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Evaluating the Viscoelastic Properties of Alligator Skin Gelatin, Alligel, Compared to Mammalian and Fish Gelatins

by

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Undergraduate honors thesis under the direction of

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Introduction

Gelatin, a gelling protein obtained from different animal sources, has been widely used in the food, pharmaceutical and biomedical industries. This versatile material is derived from denatured collagen. Depending on its source, collagen exhibits slightly different characteristics resulting in various types of collagens such as collagen type I and II (Al-Nimry et al. 2021). The most abundant type of collagen, collagen type I, is primarily found in the skin, tendon, and bone tissue. Collagen, on its own, cannot be used for a variety of applications due to its hydrophobic nature. On the other hand, gelatin, which is made by the “partial hydrolysis and crosslinking of collagen molecule,” portrays an increasing demand due to its many applications, hydrophilic nature, physical properties, thermos-reversibility, degrading and storing capacities (Al-Nimry et al. 2021)

Porcine skin gelatin is the most produced gelatin in the world with around 46% of the overall production. Gelatin is also produced from other animal sources such as bovine hides which accounts for 29.4% and many other marine animal species, such as fish and squid species, which only accounts for 1.5% (Al-Nimry et al. 2021). However, the most produced gelatins cannot be used in many parts of the world including South Asia and the Middle East in which religions like Islam, Judaism and Hinduism prohibits the consumption of porcine or bovine products. Therefore, there is a demand for other types of gelatins that can have similar characteristics and applications and can be used broadly across the globe.

Fish gelatin, for example, exhibits good gelation characteristics especially if chemical cross-linkage is added. Much of the collagen used in the making of this gelatin is derived from by-products and waste of the fishing industry. The use of this waste not only impacts positively the economy of that industry but also environmental preservation. The fish industry is a very apparent industry in the United States. However, in south Louisiana, the alligator industry is also a popular industry for its meat and leather production and there could be a potential for gelatin production. This industry is primarily practiced through intensive cultivation of alligators in enclosed systems (Hamilton 2020). In fact, 98% of American alligator production takes place in Louisiana and Florida, and they are done in alligator ranch facilities. The main product of intensive alligator production is hides that are usually converted into leather products (Hamilton 2020). This process produces a lot of waste from the animal, since the other parts of the alligator body are not being used in any other applications. In an attempt to use the waste products from the alligator industry, a potential application for alligator is being investigated. Just like fish gelatin, alligator gelatin could be used in most of the gelatin applications regardless of religious and ethical concerns. Further, use of alligator gelatin could have a positive impact in the alligator industry for both environmental and economic purposes. Some possible gelatin applications for alligator are described by Al-Nimry et al. (2021) and are listed in Figure 1. Most edible gelatin can be applied in many industries such as food industry as a biofilm for food preservation or food texture enhancer; the cosmetics industry in hair gels creams and lotions; biomedical industry for gene therapy, wound dressing and wound healing, tissue engineering; and the pharmaceutical industry in which can be used in plasma expanders and capsule shells.

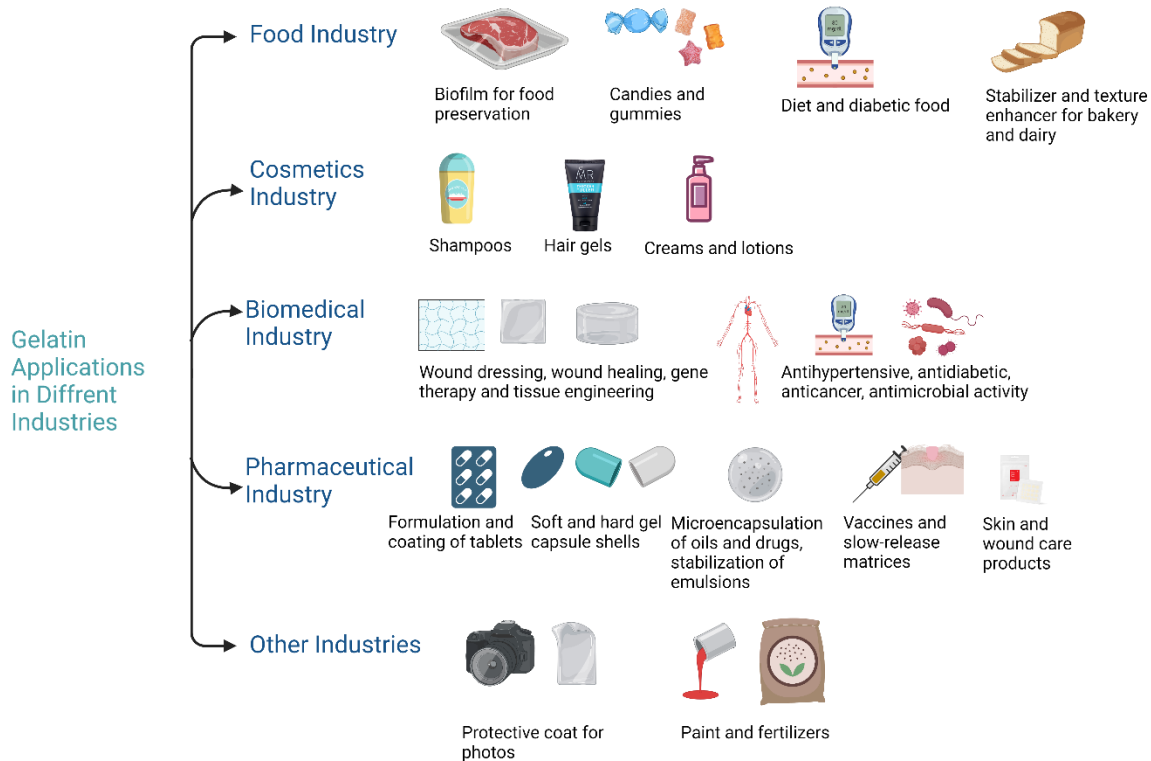


Figure 1. Gelatin applications in the different industries as described by Al-Nimry et al. (2021) and created with BioRender.com

According to Leak et al. (2003), collagen protein content is high in alligator gelatin meat ranging from 21.2% to 18.9%, which is higher than the approximate protein value of beef products of 15 to 20% and similar to some porcine products that range from low 8.45 to 22.05% of protein content (Seong et al. 2014). Alligator products also showed similar characteristics to porcine products in its fat content. However, when compared to both bovine and porcine fatty acids products, alligator fatty acids showed to have a unique type of fat which has high values of linoleic acid and low values of palmitic and stearic acid (Leak et al. 2003). This leads us to hypothesize that alligator product contents are similar to mammalian products and could be used as a replacement. Due to the possible unique differences in fat composition, we **hypothesized that the gelatin derived from the alligator skin has similar or better 1) mechanical properties, 2) stability for gelatin with temperature changes, and 3) biomaterial production compared to those of porcine skin gelatin.** Further, we suggest that we can use chemical crosslinking with mTG to enhance the physical properties of alligator gelatin for use as a biomaterial. Chemical crosslinking has been shown to enhance mechanical properties of biomaterials in a great and adaptable way for tissue engineering applications (Stachel et al. 2010). For instance, the use of microbial transglutaminase (mTG) chemically crosslinks certain amino acids such as lysin and glutaminase in most gelatins, therefore, increasing their bonding strength (Stachel et al. 2010). This signifies an overall increase of the material viscoelastic properties, making the material more stable and stronger for hydrogel and scaffold fabrication. This hypothesis will be tested through the following goals:

1. Test mechanical strength of AG against standard gelatins PG and FG.

2. Test thermos-reversibility through the evaluation of changes in strength under varying temperatures of AG against standard gelatins PG and FG.
3. Determine effects of crosslinking on AG stiffness compared to PG and FG.

Materials and Methods

Materials:

- Porcine gelatin type A, cat. # 901771, MP Biomedicals.
- Gelatin from cold-water fish skin, lot # SLCC7086, Sigma-Aldrich Co.
- Alligator gelatin lyophilized, provided, and developed by Dr. Jack Losso at Louisiana State University).
- DIUF water (Fisher).
- Tris-HCl Buffer (20 mM) pH 8.5.
- Transglutaminase preparation active RM 100 cat. #011020A, 50 U/g, Ajinomoto Health and Nutrition North America Inc– stored at 4 C.

Physically Entangled Gelatin Synthesis

Each gelatin precursor (porcine gelatin (PG), alligator gelatin (AG), and fish gelatin (FG)) was prepared at 66.7 mg/mL in DIUF water in a 1.5 mL Eppendorf tube. Gelatin solutions were vortexed to wet gelatin, which were then left at 60°C for 30 min. Each gelatin precursor of 50 μ L was pipetted to a custom polydimethylsiloxane (PDMS) mold (8 mm diameter and 1 mm height) to form physically entangled gels. These gels were incubated at 4°C for 16 h.

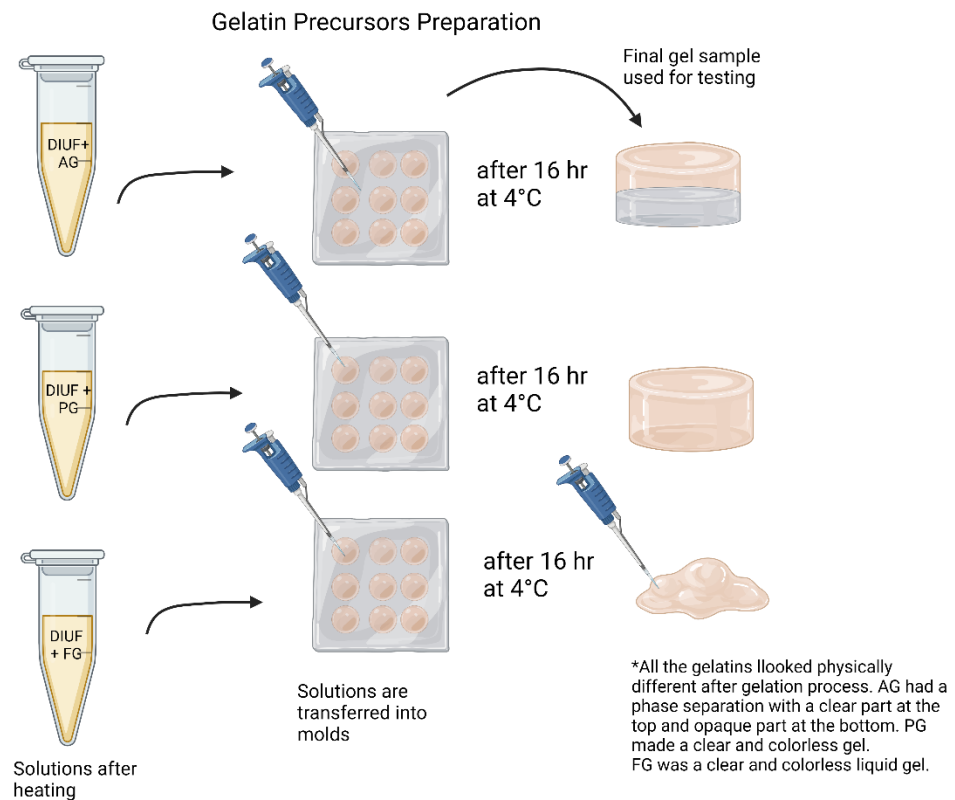


Figure 2. Flow diagram of physically crosslinked gelatin synthesis. AG= Alligator Gelatin, PG= Porcine Gelatin, FG= Fish Gelatin, DIUF = Distilled water. This image was created using BioRender.

Covalently Cross-linkage of Gelatin using Microbial Transglutaminase (mTG)

Each gelatin precursor was prepared at 66.7mg/mL, using Tris-HCl Buffer pH 8.5 instead of DIUF water, in 1.5 mL Eppendorf tubes. Gelatin solutions were vortexed and sonicated to wet gelatin, which were then left at 60°C for 30 min using a heating block. After this 30 min period, temperature was set to 37°C. Previously weighed powdered mTG was added to gelatin precursors to reach the final concentration of mTG at 20 mg/mL. This mixture was vigorously mixed for 3 m and cast in the PDMS mold. When transferring gelatin/mTG solution into the molds, it is advised to cast within 10 min before the mTG-gelatin precursors are crosslinked. Each mTG-gelatin precursor of 50 μ L was pipetted to the PDMS mold, which were also incubated at 4°C for 16 h.

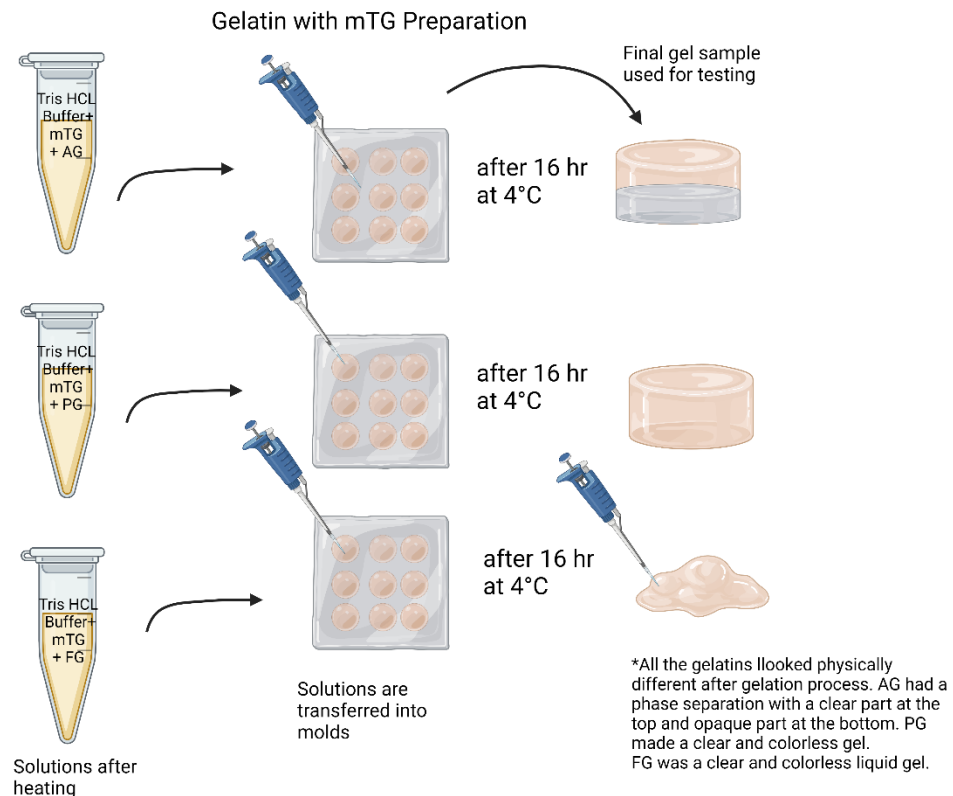


Figure 3. Flow diagram of chemically crosslinked gelatin synthesis using microbial transglutaminase (mTG). AG= Alligator Gelatin, PG= Porcine Gelatin, FG= Fish Gelatin This image was created using BioRender.

Oscillating Rheometry

Frequency Sweeping:

Using a TA Discovery HR-2 rheometer, a frequency sweep test was performed with an 8 mm parallel plate. Samples were tested at 2% strain from 0.1 to 10 Hz. Storage (G') and loss (G'') moduli were assessed.



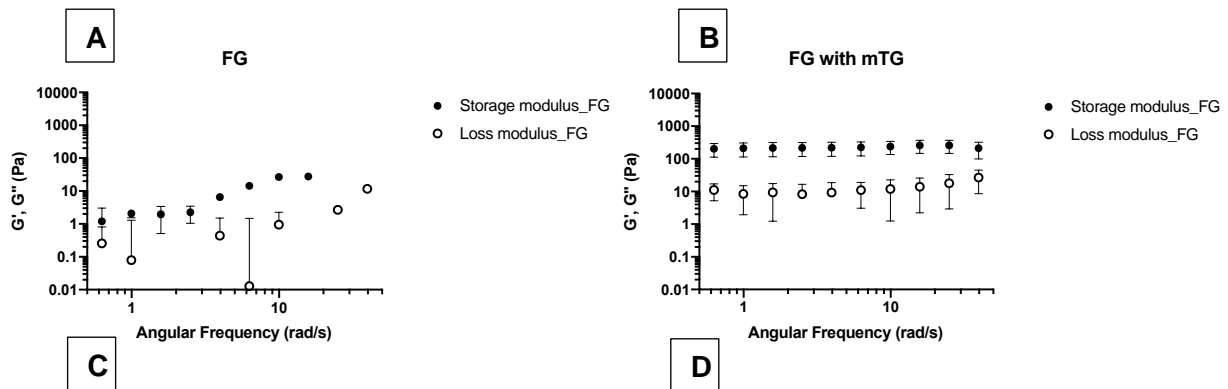
Figure 4 . TA Discovery HR-2 rheometer used for experiments.

Temperature Flow Ramp:

Using an 8mm parallel plate, a temperature flow ramp was assessed for all samples in which the rate of temperature change was 5°C/min from 20 to 45°C for the heating phase. This heating phase was immediately followed by a cooling cycle from 45 to 20°C. The oscillating frequency and the strain were fixed at 1 Hz and 2%, respectively. Both heating and cooling curves were obtained for each material.

Results and Discussion

Frequency sweep of gelatin samples showed similarities between porcine gelatin and alligator gelatin with mTG.



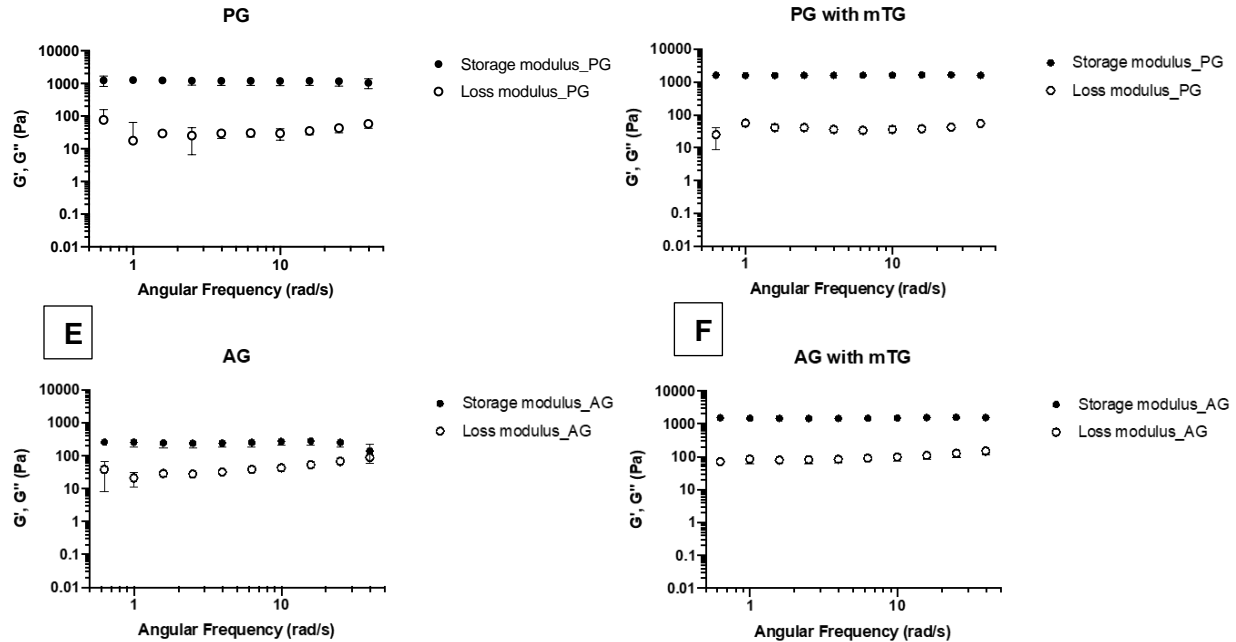


Figure 5. A and B. Fish gelatin (66.7 mg/mL) with and without mTG (20 mg/mL), **C and D.** Porcine gelatin (66.7 mg/mL) with and without mTG (20 mg/mL) and **E and F.** Alligator gelatin (66.7 mg/mL) with and without mTG (20 mg/mL) after 16-hr incubation at 4 °C subjected to a frequency sweep at 2% strain and increasing angular frequency 0.1- 10 Hz.

Initial characteristics for FG in the PDMS molds for both cases with and without mTG, FG was liquid, so the samples needed to be pipetted (Figure 1). For AG and PG gels with and without mTG, the handling was done with a spatula. AG samples exhibited a clear gel on top and an opaque portion at the bottom (Figure 1) (**see images of phase separation examples in Appendix A**).

Next, we sought to determine differences in stiffness to see if AG had similar or better properties than PG and FG. To do this, gelatin derived from porcine, fish and alligator skin were cast with a mold and then gelatin samples were transferred from the PDMS molds onto the stage of the rheometer and a frequency sweep test was performed.

For gelatin precursors without mTG, the PG samples exhibited a G' of approximately 1kPa which remained constant throughout the frequency sweep test. The AG storage modulus values were around 500 Pa at frequency ranges of 0.1 to 10 Hz. Finally, FG constituted the last and lowest set of values for both G' and G'' , in which G' had values lower than 100 Pa for the same frequency. These results demonstrate that PG exhibited the highest stiffness when physically crosslinked. When physically crosslinked (without mTG) AG has intermediate properties between FG and PG. Unlike fish gelatin, alligator gelatin generally conserves its strength through increasing frequencies, in which fish gelatin showed to increase its strength. However, the AG samples, especially without mTG, when subjected to high frequencies showed to decrease suddenly in strength and loss modulus and storage modulus become the same value.

We next wanted to determine the effects of crosslinking on the different gelatin types. When mTG was added to all gelatin precursors, there was an increased in strength based on the values of their storage moduli. At 10 rad/s, PG-mTG samples showed an increase of around 200 Pa from the G' value of PG at the same frequency. Similarly, AG-mTG samples doubled their G' value to 1.2 kPa at the same frequency. Finally, FG-mTG samples tripled its strength from 100

Pa of G' (PG without mTG) at 10 rad/s to around 300 Pa at the same frequency. These results showed that although PG is still the standard and the most stable gelatin, AG exhibits similar characteristics when mTG is applied being around the same value for G' (approximately 1.2 kPa) when covalently crosslinked by the enzyme.

Temperature flow ramp of gelatin precursors showed that alligator gelatin behaves in a similar manner to porcine gelatin when subjected to temperature changes.

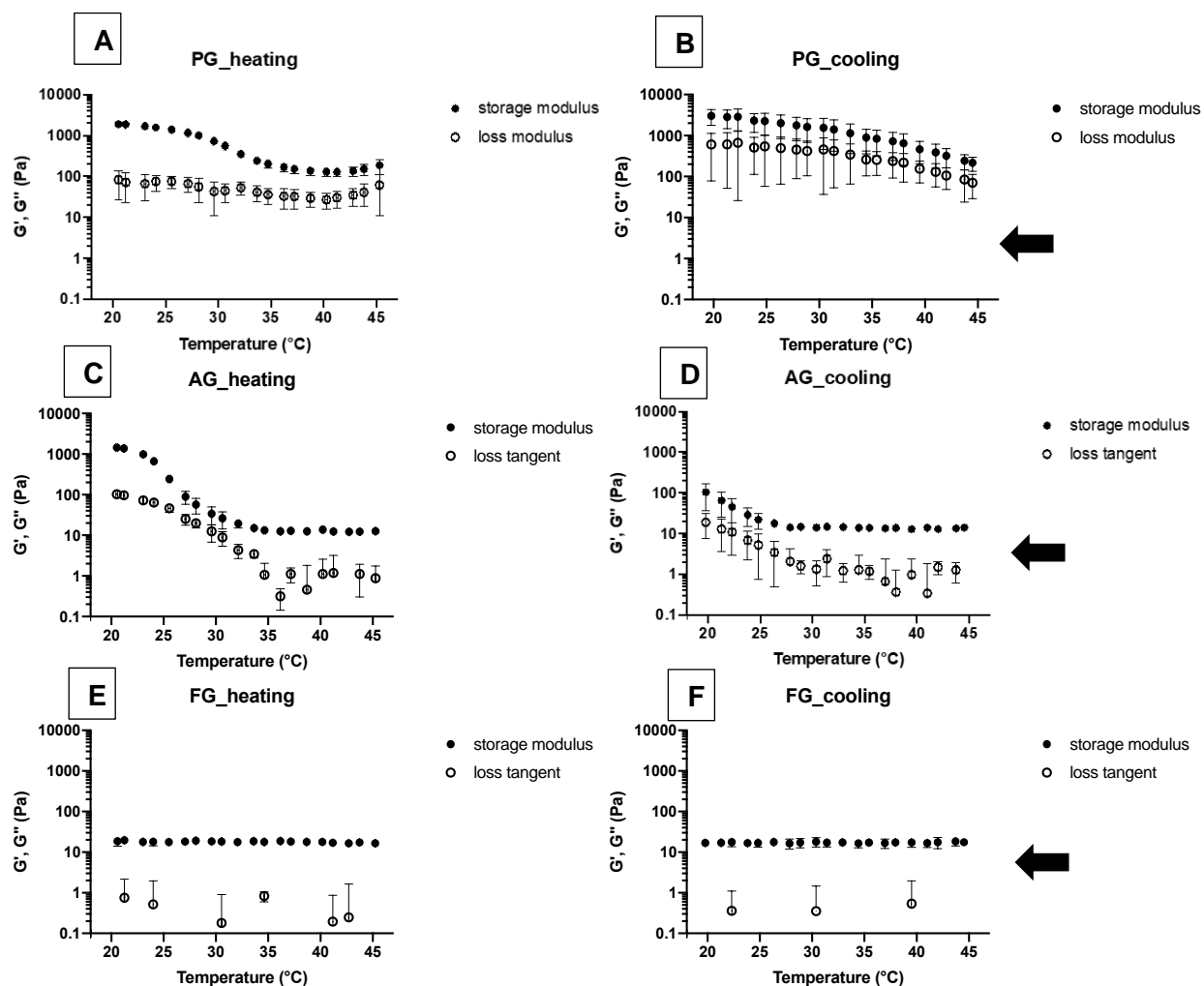


Figure 6. A and B. Porcine gelatin gels (PG); **C and D.** Alligator gelatin gels gelatin gels; **E and F.** Fish gelatin gelatin gels subjected to a temperature change rate of 0.5 $^{\circ}\text{C}/\text{min}$ and fixed parameters of 2% strain and angular frequency of 1 Hz, showing heating and cooling curves.

To determine thermo-reversibility of gelatin precursors from the different sources porcine, fish and alligator, a temperature flow ramp was performed, and heating and cooling curves were obtained based on the G' and G'' values. The gelatin samples were put on the stage using a spatula, except for the FG that was liquid at all times. FG with mTG was more viscous than its precursor. The PG and AG samples with mTG were stronger than their gel precursors. After the samples were subjected to a rate of changing temperature (0.5 $^{\circ}\text{C}/\text{min}$) at constant 2% strain

and frequency of 1 Hz, the heating and cooling curves were obtained. The cooling curves are read from right to left since the temperature changed from 45 to 20 °C. PG samples decreased in strength from initial 1.2 kPa to the low 200 Pa as temperature increased to 45 °C, in which the loss modulus and the storage modulus were approaching each other. The heating curve of PG exponentially decreased and then started to become constant at around 37 °C. PG was later subjected to a decrease in temperature in which the material underwent physical crosslinking due to the temperature decrease once again. The PG samples were reconstituted to the original gel strength of 1.2 kPa. In this instance, the loss modulus followed the storage modulus very closely. AG gels were subjected to an increase in temperature from 20 to 45 °C, in which the heating curve exponentially decrease until it hit a point (melting point) at around 35 degrees in which the strength of the material became constant at around 80 Pa. In this stage, the AG was in a liquid state. Later, this melted gelatin was subjected to a decrease in temperature in which the gelatin underwent physical crosslinking until it reached a gel strength of around 100 Pa. For the FG, G' was constant at around 20 Pa for both curves, exhibiting a liquid state only for temperatures higher than 20 °C.

When subjected to temperature changes, gelatin acts as a thermo-reversible material in which they become liquid when heated and form a gel when cooled (Al-Nimry et al. 2021). For temperature curves, it is important to evaluate changes in elastic and loss moduli, as they determine the gelling ability of the gelatin (S. M. Cho et al. 2005). This experiment did not run for enough time for the AG to make a gel, and the sample was still somewhat liquid, since the time factor had to be kept constant for all samples and all assessments. However, the point where the curve started to change exponentially when the gelling point was reached at around 25°C shows phase changes with decreasing temperature, suggests there may be thermo-reversible behavior. This is evident as the material shows a slight thermo-reversible behavior in the sense that it gains physical strength, as in G' increases in figure 3-D, with physical crosslinking of the gelatin. Further, AG demonstrates a more similar thermo-reversible behavior to porcine gelatin than fish gelatin, which stays unaffected by temperature changes in the range of 20 to 45 °C. However, when cooling PG, the material fully recovers its original strength of over 1.2 kPa. AG exhibits loss in strength when the material goes from liquid to solid state once again, with a difference in G' of around 900 kPa. The thermostability of the gelatin is attributed to mainly two factors which are the amino acid composition which is species-specific, and the molecular weight distribution, which is dependent on the gelatin processing treatment (Gomez-Guillen et al. 2011). Both factors may have affected the behavior of AG. For the FG, G' was constant at around 20 Pa for both curves, exhibiting a liquid state only for temperatures higher than 20 °C. Its gelling ability needs to be reinforced at higher temperatures if a gel wants to be made from FG. This gelatin does not act as a thermos-reversible material at these temperature ranges since their gelling points are around 8-10 degrees C (Al-Nimry et al. 2021).

mTG did not enhance thermal properties of alligator gelatin precursor but brought much more stability to the porcine one.

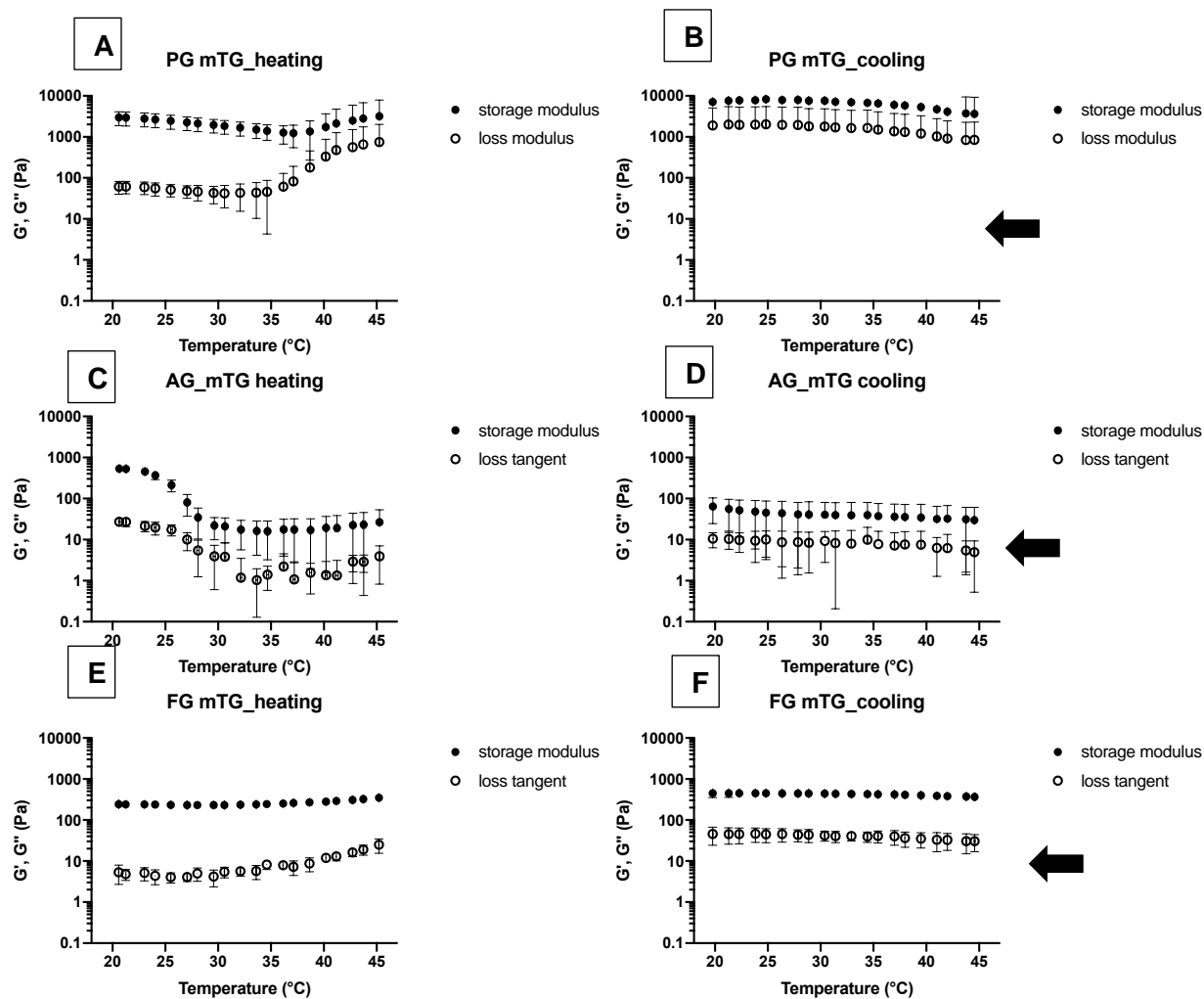


Figure 7. A and B. Porcine gelatin gels with mTG (PG-mTG); **C and D.** Alligator gelatin gels with mTG (AG-mTG); **E and F.** Fish gelatin gels with mTG (FG-mTG) subjected to a temperature change rate of 0.5 $^{\circ}\text{C}/\text{min}$ and fixed parameters of 2% strain and angular frequency of 1 Hz, showing heating and cooling curves.

PG, AG and FG samples were physically crosslinked with temperature changes during the 16hr incubation at 4 $^{\circ}\text{C}$ and chemically crosslinked with mTG. When crosslinked with mTG, PG showed a more stable thermal behavior, as its heating and cooling curves showed high strength of over 1kPa. The curves did not exhibit high slopes. Instead, it looked constant as temperature changed. When PG-mTG samples were cooled again after heating, there was not a significant loss in strength, since it returned to its original value of around 1kPa at 20 $^{\circ}\text{C}$. AG-mTG went from a high G' to a low G' at around 25 $^{\circ}\text{C}$ when heated. When subjected to a decrease in temperature, the data exhibited minimal slope in which AG-mTG maintained the same value of approximately 100 Pa when it was done heating. Finally, FG-mTG showed a significant increase in G' values to around 500 Pa. However, the material stayed very stable in a liquid but more viscous state.

When compared to the temperature curves of the precursors without mTG, there was not any significant difference in the thermos-reversible ability of alligator gelatin. In fact, chemical crosslinking did not make the material more stable when subjected to temperature changes. FG

with mTG, showed a similar behavior to its precursors without mTG, but acting as a more viscous material. The chemical cross-linkage did not help much in improving thermo-reversible characteristics at those same temperature ranges. However, this was not the case for PG-mTG, in which chemical crosslinking did not only help make the material stronger in terms of its gelling ability (G') but also in its lack of phase changing when temperature was raised or lowered (between 20 and 45 °C). Its G' value ranged from 9 kPa to 10 kPa when it was heated and cooled. In fact, PG-mTG gained more strength when cooled again after the heating cycle.

Conclusions

AG shows intermediate mechanical properties between the widely used PG (standard) and FG when subjected to constant strain and increasing shear stress. In addition, when AG was subjected to temperature changes, it behaved more similar to porcine gelatin than FG. However, AG exhibited a significant loss in strength when the material is heated and cooled again. To evaluate if these characteristics could be improved for the material to behave more like the standard PG, mTG was applied to each sample and an additional chemical crosslinking of each material was obtained. mTG altered the mechanical properties of all gelatin precursors, increasing its G' and G'' values. AG-mTG doubled its original value with the addition of chemical crosslinking. PG-mTG samples increased in strength by 1/8 of its value. FG-mTG solutions became more viscous, tripling its physical crosslinked G' value.

Physical properties needed to be evaluated under varying temperature conditions. This allows us to identify the thermos-reversibility properties of each gelatin. PG with and without mTG was the most stable material in terms of thermal properties. Its viscoelastic properties decreased as the material melted but formed a strong gel with nearly no losses when it was cooled again. In terms of mechanical strength under the evaluated conditions, AG followed a similar path to porcine gelatin, but with a lower G' value. However, AG with and without mTG exhibited losses in strength when the material was subjected to decreasing temperatures after heating. The material could not recover its original state. Overall, AG behaves similar to PG.

In addition, FG did not have much change in G' and G'' values when subjected to either shear stresses or temperature changes, as it is mostly in liquid state at temperatures higher than 20 degrees Celsius. Nevertheless, chemical crosslinking with mTG added more strength and more stability to the material, as it increased its overall viscosity and gelling ability.

Our first hypothesis was confirmed. AG does show similar viscoelastic properties to porcine gelatin when subjected to high frequencies and heating. The gels are not completely colorless and clear as the PG gels and there is an evident phase separation of the gelatin. The AG gels are suitable for biomaterial production due to its mechanical strength. However, their applications are limited to where a fully transparent gel is not required. In addition, they differ on their thermal properties, and this could be attributed to the treatment process of the gelatin or the species-specific molecular structure of the collagen from which the alligator gelatin is derived from. Lastly, chemical crosslinking with mTG did not improve AG's behavior under temperature changes but increased its overall gel strength under constant temperature conditions. This is distinct from PG as chemical crosslinking was shown to enhance both the physical and thermal properties of PG.

AG is a material that could be used for a variety of applications including tissue engineering and pharmaceutical applications. They form strong gels when chemically and physically cross-linked, exhibiting similar mechanical properties as the standard PG. Further research must be done to enhance the material's thermal properties.

Future Work

For future work, amino acid and compositional analysis of AlliGel must be performed to evaluate stability issues encountered with thermos-reversibility. The AG and AG-mTG gels also showed phase separation, clear gel on the top and opaque gel at the bottom, making it a heterogeneous material. This can be an obstacle for its applications in industries where a colorless material is needed.

However, AlliGel offers similar characteristics to PG, especially when additional methods of crosslinking are added. It would be a good material to use for hydrogel making in tissue engineering applications. The material is overall stable at room temperature and lower and could be used for hydrogel scaffolding as it maintains its shape overtime.

If a compositional analysis and cytotoxicity assays are performed, there is a possibility for it to be used in other industries like food industries.

In the future, other types of chemical crosslinking methods such as the use of reagents like glyceraldehyde, disocyanates and genipin or additional physical crosslinking like UV radiation (Stachel et al. 2010).

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Appendix

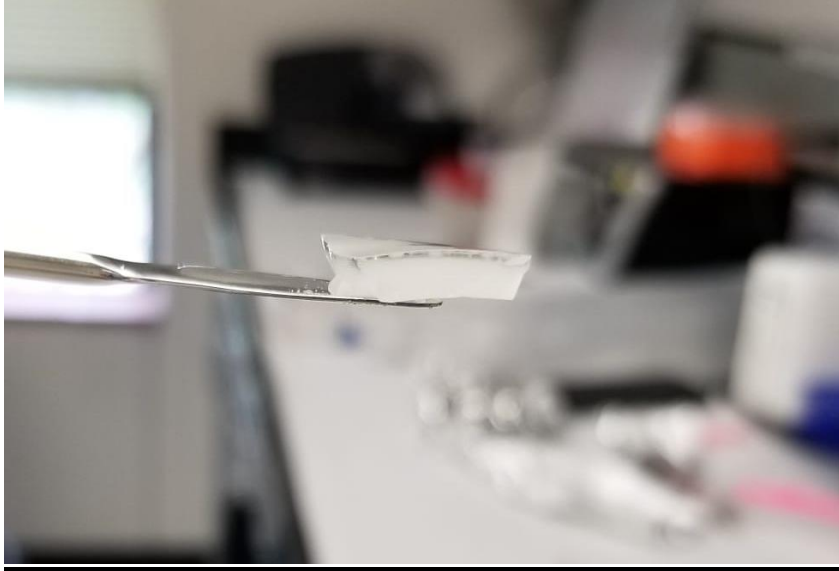


Image 1. Alligator gelatin gel after 16hr incubation at 4°C.

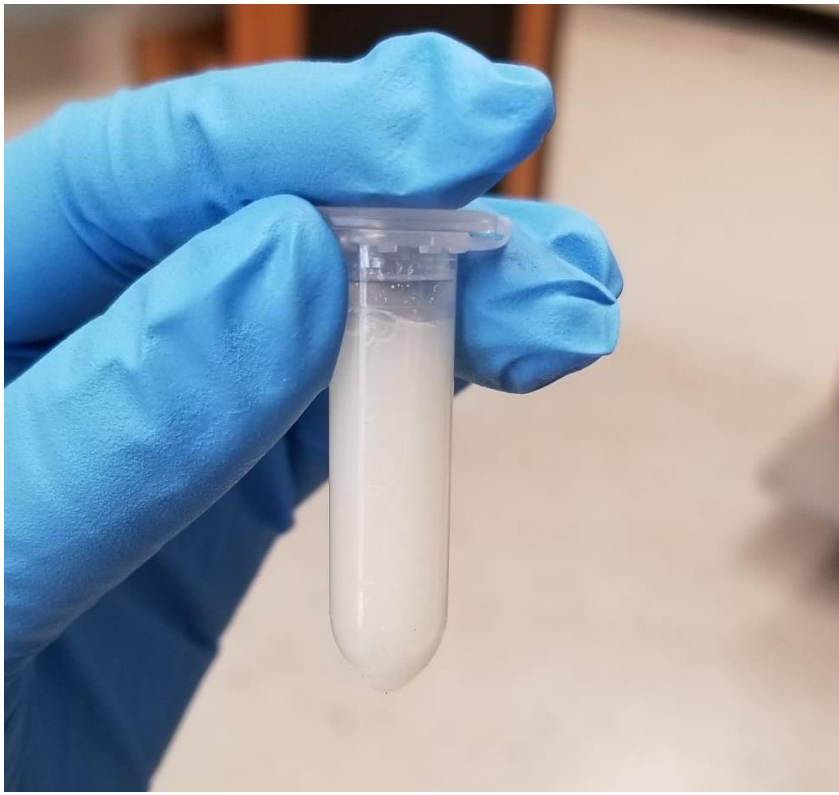


Image 2. Alligator gelatin after mixing with vortexer and heated at 60 °C for 30 min.



Image 3. Alligator gelatin with mTG after mixing with vortexer, heated at 60 °C for 30 min, and rested for 5 minutes at 37°C.