# Preparation of Biobased Sponges from Un-tanned Hides

by

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## Abstract

One of our research endeavors to address ongoing challenges faced by the U.S. hide and leather industries is to develop innovative uses and novel biobased products from hides to improve prospective markets and to secure a viable future for hides and leather industries. We had previously investigated the production of nonwoven, green composites, and films from collagen fiber networks, which were extracted from un-tanned hides and from tannery solid wastes, such as splits or trimmings. Recently, we investigated the preparation of biobased sponges from un-tanned, specifically limed hides, which have potential commercial applications in medical care. We also investigated the effects of processing steps such as bating and alkaline treatments using sodium hydroxide (NaOH) on the morphology and viscoelasticity of resultant sponges from un-tanned hides. Results showed that the treatments of fiber networks have significant effects on the properties of resultant sponges. The dynamic mechanical analysis showed alkaline treatments yielded stiffer sponges than limed and bated samples. SDS-PAGE analysis showed the molecular weights of sponges were maintained in the range as those of commercial collagen samples. Lastly the resultant sponges are in the desirable apparent density range for a potential application as a medical sponge.

## Introduction

Improving utilization of waste is one of major challenges that the U.S. hides and leather industries are currently facing. We have addressed this challenge by developing innovative uses and novel biobased products from the solid fibrous wastes generated from tanneries.[1-4] We hypothesized that those solid fibrous wastes from a tannery can be purified into collagen fiber networks and utilized in making useful biobased products such as green (i.e. renewable and degradable) composites and biobased films, all of which have great market potential. Earlier studies were devoted to understanding the effects of dehydration on the morphology and physical properties of the fiber networks derived from un-tanned hides, which will be the starting material for constructing nonwoven and green composites.[4] Five dehydration methods were investigated and observation showed solvent- and freeze-drying yielded the lowest apparent density indicating a higher degree of separation in the fibrous networks that will be favorable for further processing into useful products. The results from comparisons showed that samples frozen and then followed by vacuum drying offer many advantages over those from the other dehydration methods in terms of economic and open fibrous structure.

Further study investigated the use of crosslinking with glutaraldehyde and other treatments to improve the integrity of fibrous structures that offer better stability and physical properties.[3] Crosslinking is a process to apply a chemical agent - like glutaraldehyde to bridge molecular chains, thereby providing stability and improved physical properties to collagen matrices. There are various crosslinking treatments currently being used today for collagen materials.[5-8] Two of the most common methods, i.e. transglutaminase and glutaraldehyde, were used to treat the collagen fiber networks. Results showed that glutaraldehyde treatment yielded a highly open structure, in which the fibers are well separated from each other.[3]

Research was carried out for preparations of nonwoven and green composites derived from fiber networks extracted from hides.[2] Nonwoven sheets were prepared using paper-making technology. They were then used as reinforced components to make composites that used gelatin as the matrix. Test results showed that finer size fibers and higher portions of gelatin yielded better tensile strength and higher stiffness of nonwoven and composites. The results of this research are useful to the production of high quality fibrous products such as high efficiency air filters or green composites. Another potential

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Manuscript received September 18, 2015, accepted for publication January 18, 2016.
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product that can be derived from un-tanned hides or tannery solid wastes is biobased films. Many reports have shown the important applications of biobased films in medical care such as wound dressing and skin repair because of the excellent bio-compatibility.[9-12] Beside medical applications, recently, there is a great interest in using biobased films for food packaging.[13-18] In a recent study, matrix biopolymer collagen obtained from tannery waste combined with guar –gum was incorporated with varying proportions of carbon nanotubes to form flexible and electrically conducting carbon nano- biocomposites for potential wide applications in biosensors and electronic devices.[19]

We recently reported that the most important step for preparing a collagen film or fiber is its dissolution.[20-26] Acid extractions using acetic acid followed by enzymatic treatments mostly by pepsin are the most popular methods for extraction of soluble and insoluble collagen from different biological sources.[21-23]

We modified a dissolution method by Nishihara, which was very effective in digesting hide fibers to make collagen solutions, and it was less detrimental to the environment.[20] The dissolution system utilized a commonly used protease, pepsin at a suitable pH as described later. A study focused on preparations of biobased films from un-tanned hides, including limed hides and delimed-bated hides, which correspond with their actual tannery waste of limed splits and their trimmings. These films could be used in the field of medicine because of their good compatibility with living tissue, and in packaging of food due to their excellent mechanical properties, and accepted edibility. The aqueous concentration of collagen extracted from the hides and the timing of the addition of glutaraldehyde during the preparation of films had significant positive effects on their mechanical properties.

To further exploit the utilization of tannery solid wastes, we recently carried out an investigation to prepare a biobased sponge from collagen solutions derived from un-tanned hides. Collagen sponges have many unique properties that are desirable in medical applications.[27-30] Theoretical as well as practical-clinical applications of collagen sponge as a biodegradable material was reviewed by Chvapil.[31] Sponges are used to stop bleeding in surgery, through the application of pressure and coagulating material such as thrombin. Collagen sponges have an important advantage in that they may remain in the body cavity since they are non-antigenic and absorbable.[28-30] It was reported that the collagen sponges of uniform porosity will quickly absorb about 70 times their weight in water.[30] The variables we studied which affected sponge formation were the treatments (e.g. liming, bating and alkaline) of hide fiber networks before dissolving into a collagen solution. This report presents the preparation of collagen sponges, their resultant viscoelasticity, and their structure and morphology by examination with SEM.

# Experimental

**Materials and Procedures**

Fleshed fresh steer hides, purchased from JBS (Souderton, PA), were processed to the lime, bate, and limed and soaked in a NaOH solution, where the soluble collagen was then extracted as previously reported.[1] In summary, the fleshed hides were sided and soaked for 1 h in 150% float with 0.15% Borron TS (TFL, The Woodlands, TX) and 0.1% Proxel (Chemtan Co. Inc., Exeter, NH) with a drum speed of 6 rpm. The float was drained and the hide was washed for 5 min; 2% sodium sulfide, 2% lime, and 1% soda ash were added in addition to 100% float. The drum was run for total of 4 h with a run time of 10 min per hour. After 4 h the float was drained, 100% float was added and washed for 5 min and then drained. A 200% float was then added to the drum with 2% lime and 1% sodium sulfide and run for 20 h at 6 rpm for 3 min per hour. After 20 h the float was drained, 100% float was added and the hide was washed for 5 min, then drained again. This step was repeated for a total of two times. Pieces were cut out of the limed hide (12” x 12”), and were either stored in a refrigerator or delimed, bated and then stored in a refrigerator. For the delimed and bated hides, after the liming step the hides were refloated to 125% with 3% ammonium sulfate, 0.15% Rohapon 6000 (TFL, The Woodlands, TX), and 0.10% Borron TS, and run for 90 min at a drum speed of 6 rpm. The float was drained, the hide was washed in a 100% float, and drained again. Samples treated with NaOH, were added in a 5% solution with 200% float and let run for 24 h, then drained. Pieces from the limed only, delimed-bated treatments and NaOH were cut into approximately 1.5” to 2” squares and allowed to air dry fully in a fume hood. They were then ground in a Wiley Mill (Thomas Scientific, Swedesboro, NJ) and sieved through a 4 mm circular screen. The collagen fibers were then stored in sealed plastic bags until the preparation of their solutions.

The process we used to extract soluble collagen from limed only, and delimed-bated hides into collagen solutions was modified from that reported by Nishihara.[20] Ground (4 mm) collagen fibers (~ 2 g) were added to 30 times their weight of water in a beaker (600 mL) to rehydrate them on a stir plate. The pH of this mixture was then lowered to 2 -2.5 by using 2N HCl. A 50 to 1 weight ratio of the sample (2 g) to pepsin (0.04 g) was added to the rehydrated collagen mixture. Two to three drops of sodium azide were next added to this mixture in order to prevent future mold growth. Several such samples were stirred for 48 to 72 h so as to solubilize the collagen fiber networks. The progress of solubilizing collagen was monitored visually as evidenced by an increase in the viscosity of its solutions. Then, 33 times the weight of the samples of 0.005N HCl (66 g) was added to the beakers, and the mixture was stirred continuously for 24 h. Additional 0.005N HCl was added so as to decrease the viscosity, and render it easier to stir. After 24 h of stirring, the collagen

solutions were filtered using a fine plastic mesh screen, then neutralized using 5N or 2N NaOH to a pH of 6-7. The mixture was centrifuged, and the collagen concentrate (precipitate) was collected, washed with water, and the mixture was centrifuged again. This collagen concentrate (precipitate) was then separated from the supernatant (wash-water), and stored in a glass jar in a refrigerator until needed for film formation studies.

Instead of using the dried ground hides for making collagen solutions, freshly limed hides can also be used. However, the limed hides will dissolve better if they are ground rather than left whole. Limed only and delimed-bated hides (~ 70% moisture) were cut into 0.5" x 0.5" squares and then ground by using a Hamilton Beach Co. (Racine, WI) Model 222 meat grinder. The delimed-bated pieces were too soft to be ground thoroughly, unlike their precursor limed hides, which were readily ground in the meat grinder, and most of their ground pieces digested with pepsin. Consequently, freshly ground limed hides were the starting material for our studies, and by extension, tanneries could utilize their waste lime trimmings as a starting material to make biobased films. The delimed-bated samples were also prepared using the ground lime stock, performed in a beaker using the same formula above. The NaOH treated ground lime stock was also prepared in a beaker and treated the same as above in a 5% solution based on the weight of the ground lime stock.

Ground lime, delimed-bated, and NaOH treated stock (2 to 10 g), and 10 times the weight in the amount of water (20 to 100 mL) were added to separate beakers (600 to 2000 mL) and these mixtures were stirred using a stir plate. A collagen solution was thus prepared from these ground stocks by using the modified pepsin dissolution method which was described above, and which was initiated by lowering the pH of this mixture to 2.0 -2.5. Collagen sponges are prepared from the collagen concentrate stored in the refrigerator; the final concentration of collagen solution was 2%. The collagen concentrate was added to freezer flasks and DI water was added to make it less viscous. The freezer flasks were then dipped and turned constantly in a bowl of dry ice and acetone to rapidly freeze the concentrates. The flasks were attached to a Labconco Freezone 1 (Kansas City, MS) lyophilizer until fully dry. After the samples were completely dry, they were then stored in sealed plastic bags and stored in a desiccator. Typical percentage yield ranged from 83 to 95% for the lime and bated samples. The NaOH samples absorbed NaCl and therefore the recovery was calculated to be over 100%.

**Dynamic Mechanical Analysis**

Dynamic mechanical analysis was performed on a DMA1 (Mettler-Toledo, LLC., Columbus, OH) in compression mode. Storage modulus (E') and loss modulus (E") were measured as the function of temperature. All samples were tested using a 1 Hz frequency. A 6.35 mm (1/4 in) die was used to cut out cylindrical samples from the sponge. The sample was then mounted into the compression holder with a gage length of 1 mm and amplitude of 10 μm. The samples were then tested from -50°C to 200°C at a heating rate of 10°C/min.

**Molecular Weight Distribution**

Protein molecular weights were estimated as described by Taylor, et al.[32] A small portion of the lyophilized sponges 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). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) was run using precast 4-15% gradient gels. A broad range (BRS) calibration standard (Bio-Rad, Hercules, CA) as Reference, ranging in size from 6,500 to 200,000 Da, was used.

**Microscopic Observations**

A scanning electron microscope (SEM) was used to compare the structural difference among the different collagen sponges. The samples were freeze fractured, and then glued to specimen holders using Duco cement. They were sputter-coated once for 30 seconds with a thin layer of gold using an EMS Q150R sputter coater (Quorum Technologies, Ltd., Laughton, East Sussex, England). Images were collected using a FEI Quanta 200F scanning electron microscope (Hillsboro, OR), and operated in the secondary electron imaging mode.

**Water Absorption Capacity**

A 6.35-mm (1/4 in) diameter punch was used to stamp out pieces of the different collagen type sponge structures. The 6.35-mm pieces were stored in a sealed plastic zip-lock bag and stored in a desiccator until needed. A piece of sponge from each treatment (i.e. liming, bating, NaOH) was weighed dry and then placed in a half full 150-ml beaker with deionized water for 20 to 120 seconds. After the desired time the 6.35-mm piece was taken out from the beaker and put directly onto a scale and weighed again. The water absorption capacity was calculated by dividing the wet weight over the dry weight.

## Results and Discussion

Porosity is an important property in a sponge structure, which is directly correlated with its apparent density. This data provides insight into the effect of preparation methods on the sponge structure. For the sake of achieving better liquid absorption, the lower the apparent density the more desirable the structure will be. Figure 1 demonstrates that the bated fiber networks yielded the lowest apparent density of sponges, indicating this treatment method provided the most open sponge structure compared to the other two treatments. Leather's typical apparent density

ranges from 0.60 to 0.90 g/cm$^3$, however, the apparent density of the collagen sponges ranged from 0.01 to 0.05 g/cm$^3$. It is desirable to be in the range 0.02 to 0.03 g/cm$^3$ such as those obtained from bating and NaOH treatments as shown in Figure 1.[30]

A cross section cut from the entire collagen sponge is demonstrated in Figure 2. It looks like similar collagen sponges reported in literature.[31] One can also observe the different layers making up the sponge caused by the shell drying before the samples were put on the lyophilizer. Figure 3 shows a closer look at the cross section of the collagen sponges demonstrating a porous structure with many voids distributed in the structure. As reported previously, porous collagen scaffolds have broad applications in tissue engineering and regenerative medicine.[27-30] The open and interconnected porous structure of the collagen sponge are required for smooth cell seeding, cell penetration, and uniform cell distribution throughout a scaffold, and accelerated cell proliferation and regeneration of new tissue. From Figure 3, the different treatments look similar; however, the average pore size was measured for each sample and resulted in bated, 92.6 μm, lime, 135.7 μm and NaOH 108.1 μm. There is some difference between the different treatments and the pore

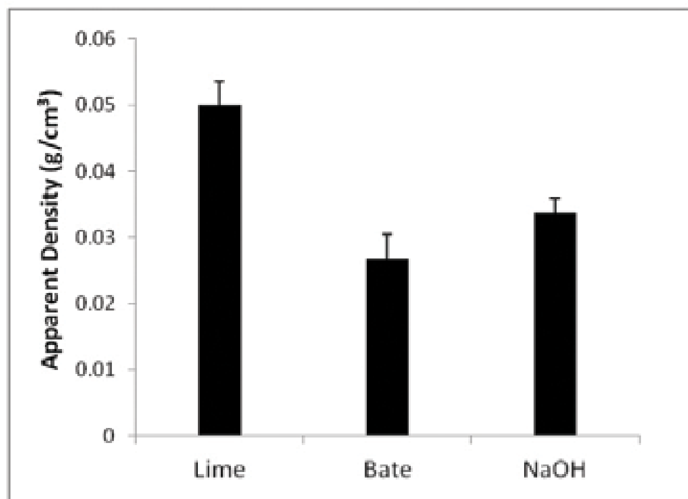


Figure 1. Collagen sponge density.

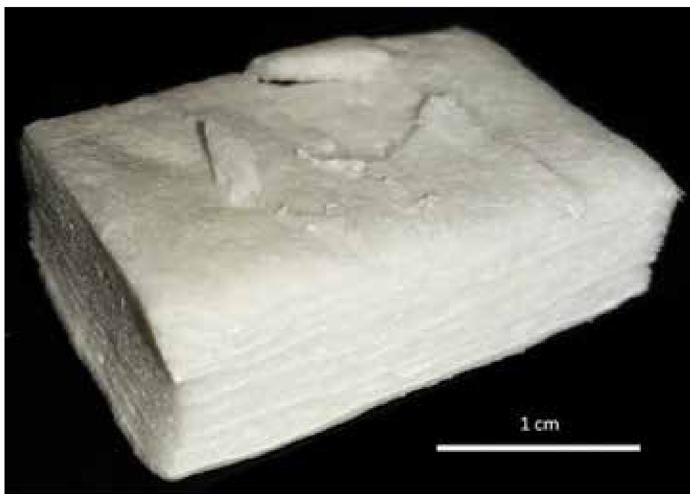


Figure 2. Stereomicroscopic view of the cross section of a limed sponge.

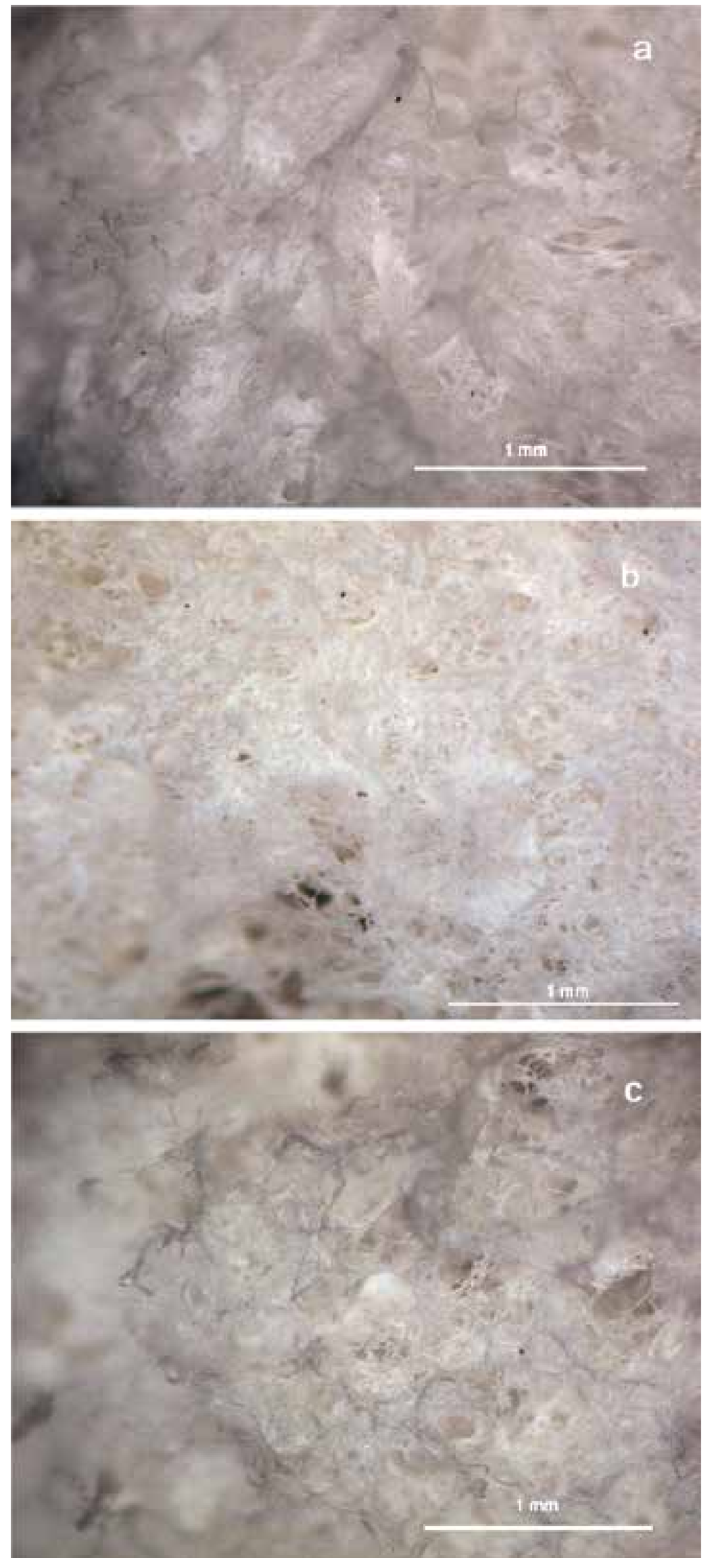


Figure 3. Stereomicroscopic view of a cross-section of collagen sponges prepared from the concentrated solution of ground hide fibers (a) limed, (b) bated, and (c) further treated with NaOH.

size can change depending on the different drying conditions and the type of drying. Further research would need to be done to match a specific application to the desired pore size needed.

The more detailed internal structure is shown in the SEM micrographs, Figure 4. It appears that the sponge is composed of both membrane and fibrous components. It is interesting to note that the sponge obtained from NaOH treatment has less of a membrane structure than those from the other two treatments. This is probably because the collagen solution from NaOH treatment has the presence of salt and this lead to a poor membrane forming ability, consequently the resultant collagen structure is dominated by the fibers. This is further confirmed in that the NaOH samples contained around 70% ash compared to the limed and bated samples which contained 1.3 and 1.2% ash respectfully.

Because the molecular weight is pivotal to the mechanical properties and dimensional stability, it is important to maintain a sufficient molecular weight of collagen during treatments of the fiber network and solution preparation. The resultant collagen sponges were nearly pure type I collagen as demonstrated in Figure 5 by SDS-PAGE analysis, comparing the limed, bated and NaOH samples to the profile of a commercial Type 1 collagen (Bovine Achilles Tendon, Sigma, St. Louis, MO) sample as

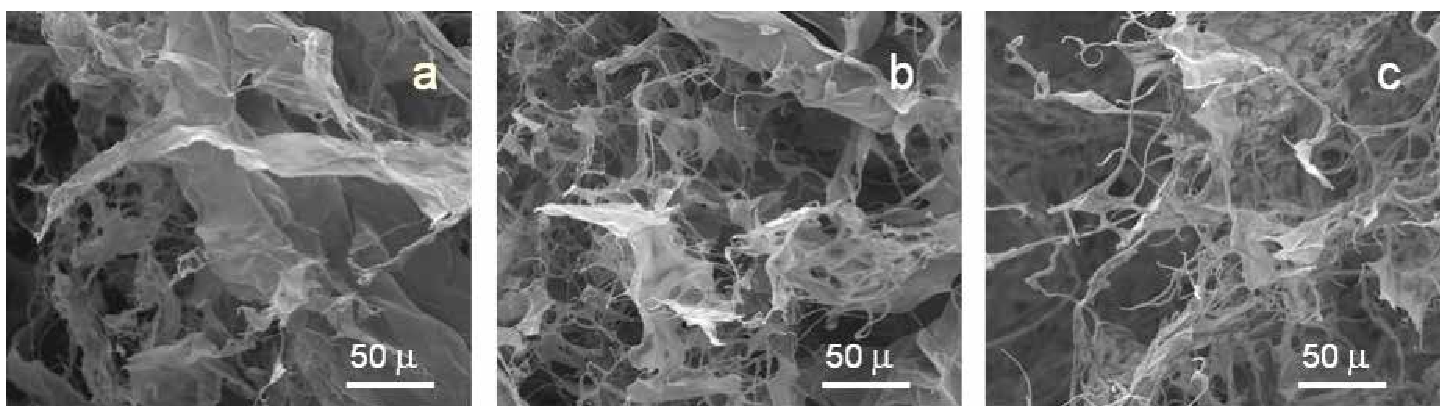


Figure 4. SEM micrographs of collagen sponge structure obtained from concentrated solution of ground hide fibers (a) limed, (b) bated, and (c) further treated with NaOH.

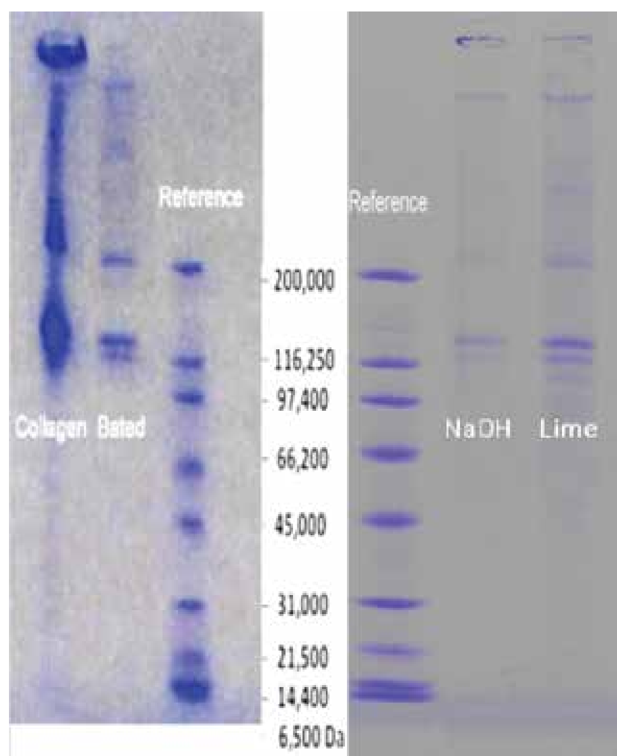


Figure 5. SDS-PAGE gels for collagen sponges prepared from lime, bate and NaOH treated collagen fiber networks compared to a commercial collagen sample and standard protein calibration.

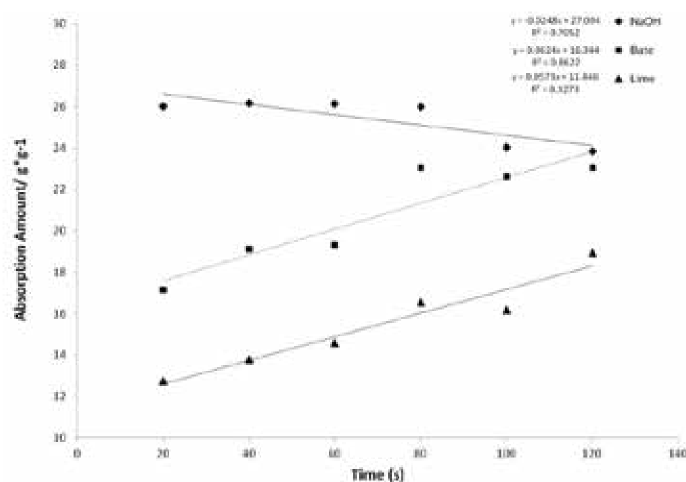


Figure 6. Water absorption Capacity as a function of time.

described previously. The bands for collagen sponges produced match the bands for the commercial collagen (marked as Collagen in the far right of Figure 5) very well, which indicates that there is little degradation occurring in the dissolution process to make the collagen sponges. In addition, we are forming aggregated collagen with no low molecular weight hydrolysates.

**Water Absorption Capacity**

One of the most important criteria of surgical sponges is the absorption capacity of aqueous liquids. The sponges have to soak up blood and other physiological fluids during their use as fast as possible. Therefore, absorption capacity should be as high as possible. Beside the surface structure, the hydrophilicity of the collagen component in the sponge may influence its absorbing capacity, which can be affected by various treatments for fiber networks. It was reported that during liming, the amide groups (Gln, Asn) are transferred into the corresponding carboxyl groups (Glu, Asp) leading to a decrease of the isoelectric point of the collagen.[33] Furthermore, long-term alkaline treatment induces the topochemical hydrolysis of some natural crosslinks.[34] The starting material in this investigation was limed hides. But, it is reasonable to expect that the additional treatments of bating and alkaline will affect the properties of biobased sponges. Bating is a further step to purify the hide before tanning in the leathermaking process by digesting undesired proteins. Figure 6, shows that water absorption increased with time for limed and bated treatments. But the NaOH treatment demonstrated a small decrease in absorption capacity with time. A reason for this phenomenon is the collagen was bound to the salt (NaCl) produced from the reaction of NaOH and HCl in the process. The salt aided in the quicker water absorption and as the NaOH sample is left in the water, the salt diffused out of the collagen and into water, therefore resulting in a decrease in the absorption capacity over time. Therefore further processing to remove the excess salt would be needed for the NaOH treated sample.

**Dynamic Mechanical Behavior**

A sponge needs some degree of stiffness for use in a load bearing application. The stiffness can be evaluated by measuring the storage modulus for the sponge samples using a dynamic mechanical analyzer (DMA). Polymeric materials such as sponges generally demonstrate a mechanical behavior that may incorporate a blend of both elastic and viscous characteristics; this is referred to as viscoelasticity.[35-38] We previously reported that besides the elasticity, the viscous component or viscosity plays an important role in determining the stress-strain curves even at the very beginning of the leather deformation.[39] The viscoelasticity is commonly measured by either dynamic or static tests.[35] In the dynamic tests as conducted in the current study, a sinusoidal variation of strain is imposed on the material and a variation of the responding stress is observed. As to the static tests (as reported in our previous papers), a constant strain or a constant stress is imposed, and the variation of the stress (relaxation) or the strain (creep) as a function of time is observed.[40] The storage modulus (E') and loss modulus (E") in viscoelastic solids such as sponges measure the stored energy (E'), representing the elastic portion, and the energy dissipated as heat (E"), representing the viscous portion. Storage modulus is known to be associated with the stiffness of materials, whereas loss modulus is linked to the plasticity of materials. The ratio of E"/E' is defined as the tangent delta or damping, which is a measure of the ability of a material to dissipate the energy. It usually peaks at the glass-transition temperature of a material, where it becomes rubber-like. The relationship between the storage and loss moduli are as follows:

$$E = E' + iE'' \qquad (1)$$

Where, E is a complex modulus (also named dynamic modulus) and i is the square root of -1.

DMA tests showed the curves of E' vs. temperature look very similar for all three sponge samples as shown in Figure 7. In the beginning, the E' decreases with temperature until around 50°C, hereafter the E' increases with temperature. Probably at 50°C, the collagen sponge starts to shrink and induced a tension and resulted in the increase of E'. It appears that limed samples show the lowest E' and further treatment by bating and NaOH increase E' significantly. A similar behavior was also observed for E", as demonstrated in Figure 8. It is worthy to note, however, that E" is much smaller that E', indicating that the elastic component in the collagen sponge is the dominant factor in governing the mechanical properties. Figure 9 demonstrates the relationship of the tangent delta vs. temperature plot to have a reversed pattern to that of the E' vs. temperature profile in Figure 7. Peak of tangent delta indicates the glass transition point, where the molecular chains start to soften up for moving. All three samples were characterized by two peak points within 10 degrees of each other,

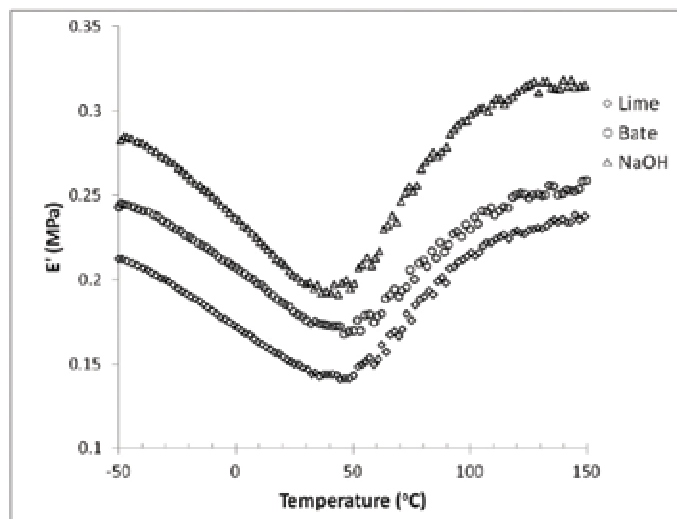


Figure 7. Storage modulus vs. temperature in Dynamic Mechanical Testing.

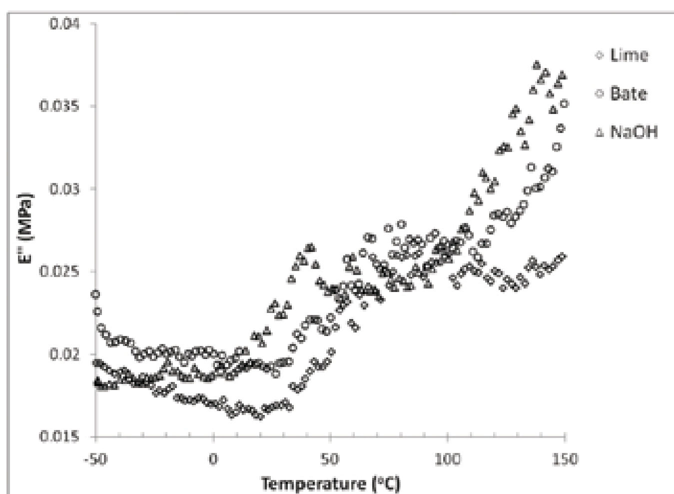


Figure 8. Loss modulus vs. temperature in Dynamic Mechanical Testing.

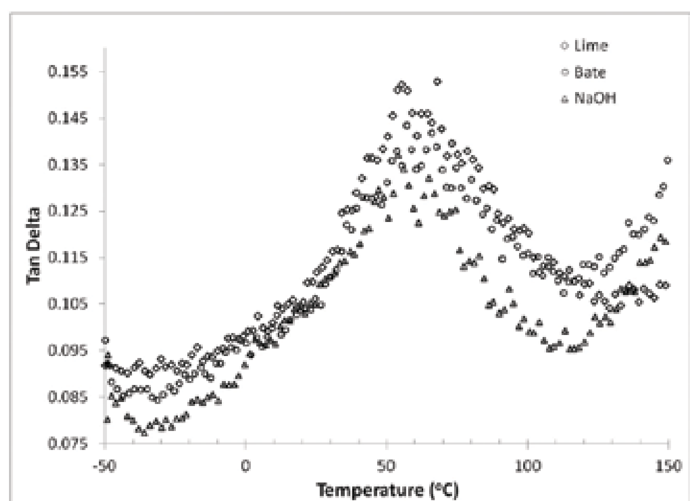


Figure 9. Dynamic loss vs. temperature in Dynamic Mechanical Testing.

the first peak for the lime, bate and NaOH sponges occurred at 55.3, 57.1, and 54.1°C respectively. Although the peak temperatures may not be significantly different from one another, it does show that the treatments had a slight shift in the tanget delta curves. This data also correlates to the absorption data, in which the limed samples had the lowest absorption capacity probably due to the fibers were more tightly compact together and resulted in a higher tan delta. Whereas the NaOH treated sample had the lowest tan delta and highest absorption rate.

## Conclusions

This study focused on preparations of biobased sponges from un-tanned hides, including limed hides and delimed-bated hides, which correspond with their actual tannery waste of limed splits and their trimmings. Observations showed that the treatments of fiber networks have significant effects on the properties of resultant sponges. Bating has a positive effect on reducing the apparent density of sponges, which are in the desirable apparent density ranges. The dynamic mechanic analysis showed alkaline treatments yielded stiffer sponges, thus indicating NaOH treatment of fiber networks is not a desirable processing step for preparing collagen sponges, in addition to the further processing that would be needed to reduce the added salt. SDS-PAGE analysis showed the resultant molecular weights were maintained in the range as a commercial Type 1 collagen. These sponges could be used in the field of medicine because of their good compatibility with living tissue.

## Acknowledgments

We thank ARS, ERRC scientists: Joseph Uknalis for SEM photos, Lorelie Bumanlag and Joe Lee for sample preparations.

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