldehyde crosslinked gelatin with polyacrylamide grafts. *J. Macromol. Sci.* **A27**, 435-443, 1990.
3. Satyanarayana, D. and Chatterji, P.R.; Hydrophilic crosslinked gelatin core with hydrophobic polymethyl acrylate grafts. Conditions of grafting and characteristics of the graft polymer. *J. Macromol. Sci.-Chem.* **A28,** 237-249. (1991).
4. Satyanarayana, D., and Chatterji, P.R.; Amphiphilic graft copolymer matrices from crosslinked gelatin. 1. Synthesis and characterization *J.M.S.-Pure Appl. Chem.* **A29**, 625-637, 1992.
5. Chen, W., Cooke, P.H., DiMaio, G. L., Taylor, M.M. and Brown E.M; Modified collagen hydrolysate, potential for use as a filler for leather. *JALCA* **96**, 262-267, 2001.
6. Sakamoto, H., Kumazaawa, Y. and Motoki M.; Strength of protein gels prepared with microbial transglutaminase as related to reaction conditions. *J. Food Sci.* **59**, 866-871, 1994.
7. Babin, H., and Dickinson, E.; Influence of transglutaminase treatment on the thermoreversible gelation of gelatin. *Food Hydrocolloids* **15**, 271-276, 2001.
8. de Carvalho, R. A. and Grosso, C.R.F.; Characterization of gelatin based films modified with transglutaminase, glyoxal and formaldehyde. *Food Hydrocolloids* **18**, 717-726, 2004.
9. Taylor, M.M., Cabeza, L.F., Marmer, W.N. and Brown, E.M.; Enzymatic modification of hydrolysis products from collagen using a microbial transglutaminase. I. Physical properties. *JALCA* **96**, 319-332, 2001.
10. Taylor, M.M., Liu, C.-K., Latona, N., Marmer, W.N. and Brown, E.M.; Enzymatic modification of hydrolysis products from collagen using a microbial transglutaminase. II. Preparation of films. *JALCA* **97**, 225-234, 2002.
11. Taylor, M. M., Liu, C. K., Marmer, W. N., and Brown, E. M.; Enzymatic modification of hydrolysis products from collagen using a microbial transglutaminase. III. Preparation of films with improved mechanical properties. *JALCA* **98**. 435-444, 2003.
12. Motoki, M., and Nio, N.; Crosslinking between different food proteins by transglutaminase. *J. Food Sci. 48, 561-566, 1983.*
13. Yildirim, M., Hettiarachchy, N.S. and Kalapathy, U.; Properties of biopolymers from cross-linking whey protein isolate and soybean 11s globulin. *J. Food Sci.* **61**. 1129-1131, 1164, 1996.
14. Yildirim, M., and Hettiarachchy, N.S.; Biopolymers produced by cross-linking soybean 11s Globulin with whey proteins using transglutaminase. *J. Food Sci.* **62**, 270-275, 1997.
15. Yildirim, M. and Hettiarachchy, N.S.; Properties of films produced by cross-linking whey proteins and 11s globulin using transglutaminase. *J. Food Sci.* **63**, 248-252, 1998.
16. Oh, J.-H., Wang, B., Field, P.D., and Aglan, H.A.; Characteristics of edible films made from dairy proteins and zein hydrolysate cross-linked with transglutaminase. *International Journal of Food Science and Technology* **39**, 287-294, 2004.
17. Taylor, M.M., Marmer, W.N., and Brown, E.M.; Characterization of biopolymers prepared from gelatin and sodium caseinate for potential use in leather processing. *JALCA* **100**, 149-159, 2005.
18. Taylor, M.M., Bumanlag, L.P., Marmer, W.N. and Brown, E.M.; Use of enzymatically modified gelatin and casein as fillers in leather processing. *JALCA* **101**, 169-178, 2006.
19. Taylor, M.M., Marmer, W.N. and. Brown, E.M.; Preparation and characterization of biopolymers derived from enzymatically modified gelatin and whey. *JALCA* **101**, 235-248, 2006.

20. Hernàndez Balada, E., Taylor, M.M., Phillips, J.G., Marmer, W.N. and Brown, E.M.; Properties of biopolymers produced by transglutaminase treatment of whey protein isolate and gelatin. *Bioresource Technology*. **100**, 3638-3643, 2009.
21. Hernàndez-Balada, E., Taylor, M.M., Eleanor M. Brown, E.M., Liu, C.-K. and Cot, J.: Whey protein isolate: A potential filler for the leather industry. *JALCA* **104**, 122-130, 2009.
22. Taylor, M.M., Marmer, W.N. and Brown, E.M.; Evaluation of polymers prepared from gelatin and casein or whey as potential fillers. *JALCA* **102**, 111-120, 2007.
23. Taylor, M.M., Marmer, W.N. and Brown, E.M.; Preparation and characterization of fillers produced from renewable resources. Evaluation of treated leather. *Proceedings, XXIXth Congress, International Union of Leather Technologists and Chemists Societies/103rd annual meeting, American Leather Chemists Association* meeting, June 20-24, Washington DC. 2007.
24. Taylor, M.M., Marmer, W.N. and Brown, E.M.; Effect of fillers from enzymatically modified proteins on mechanical properties of leather. *JALCA* **103**, 128-137, 2008.
25. Taylor, M.M., Lee, J. Bumanlag, L.P., Hernàndez Balada, E., Cooke, P.H. and Brown. E.M.; Treatment of low-quality hides with fillers produced from sustainable resources. Effect on properties of leather. *JALCA* **104**, 324-334, 2009.
26. Haslam E.; Vegetable Tannage - Where Do the Tannins Go? *J. Soc. Leather Techn. Chem.* **81,** 45-51, (1997.
27. Slabbert N.P.; The basics of practical tanning systems reconciled with vegetable tanning theories. *JALCA* **94,** 1-7, 1999.
28. Strauss, G., and Gibson, S.M.; Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. *Food Hydrocolloids* **18**, 81-89, 2004.
29. Cao, N., Fu, Y., and He, J.; Mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid. *Food Hydrocolloids* **21**, 575-584, 2007.
30. Balange, A. and Benjakul, S.; Enhancement of gel strength of bigeye snapper (*Priacanthus tayenus*) surimi using oxidised phenolic compounds. *Food Chemistry* **113**, 61-70, 2009.
31. Balange, A. and Benjakul, S.; Effect of oxidised phenolic compounds on the gel property of mackerel (*Rastrelliger kanagurta*) surimi. *LWT - Food Science and Technology* **42**, 1059-1064, 2009.
32. Balange, A. K. and Benjakul, S.; Effect of oxidised tannic acid on the gel properties of mackerel (*Rastrelliger kanagurta*) mince and surimi prepared by different washing processes. *Food Hydrocolloids* **23**, 1693-1701, 2009.
33. Kosaraju, S. L., Puvanenthiran, A., Lillford, P.; Naturally crosslinked gelatin gels with modified material properties. *Food Research International* **43**, 2385-2389, 2010.
34. Zhang, X., Do, M.D., Casey, P., Sulistio, A, Qiao, G.G., Lundin, L., Lillford, P., Kosaraju, S.; Chemical modification of gelatin by a natural phenolic cross-linker, tannic acid. *Journal of Agricultural and Food Chemistry* **58**, 6809-6815, 2010.
35. Li, J.-H., Miao, J., Wu, J.-L., Chen, S.-F.,Zhang, Q.-Q.; Preparation and characterization of active gelatin-based films incorporated with natural antioxidants. *Food Hydrocolloids* **37.** 166-173, 2014.
36. Mathew, S., and Abraham, T.E. Characterisation of ferulic acid incorporated starch–chitosan blend films. *Food Hydrocolloids* **22**, 826-835. 2008.
37. Taylor, M.M, Lee, J., Bumanlag, L.P., Latona, R.J., Brown, E.M., and Liu, C.-K.; Preparation and characterization of polyphenol-modified gelatin products. *JALCA* **107**, 51-59, 2012.
38. Taylor, M.M., Lee, J., Bumanlag, L.P., Latona, R.J., Brown, E.M., and Liu, C.-K.; Preparation and evaluation of tara-modified proteins. *JALCA* **108**, 16-22, 2013.
39. Taylor, M.M., Lee, J., Bumanlag, L.P., Latona, R.J., Brown, E.M., and Liu, C.-K.; Biopolymers produced from gelatin and whey protein concentrate using polyphenols. *JALCA* **109**, 82-88, 2014.
40. Taylor, M.M., Medina, M., Lee, J., Bumanlag, L.P., Brown, E.M., and Liu, C.-K.; Treatment of wet blue with fillers produced from quebracho-modified gelatin. *JALCA* **107**, 416-421, 2012.
41. Taylor, M.M., Medina, M., Lee, J., Bumanlag, L.P., Latona, N.L., Brown, E.M., and Liu, C.-K. Treatment of hides with tara-modified protein products. *JALCA* **108**, 438-444, 2013.
42. Olde Damink, L.H.H., Dijkstra, P.J., Van Luyn, M.J.A., Van Wachem, P.B., Nieuwenhuis, P., and Feijen, J.; Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. *Biomaterials* **17**, 765-773, 1996.
43. Liang, H.C., Chang, W.H., Liang, H.F., Lee, M.H., and Sung, H.W.; Crosslinking structures of gelatin hydrogels crosslinked with genipin or a water-soluble carbodiimide. *Journal of Applied Polymer Science* **91**, 4017-4026, 2004.
44. Tropini, V., Lens, J.-P., Mulder, W.J. and Silvestre, F.; Wheat gluten films cross-linked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. *Industrial Crops and Products* **20**, 281-289, 2004.
45. Piotrowska, B., Sztuka, K., Kolodziejska, I., and Dobrosielska, E.; The influence of transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) on the properties of fish-skin gelatin films. *Food Hydrocolloids* **22**, 1362-1371, 2008.

46. Sztuka, K., and Kolodziejska, I.; The influence of hydrophobic substances on water vapor permeability of fish gelatin films modified with transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). *Food Hydrocolloids* **23,** 1062-1064, 2009.
47. Staroszczyk, H., Pielichowska, J., Sztuka, K., Stangret, J., Kolodziejska, I.; Molecular and structural characteristics of cod gelatin films modified with EDC and TGase. *Food Chemistry* **130**, 335-343, 2012.
48. Staroszczyk, H., Sztuka, K., Wolska, J., Wojtasz-Pajak, A., Kolodziejska, I.; Interactions of fish gelatin and chitosan in uncrosslinked and crosslinked with EDC films: FT-IR study. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **117,** 707-712, 2014.
49. Kolodziejska, I., Piotrowska, B., Bulge, M., and Tylingo, R.; Effect of transglutaminase and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide on the solubility of fish gelatin–chitosan films. *Carbohydrate Polymers* **65,** 404-409, 2006.
50. Kolodziejska, I., and Piotrowska, B.; The water vapour permeability, mechanical properties and solubility of fish gelatin–chitosan films modified with transglutaminase or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and plasticized with glycerol. *Food Chemistry* **103,** 295-300, 2007.
51. Sztuka, K., and Kolodziejska, I.; Effect of transglutaminase and EDC on biodegradation of fish gelatin and gelatin-chitosan films. *Eur Food Res Technol* **226**, 1127-1133, 2008.
52. Pajak, A., and Kolodziejska, I.; Interactions of fish gelatin and chitosan in uncrosslinked and crosslinked with EDC films: FT-IR study. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **117**, 707-712, 2014.
53. Wang, X. H., Li, D.P., Wang, W.J., Feng, Q.L., Cuia, F.Z., Xu,Y.X., Song, X.H., van der Werf, M.; Crosslinked collagen/chitosan matrix for artificial livers. *Biomaterials* **24.** 3213-3220, 2003.
54. https://www.lifetechnologies.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/carbodiimide-crosslinker-chemistry.html#/legacy=www.piercenet.com (accessed 08/10/15)
55. Hesselmans, L. C. J., Derksen, A.J., van den Goorbergh, J.A.M.; Polycarbodiimide crosslinkers. *Progress in Organic Coatings*, **55,** 142-148, 2006.
56. Taylor, M.M., Marmer, W N., and Brown, E M.; Molecular weight distribution and functional properties of enzymatically modified commercial and experimental gelatins. *JALCA* **99,** 129-140, 2004.
57. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/2/n7285pis.pdf (accessed 06/22/15)