Literature Collective

Table of Contents

Collagen

Collagen Fabrics as Biomaterials (Cavallaro et. al)

John F. Cavallaro[1], Paul D. Kemp[2] - *Department of Matrix Engineering, Organogenesis Inc., 150 Dan Road, Canton, Massachusetts 02021*
Karl H. Kraus - *Department of Surgery, Tufts University School of Veterinary Medicine, 200 Westboro Road, North Grafton, Massachusetts 01536*

Overview

Collagen threads were manufactured by extruding collagen into a buffered solution of polyethylene glycol - which helps to stabilise its structure - followed by rinsing and air drying. The resulting thread could be used as tissue and ligament replacements.

Important Terms

- Extrusion: a process in which a cell exports large particles or organelles
- Buffered solution: an aqueous solution consisting of a mixture of a weak acid and its conjugate base, or vice versa
  - Buffer: a solution that can resist pH change upon the addition of acidic or basic components; able to neutralise small amounts of added acid or base - maintaining the pH of the solution as relatively stable
  - Conjugate base: the product when acid dissociates into its ions in water and - as a result - loses a hydrogen ion; the species that accepts a proton or hydrogen
- Polyethylene glycol: a synthetic resin made by polymerising ethylene glycol; helps to stabilise collagen structure without deactivating biological properties
  - Polymerisation: a process of reacting monomer molecules together in a chemical reaction to form a polymer chain
  - Ethylene glycol: an organic compound mainly used as a raw material in the manufacture of polyester fibres and for antifreeze formulations
- Collagen: main structural protein found in the extracellular matrix; most abundant protein in mammals
  - Type I collagen: the most abundant collagen and the key structural composition of several tissues
  - Extracellular matrix: helps cells bind together; regulates cellular functions (i.e. adhesion, migration, proliferation, differentiation)
- Proteoglycans: proteins that are heavily glycosylated
  - Glycosylation: reaction in which a carbohydrate is attached to a hydroxyl or other functional group of another molecule; adding a sugar molecule to an organic molecule
- Dialysis: process of removing excess water, solutes, and toxins

---

[1] His LinkedIn can be accessed here.
[2] To whom all correspondence should be addressed.

- ❖ Coagulation Bath: a liquid bath that is used to harden viscous polymer strands into solid fibres after extrusion through a spinneret; used in wet spinning processes
  - ➢ Spinneret: a cap or plate with multiple small holes through which a fiber-forming solution is forced; after exiting the spinneret, streams of viscous polymer enter an air or liquid that lead to a phase inversion - thus allowing the polymer to solidify
- ❖ Laminar flow: fluid particles following smooth paths in layers, with each layer moving past adjacent ones with little to no mixing

Key Concepts

1) Wet spinning is a process in which polymer powder is dissolved in a solvent, with the solution extruded through a spinneret into a coagulant — the polymer solution coagulates to form fibres.
2) Cross-linking is a bond that links one polymer chain to another.

Background

The biomaterial surrounding implants can contribute to how well the body receives it; biologically derived materials are researched as a viable biomaterial, as they interact with the body in such a way that they can facilitate cell attachment and function. Because prosthetics function under significant loads, the binding collagen must maintain more ordered assemblies - thus introducing the experiment in question, and the thread it needed to produce.

Purpose of the Experiment

In the experiment, the scientists used a biomimetic approach in designing their implants, attempting to maintain as much of the biological and structural organisation of collagen as possible - thus allowing a seamless graft between the implants and the body. They wanted to create a "novel method to make strong, continuous collagen threads at rates that permit the formation of collagen fabrics" (Cavallaro et. al.).

Methods

1. **Deriving the collagen**
   Type I collagen was extracted from cow tendons.

2. **Coagulation bath**
   The collagen was extruded into a buffered solution of polyethylene glycol (20% PEG 8000 in phosphate buffer at pH 7.55 - relatively neutral), which allowed its structure to stabilise. The collagen gelled upon contact with the aforementioned coagulation bath.

3. **Rinse bath**
   As the thread accumulated, it was transferred into a rinse bath, which was a dilute phosphate-buffered saline - which helps to maintain a constant pH.

4. Isopropanol bath

The thread was then partially dehydrated in a 70% isopropanol bath.

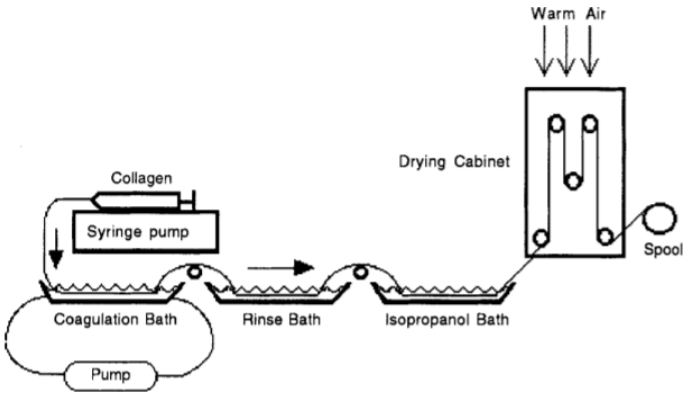
5. Drying

After coming out of the isopropanol bath, the thread was dried under tension in a cabinet with heated air blowers.

6. Spinning

The thread was spooled onto a level winding device — the tension on the thread during drying was such that the length of the thread doubled before drying. The scientists found that the thread could be produced continuously once threaded through the entire system, with production rates over 100 metres per hour.

7. Schematic Diagram



8. Cross-linking

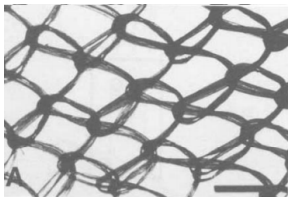
The threads were crosslinked by standard collagen crosslinking agents.

9. Thread testing

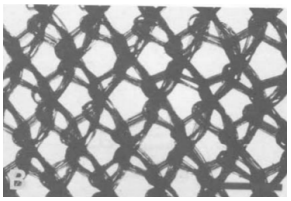
The following criteria were measured and evaluated: tensile strength, elongation, load at break, size, mass per length, and shrinkage temperature.

10. Fabrication and knitting patterns

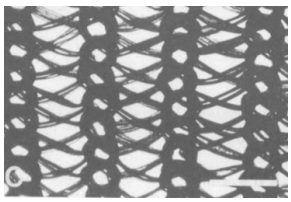
The collagen threads were woven in multiple different patterns:



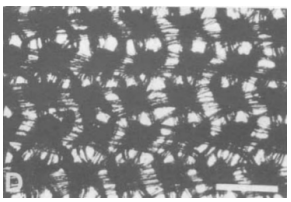
single bar, open mesh fabric



double bar, open mesh fabric



another double bar fabric



triple bar knit - designed for high bulk and low extensibility

Results

1. Shape

The threads were ultimately flattened - not round - in the cross-section, due to contacting the wet thread on the pulleys in the drying cabinet.

2. Mechanical strength

The mechanical properties of collagen threads depended on the degree of covalent crosslinking, but tensile strength and shrinkage temperature were affected at different rates.

Table II. Ultimate tensile strength (UTS) of collagen threads produced by the method described ($n = 10$) and reconstituted collagen fibers published elsewhere.[22]

	UTS (MPa)
Dry collagen thread (non-XL)	224 ±19
Dry collagen thread (EDC XL)	197 ±18
Dry collagen thread (glutaraldehyde XL)	175 ±19
Wet collagen thread (non-XL)	1.2 ±0.2
Wet collagen thread (EDC XL)	23.9 ±2.7
Wet collagen thread (glutaraldehyde XL)	27.7 ±3.1
Wet collagen thread (non-XL)[22]	2.4 ±0.46
Wet collagen thread (cyanamide XL)[22]	17.4 ±3.08
Wet collagen thread (glutaraldehyde XL)[22]	44.1 ±6.62

XL = crosslinked. Standard EDC and glutaraldehyde crosslinking were carried out as described in the text.

3. Fabric production

To produce fabric, thread needs to be both strong and flexible. The rinsing step of the thread-making process allows for both; while unrinsed collagen threads can bear approximately the same load and tensile strength as rinsed threads, they are much less flexible and cannot be knitted.

4. Knitting v. Weaving

Knitting is a versatile technique for producing strong and porous structures — as a result, collagen fabrics can be tailor-made to suit a wide variety of applications, with various requirements. The main advantage of knitting over weaving is that the former introduces closed loops at the yarn crossover points, meaning that it allows the material to hold sutures. Weaving relies upon parallel threads, which increases the risk of fraying.

5. Conditions necessary for successfully manufacturing thread

The following factors play a large role in ensuring the success of the thread-making process: the collagen solution cannot have any bubbles, since bubbles form weak spots, and, by extension, breaks in the thread; the flow of the coagulation bath must be steady, as it can drastically affect the thread mass per length; the bath cannot be too fast or too slow, so that the amount of draw at the needle orifice is precise; and the bath must have a laminar flow, in order to prevent the thread from tangling. Breaks need to be repaired quickly, and reparations involve knotting the two ends of the thread.

Takeaways

1) We need to think about which type of collagen we want to mimic - most likely Type I, as it is the most abundant and key in structural composition. Something to look into as well is how different types of collagen might respond to different spinning techniques or coagulation baths - in addition to which types of collagen will be the most practical for us to model.
2) For our team to be able to use the wet-spinning process outlined in this publication, the collagen we create will need to be a polymer — as the collagen used in the experiment was derived from cows, the scientists were able to use the fact that collagen is a naturally occuring matrix polymer.
3) We have several options for how we can knit the collagen threads such that their function is optimised; depending on what our final product is (clothes, masks, etc.), we can alter our knitting patterns to accommodate for any unfulfilled criteria.

Reference

Cavallaro, J. F., Kemp, P. D., & Kraus, K. H. (1994). Collagen fabrics as biomaterials. Biotechnology and Bioengineering, 43(8), 781–791. doi:10.1002/bit.260430813

Collagen fibres by thermoplastic and wet spinning (Meyer et. al)

Michael Meyer[3], Hagen Baltzer - *a Forschungsinstitut fuer Leder und Kunststoffbahnen, Meissner Ring 1-5, D-09599 Freiberg, Germany*
Katrin Schwikal - *Thüringisches Institut für Textil-und Kunststoff-Forschung e.V. Breitscheidstr. 97, D-07407 Rudolstadt, Germany*

Overview
Two different techniques - wet spinning of collagen dispersions and melt spinning of thermoplastic collagen - were used to manufacture collagen threads.

Important Terms
- Collagen dispersions: a result of the few cases in which the raw material that collagen comes from is purified and minced; show higher solid contents
- Thermoplastic: substances that become plastic on heating and harden on cooling, and are able to repeat these processes
- Formaldehyde: chemical compound made of hydrogen, oxygen, and carbon
- Glutaraldehyde: disinfectant, medication, preservative, and fixative
- Hemostyptic: contracting the tissues or blood vessels
- Isolable: capable of being isolated or disjoined
- Alkaline: having a pH above 7
- Homogenous mass: a mass that exhibits the same physical properties at every point throughout the mass
- Loft drying: a process in which substances are dried under carefully regulated atmospheric conditions in a loft
- Unifilar: having or involving only one thread or fibre
- Cytotoxicity: quality of being toxic to cells
- Single-screw extruder: a machine used to form plastic product into the required shape
- Plasticiser: a substance added to a material to make it softer and more flexible, to increase its plasticity, to decrease its viscosity, or to decrease friction during its handling in manufacture

Key Concepts
1) Wet spinning is a process in which polymer powder is dissolved in a solvent, with the solution extruded through a spinneret into a coagulant — the polymer solution coagulates to form fibres.
2) Melt spinning utilises a rapid cooling system to transform melted base materials into long strands or filaments. It is used for polymers that can be melted, which then solidify after being extruded from the spinneret.
3) Cross-linking is a bond that links one polymer chain to another.

[3] Corresponding author, with email address michael.meyer@filkfreiberg.de.

4) The process of splitting (via splitting machine) is used to divide or split textile in its thickness.
5) A screening method extracts, isolates, and identifies a compound in a simple with the least manipulation of the sample, and the smallest number of steps; it provides a yes/no response.

Background

Collagen is biocompatible, biodegradable, and hemostyptic. Since it can integrate well with cells, it is widely used (a) in manufacturing medical devices like sponges to coat implants and (b) as a solution in plastic surgery - specifically when grafting implants. Usually extracted from mammals, the raw material is then treated with acid to obtain acid-soluble collagen - meaning that it is soluble in acidic solutions. Generally, it is difficult to manufacture long collagen fibres, as it does not consist of isolable long fibres.

Purpose of the Experiment

The intent of the experiment was to "[manufacture] collagen threads with high yardage by two different but simple technologies[,] namely wet spinning of collagen dispersion coupled with precipitation of the material[,] as well as thermoplastic melt spinning. The resulting fibres… were characterised regarding their processing properties as well as regarding their structural, textile, physical[,] and biochemical properties" (Meyer et. al.).

Methods

1. **Deriving the collagen**

   Pig and cow skins were washed and soaked in sodium hydroxide at a pH of 12.5 overnight - the result being swollen collagen. The swollen skins were split twice on a splitting machine.

2. **Collagen dispersions**

   The alkaline-treated skin splits were used to prepare collagen dispersions. They were then treated to get homogenous collagen masses.

3. **Wet-spinning the collagen dispersions**

   The collagen dispersions became threads after being processed through a cylinder spinning system.

4. **Coagulation of the result**

   The spun thread solidified in a coagulation bath containing ethanol and acetone mixtures at different compositions; they were then blow-dried.

5. **Preparing collagen for thermoplastic spinning**

   The split skins were soaked in boiling water for 10 minutes, drained of excess water, loft dried, and were then ground to a powder in a centrifugal mill. The resulting powder was

mixed with substances such that the final result was granules that could be fed to an extruder.

6. **Melt-spinning the collagen granules**
Melt-spinning the collagen granules, coupled with a spinning pump, resulted in unifilar fibres by extrusion through nozzles with diameters of 0.3 and 0.5 millimetres.

7. **Testing the resulting fibres**
The resulting fibres were tested for tensile strength, and elongation at break of the fibres, fineness, and x-ray diffraction.

8. **Cross-linking**
The fibres were cross-linked in gas phase - the benefit being that they avoided wetting in aqueous solutions during conventional cross-linking processes.

9. **Enzymatic digestion**
To understand the fibres' degradation behaviour, samples were digested enzymatically.

10. **Cytotoxicity**
Cytotoxicity was measured by a screening method.

Results

Collagen dispersion and thermoplastic collagen were processed to form threads through wet and melt spinning techniques, respectively.

1. **Collagen dispersion**
The collagen dispersion was prepared and formed to threads via wet spinning. The stability of the fibres were influenced by the following factors: the collagen source (pigs v. cows, in addition to age of the animals), the isolation method, the swelling behaviour, the solid content of the mass, the composition of the coagulationbath, and the spinneret design. The older the age of the cattle, the collagen showed a higher natural cross-linking degree.

The tenacity of the fibres made from collagen dispersions depended upon the composition of the coagulation bath:

Table 1
Textile physical properties of threads made from collagen dispersions depending on the mixture of the precipitation agents ethanol and acetone (3.8% dry matter content; pH 4.0; cylindrical spinneret).

Sample	EtOH: acetone	Thread properties					
		Thickness [µm]	Fineness [tex]	Tenacity [cN/tex]	sd [%]	Elongation [%]	sd [%]
P1	9:1	89	9.8	8.48	12.5	20.3	19.47
P2	8:2	118	11.8	8.30	16.5	18.8	24.3
P3	7:3	140	19.7	5.75	25.5	13.1	36.6

The best fibre stability was produced when adding 10% (P1) and 20% (P2) acetone.

The mechanical properties of the wet spun fibres also depended on the spinneret design:

Table 2
Textile physical properties of threads made from collagen dispersions depending on spinneret design and dry matter content (pH 4.0; precipitation agent ethanol:acetone 9:1).

Sample	Dry matter content	Spinneret design	Thread properties					
			Thickness [µm]	Fineness [tex]	Tenacity [cN/tex]	sd [%]	Elongation [%]	sd [%]
P1	3.8	cylindrical	89	9.8	8.48	12.5	20.3	19.5
P4	11.1	cylindrical	179	28.1	7.12	12.5	28.5	15.9
P5	3.8	conical	170	20.0	4.65	22.5	14.6	35.0

The cylindrical shape led to a tenacity which was twice as much that of a conical design.

Increasing dry matter content led to higher thickness of the fibres - the tenacity decreasing slightly with increased thickness.

2. Thermoplastic collagen

The prepared collagen granules were fed into a single-screw extruder, melted, and pressed through a spinneret to form thread through melt spinning. In most cases, this process was enough to wind up a long - dubbed "endless" by the paper - thread, but the fibres were not as stable as the ones produced by the dispersions. Dry threads of thermoplastic collagen proved to be brittle, but the breaking could be prevented by adding glycerol as a plasticiser.

With increasing glycerol concentration, the tenacity of the threads decreased, while the elongation increased:

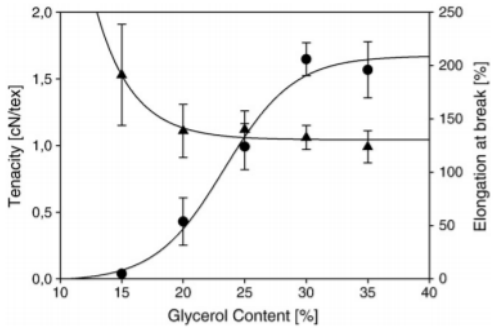


Fig. 4. Tenacity (●) and elongation at break (▲) of TC fibres depending on the glycerol content.

The most stable spinning conditions were achieved using glycerol contents between 25% and 35%.

As cross-linking is often used to improve the collagen's mechanical stability and stability against enzymatic digestion and resorption, the scientists attempted to cross-link the thermoplastic collagen with formaldehyde (FA) and 1-ethyl-3-carbodiimide (EDC):

Table 3
Textile physical properties of TC fibres cross linked by different cross linking agents.
EDC – 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

Sample		Properties of threads					
Nr.	Post treatment	Thickness [µm]	Fineness [tex]	Tenacity [cN/tex]	sd [%]	Elongation [%]	sd [%]
TC1.0	–	315	152	0.68	17.8	125	17.8
TC1.1	EDC	401	131	2.85	51.7	39	32.7
TC1.2	formaldehyde	333	127	2.52	41.3	54	56.1

The low stability of the fibres was improved 3.5 - 4.5 times through cross-linking with EDC.

3. Comparative Measurements

The fibres were compared in regards to nativity of the collagen (collagen dispersions performed better), cross-linking degree (both, when crossed with formaldehyde, showed high levels of cytotoxicity), and susceptibility to enzymatic degradation (collagen dispersions performed better, while thermoplastic collagen - without the help of cross-linking - completely degraded).

4. Conclusions

	Pros	Neutral	Cons
Collagen Dispersions Wet Spinning	• Achieved higher tenacity	• Quality depends on coagulation bath and spinneret design	• Much more laborious
Thermoplastic Collagen Melt Spinning	• Many options for enhancing properties (cross-linking, stretching filaments) • Much easier	• Quality depends on glycerol concentration and draft of fibres	• Low mechanical stability (possible to overcome)

Takeaways

1) Ideally, we should be able to forgo all of the elaborate processes the scientists went through to derive collagen; tailoring our product to each one of the two thread-spinning processes outlined in this paper would allow us to create the best product possible.
2) We probably will not be able to use melt spinning, because it seems to be a much longer and more tedious process; to utilise it, we must create collagen granules, in addition to gaining access to a melt-spinning machine. However, it seems to produce the best results in regards to the length of thread, and, despite its low mechanical stability, it can be reinforced through cross-linking or other processes — it is something we need to consider as a viable option.
3) Wet spinning would give us the best tensile strength, but it would be difficult to manufacture long threads; ultimately, we need to weigh which criteria are more important to our project and what we hope to accomplish with it.

Reference

Meyer, M., Baltzer, H., & Schwikal, K. (2010). Collagen fibres by thermoplastic and wet spinning. Materials Science and Engineering: C, 30(8), 1266–1271. doi:10.1016/j.msec.2010.07.005

Prospective Research Regarding Collagen Threads
J.F. Cavarallo, P.D. Kemp, K.H. Kraus, Biotechnol. Bioeng. 43 (1994) 781. - Collagen Fabrics as Biomaterials
D. Zeugolis, G. Paul, G.E. Attenborow, J. Appl. Polym. Sci. 108 (2008) 2886.
D.I. Zeugolis, G. Paul, G.E. Attenborow, Acta Biomater. 4 (2008) 1646.
D. Zeugolis, G. Paul, G.E. Attenborow, J. Biomed. Mater. Res. A 89 (2008) 895.
D. Zeugolis, G. Paul, G.E. Attenborow, J. Biomed. Mater. Res. B Appl. Biomater. 85 (2007) 343.
M.P. Vasilev, L.A. Volf, V.V. Kotetskin, Z.I. Pukhva, A.I. Meos, Khimicheskie Volokna 1 (1972) 34 translated from.
L.A. Chanukov, M.P. Vasilev, L.A. Volf, Sowjetische Beiträge zur Faserforschung 16 (1976) 193
K. Bienkiewicz, U. Grzegorzewska, K. Paryska, S. Pilawski, Leder Schuhe Lederwaren 6 (1984) 278
M. Meyer, R. Mühlbach, D. Harzer, Polym. Degrad. Stab. 87 (2005) 137.

Engineered Recombinant Bacterial Collagen as an Alternative Collagen-based Biomaterial for Tissue Engineering (An et. al)

Bo An, David L. Kaplan, Barbara Brodsky

Important Terms:

Hydroxylation: What you need to know is that hydroxylation is the process of adding OH to a molecule. This strengthens the molecule by increasing molecule size but more importantly, adding hydrogen bonds to the molecule. Human collagen needs hydroxylation in order to be a strong substance.

Hydroxylase: In organic systems, hydroxylases are enzymes. They act as a catalyst for the hydroxylation reaction. Being a catalyst basically means they enable the reaction of hydroxylation. This becomes important in the study; the scientists explain that despite a lack of hydroxylase, the collagen alternatives maintained structural soundness.

Scl1 and Scl2: These two things are proteins. These are the proteins that are collagen-like in terms of structure and functionality. They are important for three reasons. One is their easy production. Another is their collagen-like features. Three is that they can definitely be used for our iGEM lab.

Streptococcus Pyogenes: It is a bacteria that is the source of Scl1 and Scl2. This is where we will get our Scl1 and Scl2 to use.

Thesis of the Experiment:

Collagen production has been tried in yeast, insect cells, and plants. Proline hydroxylation has proven to be a significant obstacle in mass production. This study aims to investigate alternative collagen-like materials that can be mass produced and avoid the hydroxylation problem. The main focus is on Scl2 as an alternative and possible improvement to human collagen.

Production:

Scl2 is highly compatible with E. coli. The produced product is already in triple helix form so does not need any modifications after production. This allows for potential mass production.

Scl2 in E.Coli	Average collagen protein production
Shaking flask culture	0.3g/L
High density fed-batch culture	9.5g/L

Data Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3539881/

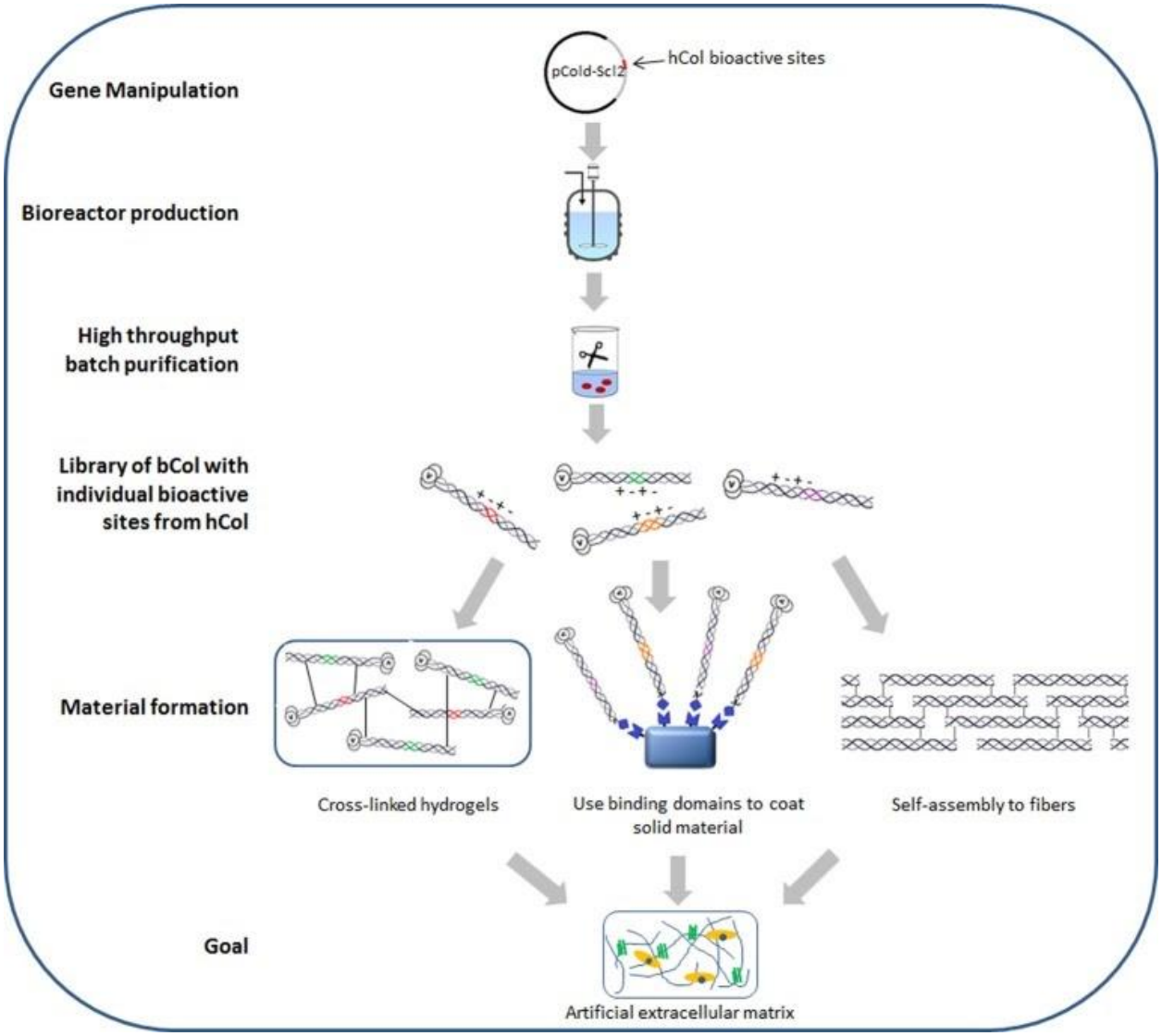
This study measured how much collagen Scl2 produced in different settings. Their study provides information of conditions necessary for maximum production levels (19g/L).

Triple Helices:

Scl2 is able to form a triple helix like collagen and have similar stability to collagen.

Modification of Scl2:

Scl2 has proper binding sites for human collagen. Studies show that a combination of the two has led to Scl2 shaping human collagen into triple helices without the need for hydroxylation. Furthermore, human collagen provides its own properties in the chimera triple helix.



<u>bCol is Scl2 and human collagen combined. hCOL is human collagen.</u>

This image displays the general process of binding human collagen and material synthesis. It starts with an Scl2 plasmid being sequenced. hCol binding sites are located within the Scl2 plasmid genome. Then, segments of hCol dna are inserted into the Scl2 plasmid.

This study runs an experiment on the binding of Scl2 to human collagen. They do so by using SPR to attach Scl2 production into a human cell which will naturally produce human collagen. I'm honestly not too sure what they did but I'm pretty sure they used antibodies to capture Scl2 for later production. They did this by simply introducing binding buffers.

Production of Human Type I Collagen in Yeast Reveals Unexpected New Insights into the Molecular Assembly of Collagen Trimers

(Olsen et. al)

David R. Olsen, Scott D. Leigh, Robert Chang, Hugh McMullin, Winson Ong, Ernest Tai, George Chisholm, David E. Birk, Richard A. Berg, Ronald A. Hitzeman, P. David Toman (dtoman@cson.com)

Journal of Biological Chemistry

1 Overview

In actual cells, several modifications are required to transform procollagen peptides into working collagen. Collagen has a triple-helix structure, i.e. a structure that resembles twisting three strands of yarn together. The following study examines the exogenous production of collagen in the yeast *Saccharomyces cerevisiae*. To examine the effect of certain domains, Olsen et. al. deleted some parts of the collagen (N or C propeptides, or both).

2 Key terms used throughout the paper

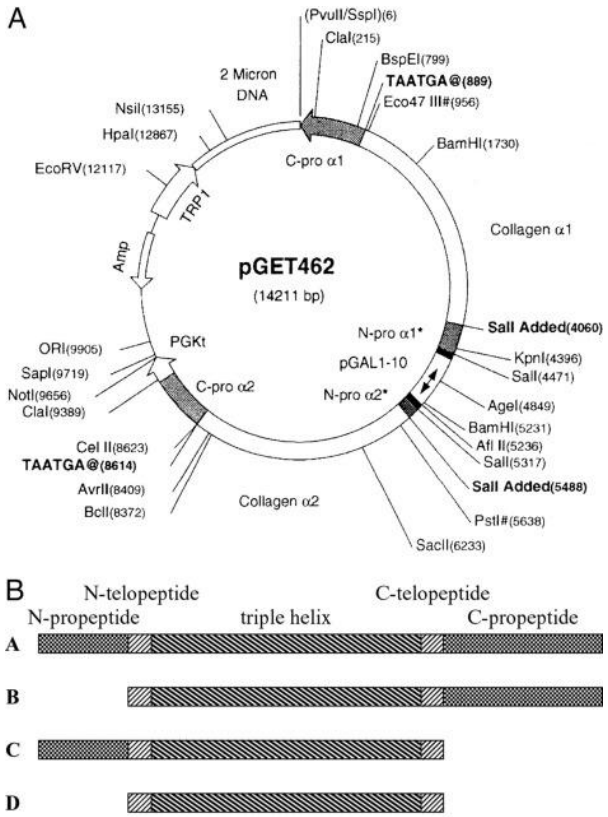
Triple helix	Triple-stranded structures spiraled around the same axis, like twisting three strands of yarn together.
heterotrimers	A molecule made up of three different monomers.
hydroxylation	Adding an -OH group into a compound
collagen types	α1 and α2 collagens are types of Type 1 collagen.
pN/pC- collagen	Procollagen without the opposite propeptides (pN collagen lacks C-propeptides)
electroporation	Making a cell membrane more permeable to DNA transfection by inducing a voltage with an electric field.
subcloning	Moving a DNA sequence from a parent vector to a destination vector
PAGE	Polyacrylamide gel electrophoresis
Bluescript IIKS+	https://www.addgene.org/vector-database/1944/
pGET737	Plasmid encoding human alpha1 and alpha2 collagen
pGET758	Plasmid with both propeptides deleted

3 Methods

3.1 Plasmid construction

pGET323 was used to create plasmids for alpha 1 procollagen lacking N-propeptides, C-propeptides, or both.

	pC-collagen homotrimer
Primer (sense)	5'-ACGCGTCGACAGCTGTCTTATGGCTATGATGAG-3'
(Antisense)	5'-TTGGAAGCCTTGGGGACCAGGTGCA-3'
PCR cycle	Denaturation: 94 °C, 1min; Annealing: 60 °C, 1 min; 72 °C; Elongation: 3 min; 35 cycles
Digestion	PCR: *Sal*I-*Apa*LI pGET323: *Sal*I and *Dra*III
Intermediate Plasmid	pDO243858
Add 2 stop codons	3' half of cDNA from*Bam*HI (nt' 2803 to cDNA of *Bam*HI site). Digestion with *Eco*47III, *Bsp*El Introduce *Not*I and *Sal*I sites: 5'-GCTGGTTTCGACTTCAGCTTCCTCCCCCAGCCACCTCAAGAGA AGGCTCACGATGGCGGCCGCTACTACCGGGCTGATGATGCCAAT GTGGTTCGTGACCGTGACCTCGAGGTCGACACCACCCTCAAGAG CCTGAGCCAGCAGATCGAGAACAT-3' (sense), corresponding antisense Oligonucleotides with 2 stop codons: 5'-GGCCGCTACTACCGGGCTTAATGAGATGATGCCAATGTGGTT CGTGACCGTGACCTCGAGG-3' (sense) and 5'-TCGACCTCGAGGTCACGGTCACGAACCACATTGGCATCATCTC ATTAAGCCCGGTAGTAGC-3' (antisense).
Final Plasmid	pDO24805
pN-collagen	pGET323 digested with *Sal*I and *Bam*HI, pGET323 digested with *Sal*I and *Cla*I, pDO24805 digested with *Bam*HI and *Cla*I.
Both deletions	5.5-kb *Sal*I-*Cla*I fragment from pGET323, 1.5-kb *Bam*HI-*Cla*I fragment from pDO248015, and the 2.2-kb *Sal*I-*Bam*HI fragment from pDO243858



3.2 Deletions

Deletion of the both N and C propeptide sequences from α2(I) procollagen cDNA was achieved by subcloning the 1.84-kb *Kpn*I-*Sac*II fragment from pGET462 (Fig 1.A) into the *Sac*II-*Kpn*I sites of Bluescript, creating plasmid **pGET758.**

Deletion of the N and the C were conducted as follows: the primers listed in Table 2 were annealed to pGET737, ligated with the chosen restriction enzymes. The portion of the plasmids containing the PCR DNA were sequenced to confirm the mutations were present.

Table 2.	Deletions of N-propeptide	Deletions of C-propeptide
Primers (Sense)	5'-AGTACGCGTCGACAGTATGATGGAAAAGGAGTTGGA-3'	5'-GGTCCTGCTGGTCCTAGGGGCCCT-3'
Antisense	5'-TCCAGGAGTTCCAGGGAAACCACG-3'	5'-AGAAGGTGCTGAGCGAGGCTGGTCTCATTAAGCCCTGTAGAAGTCTCCATCGTAACC-3'

Restriction Enzymes	*Sal*I and *Pst*I into *Sal*I-*Pst*I-digested pGET758, creating DO243861.	*Avr*II and *Cel*II into *Sal*I-*Pst*I-digested pGET, creating DO248050.
New Plasmid for Promoter Reconstruction	845-base pair *Sal*I fragment from pGET758 cloned into *Sal*I site in pDO243861: **pDO243863**	
Type I pC collagen heterotrimer	Ligate 5.494-kb*Nsi*I-*Age*I fragment from pDO243858, the 1.213-kb*Sac*II-*Age*I fragment from pDO243863, and the 6.922-kb *Nsi*I-*Sac*II fragment from pGET737.	2.3-kb*Cel*II-*Sac*II fragment from DO248050 cloned into *Sac*II-*Cel*II sites in pGET462, creating pDO243869.
Final Plasmid	pDO243865	pDO248053

3.3 Transformation:

The following plasmids were transformed into yeast GY5372 to create their respective strains at 30°C. (pH of 6.5, aeration at 1 air volume/liquid volume/min, and 20% dissolved oxygen).

Plasmid	Yeast Strain	Temperature (°C)	New Strain
pGET462	GY5372	30	CYT89
pDO243873	GY5372	30	CYT87
pDO243865	GY5372	30	CYT90
pDO248053	GY5372	30	CYT59
pDO248053	GY5196	20	CYT38

Yeast strains were transformed by electroporation and grown in 2% glucose, 0.67% yeast nitrogen base with ammonium sulfate, and 0.5% casamino acids. For protein expression, a similar medium was used with 0.5% glucose and 0.5% galactose as the carbon source and 1% sodium citrate (pH 6.5) as the buffer. The yeast was fermented in media and harvested once cell growth ceased. Debris and waste were filtered out until collagen was isolated and precipitated out of the solution.
**GY5196 strains contained 2% galactose as the sole carbon source during induction, and 34 µg/ml uracil was also added.

3.4 Digestion Assays

Proteases, or enzymes that cleave and break proteins, were added in order to ensure structural consistency. Samples were incubated at different temperatures (20, 25, 30, 35, 40 and 45 °C) for 15 minutes. Then, they were digested with pepsin at those temperatures for 2 minutes and neutralized with NaOH. Reaction products are analyzed by SDS-PAGE.

3.5 Analysis

Samples were diluted and analyzed by spectropolarimetry at the expected melting temperature. In addition, purified collagen aliquots were dried, subject to vapor phase hydrolysis overnight, and analyzed with the AccQ-Tag kit. Collagen fibers were formed by dialysis, diluted, transferred to formvar/carbon-coated grids, washed with water, and air-dried. Diameters were measured and analyzed with averages and regressions to the mean.

4 Results

Even without C-propeptides, collagen was expressed by yeast, with a 5.9-fold higher expression than the procollagen controls. pN-collagen is thought to be a component of the triple helix. The strain with both propeptide deletions actually had the highest expression levels, 18-fold higher than procollagen.

Folding of the triple helix was observed even without prolyl hydroxylase, as the SDS-PAGE suggested both digestion with and without hydroxylation formed the correctly aligned structure. The procollagen propeptide domains are sensitive to pepsin, trypsin, and chymotrypsin, however, the final triple helix is resistant.

Fibril formation showed typical banding patterns of human tissues, with a periodicity of 67 nm, with a mean diameter of 275 +- 97 nm and a length of 134to 470 nm. The melting temperature was found to be 48°C.

5 Takeaways

Type 1 collagen does not require C-propeptides to assemble into a triple helix structure. In addition, correct folding of Type 1 collagen occurs without prolyl hydroxylase expression. Olsen et. al provides invaluable proof of concept for procedureswe will use to biofabricate collagen and sericin. It may also result in simplification of our procedures and suggests yeast as a viable candidate as our host cells.

6 Reference

Olsen, D. R., Leigh, S. D., Chang, R., McMullin, H., Ong, W., Tai, E., … Toman, P. D. (2001). Production of Human Type I Collagen in Yeast Reveals Unexpected New Insights into the Molecular Assembly of Collagen Trimers. *Journal of Biological Chemistry*, *276*(26), 24038–24043. https://doi.org/10.1074/jbc.m101613200

Additive Biomanufacturing with Collagen Inks (Chan et. al)

Weng Wan Chan, David Chen Loong Yeo, Vernice Tan, Satnam Singh, Deepak Choudhury, May Win Naing

Technology and Research (A*STAR),Singapore.
Deepak_choudhury@bti.a-star.edu.sg (D.C.);
winnaingm@simtech.a-star.edu.sg (M.W.N.);
chan_weng_wan@bti.a-star.edu.sg (W.W.C.);
david_yeo@bti.a-star.edu.sg (D.C.L.Y.);
vtan026@e.ntu.edu.sg (V.T.);
Satnam_Singh@bti.a-star.edu.sg (S.S.)

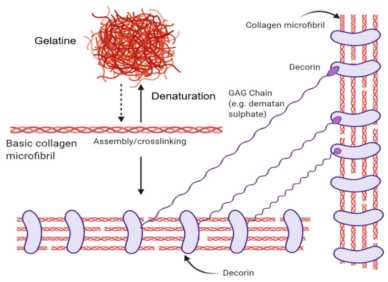


Figure 1. The structural forms of collagen and their native interactions. The basic collagen unit is a triple-helix microfiber that denatures into gelatine or can be assembled into collagen fibrils. Decorin proteins wrap around collagen fibrils in their native context and bind with glycosaminoglycan chains such as dermatan sulphate. Created with BioRender.com.

Key Vocabulary:

- Thrombogenicity refers to the tendency of a material in contact with the blood to produce a thrombus, or clot.

Key Concepts:

Collagen is found in soft and hard connective tissue in mammals. It is an abundant protein and has three varitient types, I, II, III. The amino acid motif is Gly-Pro-X (X is any amino acid) and they form a triple helix structure. The triple helix structures are strong and resistant to enzymatic degradation, however heat results in the helix to separate and form gelatin. A mutation that leads to the misplacement of the glycine makes the helix unstable. Gelatin can help with cell attachment.

Collagen is used for tissue engineering and a popular application is “using collagen scaffolds as a dermal regeneration template for severe wounds and other trauma such as burns”; these are commercially available and are preserved in powder forms. Recently, collagen has been tested for making artificial leather and bio-artificial muscle.

Some downfalls of using collagen are: poor mechanical properties, thrombogenicity, contamination, and source & batch variation. These make the usage of collagen for a potential biotechnical aspect extremely difficult to do so. Biomaterial printing is making collagen biomaterial products more effective.

The difference between bioinks and biomaterials is that in “”cells are introduced with the materials and printed, even in situ” and biomaterial scaffolds are “printed along before cellular components are added”.

“Depending on the sources of collagen, extraction steps and crosslinking methods (chemical, physical), the resultant properties will differ”

Methods:

Collagen Extraction & Sources:

Collagen can be extracted from animal skin, tendons, and tissues, which are abundant in type I and type II collagen. Cells cultured in vitro can also synthesize collagen, such as fibroblast and chondrocytes. Recombinant collagen can be produced by bacteria, yeast, transgenic corn, or silk worms. However, collagen extraction from animals is the highest yield and most cost effective method. Another important thing to consider is that collagen differs from species to species, but also every individual animal as well. Age, gender, activity and physical states of the animal play a huge role in the crosslinking king of the collagen. This leads to variability within the collagen collected, making it difficult to reproduce the experiments for accuracy.

Collagen extraction is done in three different steps: pre-treatment, extraction, and purification. Pre-treatment entials of using alkali solutions to remove non collagen proteins. Then the pre-treated tissue is added into a dilute acidic solution, to disrupt the weaker hydrogen bonds. Then the pre-treated tissue is added into a proteolytic enzyme solution, which will cleave the non helical telopeptide. Then salt precipitation, centrifugation, and dissolution in acetic acid is done. During this process, gelatin can occur and smaller peptide chains are achieved.

Methods of Collagen Crosslinking:

Chemical Crosslinking:

Glutaraldehyde (GA) is used in varying concentration and treatment times (20 minutes up to 24 hours) with increasing the concentration and treatment time to increase collagen crosslinking. THe same goes for Hexamethylene di-isocynate (HDI) and Carbodiimides (EDC). Similarly, plant extracts such as tannic acid and genipin are also good crosslinking agents.

Physical Crosslinking:

Dehydrothermal treatment (DHT) and ultraviolet irradiation (UV) are used to create covalent bonds between the intermolecular collagen structures. The DHT takes several days under a vacuum under high temperatures. One risk is that increasing the temperature and treatment time will increase the mechanical properties of the collagen, but it will denature it as well. UV irradiation usis free radical formations to stabilize the collagen structure, and it does improve the mechanical properties, but denatures the triple-helix structure. Similarly, Gamma irradiation similar to UV crosslinking denaturing increases as irradiation increases.

Structural Analysis:

Differential scanning calorimetry determines the collagen thermal stability by measuring heat flow differences.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis is used to visualize the molecular size distribution of collagen protein fragments similar to separating DNA fragments in Gel Electrophoresis.

Circular Dichroism (CD) is an absorption spectroscopy that determines the presence of secondary and tertiary collagen structures by measuring the difference in absorption of the left circularly and right circularly polarised light.

Raman spectroscopy is a label-free and non-describe method used to determine the bonds and protein structures present in collagen using inelastic light scattering through a laser source.

FTIR is a spectroscopy method that determines the bond and protein structures present in collagen using infrared radiation.

These are some methods that will allow us to test for the composition and mechanical properties of the collagen in order to "tweak" it to fit our needs.

Morphological Analysis Methods:

Scanning Electron Microscopy images surface topography of collagen samples and confocal microscopy are used for structural visualization as well. Transmission electron microscopy and atomic force microscopy visualizes banded collagen fibril structures.

Chemical Assay Methods:

- Hydroxyproline is a colorimetric assay for quantifying hydroxyproline in collagen.
- Sircol assay is a colorimetric assay to quantify collagen, binding to the [Gly-X-Y]n helical structure in collagen
- 2,4,6-Trinitrobenzene sulfonic acid (TNBS) assay is a colorimetric assay used to quantify free
- primary amines found in collagen
- Ninhydrin assay is a colorimetric assay to quantify free primary amino groups.
- Western blot is a method used to identify the type of collagen following SDS-page analysis.
- Mass spectroscopy identifies proteins

Non-Additive Manufacturing for Collagen-Based Ink Printing Applications:

Collagen sponges that are used in tissue engineering have a lot of pores and they are often used with drugs, growth factors, and bio-additives to enhance the scaffold bioactivity. When the collagen is made into thin-film collagen via evaporation, micropatterns can be designed by stacking the collagen film by layer which can influence osteoblast cell orientation.

Electrospinning:

The solvents used in electrospinning can significantly denature collagen, such as "fluoroalcohols used in electrospinning such as 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) cause a loss of collagen's triple helical structure". Instead solvents such as acetic acid/DMSO and PBS/ethanol should be used.

Additive Biomanufacturing

Extrusion:

Biomaterial inks are loaded into a syringe and printed as filaments onto a stage via a mechanical or pneumatic dispensing system. Often combining the collagen with other polymers allows for more self-supporting structures. "A process unique to extrusion bioprinting known as freeform reversible embedding of suspended hydrogels (FRESH), non-self-supporting collagen ink formulations can print complex collagen scaffolds which are then self-assembled and collected from the hydrogel suspension". This method can be combined with inkjet bioprinting for a seamless one step process to produce cell-laden 3D skin tissue.

Inkjet Printing:

Liquid biomaterials are loaded into a cartridge and deposited onto a substrate via droplets. The propulsion of droplets is due to the differences of pressure that can be induced via thermal, acoustic, or piezoelectric elements. Due to the low-viscosity inks that are used in the system, additional processing steps are required to make 3D structures.

Laser-Assisted Printing:

A layer of biomaterial is deposited onto a substrate via laser-induced forward transfer. A

cultured, resulting in complex cellular patterns [122,123]. Additionally, inkjet bioprinting was applied to create in vitro cancer model microtissue arrays for drug testing and studying tumor progression [124]. Moreover, by controlling the thickness of the collagen gels printed via inkjet printing and seeding cells between the layers of the 3D construct, cell aggregates have been shown to fuse together, demonstrating potential for organ printing [125].

heterogeneous 3D structure is obtained by coating the donor film with different materials and using the laser beams on different locations. This technique has been used to recreate skin substitutes and corneal strom-like tissue.

Stereolithography Printing:

A reservoir of photo-sensative ink is exposed to a predefined light pattern and crosslinked layer by layer onto a platform to produce a 3D structure. The use of light patterns allow for high resolution

and accuracy. SLA accommodates inks with greater viscosities. Some downfalls are that the photo-curing agents can be cytotoxic if residual components remain and can not incorporate multiple ink formulations.

Relevance to our Project:

1. Gelatin can help with cell attachment.
2. Some downfalls of using collagen are: poor mechanical properties, thrombogenicity, contamination, and source & batch variation
3. "Depending on the sources of collagen, extraction steps and crosslinking methods (chemical, physical), the resultant properties will differ"
4. For chemical crosslinking, increasing the concentration and time increases the crosslinking
5. Physical crosslinking often leads to the denaturing of the collagen
6. These are some methods that will allow us to test for the composition and mechanical properties of the collagen in order to "tweak" it to fit our needs.

Summary Chart Courtesy of the Article:

Table 1. Applications of additive bioprinting methods for collagen-based inks.

Bioprinting Method	Collagen-Based Ink Formulation	Outcome	Ref.
Extrusion	Methacrylated type I collagen; Sodium alginate	Fabrication of structures that resembles native human corneal stroma with cell-laden bioink via extrusion bioprinting.	[116]
Extrusion	Collagen Type I; Alginic acid sodium salt from brown algae; $CaCl_2$ solution	Core-sheath coaxial extrusion of alginate/collagen bioink with $CaCl_2$ allows creation of scaffolds with low collagen centration despite its low viscosity.	[114]
Extrusion	Rat tail type I collagen; Gelatin (type A); Sodium alginate	Extrusion bioprinting of collagen scaffold via gelatin/alginate system with controllable degradation time based on amount of sodium citrate during incubation.	[113]
Extrusion	Type I collagen was extracted from tendons obtained from rat tails	Identified storage modulus as the best predictor of collagen bioink printability during deposition.	[117]
Extrusion	PureCol Purified Bovine Collagen Solution; Soldium alginate (low viscosity)	Fabrication of interwoven hard (PLLA) and soft (bioink) scaffolds which support cell attachment and proliferation using a modified desktop 3D printer.	[135]
Extrusion	Methacrylated COL I; Heprasil; Photoinitiator	Successful bioprinting of liver model. Printed primary hepatocytes retained function over 2 weeks exhibiting appropriate response to toxic drugs.	[41]
Extrusion	Lyophilized Atelo-collagen, Matrixen-PSP	Pre-set extrusion bioprinting technique is able to create heterogeneous, multicellular and multi-material structures which perform better than traditional bioprinting.	[112]
Extrusion	Collagen Type I extracted from rat tails; Pluronic® F127	Fabrication of 3D constructs without chemical or photocrosslinking before and after printing via thermally-controlled extrusion.	[115]
Extrusion	Lyophilized sterile collagen, Viscoll	Formation of scaffolds which support spatial arrangement of tissue spheroids as well as support cell adhesion and proliferation.	[47]
Extrusion	Type-I collagen, Matrixen-PSP; Tannic acid	Fabrication of 3D porous structures which support cell migration and proliferation for long periods of culture. Determined optimal tannic acid crosslinking.	[67]
Extrusion	Collagen Type I; Sodium Alginate	Improved mechanical strength and bioactivity via the addition of collagen. Higher cartilage gene markers expressed, preservation of chondrocyte phenotype.	[42]
Extrusion	Type-1 collagen, Matrixen-PSP	Established a crosslinking process using tannic acid. High printed preosteoblast viability and well-defined pore size and strut dimensions for bone regeneration.	[68]
Extrusion	Type-I collagen, Matrixen-PSP; Decellularised extracellular matrix (dECM); Silk Fibroin(SF)	Hybrid collagen/dECM/SF scaffold with enhanced cellular activity and mechanical properties. Enhanced cell differentiation, mechanical properties, amenable for hard tissue regeneration.	[59]
Extrusion	Atelocollagen Type I powder	Novel self-assembly induced 3D printing to produce macro/nano porous collagen scaffolds with reasonable mechanical properties, excellent biocompatibility and mimicking native ECM.	[58]
Extrusion	Type-I collagen, Matrixen-PSP; Polycaprolactone (PCL); Hydroxyapatite (HA)/β-tricalcium-phosphate (TCP); Platelet-rich plasma(PRP)	Fabrication of collagen/PCL biocomposites loaded with bio-additives via 3D extrusion printing. Collagen/PCL biocomposites allow controlled release of HA/TCP bio-additives, which promote osteogenesis. PRP biocomposites demonstrate increased mineralisation.	[46]

Table 1. *Cont.*

Bioprinting Method	Collagen-Based Ink Formulation	Outcome	Ref.
Extrusion	Type-I collagen, Matrixen-PSP	Genipin crosslinking allowed fabrication of 3D cell-laden porous scaffold (Cellblock) with mechanical stability, pore size and osteogenic (bone tissue regeneration) potential.	[70]
Extrusion/Inkjet	Lyophilized collagen type 1 sponge derived from porcine skin	Development of a one-step process to produce a 3D human skin model with functional transwell system. Cost-effective compared to traditional transwell cultures.	[118]
Inkjet	Type I rat tail collagen; poly-d-lysine	Fabrication of neuron-adhesive patterns by printing cell-adhesive layers onto cell-repulsive substrates.	[123]
Inkjet	Collagen (Calf skin)	Cell aggregates printed between layers of collagen gels suitable for tissue engineering.	[125]
Inkjet	Collagen (rat-tail); collagen (calf skin)	Low-cost, high-throughput surface patterning with collagen and potentially, other proteins.	[122]
Inkjet	Collagen Type I	Fabrication of in vitro cancer microtissues via collagen inkjet printing. Four individual microtissues within one 96-well plate well, maintained for up to seven days.	[124]
Inkjet	Collagen: Type I rat tail collagen; Fibrinogen; Thrombin	Collagen bioinks and Fibrin/Collagen bioinks unsuitable for in situ inkjet bioprinting.	[136]
Inkjet	Type I acidic collagen; Agarose (low gelling temperature)	Fabrication of 3D corneal stromal structure with optically properties similar to native corneal stroma. Potential as a clinical or experimental model.	[120]
Inkjet	Acidic collagen solution; Agarose (low gelling temperature)	MSC branching, spreading and osteogenic differentiation controlled by collagen concentration; Osteogenic potential (bone tissue engineering).	[121]
Laser-assisted	Collagen Type I (Rat-tail)	Fabrication of cell-laden skin tissue using laser-assisted bioprinting, in vivo potential. Skin tissues consist of: a base matriderm layer, 20 layers of fibroblast and 20 layers of keratinocytes.	[130]
Laser-assisted	Collagen (Rat-tail)	Multicellular collagen skin tissue constructs printed using laser-assisted bioprinting. Keratinocyte and fibroblast layers did not intermix after 10 days. Mimics tissue-specific functions (e.g. gap-junction).	[129]
Laser-assisted	Type I collagen (rat) solution; Nano hydroxyapatite (nHA)	In situ printing of cell-laden collagen-based ink via laser assisted bioprinting allow bone regeneration (mouse calvaria defect model). Contact free printing method is sterile with clinical potential.	[126]
Laser-assisted	OptiCol™ human Col I; Ethylenediaminetetraacetic acid (EDTA) human female AB blood plasma; Thrombin from human plasma	Fabrication of 3D cornea tissue using novel human protein bioinks via laser assisted bioprinting. Novel bioink is biocompatible, without requiring additional crosslinking. First study to demonstrate laser-assisted bioprinting for corneal applications using human stem cells.	[131]
Stereolithography (SLA)	Collagen methacrylamide(CMA) synthesized using Type-I collagen; Irgacure (I2959)	Free-form photolithographic fabrication; photopatterned hydrogels retain structure after 24 h. CMA retains native collagen self-assembling properties; hydrogels biocompatible in vivo.	[134]

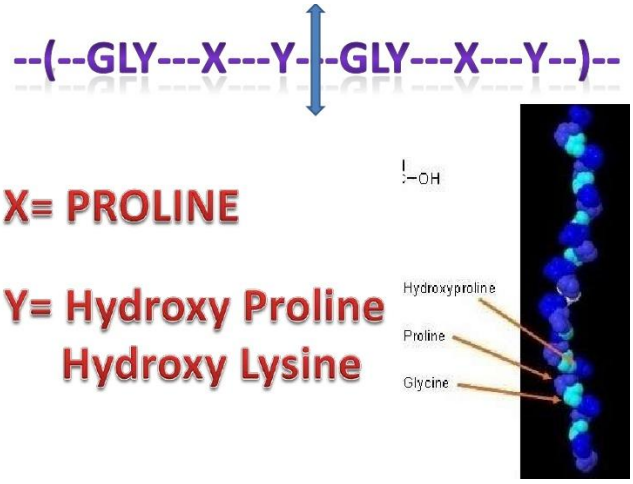
Three Decades of Research on Recombinant Collagens: Reinventing the Wheel or Developing New Biomedical Products? (Fertala)

Andrzej Fertala

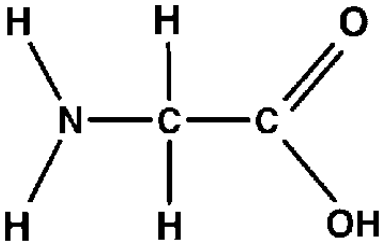
Key Terms

Recombinant (collagens): Basically human made collagens

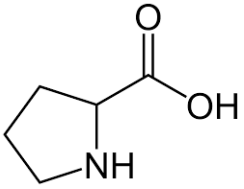
Gly-X-Y: Collagen fibres are made of this peptide. Gly-X-Y is more of a formula. X and Y can be different amino acids but collagen typically uses Proline for X and Hydroxyproline for Y.



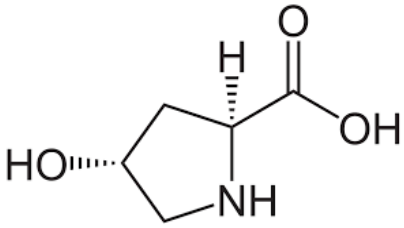
(Gly) Glycine:



(X) Proline:



(Y) Hydroxyproline:



Hydroxylation is essentially a reaction that adds an OH to a certain compound. In this case, OH is added to Proline. OH is capable of hydrogen bonding which is an extremely strong bond which constitutes collagen tensile strength.

The article talks about hydroxylation methods using different substances, techniques, X and Y inputs, and more.

Key Notes

- Go to 2.1 for hydroxylation
- Go to 3.1 for utilization of collagen
- Go to 3.2 for collagen side effects
- Go to 4.2 for expression systems
- Go to 4.3 for collagen modification and electrospinning fibers

Summary

This study highlights the methods and technologies developed to produce and utilize recombinant collagens. The beginning, part 2, is more about the properties of collagen.
Part 3 is about utilization of collagen. Part 4 is about recombinant collagens.

2.1 Biosynthesis of Triple-Helical Collagen Molecules

- All collagen types will have Gly-X-Y repeating
    - Some will be uninterrupted Gly-X-Y repeating
    - Others will be Gly-X-Y repeating with amino acid sequences that interrupt it; this makes the collagen more flexible
- P4H (prolyl-4-hydroxylase) hydroxylases (verb) proline residues present at the -Y- position.
- LH (lysyl hydroxylase) hydroxylases (verb) many lysine residues present at the -Y- position.
- Depending on our method of choice, hydroxylases may be necessary to strengthen our product. If so, we will need to analyze which hydroxylase is appropriate.

2.2 Diverse Architectures of Collagen-Rich Matrices

- All collagens are triple helices but the large formations they create are wildly different. Ex. collagens make transparent forms, cylindrical muscle forms, or more.

2.3 Self-Assembly of Collagens: Paradigms of CollagenI Fibrils, Collagen VII Anchoring Fibrils, and Collagen IV Networks

- NC1 and NC2 peptides flank the collagen triple helix. These peptides are used for folding triple helices and more.

3.1 Applications of Collagens

- Fabricating material for tissue engineering, wound healing, drug delivery (carrying the medicine by binding to it), fiber weaving.
- With proper modifications, synthetic collagens can fill the roles of rare collagen types. For context, there are different types of collagens that are used for different roles in the body.

3.2 Potential Limitations of Collagens Isolated from the Natural Sources

- Transmission of prion disease is a concern, especially for animal sources of collagen.
- 2-4% of patients show reactions to injection of exogenous(made outside of the body) collagen.
- Autoimmunity (immune system attacks body cells) is a concern with introduction of collagens that are similar to human collagens.

4.1 Prerequisites for Engineering Mammalian Recombinant Collagens

- Human collagen gains its attractive features from hydroxylation. This requires certain enzymes that modify the collagen. Synthetic collagens need to take this into account.
- Some synthetic collagens side-step this problem. Scl1 and Scl2 are an example; they do not require hydroxylation and still reach desirable fiber strength.

4.2 Collagen Expression Systems

- Mammalian cells
    - They can be used to produce native collagen cells. For example, a pig cell would produce pig collagen.
    - This is special because the enzymes that modify collagen would be present and naturally pair with the collagen.
    - This system has a relatively low yield and potentially high cost.
- Plants
    - Companies mass produce with plants (CollPlant Ltd.)
- Bacteria, yeast, insect cells
    - Compatible with mass production
- Chemical method (completely man made)
    - Lacks the strength of natural collagen

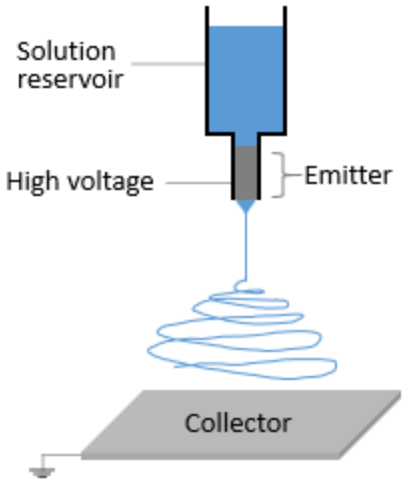
Table 1. A summary of the expression systems for production of recombinant collagens.

Expression System	Examples of Collagen Constructs	Requirement for Co-Expression of P4H (N = No, Y = Yes)	Industrial-Scale Production (N = No, Y = Yes)	Commercial Evaluation (N = No, Y = Yes)
Mammalian cells (HT1080, CHO, HEK293, NIH3T3)	Native-like human procollagens, including procollagen I, procollagen II, collagen VI, procollagen VII. Fragments of procollagens, including mini-collagen II, mini-collagen I homotrimer, mini-collagen VII, C-terminal propeptides of procollagen III, and fragments of collagen IV	N	N	N
Insect cells	Native-like collagens including collagen I, collagen II, collagen III, collagen IX, collagen	N	N	N
Mammary glands of transgenic mice	Collagen I homotrimer	N	N	N
Escherichia coli	Human-derived mini-collagen III, collagen fragments, including C propeptide of collagen XVIII, and fragments of collagen I	Y	N	N
Escherichia coli	Collagen fragments stabilized by bacterial collagen-like sequences	N	N	N
Yeast cells	Native-like human collagen I, collagen III, gelatin	Y	Y	Y
Transgenic plants	Native-like human collagen I	Y	Y	Y

4.3 Recombinant Collagen Variants

- The general idea is modifying collagens to fit specific goals. For example, a collagen that is more heat resistant.

- Collagen modification uses fragments of the full collagen molecules. This is because full collagens are hard to program. They have very specific binding interactions.
    - Modified human collagen, chitosan, and polyethylene oxide are mixed together.
    - They then do an electrospinning procedure. This makes fiber.
    - Electro Spinning 101:



    - They then connect fibers through crosslinking.

4.4 Proposed Biomedical Applications of Recombinant Collagen Constructs

- Bone graft, sponge, cell support…

6. Constraints on Implementing Recombinant Collagen Technology in Clinical Applications

- No consensus on large scale production system that follows guidelines
- No consensus on collagen type in demand
- Few notable cases of collagen succeeding in clinical trials

Bioengineered Collagens (Ramshaw et. al)

John AM Ramshaw, Jerome A Werkmeister, Geoff Dumsday

Takeaway:
Establishing Commercial Availability has important info on production sources. An Emerging System talks about Scl2's features.

Establishing Commercial Availability

- Collagen is structured by repeating Gly- Xaa- Yaa amino acids. More info on this from here.
- Prion disease is brought up as a concern from mammalian sources again.
    - The solution is to use non mammal sources: jellyfish, chickens
- Mammalian collagens have the disadvantage of needing P4H, which is the enzyme that hydrolyzes the collagen.
- Examples of collagen production sources tested for production:
    - HT1080 mammalian cell
    - HEK 293-EBNA mammalian cell
    - Baculovirus insect cell
    - E.coli
    - P.pastoris yeast
    - Bombyx mori silkworms
- Mammal cells and insect cells have P4H systems inside of them. Cells also produce full length collagens.
- E.coli has issues expressing all necessary genes of Collagen.
- Yeast is the most promising because it can produce full length hydroxylated collagens. It also allows modified gene production.
- Silkworms conditioned to produce collagen in their cocoons.

Production systems	Yield	Comments
Mammalian cells:		
HT1080	≤1 mg/L	Mainly type II collagen and variants
HEK293	≤80 mg/L	Used to produce collagen types V, VII, VIII, X, XVI
CHO	<0.5 mg/L	Type IV collagen
COS-1	<0.5 mg/L	Type III and XVII collagens
Insect cells		
Baculovirus	≤40 mg/L	Best yield for homotrimer, type III collagen and less heterotrimer type I collagen
Bacterial cells		
E. coli	≤14 g/L	No hydroxylation
Yeast cells:		
S. cerevisiae	≤0.4% protein	Incomplete hydroxylation
P. pastoris	≤1.5 g/L	Fully hydroxylated. Commercially available
H. polymorpha	≤0.6 g/L	For a 14 kDa fragment. No hydroxylation
Transgenic animals		
Mouse (milk)	≤8 g/L	For a 37kb fragment. Much lower yields for full-length constructs
Silkworm (*Bombyx mori*) (cocoon)	≤4.2 mg per cocoon	Probably not triple-helical, but rather as gelatin
Transgenic plants		
Plant seeds (Barley, Rice, Maize)	≤140 mg/Kg of seed (barley)	For a 45 kDa fragment. Considerably less for full-length collagen. Low hydroxylation
Plant leaves (Tobacco)	≤20 g/L of soluble protein	Yield can depend on hydroxylation status

An Emerging System

- Brings up Streptococcus Pyogenes (Scl2).

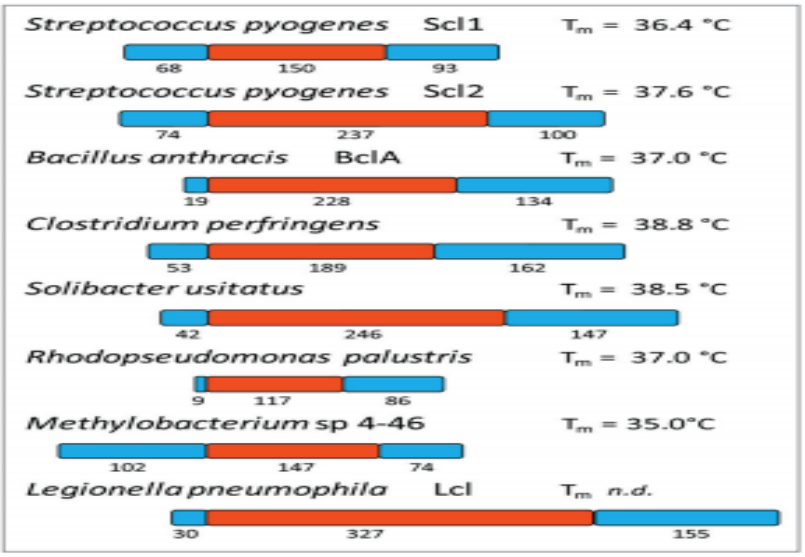


Figure 2. A schematic illustrating the structures and melting temperatures, T_m, of bacterial collagens, including the non-triple-helical terminal domains (blue) and the central triple helical domain (red) for which a triple-helical structure has been established.

- pColdIII vector used for Scl2 expression in E.coli
- 19g/L is the best yield for Scl2 with the pCold vector
- Apparently, Scl2 does not have binding sites that allow for modification of collagen.

- Scl1 on the other hand has binding sites
    - Scl1 reacts with integrins a2B1 and a11B1
    - These are combined through GLPGER sequence
        - This study goes into the collagen modification with GLPGER (it has all of the DNA sequences and stuff)
- S.pyogenes (I assume Scl1) can bind with GAGAGS motifs to enable silk binding. This allows for more complex structures.

Sericin

Development of ethyl alcohol-precipitated silk sericin/polyvinyl alcohol scaffolds for accelerated healing of full-thickness wounds (Siritienthong et. al)

Tippawan Siritienthong, Juthamas Ratanavarapornb, Pornanong Aramwit

Key Concepts

- Ethyl Alcohol can be used to precipitate sericin to form stable Sercin/Polyvinyl Alcohol scaffolds without crosslinking
- Silk sericin is a promising implant for tissue prosthetics in the human body
- While sericin itself is quite fragile, the scaffold formed by combining with PVA and EtOH precipitation is quite strong, and no crosslinking is needed

Materials and Methods

In the experiment they used fresh cocoons of actual silkworms, Polyvinyl alcohol, Ethyl alcohol, glycerin, and some other stuff. They concentrated a solution of sericin from the silk cocoons, mixed PVA and glycerin with the Sericin, then froze it, and then they soaked it in ethyl alcohol solutions. To test the actual properties of the scaffolds they used a compression machine, scanning electron microscope, water swelling test??, and tested release of sercin from the scaffolds. They tested the effectiveness of wound healing in rats, by basically giving the rats cuts and applying the scaffolds with bandages.

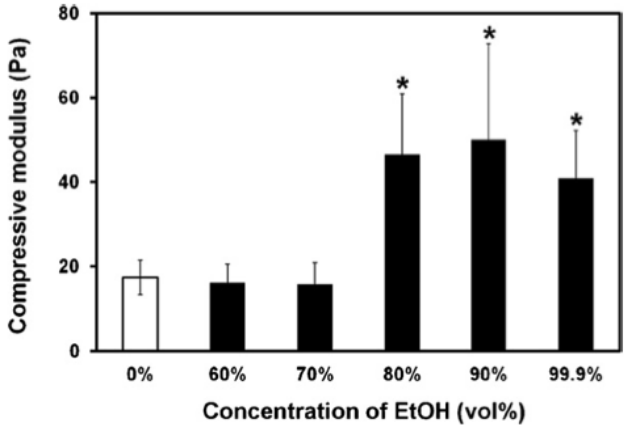


Fig. 2. Compressive modulus of EtOH-precipitated sericin/PVA scaffolds. Concentration of EtOH: 0, 60, 70, 80, 90, and 99.9 vol%, $*p < 0.05$, significant against the non-precipitated sericin/PVA scaffolds (0 vol% EtOH).

Results

Their results found that the scaffolds soaked in higher concentration ethyl alcohol showed to be denser and have a higher compressive modulus. When the scaffold was precipitated with EtOH, sericin could be precipitated much easier in the presence of EtOH. This meant that the secondary structure could change to beta sheet structure and then the structure became more compact. This caused the scaffold to become denser and thicker. Higher concentrations of EtOH showed better physical properties like compressive modulus.

Most of the results were in terms of the healing propertiesof the mice they stabbed, they did find that sericin accelerates wound healing, and when it did create a strong scaffold strong healing properties were shown to occur. Wound size did actually decrease much more for the scaffolds with sericin vs control group. They found that 70% concentrated EtOH precipitated scaffolds were most effective for healing of the wounds, because of thebalance between better scaffold structure and better cell functioning.

Preparation of New Natural Silk Non-woven Fabrics by Using Adhesion Characteristics of Sericin and Their Characterization (Lee et. al)

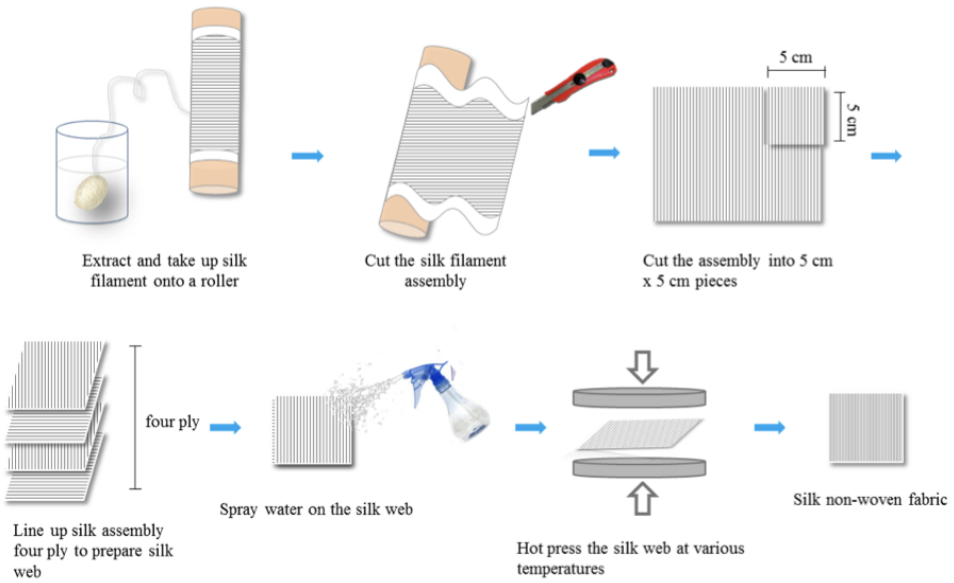
Ji Hye Lee, Yeon Su Baea, Su Jin Kima, Dae Woong Songb, Young Hwan Parkb, Do Gyu Baea,c, Jin Hyun Choi, In Chul Um*

Key Concepts

The scientists created fabric by wetting and hot press treatments on sericin, it was a non woven silk fabric. The fabric became stronger with a higher percentage of sericin within it. Electro-spun regenerated silk is a common method of creating silk fabric for biomedical purposes, but it is usually not cost efficient and not very easy to make good. Electrospun silk is also very weak, and the mechanical properties are quite poor. Generally nonwoven natural silk has been proven to be ineffective also, so natural silk filaments combined with sericin was used to press and create a non woven silk fabric that was more effective.

Materials and Methods

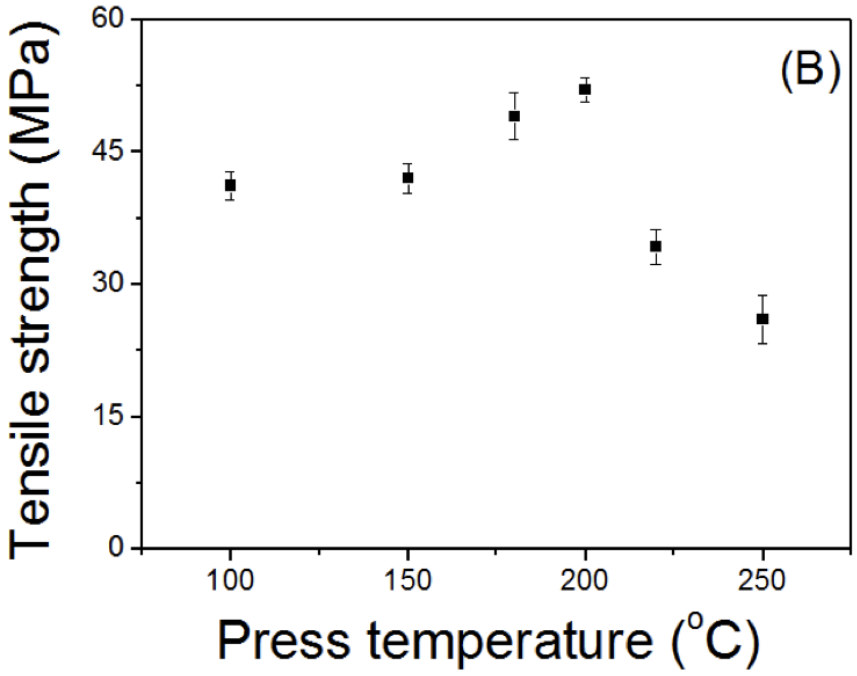
They used silk filaments, cut it up, into squares, and then used water and then a hot presser to fabricate the non woven fabric. They tested fabrics that had different amounts of sericin, and tested mechanical quality. They used a spectrometer to look at the silk protein molecular structure. They also used electron microscopy to look at the silk fabric. Tensile strength tests were conducted, and some other mechanical tests were conducted.



Results

They eventually concluded that creating a silk web of silk filaments that was connected by sericin creates a fabric with a stronger tensile strength and regular silk non-woven fabric. The new type of silk web that they made has good mechanical properties and cell viabilities, and so has applications in both cosmetic (clothing) and biomedical places.

They observed that the physical structure and tensile strength of the fabric they developed increased all the way up to 200deg celsius, and after that I think the fabric just started burning. Increased Sericin content within the fabric increased the tensile strength and Young's modulus.



Properties of Sericin Films Crosslinking with Dimethylolurea
(Turbiani et. al)

Authors: Franciele R. B. Turbiani, Jose Tomadon Jr Fernanda L. Seixas, Gylles Ricardo Stroher, Marcelion L Gimenes.
Universities: Federal Technology University, State University of Maringa
Contact: francieler@utfpr.edu.br
Publication: 11th Brazilian Polymer Congress (11º CBPol Congresso Brasileiro de Polímeros)

1. Key Concepts

1.1 Sericin

Sericin is a natural silk protein that can be extracted from the silk polymers of the *Bombyx Mori* moth.

Sericin is composed of 18 different amino acid bases, 32% of which is serine. In its natural state, Sericin has a random coil secondary structure. It can be easily converted into a β-sheet conformation through repeated moisture absorption and mechanical stretching.

There are three types of Sericin that can be extracted from silk polymers. Sericin A is insoluble in water and is the outermost layer. Sericin B, which composes the middle layer, is nearly identical to Sericin A but with the addition of tryptophan. Sericin C can be separated from the fibroin of silk with the addition of a hot, weak acid, and is identical to Sericin B but with the addition of proline.

Sericin has high elasticity and high tensile strength. Sericin has a natural affinity for keratin. It has a natural infection resistance and is highly biocompatible. Sericin has anti-aging and anti-wrinkle properties.

For the experiment, it is key that Sericin is a highly hydrophilic macromolecule, meaning it will behave like polysaccharides in Gelification (see below), ensuring it will form a film.

To quote from the experiment:

> "While manufacturing silk, sericin is removed as waste during the degumming process. If sericin can be utilized to make biofilms as a value-added product developed from the wastes, it will represent a significant source of profit, not to mention the beneficial effect of waste reduction for pollution prevention."

1.2 Degumming

Through the process of degumming, Sericin can be removed from Silk. In degumming, silk cocoons are placed in hot water and Marseille soap, freeing the silk filaments.

Alkaline sericin degumming solution is seated to boiling and is continued for 45 minutes in a dyeing machine. Alcohol ratio applied at 1 part to 30 parts.

1.3 Dimethylolurea

Classified as an irritant and health hazard in a laboratory setting, dimethylolurea is an Oxymethurea with a molecular weight of 120.11g/mol. It is used in general adhesives, anti-wrinkles, automotive care, construction, and paint fillers.

Do not come into physical contact with or inhale this chemical.

1.4 Biofilms

Biofilms are symbiotic cross-feeding groups of microorganisms. In biofilms, the microorganisms stick to each other and to the surface. The cells are embedded in a slimy extracellular matrix composed of extracellular polymeric substances, natural polymers with a high molecular weight that are secreted by the microorganisms. These EPS's are mostly composed of polysaccharides and proteins.

1.5 Gelification

Gelification is the process where water is introduced into a substance (specifically hydrocolloids), which causes it to turn into a gel. Examples of this include agar agar and gelatin.

Those hydrocolloids are evenly dispersed in water. Because they are very polar, the hydrocolloid semi-immobilizes water, creating a very viscous liquid/soft solid. When you add heat, however, the water molecules are freed and the solution behaves more like a liquid.

2. Methods

2.1 Sericin Extraction

1) The Bombyx mori silkworm cocoons were first cut into small pieces and then immersed in distillate water with 3% w/v (weight over volume).
2) Aqueous solutions were extracted with hot water under pressure at 120 degrees celsius for 60 minutes. An autoclave was used here. Autoclaves are a high-temperature steam sterilizer commonly used to sterilize healthcare tools.
3) The solution was deep-frozen at negative 20 degrees celsius. This encourages the precipitation of sericin.
4) The solution was defrosted and filtered under vacuum.
5) The concentration of sericin was adjusted to 2 wt% (mass fraction)

6) Dimethylolurea was added as a crosslinking agent and glycerol (0.6g per g of sericin) was added as a plasticizer.

2.2 Preparation of Sericin Films

1) The sericin films were made using casting
    a) First, the liquid is poured into a mold
    b) The liquid hardens as it cools, allowing the mold to give it shape
2) Solution is first stirred for about 1 hour
3) Temperature raised to 70 degrees celsius
4) DMU solution was added
    a) The experiment in question tested the different values of DMU at 0.1g/g, 0.2g/g, 0.4g/g, 0.6g/g and 0.8g/g.
    b) See results for more information
5) 70 grams of the solution was poured into polystyrene Petri dishes (diameter of 15 centimeters. Petri dishes served as a mold
6) Solution was dried in a convection oven at 40 degrees celsius for 18 hours.
7) Films were then placed in desiccators (humidity control apparatus) and kept at 52% relative humidity at room temperature for three days.

3. Results

3.11 Solubility in Water (Sw)

Moisture weight fraction (ω) was gravimetrically determined in a vacuum oven at 105 degrees celsius for 24 hours. Disks 2.5 CM were then cut out and weighed (M0). These disks were immersed in 50 mL of distilled water. Agitation of 175 rpm at 25degrees celsius for 24 hours. Dry matter (Mf) determined in the same way as ω. Finally, the solubilized matter was calculated as a function in the equation below.

$$S_W = [m_o(1-\omega) - m_f] / m_o(1-\omega)$$

3.12 Mechanical Properties

Tensile strength and percentage tensile elongation were measured at room temperature using a TA.XT2. More information on that can be found here. The method used was the standard ASTM method D882. During ASTM D882 testing, specimens are pulled in tension until failure. More info on that here, as it dives into some physics vocabulary that I am not familiar with.

3.13 Water vapor permeability coefficient (WVP)

The WVP of the films was determined gravimetrically at 25 degrees celsius according to method E96-95, where the testing chamber was maintained at a constant 73.4 degrees Fahrenheit and 50 ± 2% relative humidity. Using corningware cups of 4.61" in diameter, the E96-95 standard used the desiccant method. More information on the ASTM E96-95 method and the Desiccant method can be found here and here respectively.

Granulated calcium chloride was used as the desiccant. Cells were placed in desiccators at a constant relative humidity of 75% using a saturated NaCl solution. Using the equation below to calculate for Water vapor permeability constant, WVP is the water vapor permeability coefficient [g mm/(m^2 24h kPA)], L is the average thickness of the film [mm], G/t is the apparent steady-state rate of weight gain [g/24h], t is the total time [days], A is the permeation area [m^2], (RH1 -RH2) is the difference of relative humidities [=0.75], and Pwis the partial water vapor pressure at 25 degrees celsius [kPa].

$$WVP = [[(G/t) \,.\, L\,]/[A \,.\, P_w \,.\, (RH_1 - RH_2)]]$$

3.14 Fourier transformed infrared spectroscopy

Infrared spectra were collected using a FTIR spectrometer Varian - series 7000 at a wave-number region of 600-2000 cm^-1, resolution of 2 cm^-1, anda scan frequency of 16 times.

3.15 X-ray Diffraction

X-ray diffraction intensity curves were measured at a scanning rate of 0.0015^o s^-1 for 2θ from 5 to 50^o with a diffractometer Shimadzu D 6000 using Copper radiation

3.16 Statistical Analysis

An analysis of variance and a Tukey Test were used. Analysis of variance (ANOVA) is an analysis tool used in statistics that splits an observed aggregate variability found inside a data set into two parts: systematic factors, which influence the given data set, and random factors, which have no influence. A Tukey Test is a single-step multiple comparison procedure and statistical test. It can be used to find means that are significantly different from each other. These tests were used to determine statistically significant differences ($p < 0.5$) among averages. The software used is Statistica 7.0.

3.2 Physical Characterizations of the Films

A) Films produced using the crosslinking method were light yellow, homogenous, transparent, and visually attractive.
B) The glycerol added extended the film flexibility.
C) Average film thickness was 0.10 ± 0.02 mm
D) Addition of Dimethylolurea reduced film flexibility.

3.21 Solubility in Water

The solubility in water of films conditioned at 52% relative humidity decreased in relation to increasing DMU content. This is likely due to the fact that increasing the DMU concentration increases crosslinking degree and that DMU's better aligned incipient polymeric structure helped the strengthening action to form the film.

3.22 Structures of Sericin Films

The introduction of DMU into sericin caused one major change. Native sericin contains both random coils and Beta sheets representing amorphous and crystalline regions respectively. The addition of DMU converted Beta sheets into random coils, thereby decreasing the amount of crystallinity.

4. Takeaways

First of all, we can use their method of developingsericin biofilms, although we can replace dimethylolurea with something more commonplace. Secondly, it's important that we know that DMU results in a more amorphous biofilm. Thirdly, to keep film mechanical resistance and flexibility, and keeping low solubility, 0.4 g DMU / g Sericin is preferred.

Production of silk sericin/silk fibroin blend nanofibers (Zhang et. al)

Xianhua Zhang, Masuhiro Tsukada, Hideaki Morikawa, Kazuki Aojima, Guangyu Zhang, and Mikihiko Miura

Important Terms & Key Concepts

Silk Sericin (SS) : a natural polymer produced by silkworm in the production of silk, the gum that coats fibers and allows them to stick to each other

Silk Fibroin (SF): insoluble protein produced by silkworm, the structural center of silk

Electrospinning: a spinning technique using electrostatic forces that is used to produce ultrafine nanofibers

Nanofibers: fibers with diameters in the nanometer range

Thesis of the experiment

Although there is much research about silk fibroin and silk fibroin/ polymer blends, there is much less on silk sericin and silk fibroin blend nanofibers. This experiment works to find a novel way to prepare silk sericin/silk fibroin nanofibers.

Methods/Procedure

They prepared SS/SF blend nanofibers by electrospinning from their blend solutions.

To prepare the blended solution, solutions of 0.45, 0.3, and 0.15g silk sericin were mixed with 0.3, 0.6, and 0.9g silk fibroin solutions. This way 75/25, 50/50, and 25/75 blend solutions were made. The solutions were then stirred and refrigerated.

The solutions were then electrospun. The electrospinning was done at a working distance of 15cm, voltage of 25kV, and a flow rate of 0.06 cm/min.

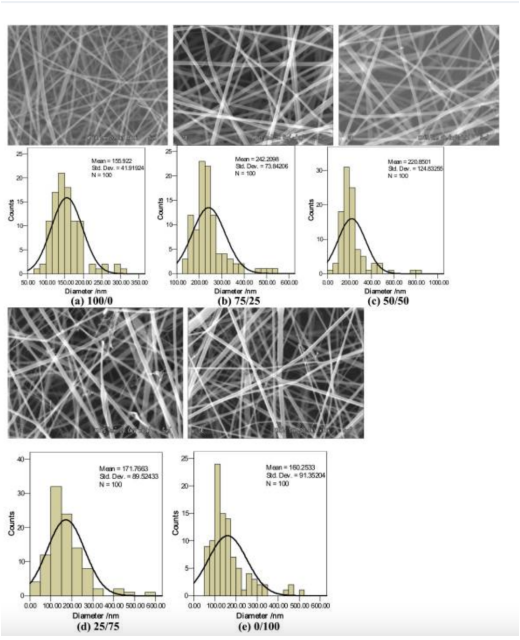
To characterize the nanofibers, a scanning electron microscope (at a resolution of 4 $cm^{=1}$ and a spectral range of 4,000-500 cm^{-1}) was used to determine the morphology and diameter.

Results

SEM microscopes show that as spun, the nanofibers display "smooth surfaces, round cross sections, and bead free sections…" The average diameter ranged from 156nm to 242 nm. **It was examined that the diameter of the SS/SF blends increased with a greater SF concentration.**

The blends with a higher SS concentration were more soluble in water, with the blends with a higher SF concentration could not completely dissolve in water.

DSC measurements were then conducted, all the mixtures showed endothermic peaks at 60-70 degrees celsius. The blends (100/0, 75/25, and 50/50) also had decomposition peaks from 138°C to 215°C, 122°C to 203°C, and 138°C to 213°C. When



the SF concentration was increased by 70-100%, decomposition peaks disappeared.

Conclusion

SS/SF blend nanofibers were prepared by creating and electrospinning a SS/SF TFA blend solution. The resulting nanofibers had smooth surfaces and round cross-sections. The average diameter of the blended solutions was thicker than those of pure SS or pure SF nanofibers. The number of beads of the nanofibers increased with increased dissolving time and increased SF content. Lastly, blends with a high SS concentration were dissolved in water.

Application of sericin to modify textile supports (Khalifa et. al)

Belhaj Khalifa, N. Ladhari & M. Touay (imenbk14@yahoo.fr)

Introduction

Finishing agents release toxic chemicals such as free formaldehydes. As a result, researchers are increasingly using biomaterials as finishing agents. This study examines sericin in fabrics as a **finishing and crosslinking agent**. Sericin makes up between 20% and 30% of the protein in silk. The adhesive protein is what holds the two silk fibroin filaments together. Sericin is made up of 18 amino acids, 70 percent of which are hydrophilic, which explains its high solubility and water absorption and desorption ability. Sericin powder was added to certain fabrics (wool and cotton) to benefit from its properties while also improving the properties of textile fabrics. This study aims to find the **optimal conditions to extract sericin**.

Textile finishing - includes preparatory treatments used before additional treatment, such as bleaching prior to dyeing; treatments, such as glazing, to enhance appearance; sizing, affecting touch; and treatments adding properties to enhance performance, such as preshrinking.

Methods

In the experiment, a Mathis LABOMAT (MATHIS AG, Oberhasli, Switzerland) was used as an autoclave to remove sericin. Cotton and wool fabrics were treated with sericin on the same unit. Freeze drying is used to remove water from the sericin solution without denaturation risk—such as from UV radiation, which is also used to remove water—and produce sericin powder.

Autoclave image
http://www.isterilizer.com/en/ezclave-n10e-full-automatic-laboratory-autoclave-sterilizer-lab

The absorbance of the 3 amino acids in sericin was 280nm. The absorbance in 280 nm is used to quantify the presence of proteins in a given liquid using spectrophotometric analysis. The treatment was evaluated by determining the sericin exhaustion rate Rexh%, as mentioned in the following equation:

$$R_{exh}\% = \frac{A_b - A_a}{A_b} \times 100$$

Ab and Aa were the bath absorbance at the wavelength 280 nm before exhaustion (without sample) and the bath absorbance after exhaustion (with sample)

Exhaustion rate - desorption and absorption of dyes and textiles

Sericin's ability to absorb water is one of its features. With Escherichia coli and Staphylococcus aureus as test species, antibacterial activity was determined using standard AATCC test procedure 147.

A jury of ten judges assessed the samples, and described how soft the fabric feels, and they rated the samples from 1 (not at all soft) to 10 (extremely soft). The untreated sample was given a 1, and the treated sample was given a 6.

Results/Conclusion

25 g/L of Na2SO4 improved up the exhaustion rate to 21.57%. In fact, sodium sulphate, more soluble, improves the migration of sericin from water to fiber.

The balance between the concentration of sericin in fiber and that of the electrolyte in the bath was reached at 95 C between 45 and 60 min.

The maximum exhaustion rate was obtained with pH3 = 3.8, when sericin and wool are overall positively charged, and results in Table 5 show that the wool samples treated with pH = 3.8 present an improved antibacterial activity.

Table 5. Evaluation of the antibacterial activity of the samples treated with different pH after 24h from incubation.

Bacterial strain	*S. aureus* Inhibition zone (mm)	*E. coli* Inhibition zone (mm)
pH 3.8		
pH 4.5		
pH 5.5		

The results showed that sericin has an affinity for wool, whereas it does not have any affinity for cotton.

This affinity for wool fiber was obtained in defined conditions with about 48% exhaustion rate for a sericin concentration of 2.5% (w/w). A percentage of sericin 5% (w/w) improved the touch of wool fabrics samples until a score of 4 points, as well as the absorption of water with a profit of 70.75%.

These findings demonstrate sericin's multifunctionality as a finishing agent, whereas other finishing agents often use toxic chemicals to achieve these same results.

Future questions/What this means for us

- Why was sericin not effective with cotton?
    - With wool: It is probable that there is a formation of the hydrogen, hydrophobic bonds and Van der Waals interactions between wool fabrics and sericin .
- Will sericin be effective with collagen?
- Will these same optimal conditions found in the study be used in our experiment?
- “Yet, it is interesting to improve the sericin exhaustion rate by using more sophisticated treatments such as grafting or cross-linking and to apply it on synthetic fibers such as polyamide and polyester.”

Cross-Linking

Cross-Linking of Collagen (Tanzer)

Review
Marvin L. Tanzer

1 Introduction

Collagen is extracellular and insoluble, and used in a variety of specialties like suture production, leather-making, and polymer chemistry. Tanzer presents three arguments:

1. The insolubility of collagen is due to cross-linking.
2. Covalent cross-links are from modified amino acids with carbonyl groups.
3. Molecular packing of collagen monomers specifies which cross-links are formed.

2 Content

Collagen *was* classified as a "fibrous" protein such as keratin, myosin, or fibrinogen, but was later discovered to be comparable to elastin in their properties of cross-linking. It is often cited as a prototype of **self-assembly systems**, or proteins that can assemble into highly ordered structures automatically.

Collagen is unique because it has an ***odd* number of polypeptide chains** (three) in a strange helical conformation, and the presence of **hydroxyproline and hydroxylysine** (amino acids). Hydroxylysine side chains may have *O*-galactosyl substitutions and residues, along with β-glycosyl residues. Collagen has high proline and glycine content with a repetitive positioning of **glycine at every third amino acid.**

Collagen solutions can polymerize into collagen fibrils, showing an increasing insolubility until they can only be dissolved by denaturation. This may be because there are **reactive carbonyl groups** which generate intermolecular cross-links.

Lathyrogens, more specifically, BAPN (beta-aminopropionitrile), inhibits collagen cross-linking: in animals, BAPN causes aortic rupture, herniations, and deformations.

However, in humans, collagen fibers become insoluble at a *lower rate* with lathyrogen administration, which may be because BAPN inhibits an enzyme that converts collagen lysine and hydroxylysine-amino groups into aldehydes.

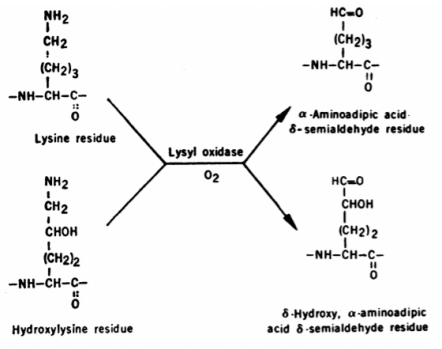


Fig. 1. Formation of collagen aldehydes by enzymatic action of lysyl oxidase on collagen molecules (5).

These aldehydes may react with other side chains to form cross-links. Cross-linking has specific criteria, and selective modification of the collagen aldehydes also prevents cross-linking, suggesting that collagen cross-linking depends on reactions between carbonyl groups and other amino acids of nearby molecules.

The two most abundant aldehydes are alpha-aminoadipic acid and delta-semialdehyde and its hydroxylated analog. These are formed by lysyl oxidase reactions and are more commonly found near amino terminals. An inner cross-link is alpha, beta-unsaturated aldol condensation product that makes the collagen quite stable, only appearing near the end of a terminal.

The aldol condensation product slowly disappears during incubation of the fibrils, and a new substance, the cross-linked histidinohydroxymerodesmosine is formed. It can potentially unite four polypeptide chains or join two or more collagen molecules. Histidine is thought to be located in specific regions that mean that the fibrils must be extremely ordered in order for them to bring bonds together.

Hydroxylysinonorleucine is a major cross-link that is made from the Schiff base formed between hydroxylysine and aminoadipic acid 8-semialdehyde. Thus the known incomplete hydroxylation of lysine at specific loci would readily give rise to the analogous, non hydroxylated Schiff base product.

Carbonyl-derived crosslinks are reducible by exogenous and endogenous reagents. Up to 25-50% of hydroxylysinorleucine and hydroxyisoleucine are reduced in vivo. Other reactions are possible involving the ketone groups.

3 Takeaways

Collagen cross-links form because two aldehyde-containing amino acids react with other amino acids in collagen. However, this only occurs when collagen molecules are assembled in naturally occurring fibrous polymers. If so, cross-linking is spontaneous and precise chemical structures and proportionality are formed.

4 Reference

Tanzer, M. L. (1973). *Cross-Linking of Collagen. Science,180(4086), 561–566.* doi:10.1126/science.180.4086.561

Enzyme-catalyzed Protein Crosslinking (Heck et. al)

Authors: Tobias Heck, Great Faccio, Michael Richter, Linda Thöny-Meyer
Affiliations: Swiss Federal Laboratories for Materials Science and Technology | Empa
Contact:Greta Faccio: https://gretafaccio.net/
Publication: Applied Microbiology and Biotechnology, https://www.springer.com/journal/253

1. Key Concepts

1.1 Cross-linking

A crosslink is a covalent structure (possibly ionic) that binds multiple polymer chains together. Polymers can be synthetic or natural (i.e. proteins -> sericin). Crosslinking takes multiple polymer chains and binds them, making the polymers more rigid and sturdy. Examples of this include the curing of thermosetting polymers (i.e. unsaturated polyester or epoxy resin) and vulcanization of rubber.

Thermosetting polymers that are cross-linked, so they can no longer be melted down into their original soft solid/viscous liquid state. Vulcanization is also irreversible.

In curing, single monomers and oligomers (a polymer whose molecules consist of relatively few repeating units), are first mixed, either with or without a curing agent. This forms a tridimensional polymeric network (a 3D combination of one thermoplastic [able to return to soft solid/viscous liquid state] and one thermoset polymer in network form).

In the first part of the reaction above, branch molecules are formed, meaning that a hydrogen atom or a single monomer is replaced by another covalently bonded chain of that same polymer. After this occurs, the remaining molecules react with the newly formed network, creating other cross-links.

As stated above, curing can occur with either a chemical curing agent or without one. Curing without an agent is described as "curing in the absence of additives" and curing with an agent is known as "curing induced by chemical additives". All curing is initiated by either heat, radiation, electron beams, or chemical additives. Examples of curing induced by additives include vulcanization, where sulfur is added to form polysulfide cross-links, as well as the drying (hardening) of paint with the addition of oxygen atoms as crosslinksin combination with oil drying agents.

Concrete cures in the absence of additives, depending on heat(?). In many cases, resins are provided as a solution/mixture with a thermally activated catalyst that induces crosslinking upon being heated. Polyesters are commonly combined with benzoyl peroxide (acne remover and water disinfectant *marketing*) to induce crosslinking. A possible issue with benzoyl peroxide is that it is a bleach and may interfere with our goal of dying our masks certain colors.
This article primarily deals with protein crosslinking. This process comprises chemical, enzymatic, or chemoenzymatic formation of new covalent bonds between the many peptide chains.

Some catalysts for protein crosslinking include transferases, hydrolases, and oxidoreductases.

1.2 Conjugation

A conjugated protein is a protein that is attached to another chemical group (think lipids, carbohydrates, nucleic acids), through either covalent bonding or other interactions.

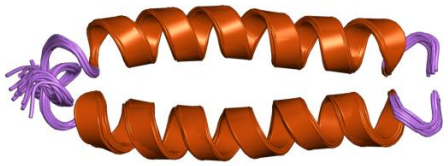
One example of protein conjugation is the Maillard reaction, where whey proteins/peptides undergo a series of complex chemical changes when they are heated in the presence of reducing carbohydrates. This is also known as glycation.

1.3 Ligation

Ligation is the process where two bases/segments are joined together. One famous process that involves ligation is in DNA replication, where DNA ligase links okazaki fragments together using covalent bonds on the lagging strand. Ligation can be employed with proteins as well to link up separate polypeptides into one long chain.

1.4 Fusion proteins

Also known as chimeric proteins, fusion proteins are proteins that are created when separate proteins that are synthesized from two or more genes are joined when the multiple genes are joined.



In the image above, two proteins have been joined into a single chimeric protein. They are attached through a linker protein and each partial protein has one subunit.

1.5 Transglutaminase

Transgluaminase is an enzyme that catalyzes the formation of an isopeptide bond (cross-linking). Specifically, it catalyzes the bond between carboxamide groups in glutamine residue and ε-amino groups of lysine residue. These residues must be bound to peptides (in our case, sericin).

1.6 Sortase A

An enzyme, Sortase A modifies its substrate through proteolytic cleavage. This sortase recognizes a Leu-Pro-x-Thr-Gly motif, which is cleaved by Sortase A between Threonine and Glycine. This enzyme also catalyzes a cell wall sorting reaction where a surface protein containing the LPXTG motif is cleaved.

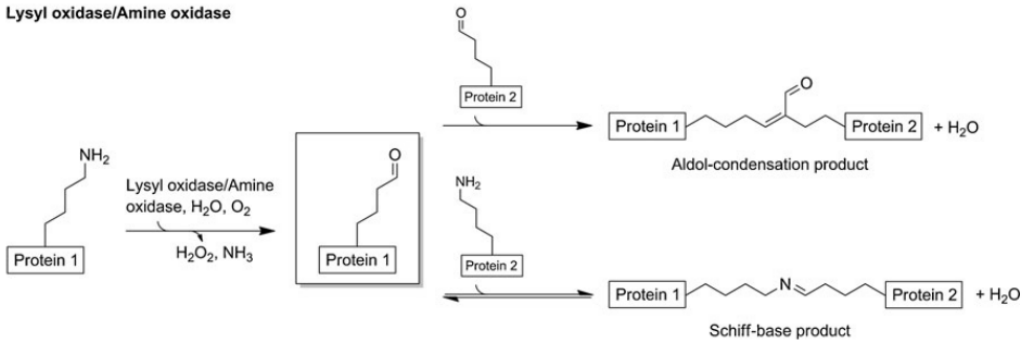
2. In Vivo

Through the attachment of ubiquitin, aka ubiquitination, at least four ubiquitin molecules are attached to target proteins. This is one of the most extensively studied crosslinking events, as ubiquitin uses covalent bonds to attach itself.

Another example of intermolecular protein crosslinking would be the crosslinking of fibrin in blood coagulation. Transglutaminase factor XIII crosslinks antiparallel fibrin chains to mechanically

stabilize clots. To do this, factor XIII takes each NH2 end of proteins, removes NH3 (ammonia), and binds the remaining N and H together. Transglutaminase can be isolated from the microbial strain Streptomyces mobaraensis.

One big crosslinking reaction is the reaction between collagen and elastin chains catalyzed by lysyl oxidases. In this reaction, deamination (the removal of a group of amino acids from a polypeptide) of lysyl side chains in collagen and elastin is combined with the synthesis of hydrogen peroxide. Below is an image of the reaction.



3. In Vitro

3.1 Transferases

The most common crosslinking reaction in vitro, they occur at the active sites of enzymes. Their main function in vivo is the crosslinking of diverse proteins, and a famous example is transglutaminase. They are dependent on calcium in eukaryotic transglutaminases, and in their enzymatic crosslinking reaction application is the network formation of food proteins. Important ones for our consideration are the formation of protein hydrogels and the modification of protein fibers.

Transferases target the glutamyl side chains of proteins (not sure of the meaning of this). They crosslink through the formation of glutamyl-lysyl isopeptide bonds (bonds between carboxyl and amino groups, commonly occurring from the joining of side chain proteins to the main core).

3.2 Hydrolases

3.21 Sortase A

Sortase A's main function in vivo is the attachment of cell-surface proteins as outlined above. They are dependent on calcium, and their main application is coupling at specific areas and immobilization of certain proteins. Not highly applicable to our experiment.

Sortase A targets the threonine-glycine peptide bond of the LPXTG sorting motif and forms those threonine-glycine peptide bonds (do not understand how this works).

3.22 Subtilisin

The main function of subtilisin in vivo is extracellular proteolysis, which means it participates in processing and activating receptors, ligands, and enzymes. It does not depend on a cofactor/metal ion, and its main purpose is the ligation of peptide fragments. This can be applied to our experiment by fixing broken bonds that may occur in the processing of sericin.

Subtilisin attacks the ends of amino acids (the free carboxyl group at the end), and the way it forms crosslinks is through the formation of peptide bonds.

3.3 Oxidoreductases

3.31 Tyrosinase

The main function of tyrosinase in vivo is the formation of melanin. It depends on copper as a cofactor, and it is involved in the network formation of food proteins (meat, fish, yoghurt, etc etc). Something to note is that this includes the formation of edible protein films.

Tyrosinase targets tyrosyl side chains in proteins, as well as phenolic compounds (compounds that are made up of one or more phenolic acids or polyphenols, including attached hydroxyl groups). They crosslink through the formation of quinone, a molecule made up of two carboxyl groups with one vinylene group.

3.32 Laccase

The main function of laccase in vivo is the degradation of lignin and biosynthesis. Like tyrosinase, it requires a cofactor of copper, and is also applied in the formation of network food proteins.

As with tyrosinase, they also focus on tyrosyl side chains in proteins and phenolic compounds. However, they form crosslinks in a much more "radical" way. They form radicals (unpaired valence electrons) and then through the subsequent coupling of the radicals (pairing of the free valence electrons) they form ionic bonds.

3.33 Peroxidase

The main function of peroxidase in vivo is the oxidation of diverse compounds. It requires heme, a precursor to hemoglobin, as a cofactor to operate. It has the same applications as laccase and tyrosinase, except it also produces protein hydrogels which can be highly applicable to our experiment.

Peroxidase targets tyrosyl side chains and phenolic compounds, and also forms crosslinks through the formation of radicals and the subsequent bindings.

3.34 Lysyl Oxidase

The main function of Lysyl oxidase in vivo is the crosslinking of collagen and elastin, as discussed above. It requires lysine tyrosinyl quinone (LTQ) and/or copper as a cofactor. The entire application of lysyl oxidase is the synthesis of protein hydrogels.

Distinctly from the previous oxidoreductases, Lysyl oxidase attacks lysyl side chains of proteins instead of tyrosyl side chains/phenolic compounds. They then form aldehydes which then go spontaneously form aldol condensations and Schiff base products (Organic Chemistry terms that I do not understand).

4. Conclusions

This article holds some major points that we can use in our experiment. Firstly, I find the opportunities posed by Lysyl oxidase and transglutaminase very ripe for consideration. They could be vital to our effort to crosslink our sericin fibers and could also open up the window into creating edible collagen films. For transglutaminase we can use Streptomyces mobaraensis to produce it, adding a Synthetic Biology aspect to our experiment.

Physical crosslinking of collagen fibers: Comparison of ultraviolet irradiation and dehydrothermal treatment (Weadock and Miller, et. al)

Kevin S. Weadock,' Edward J. Miller,' Lisa D. Bellincampi,' Joseph P. Zawadsky,' and Michael G. Dunn

Key Terms

- *UV irradiation* - exposing the collagen fibers to UV light (254 nm) for varying amounts of time and at different intensity in order to increase tensile strength
- *DHT treatment* - Dehydrothermal (DHT) treatment can physically cross-link collagen fibers. This is theorized to be better than other chemical methods because they can be toxic to collagen cells

Background

- Traditionally, chemicals are used to cross-link collagen fibers
  - Can be cytotoxic (kills cells)
  - Sometimes can be done quickly, but the drawbacks generally outweigh benefits
- UV irradiation and DHT treatment
  - Both are physical methods to crosslink the fibers
  - UV is faster than DHT treatment (3-5 days)
  - Both result in partial fragmentation of collagen α-chains

Methods

- **Making collagen** (it takes a day or two)
  - Insoluble type I collagen from bovine corium was obtained from Devro, (Somerville, NJ). A 1% (wh) dispersion at pH 3 was prepared, blended at low speed for 1 min, every 10 min for 30 min, in a Waring blender, and degassed by centrifugation at 5000 rpm for 15 min. A syringe pump (Sage Instruments; Model 341B) was used to extrude the dispersion through polyethylene tubing (inner diameter 860 p,m) at a rate of 1 ml into fiber formation buffer at pH 7.5 and 37°C. This buffer was composed of 135 mM NaCl, 30 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) and 30 mM sodium phosphate dibasic heptahydrate. The extruded collagen fibers (20 cm in length, 75 p,m in diameter) were rinsed in a series of isopropanol (16 h) and distilled water (1 h) prior to drying under tension (their own weight) at room temperature for 16 h.
- **UV irradiation**
  - Used five 15 W UV bulbs for 15, 30, 60, 120, or 240 min
  - 6 inches away from the fibers
- **DHT**
  - Fibers placed on aluminum foil in a vacuum oven (0.1 μm was applied for 1 h)
  - Left at 110°C for 3-5 days
- **Strength testing**

- Tested individually w/ 10 fibers/group and in bundles of 10 fibers
- Soaked in phosphate-buffered saline for 15 min
- Cross-sectional area calculated
- Elongated at strain rate of 1000% strain/min until they broke

Results

- Crosslinking using UV and DHT both increased ultimate tensile strength (UTS)
- DHT
  - DHT-treated fibers significantly higher UTS after 3 days of treatment
  - 5 days treatment was increased the UTS even more
- UV irradiation
  - UTS increased after 15 min
  - 30 minutes was even better
  - >30 min made no difference (same as after 5 days)
- Pepsin solubility testing:

TABLE I
Amount of Collagen Solubilized by Each Extraction Procedure as a Function of Collagen Fiber Crosslinking Treatment

Fiber treatment	0.5 M Hac Solubilized	Pepsin Solubilized	Total Solubilized
Uncrosslinked	7%	68%	75%
UV 15 min	3%	6%	9%
UV 30 min	7%	14%	21%
UV 240 min	8%	8%	16%
DHT 3 d	3%	10%	13%
DHT 5 d	3%	3%	6%

Data are expressed as a percentage of the original dry weight of the collagen fiber sample. Uncrosslinked samples were highly soluble in pepsin. Crosslinking by UV irradiation or DHT treatment substantially decreased the pepsin solubility of the collagen fibers.

Summary

- UV irradiation is the best option
- Strength comparable to a human ACL tendon
- Partial fragmentation may affect biocompatibility and resorption of fibers

Potential of 2D crosslinked sericin membranes with improved biostability for skin tissue engineering (Nayak et. al)

Sunita Nayak & Sarmistha Talukdar & Subhas C. Kundu (kundu@i3bs.uminho.pt)

Introduction

In the silk processing industry, the majority of sericin is wasted. The aim of this research is to improve the mechanical strength and stability of sericin, which is derived from silk cocoons during manufacturing, so that it can be used as a biocompatible natural biopolymer in biomedical applications. *The cocoon of the non-mulberry tropicalsilkworm Antheraea mylitta was crosslinked using glutaraldehyde as the crosslinking agent, resulting in crosslinked sericin membranes.*

In vivo studies showed sericin peptides with lower immunogenicity without generating significant levels of inflammatory mediators in studies with rats. Sericin is antibacterial, resists oxidation, UV-resistant, absorbs and releases moisture easily, with anti-tyrosinase activity and can be crosslinked, co-polymerized and blended with other macromolecular materials to produce improved biodegradable materials.

The highly hydrophilic nature of sericin is suggested to contribute towards the wound healing effect by maintaining a moist environment and absorbing excess exudate from wounds. The protein also has photoprotective properties against ultraviolet B (UVB) irradiation. Apart from its antiapoptotic nature, it can also prevent oxidative stress and tumorigenesis. These properties support the application of sericin protein for cell culture and other tissue engineering applications. This study intends to investigate 2D crosslinked silk sericin protein membranes of the non mulberry silkworm A. mylitta.

Evaluation of methods

- Physical and structural characteristics of the membranes were analyzed using scanning electron microscopy, atomic force microscopy, Fourier transform infrared spectroscopy and X-ray diffraction along with swelling and degradation studies. The secondary structure of the membrane indicates that crosslinking provides a more integrated structure that significantly improves the stability and mechanical strength of the membranes.
- The protein sericin was extracted from the cocoons of the A. mylitta silkworm, as mentioned elsewhere. Split cocoon parts were boiled for an hour in a solution of 0.02 M Na_2CO_3.The resulting solution containing sericin proteins was dialyzed against water for two days with daily water changes using a 3-kDa cellulose membrane.
- The dialyzed sericin solution was concentrated up to 2% w/v against polyethylene glycol. The sericin protein hydrolysates thus obtained were visualized following 8% SDS polyacrylamide electrophoresis gel under non reducing condition and stained with Coomassie brilliant blue R 250.

- 5 ml sericin (2 percent w/v) was crosslinked with 100 l glutaraldehyde (Sigma) (25 percent w/v) and 20 l 12 N HCl)group-activating agent). The sericin membranes were poured onto teflon-coated plates, dried overnight, and then removed. The membranes were cast on tissue culture plates for cell culture experiments. The membranes were washed multiple times with deionized water to remove glutaraldehyde and treated with 70% (v/v) ethanol to cause insolubility and crystallinity for subsequent studies.
- Cell culture studies were conducted on crosslinked sericin 2D-coated 12-well tissue culture plates with culture area 3.5 cm2 /well
- Fibroblast cells AH927 were cultured on both uncoated and sericin coated (0.5, 1.0, and 2.0 wt%) plates for days 1 and 3
- The electrophoretic profile of extracted sericin protein from the cocoons of A. mylitta by the alkaline method shows a distributed smear throughout the gel lane from a higher to lower protein range, whereas the electrophoretic profile of protein isolated from the middle silk glands from A. mylitta shows distinct protein bands that range from higher molecular weights greater than 205 kDa to the smaller ones of 20 kDa

Conclusion

The study describes silk protein sericin as a biocompatible natural biopolymer, which is currently underutilized and a discarded waste of the silk industry. Crosslinking of sericin with glutaraldehyde produces stable membranes with superior surface topology, mechanical strength and swelling ratio. Fabricated crosslinked membranes with slow degradation and improved fibroblast cell attachment and viability could give silk sericin protein material in the form of membranes a new dimension for fast and successful skin repair. The findings suggest that the crosslinked sericin membrane may be useful as a biopolymeric graft material. Sericin is amorphous in nature and crosslinking with glutaraldehyde results in the chemical modification of proteins with crosslinking agents that reinforce chemically, joining of two or more molecules by a covalent bond, making compact structures resulting in increased integrity and stabilization of protein.

Fabrication of Chitosan/Silk Fibroin Composite Nanofibers for Wound-dressing Applications (Cai et. al)

Zeng-xiao Cai, Xiu-mei Mo, Kui-hua Zhang, Lin-peng Fan, An-lin Yin, Chuang-long He, Hong-sheng Wang

Key Vocabulary:

- Chitosan: a naturally occurring carbohydrate with numerous applications in the biomedical setting
- Electrospinning: a method used to fabricate fibers in the nanometer scale, it depends on high electrostatic forces.
- scanning electron microscopy: Scanning the surface with focused beams of electrons and studying how the electrons are interacting with the sample. This can give insight into the surface characteristics and composition.
- Turbidity Measurement: how light is scattered in a water sample againges the amount of light scattered in a reference solution
- MTT assay: a colorimetric assay for assessing cell metabolic activity

Introduction:

Through the use of electrospinning, Chitosan and Silk Fibroin were fabricated and the composition was tested through scanning electron microscopy. The mechanical properties and fiber diameters were observed in comparison to the chistsan to silk fibroin ratio. The composite nanofibers had differing antibacterial effects depending on the bacteria. The composite nanofiber was essentially being evaluated for its effectiveness for wound healing applications.

Chitin is the most abundant natural biomaterial which is found in crustacean shells. It has been applied in fields of agriculture/food industry, biotechnology, and pharmaceutical industries. The unique structure of chitin makes it non-toxic, biodegradable, and antibacterial.

Some current techniques to fabricate microscale fibers include: self assembly, template-directed synthesis, phase separation, and electrospinning. Electrospinning is the most effective due to its simplicity and ability to fabricate various synthetic and natural fibers into ultrafine fibers. The fibers produced from electrospinning are effective snesores, wound dressers, tissue engineering scaffolds, and controlled release carries, "due to their high surface to volume ratio, high porosity and good inter-pore connectivity". However, chitosan can not be directly electrospinning and it has to be combined with other polymers, such as silk fibroin.

Silk fibroin comes from the cocoons of *Bombyx mori* silkworms. Collection of the fibroin is relatively easy and has useful characteristics for the biotechnology industry, such as good air permeability,

biodegradation & biocompality, and low inflammatory reaction. This makes it a remarkable candidate for wound dressings or fibers used in the medical setting.

Morphology of CS/SF Blend Nanofibers:
The average diameters of electrons spun by composite nanofibers are "249.7 ± 157.1 nm, 214.0 ± 108.7 nm and 185.5 ± 114.7 nm", with the chitosan weight ratios increasing from 20, 50, to 80%. Furthermore, the diameters decreased when the chitosan weight increased. The voltage, collecting distance, feed rate and solution concentration were kept constant.

Crosslinking of Fibers:
The composite nanofibers are extremely water soluble. Thus to combate this, the nanofibers were crosslinked. The procedure is as follows, "Crosslinking of silk fibroin and chitosan with GTA involves the reaction of free amino groups of chitosanand amino acid of the silk fibroin with the aldehyde groups of GTA [23]. After crosslinking, the membranes were water insoluble". This is extremely relevant since substances in the biomedical industry should be insoluble in water if they are used for the human body.

Mechanical Properties:
Increased silk foroin content increased the strength of the cross-linked composite nanofibers.

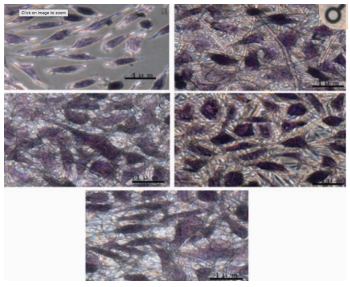
Mechanical Properties of Cross-Linked CS/SF composite nanofibrous membranes.

Crosslinked CS/SF (wt/wt)	0:10	2:8	5:5	8:2
Tensile stress (MPa)	10.3 ± 0.24	1.2 ± 0.13	1.1 ± 0.22	1.0 ± 0.21
Ultimate strain (%)	2.8 ± 0.22	3.8 ± 0.21	2.5 ± 0.25	1.3 ± 0.20

Data Table 1: stress and strain of the composite nanofibrous membranes at break

In Vitro Application of Antibacterial Activity:
The ability of the nanofibrous membranes to be antibacterial was measured through optical density. It was found that increased chitosan content allowed for increased antibacterial activity for E. coli and S. aureus strains. However, the antibacterial effect depends on the type of bacteria.

Cell Morphology and Proliferation:



The CS/SF composite was beneficial to the fibroblast development and that cells can function and attach on the membranes. These properties were assessed using a MTT assay in vitro. The composite promoted cell development.

Key Results and Findings:
It was found that, "fiber diameters decreased with increasing percentage of chistaon". Furthermore,

Materials:

- Chitosan purchased from sigma-Aldrich Chemical Company
- Bombyx mori Silkworms supplied from Jiaxing Silk
- Solvents: 1,1,1,3,3,3,-hexafluoro-2-propanol and 2,2,2-trifluoroethanol

Methods:

1. Raw silk boiled three times in NaCO3 solution
2. Degummed SF dissolved in ternary solvent for one hour at 65 C
3. Solution was dialysed with cellulose tubular membrane for 3 days
4. SF dissolved in HFIP and Chitosan dissolved in HFIP/TFA with volume ratio of 9/1
5. Electrospinning performed under room temperature, with dispensed rate of .8 mL/h and 20 kV
6. CS/SF nanofibers crosslinked in 10 mL of 25% glutaraldehyde (GTA) aqueous solution for 24 hours and dried by vacuum
7. Scanning Electron Microscopy was performed
8. Mechanical Properties, such as tensile stress-strain were collected through tests
9. E. coli and Staphylococcus aureus grown on agar culture and after cultivation placed iwt the membranes
10. Hematoxylin and eosin staining was performed to investigate cell attachment and proliferation

Results:
The diameter of the nanofibers increased with increasing silk fibroin content. The addition of silk fibroin made the fiber stronger. The MTT assay provided data that the composite nanofibers promote cell attachment and proliferation. Inhibition of gram negative Escherichia coli bacteria was demonstrated through the MTT assay and the antibacterial properties increased with increasing chitosan.

Takeaways:
Chitosan is particularly useful for biomedical applications because it has "excellent antibacterial properties". Furthermore, it is biodegradable and biocompatible, which would make it an excellent asset in the clinical setting. Furthermore it is extremely useful to know that increasing the chitosan in the composite included increased E.coli Bacteria inhibition, and made the fiber overall stronger. This ratio of the chitosan and silk fibroin with crosslinking is highly insoluble and quite durable. Some further steps to take are to research if there are other types of bacteria that can be tested for antibacterial properties of the composite nanofibers.

Physical crosslinking of collagen fibers: Comparison of ultraviolet irradiation and dehydrothermal treatment (Weadock et. al)

Kevin S. Weadock,' Edward J. Miller,' Lisa D. Bellincampi,' Joseph P. Zawadsky,' and Michael G. Dunn

KEY TERMS

- *UV irradiation* - exposing the collagen fibers to UV light (254 nm) for varying amounts of time and at different intensity in order to increase tensile strength
- *DHT treatment* - Dehydrothermal (DHT) treatment can physically cross-link collagen fibers. This is theorized to be better than other chemical methods because they can be toxic to collagen cells

BACKGROUND

- Traditionally, chemicals are used to cross-link collagen fibers
  - Can be cytotoxic (kills cells)
  - Sometimes can be done quickly, but the drawbacks generally outweigh benefits
- UV irradiation and DHT treatment
  - Both are physical methods to crosslink the fibers
  - UV is faster than DHT treatment (3-5 days)
  - Both result in partial fragmentation of collagen α-chains

METHODS

- Making collagen (it takes a day or two)
  - Insoluble type I collagen from bovine corium was obtained from Devro, (Somerville, NJ). A 1% (wh) dispersion at pH 3 was prepared, blended at low speed for 1 min, every 10 min for 30 min, in a Waring blender, and degassed by centrifugation at 5000 rpm for 15 min. A syringe pump (Sage Instruments; Model 341B) was used to extrude the dispersion through polyethylene tubing (inner diameter 860 p,m) at a rate of 1 ml min into fiber formation buffer at pH 7.5 and 37°C. This buffer was composed of 135 mM NaCl, 30 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) and 30 mM sodium phosphate dibasic heptahydrate. The extruded collagen fibers (20 cm in length, 75 p,m in diameter) were rinsed in a series of isopropanol (16 h) and distilled water (1 h) prior to drying under tension (their own weight) at room temperature for 16 h.
- UV irradiation
  - Used five 15 W UV bulbs for 15, 30, 60, 120, or 240 min
  - 6 inches away from the fibers
- DHT
  - Fibers placed on aluminum foil in a vacuum oven (0.1 μm was applied for 1 h)
  - Left at 110°C for 3-5 days
- Strength testing

- Tested individually w/ 10 fibers/group and in bundles of 10 fibers
- Soaked in phosphate-buffered saline for 15 min
- Cross-sectional area calculated
- Elongated at strain rate of 1000% strain/min until they broke

Results:

- Crosslinking using UV and DHT both increased ultimate tensile strength (UTS)
- DHT
  - DHT-treated fibers significantly higher UTS after **3 days** of treatment
  - 5 days treatment was increased the UTS even more
- UV irradiation
  - UTS increased after 15 min
  - 30 minutes was even better
  - >30 min made no difference (same as after 5 days)
- Pepsin solubility testing:

TABLE I
Amount of Collagen Solubilized by Each Extraction Procedure as a Function of Collagen Fiber Crosslinking Treatment

Fiber treatment	0.5 M Hac Solubilized	Pepsin Solubilized	Total Solubilized
Uncrosslinked	7%	68%	75%
UV 15 min	3%	6%	9%
UV 30 min	7%	14%	21%
UV 240 min	8%	8%	16%
DHT 3 d	3%	10%	13%
DHT 5 d	3%	3%	6%

Data are expressed as a percentage of the original dry weight of the collagen fiber sample. Uncrosslinked samples were highly soluble in pepsin. Crosslinking by UV irradiation or DHT treatment substantially decreased the pepsin solubility of the collagen fibers.

  -

Summary:

- UV irradiation is the best option
- Strength comparable to a human ACL tendon
- Partial fragmentation may affect biocompatibility and resorption of fibers

Human Health and Environmental Risks Posed by Synthetic Biology R&D for Energy Applications: A Literature Analysis (Hewett et. al)

Joel P. Hewett, Amy K. Wolfe, Rachael A. Bergmann, Savannah C. Stelling, and Kimberly L. Davis (johewett@gmail.com)

Overview:

This analysis aims to be a comprehensive review of risk-related synthetic biology literature. The researchers found 44 discrete risk issues (18 related to human health and 26 related to the environment) after reviewing more than 200 articles. The human health risks were categorized in these categories: allergies, antibiotic resistance, carcinogens, and pathogenicity or toxicity. The Environmental risk issues were categorized in these groups: change or depletion of the environment, competition with native species, horizontal gene transfer, and pathogenicity or toxicity. As the field of synthetic biology is rapidly growing, there has been an increase in the number of biotechnology tools such as CRISPR. The problem is, these tools created new risk-related questions.

Methods:

The researchers reviewed more than 200 documents in multiple databases such as Google Scholar as well as studies by research organizations such as the National Research Council. They cross-referenced bibliographies and citations against their literature base. They also made sure the research papers were focused on synthetic biology and risk. When finding papers, the researchers made sure to thoroughly review the paper to eliminate any bias. They categorized the target organisms in 3 main groups: Microbes, Algae, and Plants.

Results:

The researchers were able to identify 44 (18 in health and 26 in the environment) discrete risk issues in total from reviewing the research papers. They then grouped the issues in different categories. The human health risk issue categories were: Allergies, Antibiotic resistance, Carcinogens, Pathogenicity or toxicity. The categories for the environmental risk issues were: Change or depletion of the environment, Competition with native species, Horizontal gene transfer, Pathogenicity or toxicity. When there was a risk issue in a research paper and the specific organism type wasn't specified, they grouped it into the unspecified category.

Human Health Risk Issues

Allergies: The risk is a genetically modified organism can express new allergens through pollen or other molecules. Also, they have a highly transmissible nature.

Antibiotic Resistance: Antibiotic resistance genes are often used as a selectable marker. However the risk is, the modified organisms continue to express the antibiotic-resistance gene and even worse, the bacterial strains can become resistant to the antibiotic.

Carcinogens: These chemicals can lead to the development of cancerous tissue.

Pathogenicity or Toxicity: Pathogens and toxins have unwanted consequences such as algal strains when used.

Environment Risk Issues

Change or Depletion of Environment: An organism that is synthetically modified could have a negative effect on the environment such as declining the diversity.

Competition with native species: A modified organism can change the environment by competing with native species. These organisms could have an advantageous trait which would allow them to have a greater chance of survival.

Horizontal Gene Transfer: Modified organisms can affect the environment through horizontal gene transfer. HGT can spread antibiotic resistance genes through the ecosystem.

Pathogenicity or Toxicity: Pathogens and Toxins are also bad for the environment too. These can lead to mutations in organisms.

	Risk Issue Category			
Target Organism[a]	Allergies	Antibiotic Resistance	Carcinogens	Pathogenicity or Toxicity
Microbes	Trigger an allergenic response via dermal, ingestive, or respiratory exposure[28]	Transfer antibiotic resistance genes into a harmful strain of bacteria, reducing the effectiveness of medical therapy[29,31,46]	Generate carcinogenic by-products[28]	Cause a microbial strain to become pathogenic to humans[20,45,47]; generate toxic by-products[13,28]
Algae	Trigger an allergenic response via dermal, ingestive, or respiratory exposure[27,28]	Transfer antibiotic resistance genes into a harmful strain of bacteria, reducing the effectiveness of medical therapy[29,31,46]	Generate carcinogenic by-products[28]	Cause an algal microbial strain to become pathogenic to humans[20,45,47]; trigger toxic algal blooms in bodies of water used for human recreation or consumption[22,28,32,33]; transfer of chemical-producing traits from engineered algae into algae located within the human food chain[20]; generate toxic by-products[13,28]
Plants	Transfer an allergen into an engineered biofuel crop that may commingle with food crops[20,29]	Transfer antibiotic resistance genes into a harmful strain of bacteria, reducing the effectiveness of medical therapy[26,44,48]	–	Develop plant pathogenicity and infect other species[19,26]
Unspecified	–	Evolve or proliferate in unpredictable ways that spread antibiotic resistance genes to other organisms[7,17,29,35]	–	Cause an organism to become pathogenic or increase a known pathogen's virulence[7,19,26]; cause an unforeseen or unexpected change in an organism's pathogenic characteristics[18,19,24,34]

Takeaways:

This literature analysis will be extremely helpful in our research process. When we choose to target a certain organism, we will be aware of the health or environmental risks of it. Knowing this information is important since we will consider biosafety when conducting our research.

Vector Cloning

Risks of Synthetic Biology

Biosafety Concerns:

One biosafety concern is the intentional or unintentional release of synthetic organisms into the environment. This is bad because it could cause displacement in the ecosystem. Also, synthetic organisms can have an advantage against native organisms. A second important issue is horizontal gene transfer. This creates high risks to the genetic structure of organisms. A third biosafety issue is the formation of antibiotic-resistant superbugs. The superbugs can escape host cells and enter the environment and since then they can reproduce.

Biosecurity Concerns:

This has mostly to do with bioterrorism. Since synthetic Biology is increasingly becoming more accessible, there have been some groups that abuse its power. For example, in 2017 there was the Horsepox virus that came from multiple DNA fragments which arrived through mail. Also drones can be employed by terrorists to spread viruses. iGem made a rule to make sure that projects can't pose any risks to communities or environments.

Ethics:

Using synthetic organisms in the environment could be a huge ethical concern. In 2010, a Human made cell caused debate on ethics of Synthetic Biology. This caused the U.S. President Barack Obama to ask the Presidential Commission for the Study of Bioethical Issues to review the ethical boundaries associated with it. In a report called "The Ethics of Synthetic Biology and Emerging Technologies", 5 ethical principles were pointed out. These were public beneficence, responsible stewardship, intellectual freedom and responsibility, democratic deliberation, and justice and fairness. After a group of synthetic biologists launched a Human Genome Project-Write federation, ethical concerns were once again voiced. The scientists later explained their federation and how it was aimed at synthesising plant and animal genomes rather than human genomes.

Takeaways:

The document lists some ways to make sure we take measures for biosafety issues.

We can use genetic safeguards which help not accidentally releasing genetically engineered microbes into the environment.

A genetic firewall - Chemically synthesized bacterial components such as xeno nucleotides do not exist in nature.This way, Synthetic organisms that depend on xeno nucleotides will not survive outside their designated environments.

Detecting or identifying synthetic organisms in the environment is important.

DNA Cloning Using In Vitro Site-Specific Recombination (Brasch et. al)

James L. Hartley, Gary F. Temple, and Michael A. Brasch

Overview

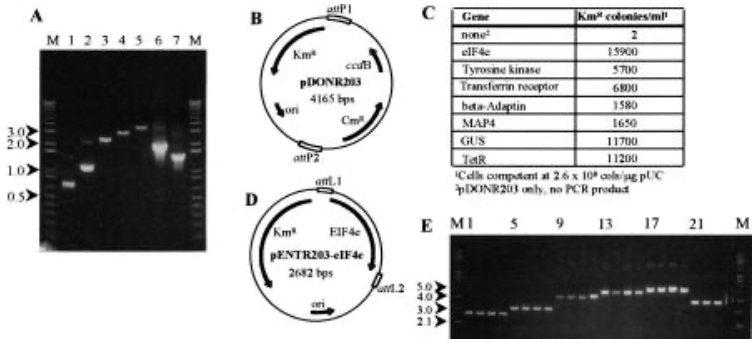
This article talks about recombinational cloning that uses in vitro site specific recombination to clone PCR products and to subclone DNA segments at a high efficiency. The subclones that are created maintain the orientation and reading frame register so new fusions are still possible.

Why this technology is different

Previously, analysis of open reading frames required the ORF to be expressed, the protein to be purified, the antibodies produced, phenotypes examined, and other steps which required subcloning into 1+ specialized vectors. This group developed a non-host specific technology.

Method

They use two starting DNAs which are an Entry Clone which carries the DNA segment to be transferred and a Destination Vector, where the DNA will be subcloned. By incubating the DNA's with recombination proteins, you create a recombination protein mediated recombination which transfers the cloned DNA segment into the Destination Vector. To get the Expression clone, they first mixed E.coli into the mixture and imposed two selection schemes. The Entry and Destination vectors are resistant to different antibiotics and the Destination Vector has a selection marker(F plasmid) to inhibit E.coli growth. a



Plasmids were expected sized and proved the high efficiency cloning of numerous PCR products with only a few hours of laboratory time. The resulting plasmids have the amplified DNA in an Entry Clone which can transfer the DNA to a number of Destination vectors.

"First, intermolecular recombination forms a cointegrate molecule, which then resolves into two daughter molecules by a second intramolecular reaction. The desired clone is obtained from this mixture of molecules by imposing antibiotic resistance selection for the desired construct and a selection (encoded by the *ccd*B gene) against starting molecules and intermediates Performing these reactions in vitro eliminates problems of plasmid segregation inherent with in vivo recombination schemes."

RC-PCR can be used for directional cloning, expression and purification of proteins, and RC compatible clones can be transferred to RC compatible vectors.

Potential Problems

- The efficiency of in vitro RC reactions decrease with increasing the size of DNA fragments involved.
- Can be minimized by using equal moles of DNA and higher incubation times

How Can we use this:

This new form of vector cloning is much more efficient and will achieve the same results in a quicker amount of time. We might have some difficulty acquiring materials like PCR primers and purified lambda recombination proteins. However, if we can do it, this process only takes a few hours to complete so there is room for some error and retesting.

Synthetic biology: Recent progress, biosafety and biosecurity concerns, and possible solutions (Wang and Zhang)

Fangzhong Wang, Weiwen Zhang
Contact: wwzhang8@tju.edu.cn

Key Concepts:

Synthetic biology is the application of engineering with biology concepts to modify or create biological systems. However, these new technologies could be possibly misused and abused, presenting risks concerning biosafety, biosecurity, and ethics. Recently, regulatory measures were established to help combat these risks.

Hewett et al. identified 44 risks in synbio from 200 documents and categorized them into four different types.

Potential Risks:

1. The principle of biosafety regarding microorganisms is preventing them from infecting humans, animals, and plants, and causing disease, which by extension could mean preventing them from being released into the environment. It is not easy to predict what a synbio-modified organism would do in a given environment compared to a non-altered counterpart, since synbio procedures are very complex (usually involving new pathways or unknown genes or even amino acids). The European Union funded some studies on this, which concluded that introducing synthetic microbes into the environment for plant growth enhancement will not have a long term effect because of competition and predation, but this also just indicates that those microbes were not successful. Genetically engineered microbes could also be much more capable of effecting greater horizontal gene transfer into an environment. Finally, since plasmids used in synbio usually contain antibiotic resistance genes, there is a risk for superbugs to escape their host cells and enter the environment, creating more superbugs in nature.
2. Biosecurity is focused on the deliberate abuse of biotechnologies or bioweapons for malicious purposes. With more capabilities from synthetic biology comes an increased risk of bioterrorism. We are not very concerned with this because we are not terrorists (I think). However, it is not difficult to get sequences of highly pathogenic microbes, in part due to academic competitions (iGEM) and amateur biological groups making access to professional knowledge easier. Luckily, the iGEM Safety and Security Program exists. (Also fun fact, the U.S. Intelligence Agency considers CRISPR-Cas9 as a potential weapon of mass destruction.)
3. Ever since the first human-made cell was created, people have argued about the ethics of the utilization of synbio for creating artificial life, since it might have negative effects on health and the environment. HGP-Write caused a public scare about ethics since people were afraid

that they could be using their synthesized human genome to inject DNA into embryonic cells or misusing it in other ways. The scientists addressed these concerns as misunderstandings of the project.

Precautionary Measures and Regulations:

1. Genetic safeguards can help prevent microorganisms from escaping into the environment. Early designs of these were toxin gene expressions, which would kill microorganisms when these genes were expressed. Another type of safeguard is a toxin-antitoxin system, where an antitoxin is needed to neutralize a toxin at the transcription and translation levels. Auxotrophy systems also can be used, where the cells require a certain non-natural amino acid to produce antidotes for toxins, such as in E. coli, where 3-iodo-*l*-tyrosine is needed against colicin E3. In engineered S. *enterica*, transcription of some genes is shut down upon the lack of presence of arabinose. However, thesemeasures might still be vulnerable to gene mutations, and auxotroph systems will not work when the targeted genes are complimented with cross-feeding from related microbes. Therefore, more recent safeguards use a combination of these techniques.
2. Genetic firewalls: xenobiology can be useful for regulating microbes as the biomaterials cannot be found in nature. This can eliminate risks regarding genetic information exchange and horizontal gene transfer. Artificial base pairs can be designed through interchangeable hydrogen bond donors/acceptors or modification of pyridine or pyrimidine rings of natural base pairs. Other methods of using artificial biomaterials include using noncanonical amino acids, and furthermore, repression of stop codons, which forces the microorganisms to use noncanonical amino acids as stop codons. This would also help prevent evolutionary escape.
3. DNA watermarks or barcodes can identify rogue microbes present in the environment. The standards of DNA watermarking are that it does not affect the phenotype of the synthetic organism, it is resistant to gene mutation and malicious attacks, it can be identified by private or governmental entities, and it is unique for each laboratory.
4. Scientific codes of conduct, which can be present at the government level, encourage life scientists to consider the ethical values of their projects, the potential risk on health, and emergency measures (which we are doing through the HP branch.

Conclusions and Takeaways:

Because of how synthetic biology is becoming easier to use, intentional or unintentional misuse can have serious implications on society, economy, and the environment. Scientists should consider these consequences and make an effort in the laboratory to prevent or combat these risks, and public dialogue should be conducted regarding the ethics of the project (just leave that to HP). On the part of the lab branch, the technologies such as genetic safeguards and genetic firewalls should be considered to control the microorganisms that we will be using to create our fabric or whatever we call it.