Greener Solutions: Improving performance of mycelium-based leather
Final Report to MycoWorks

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Abstract

In this report we propose several strategies to improve the strength, flexibility, and durability of MycoWorks' mycelium-based, leather-like material, while striving to uphold MycoWorks' core mission to achieve a sustainable, biodegradable product. Our strategies can improve the technical performance of the current material while decreasing the health and environmental risks associated with the production of animal leather. We propose three methods to increase the strength, and potentially the flexibility, of the MycoWorks product by cross-linking the chitosan in the material. We also propose a strategy to apply a moisture barrier to the material to prevent leaching of the plasticizer. We evaluate the technical performance, feasibility, and the health and environmental hazards of our strategies using rigorous frameworks. Our frameworks cover a wide range of performance features, feasibility considerations, and health endpoints. Our genipin cross-linking and corn zein coating strategies can be implemented immediately and we recommend them with no reservations.

Introduction

Background

Humans have been converting animal hides into leather for millennia to make use of the material's desirable qualities such as strength, flexibility, durability, and pleasant look and feel. These qualities make animal leather highly versatile, rendering it useful in the production of clothing, furniture, automobiles, books, and other consumer products. To meet the demand for such products, over 350,000,000 cow hides are used for leather production annually, in addition to the hides of sheep, goats, and other animals (Food and Agricultural Organization of the United Nations, 2016). For the purposes of our discussion when we mention conventional or animal leather we are referring to bovine leather.

Although useful, animal leather presents many environmental, social, and health-related problems throughout its lifecycle. These issues begin as early as the procurement of the raw material. Cattle are often raised in tight quarters, a situation which presents animal welfare issues such as excessive antibiotic use, which may exacerbate the rise of antibiotic resistance (Mathew, Cissell, & Liamthong, 2007). Cattle production is also highly carbon, water, and land intensive, giving cow hide production a rather large ecological footprint (Ridoutt, Page, Opie, Huang, & Bellotti, 2014).

A series of chemical treatment processes collectively referred to as "tanning" convert animal hide to strong and supple leather. The most common tanning method is chromium tanning, although other processes such as aldehyde tanning and vegetable tanning are also used. Chromium uses large quantities of water and trivalent chromium salts to cross-link the collagen in the hide. Trivalent chromium is a skin sensitizer and allergen (Hedberg, Lidén, & Odnevall Wallinder, 2015). Further, it may change its valence state to become hexavalent chromium, a much more toxic chemical known for its potent carcinogenicity (Hedberg & Lidén, 2016). In addition to presenting significant occupational health hazards, chromium compounds may leach out of leather products long after production, placing consumers at risk (Hedberg & Lidén, 2016).

Chromium tanning and other leather tanning processes tend to have relatively low uptake rates, leading to the formation of significant quantities of contaminated wastewater. Current methods to remove chromium from wastewater result in large quantities of solid waste (Aravindhan, Madhan, Rao, Nair, & Ramasami, 2004). Given that the waste associated with leather production is difficult to remediate before its disposal, waters near leather tanning facilities may be polluted with the waste products, significantly damaging normal ecosystem function (Dixit, Yadav, Dwivedi, & Das, 2015).

Conscious consumers have long acknowledged the issues inherent in leather manufacturing. Demand for a safer, more environmentally-friendly alternative led to the development of synthetic ("vegan") leather. While vegan leather circumvents some of the problematic aspects of cattle farming, it fails to address concerns of carbon intensiveness and health problems. The material is synthesized from polyvinyl chloride (PVC), a petroleum-based polymer. PVC's petroleum-based roots raise concerns of sustainability and ecological footprint. Further, PVC is relatively brittle, necessitating the use of plasticizers. Dibutyl phthalate is usually the plasticizer of choice for vegan leather, but its use is problematic because it disrupts hormonal signals and exerts toxicity on the male reproductive system (Dixit, Yadav, Dwivedi, & Das, 2015).

It is clear that leather products currently on the market perform unsatisfactorily with regards to environmental and health-related outcomes. While problematic, this is unlikely to diminish the demand for leather. Therefore there is a significant need for a more sustainable alternative. Innovative companies who take up this challenge have a significant business opportunity and a chance to move a multibillion-dollar industry in a more sustainable direction.

MycoWorks

MycoWorks is a startup in San Francisco, California that produces a mushroom-based leather alternative. The material has a similar look and feel to conventional leather while using a manufacturing process with significantly fewer environment and health hazards. The material is a composite of mushroom mycelium and cotton cellulose, which makes it biodegradable and eliminates the concerns associated with raising animals for leather production. The current manufacturing process uses minimal chemical additives, using only small amounts of water and polyethylene glycol (PEG). Further, the material has a relatively low carbon footprint, with the potential for a carbon negative manufacturing process with some refinement.

MycoWorks' material already shares many of the properties of conventional leather and has been used in the manufacture of products such as purses, wallets, and phone cases. In order to match animal leather's versatility there are several key targets for improvement. The primary targets are improvements in strength, flexibility, and durability.

The garment industry has developed many strategies to achieve strength, flexibility, and durability. Unfortunately, many of them also involve the use of harmful chemicals. MycoWorks prioritizes environmental sustainability and worker and consumer health, which makes many of the traditional strategies for adding strength and flexibility incompatible with their core values. It is therefore necessary to identify chemicals and strategies which will enable MycoWorks to improve their material without compromising the comparative safety and environmental friendliness of their processes. Our strategies will enable MycoWorks to continue to grow and shift the leather industry in a more sustainable direction.

Restriction criteria for strategy development

To focus the direction of our research we employed several restriction criteria when selecting and developing the strategies proposed in this report. The most important constraint was that strategies be implementable during the post-harvesting portion of the current manufacturing process (Fig. 1).

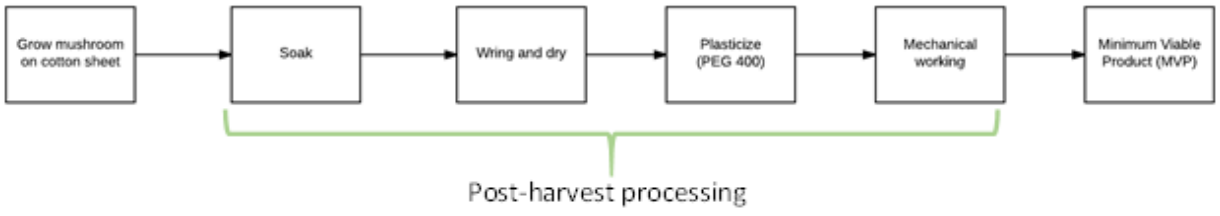


Figure 1. Current MycoWorks manufacturing steps to produce the minimum viable product (MVP)

The first step of manufacturing the MycoWorks minimum viable product (MVP) is to seed *Ganoderma lucidum* spores onto sheets of felted cotton. The *Ganoderma* mycelium fibers grow among the cotton

fibers and form a composite material which is then harvested. MycoWorks has optimized the growth and harvest process and requested that we focus on the downstream manufacturing steps.

The current post-harvesting process has four steps: soaking, drying, plasticizing, and mechanical working. The purpose of the soaking and drying steps is to ensure the material has uniform moisture content throughout. Polyethylene glycol 400 (PEG 400), a hydrophilic low molecular weight polyol, is applied and maintains pliability by ensuring that the internal moisture does not evaporate. Lastly, the material is manipulated mechanically to produce the appearance of leather.

This production process works well for the current manufacturing scale, in which sheets ranging from one by one foot, to sheets of several feet are manufactured. Therefore the second restriction criterion was that the strategies be practical for the current scale of operations, usable in the short term, and applicable at an increased manufacturing scale.

The final restriction criterion was that the solutions comply with MycoWorks' core mission to achieve a sustainable, biodegradable product. We prioritized strategies accordingly, employing naturally-sourced ingredients where possible. The goals of improving strength, durability, and flexibility, as well as the three restriction criteria we applied, helped inform the development of three cross-linking strategies and one moisture barrier strategy. With sufficient optimization MycoWorks can implement any of these strategies in the near future.

Composition of the MycoWorks material

The fungal species that MycoWorks uses is *Ganoderma lucidum*, which uses wood as its primary food source. The fruiting body is composed of mycelium. On the microscopic scale the thread-like mycelium grows in a branched and unruly manner, resulting in a soft and spongy substance. In turn, a major component of mycelium is chitin, one of the most common polysaccharides found in nature (Fig. 2).

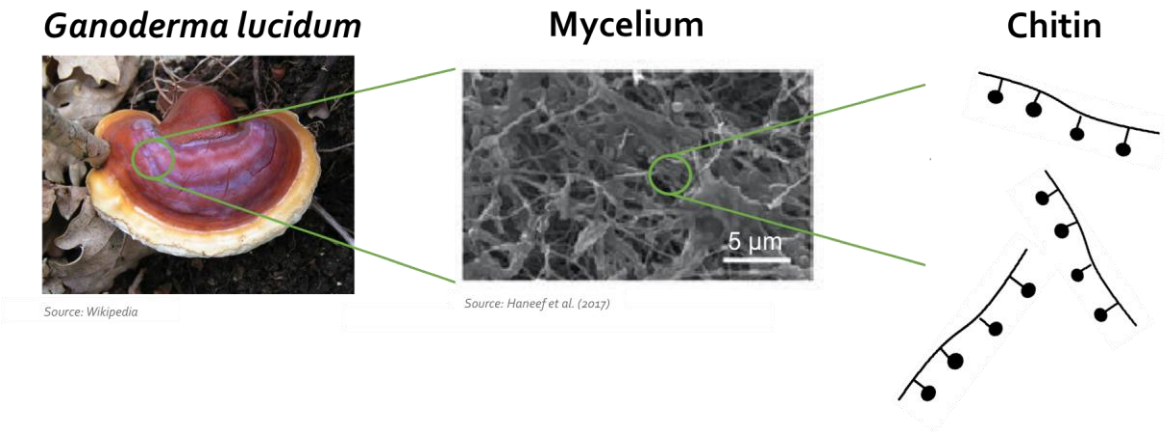


Figure 2. Major components of the *Ganoderma lucidum* mushroom. Microscope image (center) (Haneef et al., 2017). Note that we represent chitin and chitosan as a simple cartoon that captures their important structural characteristics: long-chain fibers with significant chemical functional groups along it.

Chitin contains hydroxyl (-OH) and acetamide (-$NHOCH_3$) groups (Fig. 3). Chitin can be deacetylated to form chitosan, a polysaccharide nearly identical to chitin. The difference between the two is that chitosan has primary amine groups (-NH2) instead of acetamide groups. When at least 50% of the

acetamide groups are deacetylated to amine groups the name of the polysaccharide shifts from ‘chitin’ to ‘chitosan.’ Deacetylation methods are discussed in Appendix D.

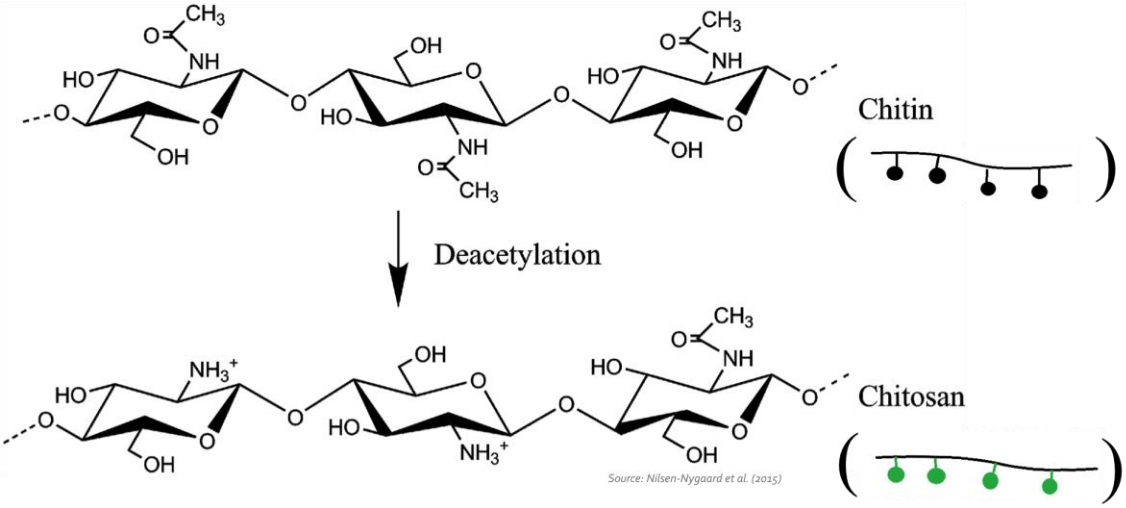
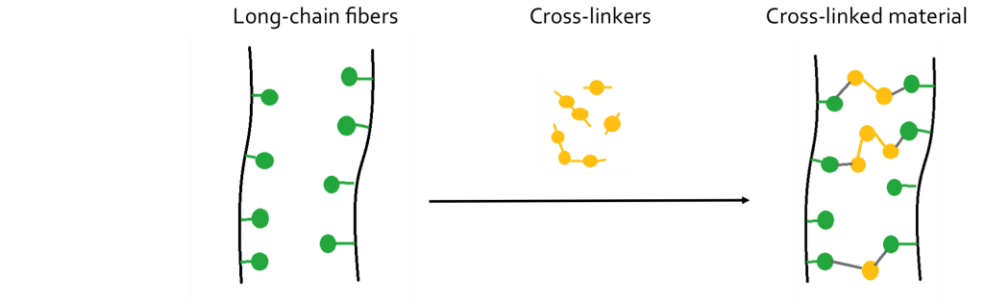


Figure 3. Chemical structures and cartoon representations of chitin and chitosan

Primary strategy development: incorporate cross-linking

Our primary approach for strategy development was to mimic cross-linking in animal leather. This is a phenomenon that occurs during the tanning step of leather and imparts the strength inherent to leather. Cross-linking involves long-chain fibers reacting with cross-linking molecules to form molecular bonds between the fibers. This produces a material that is stronger and sometimes more flexible than the original material (Fig. 4). In leather the long-chain fibers are composed of collagen, which is cross-linked by chromium(III) compounds or aldehydes.

We propose to cross-link the chitosan fibers in the MycoWorks material using a variety of cross-linkers. These constitute the three cross-linking strategies presented in this report. We believe that the resulting material will be stronger and potentially more flexible than the current MVP.



Animal leather:	Collagen	Chromium(III) compounds or aldehydes	Leather
MycoWorks material:	Chitosan	**Our proposed strategies**	A stronger, more flexible material

Figure 4. Illustration of cross-linking components for animal leather and for the MycoWorks material

All of our proposed cross-linking strategies act on chitosan, not chitin. Chitosan has readily reactive primary amine functional groups that form amide bonds during cross-linking, whereas chitin has far less reactive acetamide functional groups. Amide bonds resist hydrolysis and confer structural rigidity at the molecular level. Cellulose – another polysaccharide present in the MycoWorks material – was not extensively considered for cross-linking since its hydroxyl functional groups present less favorable reactivity for cross-linking.

Secondary strategy development approach: introduce a moisture barrier

Our secondary approach was to introduce a moisture barrier as a coating to the MycoWorks material. PEG 400 is a successful plasticizer but it leaches out when the material is washed with water. Furthermore, the plasticizing performance is dependent on the ambient humidity. A moisture-resistant coating applied to the MycoWorks material would seal in the plasticizer, reducing its leaching out.

Baselines for guiding strategy development & evaluation

We established baselines to guide the development and evaluation of our proposed strategies. In terms of technical performance, we propose strategies that improve technical performance over the MycoWorks MVP. As mentioned above, the main targets for technical performance improvement are strength, flexibility, and durability. In terms of hazards, we propose strategies that present fewer hazards than the animal leather production process does.

Proposed Strategies

Cross-linking strategies

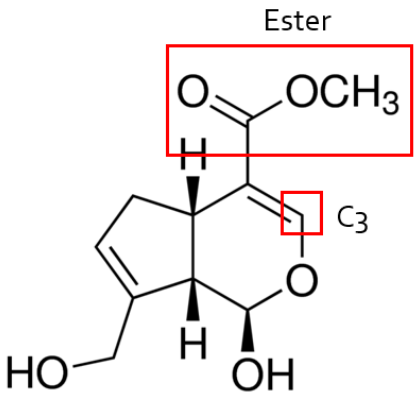
Direct chemical replacement: natural cross-linkers

The simplest approach to inducing cross-linking involves the application of a single compound, such as an aldehyde or tannin, in solution. Formaldehyde and glutaraldehyde have been used extensively in leather tanning and other applications where cross-linking is required, however both are hazardous to varying degrees. Formaldehyde is a known carcinogen and has been banned from the tanning industry (Covington, 1997; Swenberg et al., 2013). While glutaraldehyde is promoted as a "greener" formaldehyde alternative due to its biodegradability, it is acutely toxic to wildlife and humans (Leung, 2001; Takigawa & Endo, 2006). Plant-based tannins have also been used in the leather industry (Covington, 1997). These tannins fall into two general tannin classes: pyrogallol and catechol. Although tannins that occur naturally in fruits and vegetables have been shown to potentially impact human health positively, the two classes of tannins used in leather processing appear to exhibit negative health effects (Chung, Wei, & Johnson, 1998). Pyrogallols exert toxicity in the liver, lungs, kidneys and gastrointestinal tract, leading to severe chronic and acute health endpoints (Upadhyay, Gupta, Prakash, & Singh, 2010). Catechols are highly reactive compounds that can damage cellular DNA and proteins, resulting in potential chronic and acute health issues as well (Schweigert, Zehnder, & Eggen, 2001).

Striving to maintain methodological simplicity, we sought to directly replace these hazardous tanning compounds with naturally-derived cross-linking compounds that exhibit minimal cytotoxicity and can biodegrade. Researchers in the bioengineering field, who develop safe and effective methods to deliver drugs and graft tissue, have identified several natural cross-linkers. They include vanillin (Peng et al., 2010), procyanidin in apple and grape seeds (K.-Y. Chen et al., 2009; Pinheiro, Cooley, Liao, Prabhu, & Elder, 2016; Slusarewicz, Zhu, & Hedman, 2010; Zhai et al., 2006), alginated dialdehyde from brown algae (Xu, Li, Yu, Gu, & Zhang, 2012), and genipin (Jin, Song, & Hourston, 2004; Pinheiro et al., 2016; Slusarewicz et al., 2010; Yoo, Kim, Kim, & Choi, 2011). Within our context, genipin is the most promising among these natural cross-linkers because it is relatively efficient (Mi, Sung, & Shyu, 2000; Slusarewicz et al., 2010) and is the primary cross-linking agent that has been experimented with chitosan (Butler, Ng, & Pudney, 2003; H. Chen, Ouyang, Lawuyi, Martoni, & Prakash, 2005; Chiono et al., 2008; Gorczyca et al., 2014; Grolik et al., 2012; Jin et al., 2004; Mi et al., 2000; Muzzarelli, 2009; Sampaio, Fook, Fidéles, Cavalcanti, & Fook, 2014; Zhang et al., 2010; Zheng, YunYu, & Al), 2009).

Genipin is a small-molecule metabolite of geniposide (Fig. 5). Found in the gardenia fruit, it is used in Eastern medicine for its anti-inflammatory, diuretic, choleretic, and hemostatic properties (Butler et al., 2003). It is also used in East Asia for food coloring (Mi et al., 2000). In the gardenia fruit geniposide is a defense chemical against herbivores and pathogens. Geniposide is categorized as an iridoid glycoside, a class of compounds that are secondary metabolites in plants. Iridoid glycosides provide defense, generally have a bitter taste, and have antifeedant and growth inhibitory activities against insects (Konno, Sabelis, Takabayashi, Sassa, & Oikawa, 2010). A side reaction occurs with genipin use that turns materials blue and so alternative compounds within this class might be explored. These potential alternatives include kutkoside and picroside-I , which may have metabolites with functional groups that may be able to also induce cross-linking in chitosan, without turning the material blue.

Source: https://www.thesynergycompany.com/



Source: Sigma-Aldrich

Figure 5. Source and chemical structure of genipin

Functional/reactive groups + chemical reactions

The functional groups of genipin involved in cross-linking are the 1) an ester and 2) the third carbon (C3) in the six-membered dihydropyran ring (Fig. 5). Both of the functional groups react with the primary amine group in chitosan and become connectors between two chitosan fibers (Fig. 6) (Butler et al., 2003; Mi et al., 2000). Although these reactions are not fully understood, it appears that they can occur in acidic, neutral, or alkaline conditions (Sampaio et al., 2014). One half of the cross-linking, Reaction A, occurs via an initial nucleophilic attack by the chitosan primary amine on C3, opening up the dihydropyran ring (Fig. 7). The formation of a secondary amine completes the reaction, leading to a heterocyclic compound of genipin bound to chitosan (Butler et al., 2003; Mi et al., 2000). The open dihydropyran ring also generates a radical oxygen (Fig. 7, blue square), which induces polymerization of genipin. This polymerization causes the chitosan-containing material to turn blue (Butler et al., 2003). The other half of the cross-linking, Reaction B, occurs via a nucleophilic substitution reaction where the ester group on genipin is substituted with a secondary amide bond. The primary amine group on chitosan attacks the ester and converts into a secondary amide (Fig. 8) (Butler et al., 2003; Mi et al., 2000). The order of Reaction A and B differs by publication (Butler et al., 2003; Mi et al., 2000).

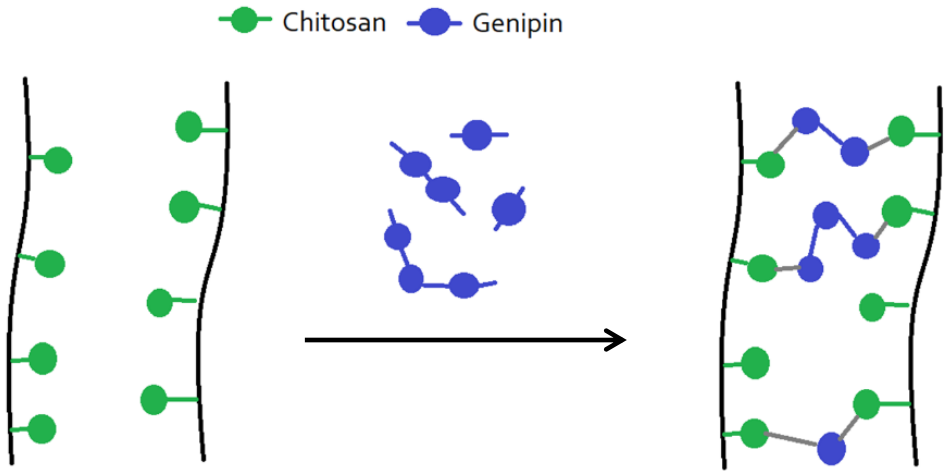


Figure 6. General reaction scheme between chitosan fibers and mono-/polymers of genipin

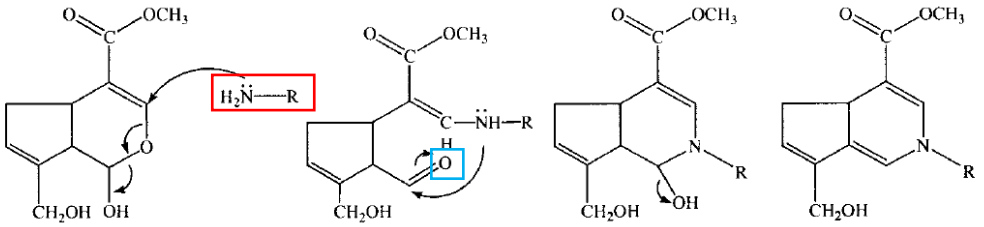


Figure 7. Reaction A – nucleophilic attack of C3 by the chitosan primary amine (red box) (Butler et al., 2003)

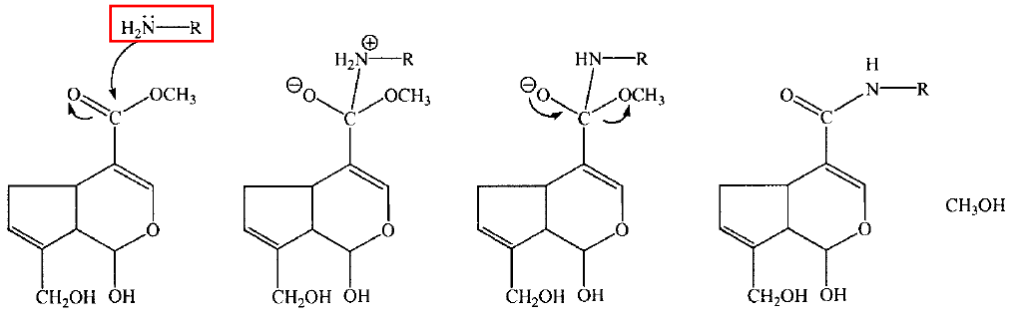


Figure 8. Reaction B – nucleophilic substition by the chitosan primary amine (red box) for a secondary amide (Butler et al., 2003)

Proposed processing steps

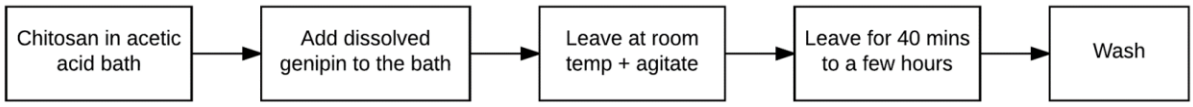


Figure 9. Proposed processing steps to achieve cross-linking via genipin

Cross-linking with genipin is a simple procedure that requires minimal energy input. Commercially available genipin powder would be dissolved in a solvent first, and then mixed into a solution with partially deacetylated MycoWorks material (Fig. 9). The mixture or bath would then be incubated for 40 minutes to several hours at room temperature with agitation. The length of the incubation period differed across experiments and would need to be optimized for the MycoWorks material. The resulting material requires washing with water or another solvent to neutralize the material to pH 7 and remove unreacted genipin. This general procedure is based on experiments conducted with chitosan powder dissolved in acetic acid, so the solvent for genipin powder is also acetic acid (Butler et al., 2003; Mi et al., 2000). The solvent selected across publications appears to be based on the polymer being cross-linked by the genipin. For example, demineralized water was used when cross-linking gelatin and chitosan (Chiono et al., 2008; Jin et al., 2004; Liu & Kim, 2012), PBS was used in composite films with collagen (Pinheiro et al., 2016; Slusarewicz et al., 2010; Yoo et al., 2011), and ethanol was used for composite films with silk fibroin (Zhang et al., 2010) and collagen/gelatin (Gorczyca et al., 2014). MycoWorks may be able to achieve cross-linking in distilled or demineralized water, so this approach should be tested first before a solvent is introduced. The solvent might also be based on the procedure used to deacetylate the chitin into chitosan.

Targets for optimization required for the use of genipin in the MycoWorks process includes: concentration of genipin, pH for the reaction, incubation time, and solvent needs. The genipin

concentration has varied across studies: 1-100 mM (Butler et al., 2003), 0.05 – 1% wt (Jin et al., 2004; Liu & Kim, 2012; Yoo et al., 2011), 0.1-1 % w/v (Mi et al., 2005; Mu, Guo, Li, Lin, & Li, 2012), 0.5-2.5% w/w to the weight of the polymer (Chiono et al., 2008; Gorczyca et al., 2014). Varying the pH at which the reaction is done affects the degree of cross-linking and the chain length of the genipin bridges, so it benefits MycoWorks to explore several solvent and pH options (Mi, Shyu, & Peng, 2005). The incubation times suggested in literature are based on the development of solid films; therefore the time needed to achieve optimal cross-linking within the MycoWorks material will likely vary.

Overall strategy benefits + challenges

The process to achieve cross-linking with genipin is relatively straightforward, with a few factors to optimize experimentally, and can be integrated easily into the current MycoWorks process and scale. All of the components of this strategy are commercially available, including any solvents that MycoWorks may want to test to vary the pH (e.g. acetic acid, phosphate buffer, Tris buffer, and sodium hydroxide). Genipin itself is biodegradable, and if no solvent besides distilled/demineralized water is necessary to achieve optimal cross-linking and performance, then the strategy is sustainable and completely biodegradable as well. Since cross-linking occurs with genipin polymers of various lengths, flexibility may also be achieved as there is varying space between bonded polymers (Butler et al., 2003; Mu et al., 2012). Thus, the performance goals of increasing strength and flexibility of the MycoWorks MVP can be achieved through this strategy. Furthermore, the varying extent of cross-linking will lead to additional desirable features such as enhanced swelling capability, resistance against enzymatic hydrolysis, and thermal stability of genipin-fixed materials (Mu et al., 2012).

The biggest downside of this strategy is that it will turn the material blue as previously described (Fig. 10). The degree of color change is a function of the genipin concentration, incubation time, pH, temperature, and exposure to air (Butler et al., 2003; Gorczyca et al., 2014; Paik, Lee, Cho, & Hahn, 2001). Increased exposure to air enhances the intensity of the bluenecolor (Butler et al., 2003). Additionally, the blue pigment is more stable in more alkaline conditions (Paik et al., 2001). Therefore, this blue side effect could potentially be minimized by performing the experiment in a contained environment with minimal airflow and in an acidic solution.

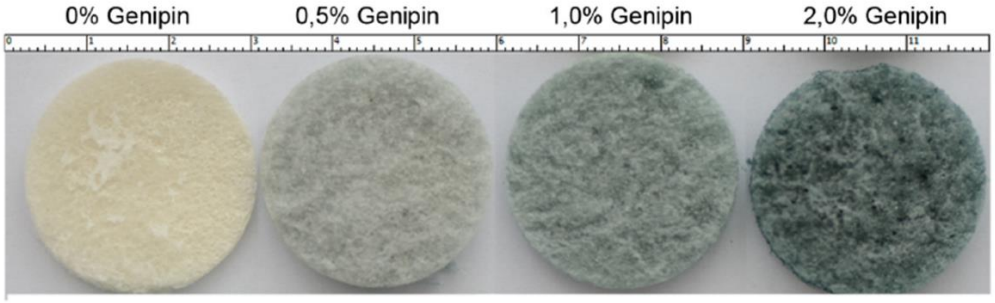


Figure 10. Varying degree of blueness with varying experimental conditions (Gorcyzca et al., 2014)

Enzymatically-driven cross-linking

Following natural cross-linkers, enzymes represent another simple approach for inducing cross-linking. We turned to bio-inspired design for leads in designing the enzymatically-driven cross-linking strategy.

Many natural processes are catalyzed by enzymes. Among these are: the hardening of insect shells, melanization (darkening) of soil, and browning of fruit. The enzyme tyrosinase is responsible for the three reactions listed, and more (Fig. 11).

Figure 11. Natural processes caused by tyrosinase driven reactions

Tyrosinase is a polyphenol oxidase, meaning that its binding site accepts and a variety of polyphenols upon which it catalyzes an oxidation-reduction reaction. The binding site accommodates two aromatic compounds and an oxygen (Mayer et al., 2006). The polyphenol oxidase class of enzymes also includes laccase and peroxidase, which may present other potential enzymatically-driven cross-linking solutions. Commercially available tyrosinase is typically produced through fermentation by genetically engineered fungi.

Functional/reactive groups + chemical reactions

Unlike the natural cross-linker strategy, tyrosinase does not actually serve as the cross-linking compound. Instead tyrosinase converts a phenolic substrate into a more reactive o-quinone compound, which serves as the cross-linker (Fig. 12). A favorable aspect of the tyrosinase-driven reaction is that tyrosinase accepts many different phenolic substrates (Mayer et al., 2006). After the o-quinones are produced, they serve directly as cross-linking monomers and polymers between chitosan fibers (Fig. 13). Enzymatically-generated quinones preferentially react with the primary amine group of chitosan (Kumar et al., 1999). While the tyrosinase cross-linking reaction is not fully understood, it appears that these reactions can occur in acidic, neutral or alkaline conditions (Mayer et al., 2006).

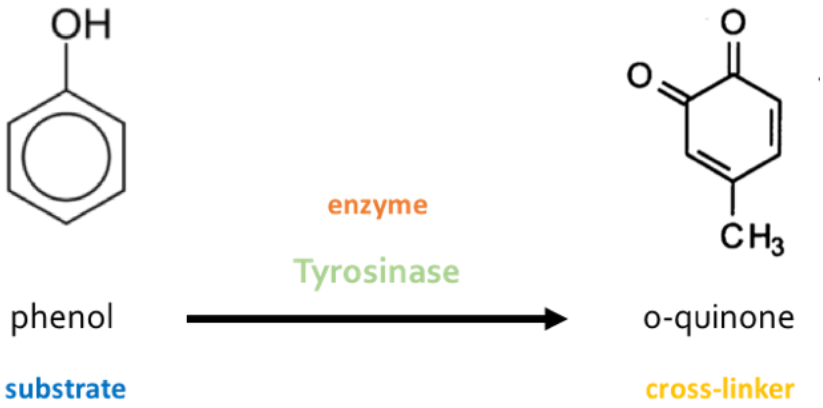


Figure 12. Chemical reaction to convert phenolic substrate into reactive o-quinone cross-linker via tyrosinase

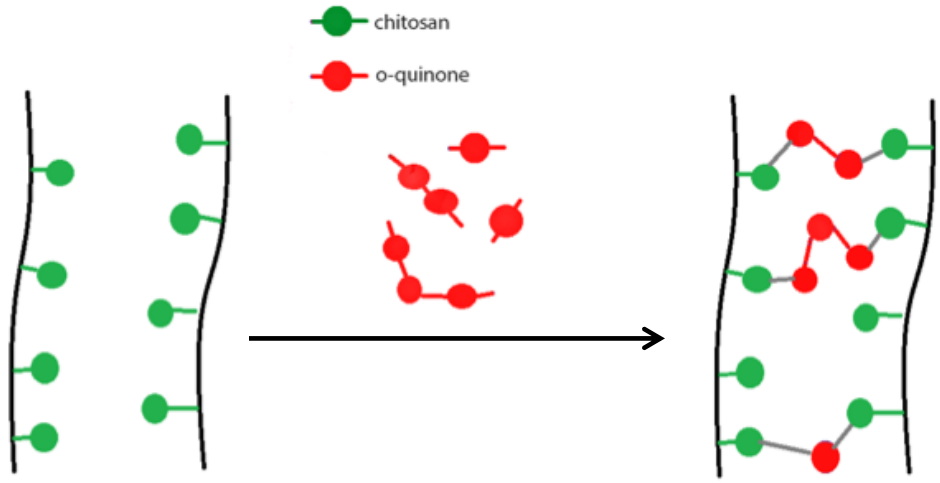


Figure 13. General reaction scheme between chitosan fibers and mono-/polymers of o-quinone

Proposed processing steps

Tyrosinase can be used to drive a cross-linking reaction in chitosan fibers through a straightforward and low energy process (Fig 14).

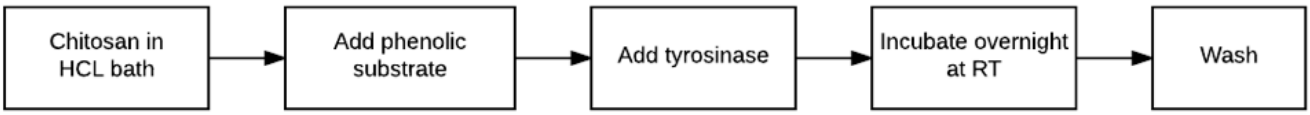


Figure 14. Proposed processing steps for enzymatically-driven cross-linking via tyrosinase enzyme (Kumar et al., 1999)

In literature this procedure was done in dilute hydrochloric acid, however given that the tyrosinase is active at different pH levels, MycoWorks is likely to be able to find alternative solvents for this strategy (Kumar et al., 1999). First, commercially available p-cresol would be mixed into a solution with the MycoWorks material. Varying levels of p-cresol will need to be tested to find the optimal amount needed for the level of crosslinking desired. Depending on the concentration of p-cresol added, a

corresponding amount of commercially available tyrosinase is then added. Afterwards, the solution containing the chitosan-phenolic substrate and tyrosinase is left to incubate overnight at room temperature. To remove low molecular weight solutes, such as unreacted phenolic substrate, a solvent can be added to neutralize the pH of the solution. This precipitates out unreacted solutes. The solvent washing step can be repeated as needed. Finally, the material is washed with deionized water.

Overall strategy benefits + challenges

The tyrosinase-driven cross-linking strategy is appealing due to the low complexity of the process. After mixing in the phenolic substrate and tyrosinase, only room temperature incubation and washing steps are required. The fundamental ability of tyrosinase to induce cross-linking in chitosan has been proven by several researchers(Kumar et al., 1999) (Y. Zhang et al., 2010).

Compared to just purchasing quinone compounds and using those directly as cross-linkers, o-quinone formation via a tyrosinase driven reaction confers favorable properties. Quinone compounds are highly electrophilic, meaning they tend to be very reactive and as a category pose significant health, environmental, and occupational hazards. In addition tyrosinase-generated o-quinones appear to preferentially react with the primary amine group of chitosan (Kumar et al., 1999). Therefore, the strategy presented here should produce o-quinone cross-linkers that specifically target the functional groups which we are interested in cross-linking.

The key challenge associated with the tyrosinase enzymatically-driven cross-linking is the lack of information on how it affects the mechanical properties of the material. Kumar et al. (1999) confirm that the strategy is viable for inducing cross-linking in chitosan; however, it is not clear whether this cross-linking will confer the level of strength and flexibility desired for the final MycoWorks material. Given that tyrosinase also drives many “browning” reactions in natural processes (such as food browning), further research is also needed into how this strategy may affect the color of the MycoWorks material.

Nanocomposite-based material

In the naturally-evolved world, arthropods layer chitin with nano-sized additives to form nanocomposites which demonstrate a wide range of physical properties. These additives include inorganic chemicals from the arthropod’s environment and proteins produced by the arthropod (Appel, Heepe, Lin, & Gorb, 2015; Raabe, Sachs, & Romano, 2005). The presence of the additives at the nanoscale greatly improves the performance of the arthropod’s chitinous structures. Stronger exoskeletons, better wings, and more resilient joints are all macroscale results of the nanoscale interactions between the additives and chitin (Appel et al., 2015; Raabe et al., 2005). MycoWorks already employs the concept of using compositing to improve strength in the manufacture of its MVP. The current composite is composed of chitin and cotton fibers. We believe that additional compositing, combined with cross-linking to fix it in place, will improve the strength of the MycoWorks MVP.

Initial forays into arthropod cuticle biomimicry led to potentially viable cross-linking strategies. Miessner, Peter, and Vincent (2001), taking inspiration from naturally occurring tanning in mollusks, used catechol and a peptide coupling reagent to induce crosslinking in dihydroxyphenylalanine (DOPA), a peptide impregnated into the material they were working with. Oh and Hwang (2013) investigated the biochemical process of sclerotization in squid beaks, which is similar to tanning, to improve the mechanical properties of chitosan. They used DOPA as the cross-linker and sodium peroxidate to oxidize

the DOPA in order to facilitate cross-linking between it and primary amine functional groups on chitosan (Oh & Hwang, 2013) Both of these strategies improved mechanical properties and looked promising, and both posed significant occupational hazards. The catechol hazards are enumerated above; DOPA is a precursor to the neurotransmitter dopamine, which makes occupational exposure a particular concern.

We sought a different method of cross-linking chitosan using the primary amine group and found work which had been done in chitosan hydrogels using chitin nanowhiskers and the blocked isocyanate cross-linker hexamethylene-1,6-di-(aminocarboxysulfonate) (HDS) (Araki, Yamanaka, & Ohkawa, 2012). Chitosan is dissolved in 5 wt% acetic acid and chitin nanowhiskers are added at various concentrations. Synthesized HDS is mixed in and the gel is allowed to solidify. The nanowhiskers fill the gaps between the chitosan fibers, forming a nanocomposite which is strengthened further through cross-linking. The resulting chitosan-chitin nanocomposite has improved elastic modulus and tensile strength. The HDS molecule is a 6-carbon chain with a blocked isocyanate functional group at each end of the molecule (Fig. 15). It catalyzes bond formation between the primary amine functional groups on chitin and chitosan, and the isocyanate groups on HDS (Fig. 16).

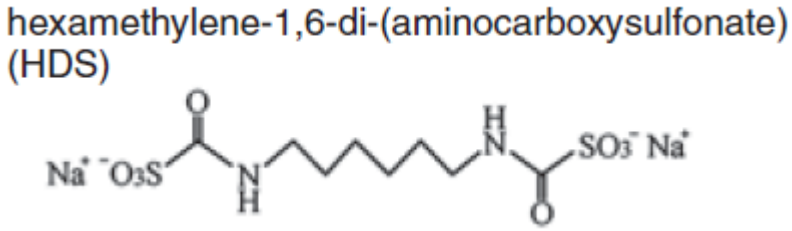


Figure 15. Structure of HDS (Araki et al., 2012)

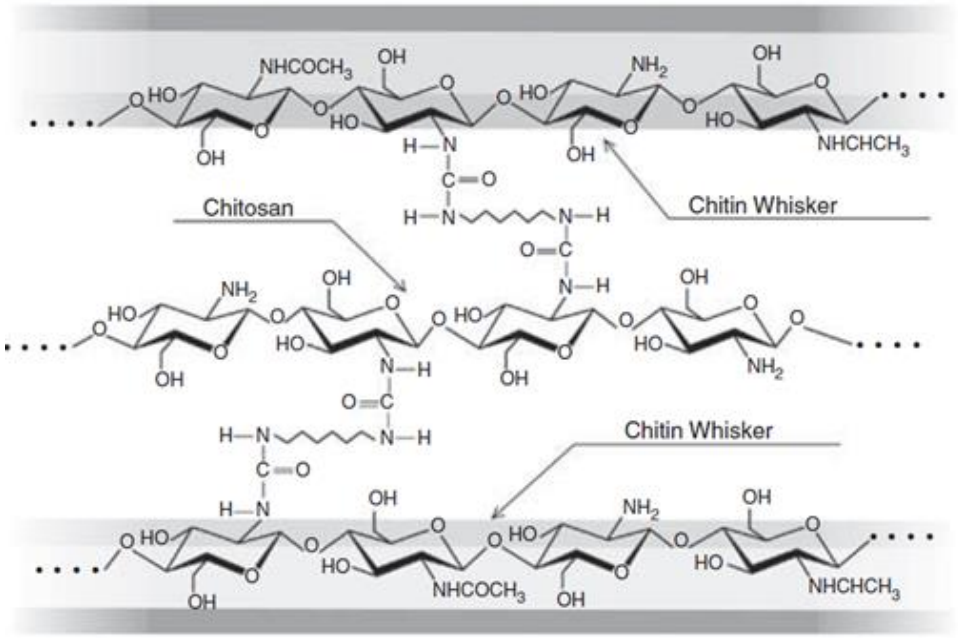


Figure 16. HDS-mediated cross-linking between chitin nanowhiskers and chitosan fibers (Araki et al., 2012)

There are some problems with HDS. First, it is not commercially available and must be synthesized in the lab from hexamethylene diisocyanate and sodium metabisulfite. Second, both of these chemicals are persistent in the environment and pose acute as well as chronic health hazards in the occupational setting. Primary amines, however, are predisposed to other types of chemical reactions. One of the most prevalent is the amide bond, which occurs between a primary amine and a carboxylic acid.

Functional/reactive groups + chemical reactions

There are many ways to synthesize amide bonds (Pattabiraman & Bode, 2011). Many of them, such as amidation by aldehydes, metal catalysts, and bromo-nitro compounds, pose health and environmental toxicity risks. Boronic acids are fairly new amidation catalysts which are efficient at forming amide bonds between primary amines and carboxylic acids in a direct and waste-free reaction (Pattabiraman & Bode, 2011). Additionally they pose fewer health and environmental concerns than other amidation catalysts (Al-Zoubi, Marion, & Hall, 2008).

The boronic acid catalyst selected is 2-iodophenylboronic acid (IPBA) (Fig. 17) and the carboxylic acid is suberic acid (Fig. 18). IPBA has has the best performance in forming amide bonds at room temperature out of 45 *ortho*-functionalized arylboronic acids tested (Al-Zoubi et al., 2008). The majority of boronic acid catalysts require temperatures as high as 110°C, however we sought to have a process which uses as little thermal energy as possible. The boronic acid catalyzes amidation by generating an active ester (Pattabiraman & Bode, 2011).

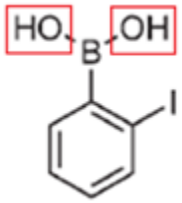


Figure 17. Chemical structure of 2-iodophenilboronic acid (Al-Zoubi et al., 2008)

Suberic acid, the cross-linker chosen for this strategy, is a linear saturated dicarboxylic acid. It has a 6-carbon chain with a carboxyl functional group (-COOH) on each end. It was chosen because it has the same carbon chain length as HDS. The carbon chain length of the cross-linker molecule is important because cross-linking using short chain carboxylic acids has been shown to cause brittleness in fabrics (Harifi & Montazer, 2012). The longer chain length allows for room to move, on a molecular level, which we believe will help maintain, or potentially increase flexibility of the MycoWorks material.

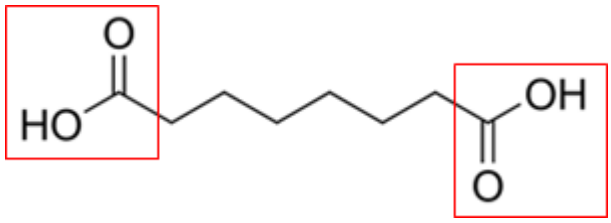


Figure 18. Chemical structure of suberic acid (Source: Sigma Aldrich)

Chitin nanowhiskers fill the spaces between the fibers in the MVP. The nanowhiskers are approximately 10nm in diameter and 300-500nm in length, allowing them to penetrate into the material (Fig.19). While chitin has predominantly acetamide groups (-$NHCOCH_3$), it also has naturally occurring primary amine functional groups, so the nanowhiskers do not need to be deacetylated (Fig. 20). Sufficient primary

amines will be available to be cross-linked. Too many primary amines on the chitin nanowhiskers could result in excessive cross-linking, which can lead to stiffness.

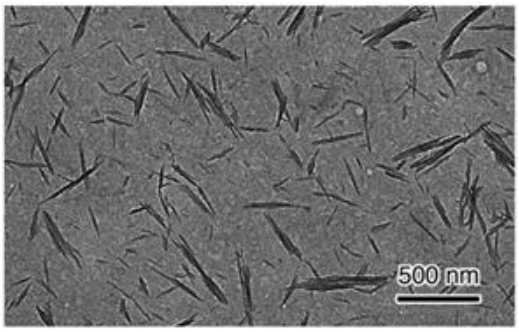


Figure 19. SEM image of chitin nanowhiskers (Araki et al., 2012)

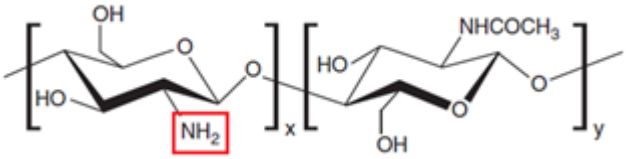


Figure 20. Chemical structure of chitin (Araki et al., 2012)

A simple thermal dehydration reaction between primary amines and carboxyl groups forms unreactive carboxylate-ammonium salts (Al-Zoubi et al., 2008; Arnold et al., 2006). The arylboronic acid overcomes the activation energy necessary to mediate amide bond formation (Al-Zoubi et al., 2008). In this strategy the amide bonds are formed between the chitin nanowhiskers and suberic acid, and the suberic acid and chitosan fibers (Fig. 21). The mechanism by which IPBA catalyzes direct amidation is not fully understood (Al-Zoubi et al., 2008; Arnold et al., 2006). The mechanism's general activity and the chemical species involved are mostly known, but specifics about why some arylboronic acids are better catalysts than others have not yet been determined.

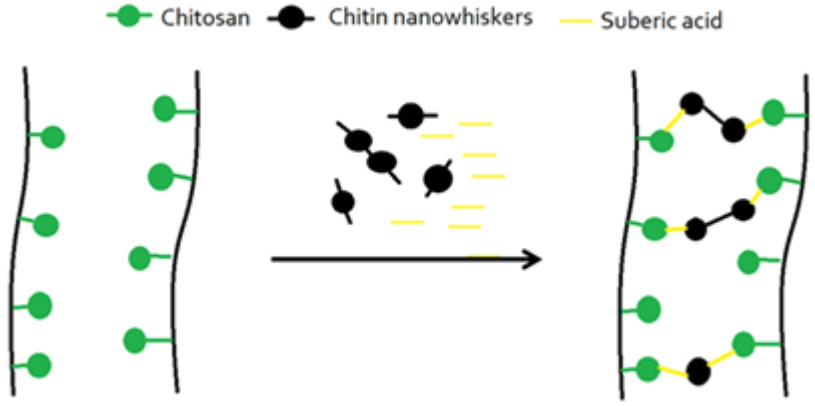


Figure 21. General reaction scheme between chitosan fibers, suberic acid, and chitin nanowhiskers

When IPBA is in solution it fluctuates between two species: a diboronate (labeled 9 in Fig. 22) and a boroxine (labeled 10 in Fig. 22). These two species provide electrophilic activation of the suberic acid carboxyl group (Al-Zoubi et al., 2008). The carboxyl groups transform into carboxylates through boron conjugation and hydrogen bonding, and the boronic acid is acylated (Al-Zoubi et al., 2008). A hydrogen bond is created between the two, forming the intermediates monoacylboronate and diacylboronate (labeled I and II respectively in Fig. 23) (Al-Zoubi et al., 2008; Arnold et al., 2006). While the question of which of these intermediates is the active acylating species remains, literature indicates that it is believed to be the diacylboronate (Arnold et al., 2006). The active intermediate oxidizes the primary amine and acylates it, forming an acylated amide. The amide and carboxylate undergo a dehydration

reaction to form a covalent amide bond, and detach from the IPBA intermediates (Al-Zoubi et al., 2008; Arnold et al., 2006).

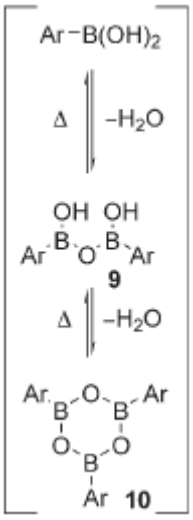


Figure 22. Arylboronic acid (Ar-$B(OH)_2$) species in solution (Arnold et al., 2006)

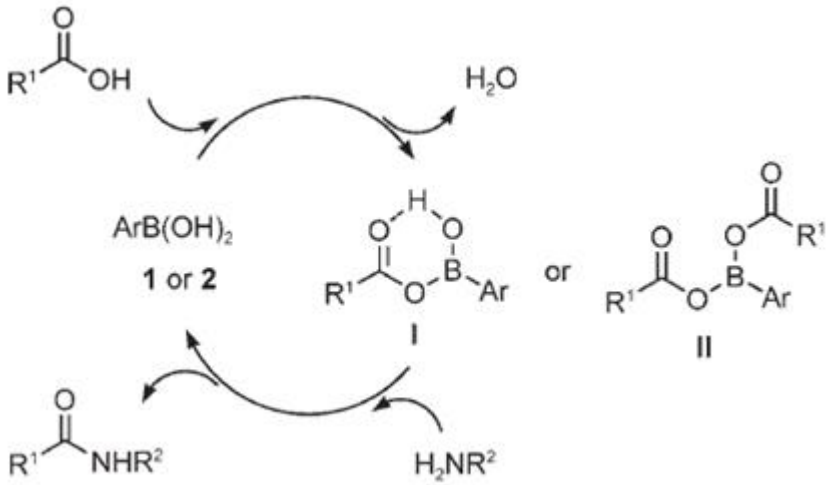


Figure 23. Proposed mechanism for direct amidation with IPBA (Al-Zoubi et al., 2008)

Use of IPBA is relatively simple compared to other catalytic amidations. The reaction does not require energy input through heating, nor does it require cooling to ensure undesirable side reactions do not occur (Al-Zoubi et al., 2008). No excess substrates are required for the reaction, and the carboxylic acid and amine are in equivalent molar concentrations (Al-Zoubi et al., 2008). Additionally, no by-products are formed and in the lab reactions the IPBA was recovered after it catalyzed the amide bonds (Al-Zoubi et al., 2008).

Proposed Processing Steps

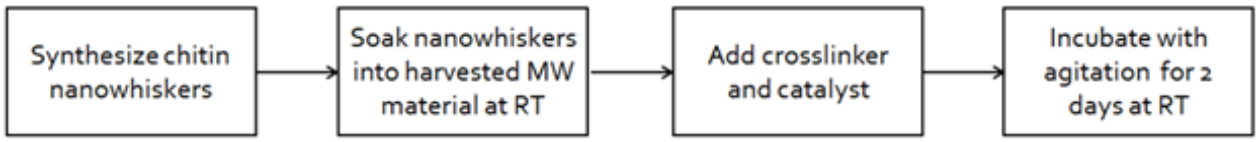


Figure 24. Proposed processing steps to achieve a cross-linked nanocomposite material

The composite-based strategy has four major processing steps. First the chitin nanowhiskers are synthesized from commercially available chitin powder. Chitin powder is extracted from shrimp shells which would otherwise be waste and is comprised of aggregates of nanofibers (Ifuku, 2014). To synthesize the nanowhiskers the powder is dispersed in dilute acetic acid and ground using a friction grinder (Ifuku, 2014). The acidic water ionizes chitin's acetamide functional group, forming electrostatic repulsive forces which aid the separation of the nanofiber aggregates into homogenous nanowhiskers with a diameter of about 10 to 20 nm, and a length of 200 to 500 nm (Araki et al., 2012; Ifuku, 2014). The nanowhiskers can be introduced into the MycoWorks material during the soaking portion of the post-harvest manufacturing process.

The second step of this strategy is to partially deacetylate the MycoWorks material. In order for chitin to form amide bonds more easily, sufficient acetamide functional groups must be converted into primary amines. If the degree of deacetylation is less than 50%, chitin retains its properties and does not become chitosan. Creatures which produce chitin use the enzyme chitin deacetylase to transform chitin into chitosan; this enzyme is not available commercially. Application of sodium hydroxide is the most common chemical method to deacetylate chitin. Treating chitin with 2M Sodium Hydroxide for 3 hours at 24°C (75°F) will result in an approximately 15% increase in deacetylated chitin molecules, which will yield accessible primary amines for cross-linking (Pires, Vilela, & Airoldi, 2014).

In the final step, the chitin nanowhiskers are cross-linked to the partially deacetylated MycoWorks material using suberic acid as the dicarboxylic acid cross-linker, and 2-iodophenylboronic acid (IPBA) as the catalyst of amide bonds (Al-Zoubi et al., 2008). For a carboxylic acid with one functional group, the ratio of carboxyl moles to amine moles is 1:1 (Al-Zoubi et al., 2008). In a reaction using a dicarboxyl the ratio of suberic acid moles to amine moles is 1:2. Because the IPBA is not consumed in the reaction it can be added at a level 10 fold lower than the molar concentration of amine (Al-Zoubi et al., 2008). An amine concentration of 0.5 mM only requires the addition of 0.05 mM IPBA (Al-Zoubi et al., 2008). Impregnating chitin nanofibers into the partially deacetylated MycoWorks material and cross-linking the two using amide bonds has been shown in literature to provide extra structure and strength (Araki et al., 2012). This strategy also potentially improves the flexibility of the material due to the 6 carbon chain length of suberic acid, which allows for more movement among the fibers of the material.

Overall strategy benefits + challenges

The strategy of cross-linking chitin nanowhiskers to the chitosan fibers of the MycoWorks MVP to form a nanocomposite has several benefits and challenges. The filler used is a renewable resource and is biodegradable, which meets MycoWork's goals of sustainability and biodegradability. The chemical process does not have side reactions or by-products which could cause potential hazards. When the amide reaction is complete there are no unreacted chemicals left in the material. Filling some of the

gaps between the chitosan fibers with the chitin nanofibers is a method proven to increase both tensile strength and elastic modulus chitosan-chitin hydrogels.

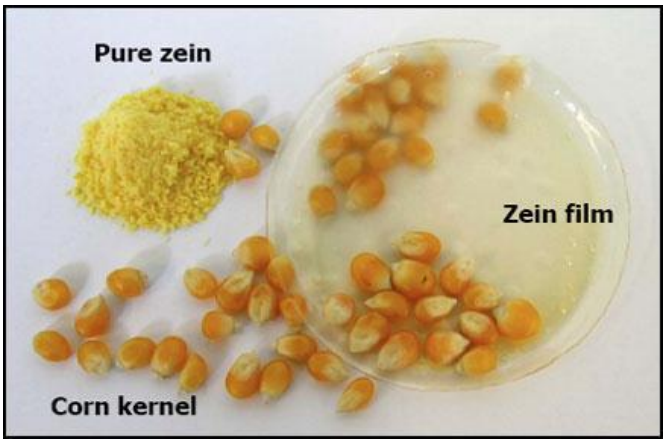
The experiments this strategy is based on were done in hydrogels and the strategy may not be fully replicable in the MycoWorks MVP. Additionally, substituting the hexamethylene-1,6-di-(aminocarboxysulfonate) catalyst and cross-linker used in the Araki (2012) study with 2-iodophenylboronic acid and suberic acid adds another unknown challenge, no direct example of this strategy has been located in literature. Lastly, the solvents used may present the need for additional experimentation. The most common solvent used in the amide reaction between IPBA and carboxylic acids is dichloromethane, which has an unacceptable occupational hazard profile due to carcinogenicity. Appendix C presents alternative solvents with better hazard profiles which may be substituted for dichloromethane.

While this strategy presents potentially lengthy challenges to optimization, it offers a promising technology for building on the existing MycoWorks composite material. If the genipin-only strategy results in material which is too stiff, an approach combining chitin nanowhiskers in conjunction with genipin may prove viable. Because genipin acts on primary amine groups, it is possible to increase the molecular distance between the chitosan fibers by cross-linking nanowhiskers with genipin and chitosan to increase the distance between the fibers.

Moisture barrier strategy: corn zein coating

There are a number of water-insoluble proteins which may be extracted from agricultural byproducts that could be used as bio-based moisture barriers for the MycoWorks material (Gennadios, 2002). Examples of such proteins include corn zein, wheat gluten, and fish myofibrillar protein. Corn zein is a protein that is a byproduct of corn gluten meal, which itself is a byproduct of corn processing. Corn zein is typically incorporated into animal feed, but is not used in human food because of its low nutritional value (Shukla & Cheryan, 2001). Wheat gluten is the protein left over after starch is washed away from wheat flour dough. Fish myofibrillar protein can be extracted from byproducts of surimi production. All of these proteins can form films that are moisture-resistant to different degrees. All merit further consideration as sources of moisture barriers for the MycoWorks material, but in this report we focus on corn zein because we found it to be the most promising of these proteins.

Corn zein is extracted from corn and produced as a powder. It is sold commercially and can easily be made to form a film (Fig. 25) (Cuq, Gontard, & Guilbert, 1998). These films are well characterized and known to be moisture-resistant, grease-resistant, and antimicrobial (Gennadios, 2002). Corn zein is used commercially to form coatings directly on foods like nuts and candies to maintain freshness, flavor, and color; for coatings on biodegradable and edible food packaging; and as casings on pharmaceutical tablets to protect the components and to achieve controlled drug delivery.



Source: Wageningen University & Research (https://www.wur.nl/en/show/Encapsulation-properties-of-corn-protein-zein.htm)

Figure 25. Pure corn zein in powder form, and corn zein film

Proposed processing steps

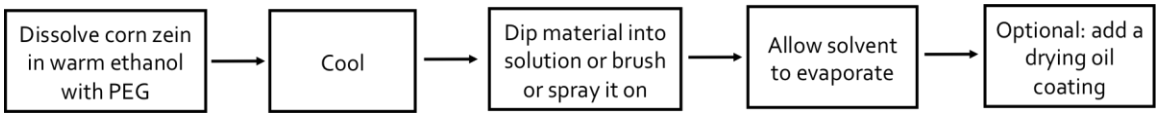


Figure 26. Proposed incorporation of corn zein coating into MycoWorks manufacturing process.

Several steps are needed to incorporate a corn zein coating into the current MycoWorks manufacturing process (Fig. 26). Corn zein powder would be dissolved in a solution of warm ethanol and PEG. After cooling, the solution could be applied to the MycoWorks material through dipping, brushing on, or spraying on. After the solvent evaporates a drying oil (e.g. as tung oil) could be added for increased moisture resistance if it is shown not to increase brittleness.

Moisture-resistance and flexibility of corn zein films

The majority of studies that we found on the properties and performance of corn zein focused on stand-alone corn zein films rather than corn zein coatings. We therefore examined studies of stand-alone corn zein films in order to assess the potential of a corn zein coating as a moisture barrier for the MycoWorks material. It is important to keep in mind that the properties and performance of a coating are not identical to those of a stand-alone film.

Corn zein films are generally brittle, requiring the addition of plasticizers. Common plasticizers include polyols such as glycerol and PEG, mono/di/oligosaccharides, lipids, and lipid derivatives. Various plasticizers can increase the film's percent elongation by 10- to 100-fold (Table 1). We recommend that MycoWorks experiment with different plasticizers in different ratios in order to achieve maximum flexibility of the corn zein film to reduce the risk of the coating flaking off due to normal wear and tear.

Type of Film	Plasticizer	Tensile Properties Test Conditions	TS (MPa)	E (%)	YM (MPa)	WVP g·mm/m²·h·kPa	WVP Test Conditions
Cast Films	none	20°C, 52% RH	10.9	3	551	0.620	30°C, 0/100% RH
	30%, Gly:PPG, 1:3	25°C, 50% RH	5.1	117	135	1.060	30°C, 0/100% RH
	22% Gly:PEG, 1:1	25°C, 50% RH	22.8	18		1.476	
Drawn Films	33%, oleic acid	25°C, 50% RH	8.9	12	267	0.040	25°C, 50/0% RH
	33% oleic acid	25°C, 98% RH	8.1	28	156	0.060	25°C, 98/0% RH
	38% oleic acid	25°C, 50% RH	5.9	44	307		
	60%, oleic acid	25°C, 50% RH	3.3	154	129		
Coated w/Tung Oil	50%, oleic acid	25°C, 50% RH	4.5	20	200	0.005	25°C, 98/0% RH
LDPE	none	25°C, 50% RH	9.0–17.0	500		0.003	38°C, 90/0% RH

Table 1. Experimentally determined features of corn zein films, adapted from Table 2.1 in Gennadios, 2002. Gly = glycerol, PPG = polypropylene glycol, PEG = polyethylene glycol. TS = tensile strength, E = percent elongation, YM = Young's Modulus, WVP = water vapor permeability. RH = relative humidity. Cast films refer to those produced by dissolving corn zein in solution (usually an alcohol) with non-fatty acid plasticizers, while drawn films result when corn zein is plasticized in solution with long chain fatty acids.

Corn zein films have been well-studied for their moisture barrier properties. The most common measure of moisture evaporation prevention is water vapor permeability (WVP). This metric describes the passage of water vapor through a film. A lower WVP signifies a smaller amount of water vapor permeating through a film over a given area, time period, and pressure. A film of corn zein without plasticizer has a WVP 100 times that of a LDPE film - a good moisture barrier - and a film with oleic acid as a plasticizer has a WVP 10 times that of a LDPE film (Table 1). When tung oil is added, the film's WVP reaches the same order of magnitude as that of a LDPE film. Tung oil comes from the seed of the tung tree and is an example of a drying oil that thickens upon drying. We note that this table does not contain measurements for films with only PEG as a plasticizer; therefore MycoWorks will need to test the moisture resistance of corn zein coating using PEG as the plasticizer. Plasticizers have been shown to negatively impact the WVP of corn zein film. Therefore if PEG is found to negatively impact the moisture resistance of the corn zein coating, further testing using different plasticizers in various ratios will need to be done to maximize the water resistance of the coating.

Overall strategy benefits + challenges

Based on the available literature about corn zein films we are confident that the moisture barrier and flexibility properties of a corn zein coating are promising enough to warrant testing on the MycoWorks material. Corn zein films also have the added benefit of grease resistance, which may confer positive physical properties not yet considered. Additionally, corn zein comes from byproducts of corn processing, keeping with MycoWorks's goal to use bio-based components that are agricultural byproducts. All materials involved in this strategy are commercially available, and a corn zein coating would be simple to integrate into the current manufacturing process. As reflected by its application in food and drug products, corn zein generally presents minimal health and environmental hazards apart from potential allergenicity.

To the best of our knowledge corn zein coatings have not been tested on textiles. Further research is needed to determine the coating's durability through washes, and to optimize the corn zein : plasticizer ratio. Another potential drawback of is that corn zein films might give the MVP a weak yellow color; however, various strategies for de-colorizing corn zein have been presented and MycoWorks could develop a de-colorization strategy if needed (Sessa, Eller, Palmquist, & Lawton, 2003).

Technical Performance Evaluation

Framework

To evaluate the technical performance across strategies, we developed the framework in Table 2.

	Categories	0	xxx	xx	x
Performance	Strength	Unknown	<= 0.5x	Similar to MycoWorks	>=2x
	Water Resistance	Unknown	Lower	Similar to MycoWorks	Higher
	Flexibility	Unknown	Lower	Similar to MycoWorks	Higher
	Longevity Through Washes	Unknown	Lower	Similar to MycoWorks	Higher
	Handfeel	Unknown	Worse	Similar to MycoWorks	Better
	Discoloration	Unknown	Negative color change	-	Positive or no color change
Feasibility	Post-processing	Unknown	No	-	Yes
	Process Complexity	Unknown	High	Medium	Low
	Material Availability	Unknown	Few commercially available materials (<10%) or hard to synthesyze	Some synthesis required	Most materials commercially available (>90%)
	Thermal Energy Requirement	Unknown	High	Medium	Room temp
	Innovation Requirement	Additional research needed	Major hurdles anticipated	Minor hurdles anticipated	Process optimization only

Table 2. Criteria used to evaluate technical performance of prospective strategies

We split technical metrics across two major categories: performance and feasibility. The performance category evaluates each strategy's impact on the performance of the MycoWorks material. Within this category, the proposed strategies are evaluated relative to the current MycoWorks material's performance as a baseline. We describe each performance metric in detail below:

- *Strength*: Ultimate tensile strength (UTS) (MPa) is used to evaluate the overall strength of the material. UTS is defined as the max elongation/tensile stress a material can withstand before rupturing.
- *Water resistance*: Ability of the material to repel water.
- *Flexibility*: Bending endurance, which is the number of bending cycles a material can withstand before failing, represents the ideal flexibility metric for the MycoWorks material. However, literature related to our proposed strategies typically does not report bending endurance as a mechanical property. Instead, elastic modulus (also known as Young's modulus), is used as a proxy for bending endurance and overall material flexibility.
- *Longevity through washes:* Ability for the properties conferred by the proposed strategy to last through multiple washing cycles.
- *Handfeel*: Subjective metric to evaluate how the proposed strategy may affect the feeling of the MycoWorks material on skin.
- *Discoloration:* Binary metric to evaluate whether the strategy will or will not discolor the material.

In addition to technical performance, we assess technical feasibility for each strategy. Technical feasibility metrics evaluate the difficulty of implementing each strategy, relative to the other strategies. Each metric is described below:

- *Post-processing:* Per MycoWorks' preference, we only evaluated post-processing strategies. Here we define post-processing strategies as strategies that can be applied to the MycoWorks MVP material. Therefore, none of the proposed strategies affect the established manufacturing process for the base MycoWorks material.
- *Process complexity:* Process complexity aggregates a few characteristics that define the complexity of the proposed strategy's process, chiefly time, number of steps, and the requirement for any advanced processing steps.
- *Material availability:* Metric to evaluate whether the constituent materials in the proposed strategy are available commercially.
- *Thermal energy requirement:* To consider the full environmental impact of each strategy, we assess the energy input needed for each strategy. Since all of our strategies only seemed to require some or no thermal processing, we only consider the thermal energy required. For example, proposed strategies that can occur at room temperature are considered to have a low thermal energy requirement.
- *Innovation requirement:* Since our proposed strategies come from academic literature, this metric evaluates whether we anticipate technical hurdles to implementing the strategy. For example, if a proposed strategy has already been shown to work directly on chitin-cellulose textile materials, then we rate this strategy to have a low innovation requirement.

Technical performance evaluation for each strategy

Based on the Technical Performance Framework in Table 2, we evaluate the proposed strategies and summarize our findings in Table 3 below.

		Technical Performance						Technical Feasibility				
	Strategy	Physical Properties				Cosmetic Side Effects		Integration				Innovation
		Strength	Water Resistance	Flexibility	Longevity Through Washes	Handfeel	Discoloration	Post-processing	Process Complexity	Material Availability	Thermal Energy Requirement	
Existing Strategies	MycoWorks MVP	xx	xx	xx	xx	xx	xxx	N/A	N/A	N/A	N/A	N/A
	Animal leather	x	x	x	x	x	N/A	N/A	N/A	N/A	N/A	N/A
	Vegan leather	xx	x	o	x	xxx	N/A	N/A	N/A	N/A	N/A	N/A
Proposed Strategies	Genipin	x	x	xx	o	o	xxx	x	xx	x	x	x
	Tyrosinase	o	o	o	o	o	o	x	xx	xx	x	xxx
	Nanowhiskers	x	o	xx	o	o	o	x	xxx	xx	x	xx
	Corn zein + PEG	xx	x	x	o	o	o	x	xx	x	xx	xxx

Table 3. Technical performance evaluation summary

Cross-linker: Genipin

The cross-linking strategy with genipin performs relatively well for technical performance and feasibility (table 3). Genipin is likely to enhance the physical properties of MycoWorks' material in priority areas including enhanced tensile strength (Jin et al., 2004; Zhang et al., 2010), compressive strength (Gorczyca et al., 2014), and hydrophobicity (Jin et al., 2004), as demonstrated from several experiments in the field of bioengineering. Tensile strength is the primary interest; and Zhang et al. showed that cross-linking

hydroxybutyl chitosan scaffolds with genipin lead to a 10-fold increase in tensile strength (uncross-linked: 2.66±0.20 MPa, cross-linked: 23.86 ±1.99 MPa, respectively). MycoWorks may be able to achieve similar results.

Increasing the concentration of genipin also improves the elastic modulus (Butler et al., 2003), but increasing the incubation time makes the cross-linked material more stiff and less ductile (Zhang et al., 2010). Optimizing the methodology is thus pertinent to achieving ideal physical properties. While we cannot know without laboratory tests whether cross-linking with genipin will improve the current materials' flexibility, this strategy could still contribute to improved flexibility since a potential increase in hydrophobicity could prevent PEG from leaching out.

With minimal materials and steps required, this strategy is very technically feasible since it can be applied post-harvesting of the MycoWorks material, all of the materials are commercially available, and the reaction can occur at room temperature. However, the cross-linking efficiency could likely be improved by increasing the incubation temperature. While there is a need to optimize experimental conditions, e.g. genipin concentration and pH, the research required to implement this strategy is minimal since it is clear that cross-linking can be achieved by mixing genipin and chitosan.

Cross-linker: Enzymatically-derived o-quinone

Enzymatically-driven cross-linking with tyrosinase is interesting from a technical feasibility standpoint but faces significant challenges regarding its potential technical performance. Specifically, we have evaluated the technical performance of tyrosinase as unknown and requiring additional experimentation and research. Enzymatically-driven cross-linking in chitosan films via tyrosinase has been shown to work (Kumar et al., 1999), but in a hydrogel context with little resemblance to the MycoWorks textile application. Besides increased viscosity in the hydrogels caused by cross-linking, no changes to other mechanical properties such as strength and flexibility are reported.

Technical performance concerns aside, we have still included the enzymatically-driven cross-linking strategy based on its highly favorable technical feasibility metrics. The tyrosinase strategy, like many enzymatic strategies, is relatively simple, with few and simple processing steps, minimal thermal energy inputs, and mild pH conditions. It accepts many phenolic substracts, making it robust and relatively low hazard.

Cross-linker: Nanocomposite

The arylboronic acid-mediated solution of cross-linking a nanocomposite has attractive technical performance characteristics. This method significantly improves the tensile strength and elastic modulus of cross-linked chitin-chitosan hydrogels. Araki et al. (2012) measured the elastic modulus and stress at break for hydrogels with increasing chitin nanowhiskers content. Chitosan hydrogels with no added nanowhiskers have demonstrated an elastic modulus of 2.53 kPa and stress at break of 3.24 kPa. Adding 2.97 wt% of chitin nanowhiskers increases the elastic modulus 7-fold and the stress at break 5.5-fold (17.8 kPa and 17.7 kPa, respectively). The results are more dramatic when the nanowhisker content is increased to 13.3 wt%. Elastic modulus improves nearly 67-fold to 169 kPa, and the stress at break improves nearly 42-fold to 135 kPa. While exact improvements in the MycoWorks MVP cannot be predicted, the magnitude of these experimental results suggests that there will be a measurable improvement in the strength and flexibility of MycoWorks' material.

From a feasibility standpoint, this is a more complex solution than the previous cross-linking strategies given the number of steps involved, the need to synthesize the nanowhiskers, and the fact that this strategy has not been implemented in a material like the MVP. Although chitin nanowhisker synthesis is simple, it adds an extra step; and working with nanomaterials may present workplace hazards that must be addressed. Additionally, the need to replace the dichloromethane solvent may extend the time required for optimization. Despite these factors, 2-iodophenylboronic acid is known to work well with a wide range of carboxylic acids and compounds containing primary amine functional groups.

Moisture barrier: corn zein coating

Overall the corn zein coating strategy's technical performance appears promising. A study of a corn zein film with PEG showed the film to have a tensile strength of 6 MPa (Gennadios, 2002, Table 1.8), only slightly lower than that of the MycoWorks MVP (7.5 - 12.1 MPa). Therefore, we predict that the MycoWorks material with a corn zein coating would have at least as much tensile strength as the MVP. Corn zein is water-insoluble, and its films are moisture-resistant, so we expect such a coating to improve the water resistance of the material. A corn zein coating would act as a moisture barrier, preventing the PEG plasticizer from washing out. A film of pure corn zein has comparable flexibility to animal leather, with Young's Modulus at 551 MPa (Gennadios, 2002, Table 2.1). Addition of the PEG plasticizer will further increase flexibility.

All the components of this strategy are commercially available, but the process to incorporate the corn zein coating would require several extra steps (potentially de-colorizing the corn zein, dissolving it, heating and applying the solution, and evaporating the solvent), making for a somewhat complex process, and a moderate thermal energy requirement.

The major technical performance drawback is that corn zein coatings on textiles have not been reported, so a significant amount of additional research will need to be done before this strategy can be employed. The material with the coating will need to be tested for strength, flexibility, and moisture resistance, since the studies we encountered deal with stand-alone films, not coatings. Further, the coating's longevity through washes must be tested. The ratio of corn zein, plasticizer, and (optionally) drying oil must be adjusted to maximize flexibility and moisture resistance.

Hazards Assessment

Framework

MycoWorks aims for the most sustainable product they can attain at every point in its lifecycle. This means that they must consider many health-related and ecological endpoints as they select strategies to improve their material. GreenScreen for Safer Chemicals (hereafter, GreenScreen) is a useful methodology to do so. Conducting a full GreenScreen is very time and data-intensive. Under our circumstances, it was untenable to fully implement this methodology. We therefore performed a modified GreenScreen, using their endpoints and framework but performing less intensive research than would be necessary for a full GreenScreen on all strategies.

GreenScreen divides chemical and ecological hazards into five broad categories: chronic human health endpoints, acute human health endpoints, ecotoxicity, environmental fate, and physical hazard. Each of these classes is comprised of several specific endpoints. For example, the category "chronic human health endpoints" is made up of the sub-categories: carcinogenicity, mutagenicity, reproductive toxicity, endocrine disruption, developmental toxicity, and neurotoxicity. For all endpoints associated with each category, see our complete hazard table in Appendix B.

We sought out information to characterize the hazard associated with each endpoint for every chemical in our strategies. We started with information-aggregating sources like the Pharos Project. Pharos collects information from authoritative scientific bodies around the world, making it easy to identify and pursue credible sources of information. We assessed the information provided by authoritative sources to assign hazard ratings to our chemicals. Where authoritative information was unavailable, we searched for academic literature that could help us assess hazard.

If a chemical did not have a rating after our sources were exhausted, we assigned the chemical a rating of “data gap”. We otherwise assigned each chemical/endpoint combination a rating of low/no hazard, moderate hazard, or high hazard. These ratings account for the degree of certainty and the potency of effect. For example, a chemical that is known for certain to exert a given health effect but that only does so at unrealistically high concentrations might be assigned a moderate rating for that health endpoint.

A detailed hazards table with ratings for every chemical and endpoint is available in Appendix B. For simplicity and interpretability, the body of the report contains summary tables for each strategy. These tables contain one rating per hazard category for each chemical. This rating is reflective of the most hazardous rating the chemical received for any endpoint within the category. For example, if a chemical received four "low hazard" ratings and one "high hazard" rating for ecotoxicity, the chemical has a "high hazard" rating for ecotoxicity in its summary table. This was done to aid health-protective decision-making by presenting the most conservative possible representation of a chemical's safety.

Our ultimate goal is to improve MycoWorks' material while maintaining its superior performance to animal leather on health and environmental endpoints. As such, we performed the process outlined above for chromium tanning and used its hazards as a baseline for comparison with our recommended strategies. We chose tanning because it is the most analogous step in the leather-making process to our strategies. We chose chromium tanning in particular because it is the most common way conventional leather is produced.

Choice of solvent is crucial to the safety of any strategy MycoWorks chooses to implement. At the same time, many of the strategies we outline in this report share common solvents. We therefore exclude them from each strategy's summary table to make the tables more clear and conducive to comparisons between strategies. For the hazards associated with the various solvents involved in our strategies, see Table 8.

Hazards assessment by strategy

Cross-linker: Genipin

Chemical/Strategy	Chronic Human Health	Acute Human Health	Ecotoxicity	Fate	Physical
Genipin	M	M	O	L	L

Table 4. Summary hazard table for cross-linking with genipin

While many data gaps still exist, the overall hazard of the genipin strategy, which includes the use of a solvent to dissolve genipin powder and facilitate the cross-linking reactions, is moderate (Table 4). The risk is greater with a solvent like acetic acid, but it is a weak acid that is primarily corrosive at high concentrations (Table 8). MycoWorks might be able to minimize the health and environmental impact of the material by using a water-based solution instead if they are able to achieve an acceptable level of cross-linking. The environmental fate and physical/chemical hazards are also low. The ecotoxicity of genipin remains a data gap, but there is potential for minimal ecotoxicity since the parent compound, geniposide, serves as a defense mechanism for the gardenia plant as previously described.

The primary human health concern of the overall strategy is acute health endpoints. No studies have been conducted for inhalation of genipin powder, but genipin could cause skin sensitization at low concentrations as illustrated by a patch test (Bircher, Sigg, Scherer Hofmeier, Schlegel, & Hauri, 2017). Solutions of dissolved genipin powder thus must be handled with care in an occupational setting. Carcinogencity and mutagencity are of probable concern at high concentrations if directly consumed (Hou, Tsai, Lai, Chen, & Chao, 2008; Yamazaki, Chiba, & Yoshikawa, 2009), but unlikely to occur in an occupational setting. Studies that examined biocompatibility and cytotoxicity of materials cross-linked with genipin or direct *in vitro* exposure to genipin also revealed good biocompatibility as well as minimal cytotoxicity (Fessel, Cadby, Wunderli, van Weeren, & Snedeker, 2014; Gorczyca et al., 2014; Tsai, Huang, Sung, & Liang, 2000; Yuan et al., 2007; Zhang et al., 2010). Risk for chronic health endpoints are thus likely minimal. Besides posing a probable occupational hazard, the application of this cross-linking strategy into the MycoWorks material will not pose a health hazard to consumers.

Cross-linker: Enzymatically-derived o-quinone

Chemical	Chronic Human Health	Acute Human Health	Ecotoxicity	Fate	Physical
Tyrosinase	L	M	O	O	L
p-Cresol	M	H	M	O	M

Table 5. Summary hazard table for tyrosinase cross-linking strategy

Enzymes generally present low hazards outside of two acute human health endpoints: allergenicity and irritation. Exposure to a foreign protein, in this case the enzyme, can cause an adverse immune response mediated by allergen-specific immunoglobulin E (IgE) antibody formation. Therefore, tyrosinase would present a potential occupational hazard but minimal hazard to the person handling the final product.

Hazards must also be considered for the phenolic substrate. In Kumar et al. (1999), p-cresol is the studied phenolic substrate. p-cresol admittedly presents hazards for chronic human health, ecotoxicity, and physical endpoints. Its main risks are severe skin burns and eye damage on contact. Fortunately, tyrosinase can accept many phenolic substrates (derivatives of phenol such as o-cresol), meaning MycoWorks can choose their phenolic substrate based on the level of hazard risk they are comfortable with. For other phenolic substrates that may represent lower hazard alternatives, see Halfon et al. (1986).

Cross-linker: Nanocomposite

Chemical	Chronic Human Health	Acute Human Health	Ecotoxicity	Fate	Physical
Chitin Nanowhiskers	L	H	L	L	L
2-iodophenylboronic acid	M	H	L	O	L
Suberic acid	L	M	L	L	L

Table 6. Summary hazard table for nanocomposite strategy

Many nanomaterials are small enough that there are concerns that they may enter the cell and exert cytotoxic effects. Fortunately, this does not appear to be the case for chitin nanowhiskers (Zeng, He, Li, & Wang, 2012). However, respirability is still a concern due to their small size. Otherwise, chitin is generally thought of as relatively low-hazard; and proposed uses of chitin nanowhiskers include food packaging and biomedical applications (Zuber, Zia, & Barikani, 2013; Zeng, He, Li, & Wang, 2012; Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Kumar, 2000; Prashanth & Tharanathan, 2007). Given the available information, we believe nanowhiskers to be relatively low hazard.

Suberic acid is also of relatively low concern. Dicarboxylic acids have potential for skin, eye, and respiratory irritation (Johnson, Pollock, & Cantrell, 2000), but typically at high concentrations. This is a lesser concern as the dicarboxylic acid increases in molecular weight (Szilagyi, 2001). Apart from these acute health endpoints and minor evidence suggesting target organ toxicity to the kidneys (Johnson, Pollock, & Cantrell, 2000), dicarboxylic acids are generally thought to be non-toxic.

There is relatively little information available for 2-iodophenyl boronic acid. Available data suggest that it is of low concern for ecological endpoints, but presents significant acute workplace hazards such as skin and eye irritation (Fail, Chapin, Price, & Heindel, 1998). We must turn to other boronic acids for additional information. There is evidence to suggest that boronic acids are relatively safe from a neurotoxicity and mutagenicity standpoint, exerting these effects but only at unrealistically high doses (Soriano-Ursúa, Farfán-García, López-Cabrera, Querejeta, & Trujillo-Ferrara, 2014; Hansen, Jolly, & Linder, 2015). Concerningly, many boron-containing compounds are reproductive and developmental toxicants (Fail, Chapin, Price, & Heindel, 1998). Although, research specific to 2-iodophenyl boronic acid is needed for these endpoints.

Moisture barrier: corn zein coating

Chemical	Chronic Human Health	Acute Human Health	Ecotoxicity	Fate	Physical
Corn Zein	L	L	O	L	L
PEG	L	L	L	M	L

Table 7. Summary hazard table for corn zein coating

Overall, the corn zein strategy presents low hazard relative to animal leather production and to the other strategies we propose. Corn zein, being a derivative of corn, has low inherent toxicity, although allergenicity could be a concern for people with a corn allergy. This would present an occupational hazard for corn-allergic workers handling the material, although the risk of allergenicity would be minimal for people handling the end product. Even the occupational allergenicity hazard is low compared to that of animal leather production: worker exposure to chromium, which is used as a tanning agent in animal leather production, can cause such severe hypersensitivity (Were, Moturi, & Wafula, 2014; Ahmed, Mushtaq, Khan, & Khan, 2013).

The overall low toxicity of corn zein is exemplified by the fact that it is edible and reflected by its Generally Recognized As Safe (GRAS) designation by the FDA. However, it is important to keep in mind that scientific consensus on hazard is not always required to establish the GRAS label for a given food or food additive, so this label alone should not be cited as evidence of lack of hazard.

PEG also presents overall low hazard, notably in the categories of carcinogenicity, mutagenicity/genotoxicity, and acute mammalian toxicity. However, data gaps exist in the categories of reproductive, developmental, and endocrine toxicity, as well as in neurotoxicity. As for ecotoxicity, PEG presents medium hazard for persistence.

Solvents

Chemical	Strategies Used/Possible	Chronic Human Health	Acute Human Health	Ecotoxicity	Fate	Physical
Acetic acid	Conventional leather, Genipin	L	H	M	L	M
Ethanol	Corn zein, Genipin	M	H	L	L	H
Hydrochloric acid	Nanowhiskers, Tyrosinase	M	H	L	L	M
Sodium hydroxide	Nanowhiskers	M	H	H	M	M

Table 8. Summary hazard table for solvents

There is substantial overlap in which strategies use which solvents. While we have attempted to characterize solvent hazard in the above table, this is difficult and this table should be interpreted cautiously. While all of our potential solvents may exert acute toxic effects (hence, the high hazard rating for all of them), they do so at different concentrations. Based on their chemical activities, ethanol and acetic acid are much less potent than hydrochloric acid and sodium hydroxide in this regard, and should be regarded as safer. Where possible, solvent concentrations should be adjusted to ensure maximum workplace safety.

Comparative Analysis

Strategy comparison: technical performance

Overall, the genipin, nanowhiskers, and corn zein strategies demonstrated the potential to improve the MycoWorks material's technical performance. Among the cross-linking strategies, genipin showed the potential to improve the MycoWorks material's strength and water resistance with minimal expected impact on the flexibility of the material. The tyrosinase strategy's technical performance impact is inconclusive based on the literature. The nanowhiskers strategy showed the ability to improve the strength and flexibility of materials similar to the MycoWorks material by a factor of 1.5. Across the remaining technical performance metrics—longevity through washes, handfeel, discoloration—we found inconclusive information except for genipin, may tint the material blue.

From a technical feasibility standpoint, the implementation of each strategy inherently implies that the existing MycoWorks manufacturing process must include additional steps and complexity. Relative to each other, though, genipin represents the simplest cross-linking strategy, followed by the enzymatic strategy and the nanowhiskers strategy. The corn zein strategy should also be a relatively straightforward moisture barrier strategy to implement.

Strategy comparison: hazard assessment

Chemical / Strategy	Chronic Human Health	Acute Human Health	Ecotoxicity	Fate	Physical Hazard
Animal Leather	H	H	H	O	M
Genipin	M	M	O	L	L
Tyrosinase	L	M	O	O	L
Nanocomposite	M	H	L	O	L
Corn zein	L	L	O	L	L

Table 9. Summary hazard table for each strategy and animal leather

Relative to our baseline (animal leather), our strategies had relatively few hazards. Hazards associated with leather tanning are outlined in detail in the introduction. Based on chromium's allergenicity, ability to pollute nearby water bodies, and potential to change valence state to the carcinogenic chromium(VI), we rate the overall hazard of animal leather tanning as high hazard.

We do not regard any of our strategies as high hazard, and perceive them all to be significantly safer than conventional leather. The most hazardous of our strategies is the nanocomposite method. The potential for skin and eye irritation is strong in this strategy, and boronic acids may present chronic health risks such as reproductive toxicity and mutagenicity.

On the other end of the spectrum, corn zein is the safest of our strategies. The only potential risk associated with corn zein is exposure of workers with corn allergies. Corn zein is used in food and pharmaceuticals, so it is otherwise relatively safe. Enzymatic cross-linking poses similar but slightly more severe risks to corn zein, with possible allergenicity and irritation. Tyrosinase and other enzymes are otherwise generally regarded as low-hazard. However, the hazards associated with the phenolic substrate must be considered. We presented p-cresol as an example, but tyrosinase may accept a variety of phenolic substrates, allowing MycoWorks to cater their substrate to their desired hazard level.

Genipin's hazards are more severe than corn zein and tyrosinase, but less so than the nanocomposite strategy. We categorize genipin as moderate hazard, mostly due to the possibility of skin and eye irritation at relatively low concentrations. This may be mitigated with proper personal protective equipment, but is still an issue nonetheless. Our next, less serious concern with genipin is its potential for carcinogenicity and mutagenicity if directly consumed at very high concentrations. Since this is unlikely, it is worth keeping in mind but is not particularly concerning.

Recommendations

Among the cross-linking strategies, genipin is our front runner because the overall process is relatively simple and can be easily integrated into the manufacturing steps MycoWorks has already established. It has great potential to address desired performance properties of increased strength, flexibility, and hydrophobicity. However, one must note that the technical performance parameters are mostly based on non-textile chitosan-based composite films without cellulose, so the properties may be different. The performance metrics were also not reported or measured consistently across studies. A foreseeable downside to this strategy so far is that it will tint the material blue, but if other coloration processes are going to be applied post-manufacturing, it may not be relevant. Further, the concentration of genipin used may be modified to mitigate this problem.

We also recommend testing a corn zein coating since corn zein films are a well-established moisture barrier. This simple addition to the MycoWorks post-harvesting process could prevent PEG from leeching out. The overall strategy is low-hazard, uses commercially available components, and is simple to implement. However, further research is needed to determine the coating's effectiveness on the MycoWorks material. If de-colorization is necessary, MycoWorks could apply proven de-colorization strategies already developed for corn zein films. Together, these cross-linking and moisture barrier strategies could lead to a durable MycoWorks material that is strong, flexible, and washable.

Next Steps + Conclusion

Next steps

To apply the recommended strategies we propose several next steps. For cross-linking with genipin, MycoWorks should understand how the material is impacted by the various steps in the procedure leading to cross-linking, e.g. introduction of the material to solvents of varying pHs, as well optimize the processing steps for their own application. Factors that should be optimized include:

- Amount of deacetylation needed to achieve an optimal amount of chitosan
- Minimum genipin concentration needed to achieve acceptable material strength
- Optimal incubation period
- Optimal pH for cross-linking to occur and to minimize blueness
- Temperature if room temperature is not sufficient

After experimenting with the methodology and varying factors such as genipin concentration, MycoWorks may also want to use a scanning electron microscope (SEM) to understand the material's molecular structure and degree of cross-linking in addition to testing the tensile strength and flexibility. Analyzing an SEM image could allow MycoWorks to identify the optimal reaction conditions to achieve the best product possible.

Additional significant additional research is needed to determine the viability of the corn zein strategy for the MycoWorks material since to our knowledge, corn zein coating has not been applied to textiles. MycoWorks should test the durability of the coating through washing and investigate the effect of temperature and humidity on the coating's performance.
The following parameters should be optimized:

- Choice of plasticizer(s)
- Corn zein to plasticizer ratio
- Coating-formation conditions
- Choice and amount of drying oil, if the drying oil is found not to increase brittleness of the material

Conclusion

In this report, we have established the groundwork for finding greener solutions to traditional tanning and plasticizing chemicals used in the leather industry. Limiting our solutions to a post-harvest processing method that would not impact the development of MycoWorks' minimum viable product, we strove to balance health and environmental safety, technical feasibility, and overall sustainability in the development and lifetime of the product. We established a general approach for finding a greener solution by identifying the functional groups of traditional chemicals and translating biological cross-linking into chemicals. This was followed by evaluative frameworks for technical feasibility and health and environmental impacts.

Through this process, we identified three cross-linking strategies and one moisture barrier strategy that have never been applied within the textile industry. While all strategies lead to the desired performance properties and exhibit lower hazards relative to existing chemicals used for cross-linking and enhancing water resistance, our understanding is limited to findings from the bioengineering and biomedical fields.

Therefore, MycoWorks must experimentally optimize the recommended strategies to understand the true impacts to their material. Combining one or more of the proposed strategies could also achieve the optimum strength, flexibility, and durability and is worth experimenting with. Moving forward, we recommend that MycoWorks use our research approach, the two frameworks, and the information contained in this report to pursue alternatives besides the two recommended here.

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Annotation: This paper contains a lot of information on the details of amide bond formation using 2-iodophenylboronic acid. The reaction is waste-free in that it is very efficient. Depending on the solvent used, there is 71% (in tetrahydrofuran) or 91% (in dichloromethane) amide bond formation between an amide and a carboxylic acid when IPBA is used. The super cool thing about this is that the reaction was done at room temperature, which is a big improvement on the energy cost of traditional methods of amide bond formation, which require much higher temperatures.

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Annotation: Zein coatings are usually prepared by dissolving zein powder in aqueous ethanol or isopropanol, and plasticizers such as propylene glycol, glycerin, and acetylated glycerides are added. The product can be dipped into, sprayed with, or brushed with the zein/plasticizer solution. The authors also provide measures of water vapor permeability and oxygen permeability of zein coatings. This book is a compiles information about films and coatings formed of different proteins including corn zein, wheat gluten, and fish myofibrillar protein.

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Grolik, M., Szczubiałka, K., Wowra, B., Dobrowolski, D., Orzechowska-Wylęgała, B., Wylęgała, E., & Nowakowska, M. (2012). Hydrogel membranes based on genipin-cross-linked chitosan blends for corneal epithelium tissue engineering. Journal of Materials Science: Materials in Medicine, 23(8), 1991–2000. https://doi.org/10.1007/s10856-012-4666-7

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Hedberg, Y. S., & Lidén, C. (2016). Chromium(III) and chromium(VI) release from leather during 8 months of simulated use. *Contact Dermatitis*, *75*(2), 82–88. https://doi.org/10.1111/cod.12581

Hou, Y. C., Tsai, S. Y., Lai, P. Y., Chen, Y. S., & Chao, P. D. L. (2008). Metabolism and pharmacokinetics of genipin and geniposide in rats. Food and Chemical Toxicology, 46(8), 2764–2769. https://doi.org/10.1016/j.fct.2008.04.033

Annotation: This study explored how geniposide is metabolized in vivo via a rat model. Geniposide is metabolized into genipin which is then metabolized into genipin sulfate. Post-metabolism, genipin sulfate was found in plasma whereas genipin was not, suggesting rapid metabolism during the first pass through the liver and intestine. 7/9 rats that received 200 mg/kg of genipin died while 8/8 rats that received 100 mg/kg of genipin all survived well throughout the study. This suggests that genipin is toxic at higher concentrations and dosage should be properly decided to ensure safety.

Ifuku, S. (2014). Chitin and Chitosan Nanofibers: Preparation and Chemical Modifications. *Molecules*, *19*(11), 18367–18380. https://doi.org/10.3390/molecules191118367

Annotation: This paper has instructions on how to produce chitin and chitosan nanowhiskers from various sources of chitin. It is useful because it details how to derive chitin nanowhiskers from commercial chitin powders. This method is simple, requiring acidic water and a grinder. There is an explanation about the underlying forces which allow this method to work. This ease of conversion was attributed also to the electrostatic repulsion effect as described above. The repulsive force caused by the cationization of amino groups facilitated fibrillation into NFs.

Jayakumar, R., Menon, D., Manzoor, K., Nair, S. V., & Tamura, H. (2010). Biomedical applications of chitin and chitosan based nanomaterials—A short review. *Carbohydrate Polymers*, *82*(2), 227–232. https://doi.org/10.1016/j.carbpol.2010.04.074

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Annotation: Good paper that explains the class of plant metabolites that geniposide is in and can be referred to if MycoWorks wants to pursue other compounds that might not cause a blue side-effect but potentially leads to effective cross-linking.

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Annotation: In this paper, the authors demonstrate an enzymatic method to form chitosan gels. The authors use the enzyme tyrosinase to convert a phenolic substrate (p-cresol) into the more reactive o-quinone, which then reacts with chitosan. The authors are not sure of the exact chemistry going on, but they have evidence that o-quinone acts as a cross-linker with the chitosan. The cross-linking results in a more viscous gel.

Kumar, M. N. V. R. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1–27. https://doi.org/10.1016/S1381-5148(00)00038-9

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Oh, D. X., & Hwang, D. S. (2013). A biomimetic chitosan composite with improved mechanical properties in wet conditions. *Biotechnology Progress*, *29*(2), 505–512. https://doi.org/10.1002/btpr.1691

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Pattabiraman, V. R., & Bode, J. W. (2011). Rethinking amide bond synthesis. *Nature*, *480*(7378), nature10702. https://doi.org/10.1038/nature10702

Annotation: Ideas for how to form amide bonds that doesn't involve diisocyanates. Looking to replace HDS, thus the catalyst for the amide bond formation needs to be a lower hazard than HDS. Several suggestions are made, though most use chemicals that are also hazardous, for example metals. Most promising is the boronic acid catalysts. 2-iodophenylboronic acid and 3,4,5-trifluorophenylboronic acid are mentioned in the section. Looking into details of the two, 2-iodophenylboronic acid is the most promising. It is also cited as being a greener solution for amidation of carboxylic acids.

Peng, H., Xiong, H., Li, J., Xie, M., Liu, Y., Bai, C., & Chen, L. (2010). Vanillin cross-linked chitosan microspheres for controlled release of resveratrol. Food Chemistry, 121(1), 23–28. https://doi.org/10.1016/j.foodchem.2009.11.085

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Sampaio, G. Y. H., Fook, A. C. B. M., Fidéles, T. B., Cavalcanti, M. E. R. R. M., & Fook, M. V. L. (2014). Biodegradable Chitosan Scaffolds: Effect of Genipin Crosslinking. Materials Science Forum, 805, 116–121. https://doi.org/10.4028/www.scientific.net/MSF.805.116

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Shukla, R., & Cheryan, M. (2001). Zein: the industrial protein from corn. Industrial Crops and Products, 13(3), 171–192. https://doi.org/10.1016/S0926-6690(00)00064-9

Annotation: The authors discuss how corn zein is currently produced and in what products it is commonly used. They explain that corn zein is not used in food items since it lacks essential amino acids such as lysine and tryptophan, and is therefore of low nutritional quality. The authors also describe the chemical composition of zein and the protein structure, as well as methods for maintaining zein in a liquid rather than gel state – this is essential for applying it as a coating.

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Annotation: The findings of this study contrasted that of some others that studied the long-term effect of taking Chinese medicine with the gardenia fruit extract for about an average of 20 years. They determined that the maximum concentration before mutagenicity occurs is 50 ppm, which is equivalent to 50 mg/L. This is another study that suggests that genipin is toxic at high concentrations.

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Xu, Y., Li, L., Yu, X., Gu, Z., & Zhang, X. (2012). Feasibility study of a novel crosslinking reagent (alginate dialdehyde) for biological tissue fixation. Carbohydrate Polymers, 87(2), 1589–1595. https://doi.org/10.1016/j.carbpol.2011.09.059

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Annotation: This manuscript studies an enzymatic method for inducing covalent cross-linking in chitosan to create hydrogels. Specfically, the authors use tyrosinase, an enzyme derived from fungus. The results indicate that tyrosinase can successfully induce cross-linking in chitosan

Zheng, C., YunYu, H., & Al), P. W. (et. (2009). Effect of temperature on crosslinking of collagen/chitosan scaffolds with genipin. Zhongguo Jiaoxing Waike Zazhi / Orthopedic Journal of China, 17(3), 217–220.

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Appendices

Appendix A: Record of Methodologies

Approach

In the animal kingdom the armored scales of fish, the strong carapaces of arthropods, and the flexible 'shells' of cephalopods demonstrate the varied applications of chitin in the naturally-evolved world. These creatures use strategies which include natural tanning, cross-linking, and the formation of nanocomposites to change the innate physical properties of chitin. On its own chitin is not very durable, strong, or flexible; its ability to be transformed on the molecular- and nano-scales makes it a malleable material for use as a base in many applications.

Knowing that many living creatures use chitin in varied ways, our first step was to look to nature for inspiration. We looked at crustaceans, jellyfish, beetles, and even kelp to get an idea of how nature builds materials which are durable, strong, and flexible. Existing work which focused on analysis and mimicry of arthropod cuticles held the most directly-applicable path of inquiry. Initial forays led us to interesting done to create chitin and chitosan films and hydrogels. Most of the techniques we researched use compounds and solvents which are problematic from health, environment, and worker exposure perspectives. We did, however, learn about the underlying chemical mechanisms driving cross-linking in chitin and chitosan. We chose to investigate the nanocomposite cross-linking strategy.

Enzyme-based solutions are generally less hazardous and more sustainable than chemistry-based solutions, so we sought out ways to induce cross-linking using enzymes. This research resulted in the tyrosinase-mediated cross-linking strategy.

We also investigated the chemical mechanisms used to cross-link proteins in leather processing. With an understanding of the chemical reactions induced by catechol and pyrogallol tannins we searched for a direct replacement which could perform the same function in chitosan. The genipin cross-linking solution was produced by this research.

Examples of search queries

Undirected queries:

- poly carbamoyl sulfonate crosslinking mechanism
- glutaraldehyde crosslinking chitin tensile strength
- malondialdehyde crosslinking tensile "strength"
- piperidine chrome tanning
- pyridine chrome tanning
- Carboxymethyl cellulose tensile strength chitin
- jellyfish mesogloea structure
- mtgase cross-linking chitin

Natural cross-linkers:

- alternative to glutaraldehyde crosslinking* vanillin
- chitosan genipin cross linking tensile "strength" strain
- chitosan genipin tensile strength

- chitosan "genipin" "tensile strength"

Enzymatic cross-linking:

- enzyme cross-linking chitosan film

Nanocomposite cross-linking:

- chitosan hydrogels
- amide bond catalysts
- green amide bond formation
- iodophenylboronic acid
- Wikipedia search for dicarboxylic acids
- green screen assessment for dichloromethane

Moisture barriers:

- protein-based food packaging coatings
- compostable food packaging coatings
- water insoluble proteins films
- corn zein coating textiles
- corn zein film moisture resistance
- corn zein film plasticizer
- drying oil moisture resistance

Appendix B: Full Hazards Assessment Table

Green Screen (v.1.2) Hazard Profile Summary Table																				
			Human Health Effects													Ecotox.		Fate		P-Chem	
			Group I					Group II													
	Chemical	CAS #	Carcinogenic (C)	Mutagenic/ Genotoxic (M)	Reproductive (R)	Developmental (incl Neuro. Tox) (D)	Endocrine Activity (E)	Acute Mammalian Tox (AT)	Systemic Tox/Organ Effects (incl. Immune) (ST)	Neurotoxicity (N)	Skin Sensitization (SnS)	Respiratory Sensitization (SnR)	Irritation/ Corrosivity Skin (IrS)	Irritation/ Corrosivity Eye (IrE)	Acute Aquatic Toxicity (AA)	Chronic Aquatic Toxicity (CA)	Persistence (P)	Bioaccumulation (B)	Reactivity (Rx)	Flammability (F)
Animal leather*	Chromium(III) Sulfate	13825-86-0	O	H	O	O	O	L	M	O	H	M	H	M	M	M	O	O	M	M
	Sulfuric acid	7664-93-9	H	L	L	M	O	H	H	O	L	H	H	H	M	H	H	L	M	M
	Acetic acid		L	L	O	L	O	M	L	L	M	M	H	H	M	L	L	L	M	M
	Sodium bicarbonate	144-55-8	L	L	M	L	M	H	M	O	O	L	M	M	L	L	H	O	M	L
Corn zein	Corn zein	N/A	L	L	L	L	L	L	L	L	L	O	L	O	O	O	L	L	L	L
	PEG	25322-68-3	L	L	O	O	O	L	O	O	L	L	L	L	L	L	M	L	L	L
	Ethanol	64-17-5	L	L	L	M	O	L	M	M	L	O	L	H	L	L	L	L	L	H
Genipin	Genipin	6902-77-8	M	M	O	O	O	O	O	O	M	L	O	L	O	O	L	L	L	L
	Acetic acid	64-19-7	L	L	O	L	O	M	L	L	M	M	H	H	M	L	L	L	M	M
Enzymatic	Tyrosinase	9002-10-2	L	L	L	L	L	L	L	L	L	M	L	M	O	O	O	O	L	L
	p-cresol	106-44-5	M	O	O	O	M	M	O	O	H	M	H	H	M	M	O	O	M	M
Chitin Nanowhiskers	Chitin nanowhiskers	1398-61-4	L	L	L	L	L	L	M	L	H	H	O	O	L	L	L	L	L	L
	2-iodophenylboronic acid	1008106-86-2	L	L	M	M	L	O	L	L	H	O	M	H	L	L	O	O	L	L
	Suberic acid	505-48-6	L	O	L	L	O	L	M	O	L	O	M	M	L	L	L	L	L	L

*There are many steps to leather production and many different types of leather production. We selected the tanning step of the process because this step confers the strength and flexibility MycoWorks aims to replicate and is therefore most analogous to our proposed strategies. Further, we chose Cr tanning instead of vegetable tanning because 90% of leather production in the US uses Cr tanning (US EPA)

Appendix C: Alternative Solvents

In literature the catalyst 2-iodophenylboronic acid catalyzes amide bond formation between carboxylic acids and primary amines while dissolved in dichloromethane. As the hazard assessment shows, this solvent is not ideal for use in an occupational setting since it is a known carcinogen. Dichloromethane is used extensively in paint and varnish strippers, thus it became a "priority product" for replacement under the California Safer Consumer Products regulations. BizNGO, a collaboration between universities, government, environmental groups, and consumer products manufacturers, used the GreenScreen comparative hazard assessment method to direct a search for safer alternatives to dichloromethane. In total eleven alternatives were identified, and their GreenScreen hazard assessments included in the report (Jacobs, Wang, & Rossi, 2015). Below we have attached the assessment for each alternative, however we strongly suggest reading the entire report as it is available for free.

GreenScreen® Hazard Assessment Results

Chemical Name	CASRN	Group I Human					Group II & II Human									Ecotox		Fate		Physical	
								ST		N											
		C	M	R	D	E	AT	Single	repeated	Single	repeated	SnS	SnR	IrS	IrE	AA	CA	P	B	RX	F
Methylene chloride	75-09-2	H	NE	DG	DG	M	*M*	vH	H	vH	vH	L	DG	H	H	M	*L*	vH	vL	L	L
Benzyl alcohol	100-51-6	L	L	*L*	M	DG	M	L	L	*M*	*H*	H	*L*	L	H	L	L	vL	vL	*L*	L
2-(2-butoxyethoxy) ethanol	112-34-5	*L*	L	L	L	DG	L	L	H	DG	L	L	DG	*M*	H	L	L	vL	vL	*L*	M
Dimethyl sulfoxide	67-68-5	*L*	L	L	L	DG	L	L	L	L	L	L	L	*M*	*M*	L	*L*	*L*	vL	*L*	M
1,3-dioxolane	646-06-0	L	*M*	*M*	M	DG	L	M	M	M	L	L	DG	*M*	*H*	L	L	*M*	vL	*L*	H
Estasol (dibasic esters mixture)	95481-62-2	*L*	L	L	*M*	*M*	L	M	*M*	M	DG	L	DG	L	*M*	M	L	vL	vL	*M*	L
d-Limonene	5989-27-5	L	L	DG	L	DG	L	L	L	DG	DG	H	DG	H	*H*	vH	H	vL	M	*L*	M
Acetone	67-64-1	*L*	L	*M*	M	DG	L	*M*	M	M	*M*	L	DG	L	H	L	L	vL	vL	L	H
Methanol	67-56-1	NA	NA	NA	H	NA	H	vH	NA	NA	NA	NA	NA	NA	NA	L	*L*	vL	vL	NA	H
Toluene	108-88-3	DG	L	H	H	M	L	M	H	M	H	L	DG	H	L	H	*H*	*H*	vL	L	H
Formic acid	64-18-6	L	L	L	*L*	DG	H	vH	*H*	vH	DG	L	DG	vH	vH	M	M	vL	vL	L	M
Caustic soda	1310-73-2	*L*	L	*L*	*L*	*L*	H	vH	*L*	*L*	*L*	L	DG	vH	vH	M	DG	*L*	vL	*M*	L

Abbreviations

C = Carcinogenicity
M = Mutagenicity
R = Reproductive Toxicity
D = Developmental Toxicity
E = Endocrine Activity
AT = Acute Toxicity
ST = Systemic Organ Toxicity
N = Neurotoxicity
SnS = Skin Sensitization
SnR = Respiratory Sensitization
IrS = Skin Irritation
IrE = Eye Irritation
AA = Aquatic Toxicity
CA = Chronic Aquatic Toxicity
P = Persistence
B = Bioaccumulation
RX = Reactivity
F = Flammability

Note

Hazard levels (Very High (vH), High (H), Moderate (M), Low (L), Very Low (VL) in italics reflect estimated (modeled values, authoritative B lists, screening lists, weak analogues, and lower confidence. Hazard levels in BOLD are used with good quality data, authoritative A lists, or strong analogues. Group II Human Health endpoints differ from Group II Human Health endpoints in that they have four hazard scores (i.e., vH, H, M and L) instead of three (i.e., H, M and L), and are based on single exposures instead of repeated exposures. DG indicates insufficient data for assigning hazard level. NE indicates no determination was made (conflicting data).

Figure ___. GreenScreen comparative hazard assessment of dichloromethane and its alternatives (Jacobs et al., 2015)

References:

Jacobs, M., Wang, B., & Rossi, M. S. (2015). Alternatives to Methylene Chloride in Paint and Varnish Strippers. *BizNGO*. Accessed November 29, 2017. http://www.biznGO.org/alternatives-assessment/methylene_report_request

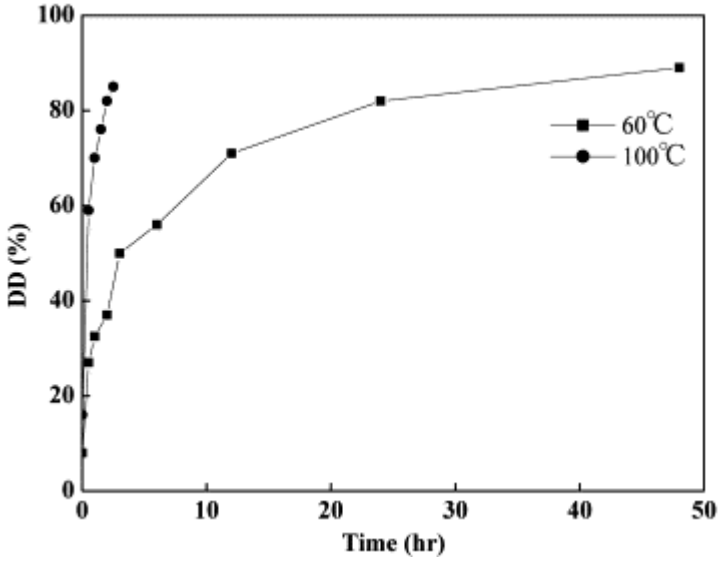
Appendix D: Potential strategies for deacetylating chitin

Although the polysaccharide chitin is theoretically composed of N-acetylglucosamine units, in nature it also contains deacetylated units, called glucosamine units, randomly interspersed. The difference between the two is that the acetamide functional group on N-acetylglucosamine has been deacetylated and is a primary amine on glucosamine. Primary amines are readily available for a variety of cross-linking chemical reactions. Thus the more glucosamine groups available on chitin the more cross-linking can occur. Once glucosamine makes up 50% or more of the polysaccharide, it is called chitosan instead of chitin.

Deacetylation of chitin is necessary to make it able to cross-link. In fungal species a subset of enzymes called carbohydrate esterase enzymes, also known as chitin deacetylases, catalyze the transformation of N-acetylclucosamine into glucosamine (Geoghegan & Gurr, 2017). This class of enzymes is not commercially available at this time.

The most common and most cost effective method to deacetylate chitin is through treatment with sodium hydroxide (NaOH). Various conditions have been employed. Below are the conditions as reported in literature. Full deacetylation of the MycoWorks MVP is neither needed nor desired. The time and temperature for incubation with sodium hydroxide should be lowered as necessary to ensure the material does not become overly acetylated.

- Chitin added to an aqueous 40% by weight sodium hydroxide solution. Heated to a high temperature, though below boiling (e.g. 80°C). It is held at this temperature until the desired degree of acetylation (DA) is reached. The longer the reaction is done, the lower the DA (Tolaimate et al., 2000) Three hours results in DA of 25%, 6 hours in DA of 3%, and 9 h ours in DA of 1%.
- If the deacetylation reaction in 40% NaOH is done at 60°C it is easier to control the degree of deacetylation, though the reaction does take longer (Min et al., 2004)



- Chitin added to 2M aqueous sodium hydroxide solution. Held at 24°C (75°F) for 3 hours. The DA decreased from 40.8% to 26.3% (Pires, Vilela, & Airoldi, 2014).
- Another study was done to determine the influence on the degree of acetylation by different time and temperature combinations (Chang, Tsai, Lee, & Fu, 1997)

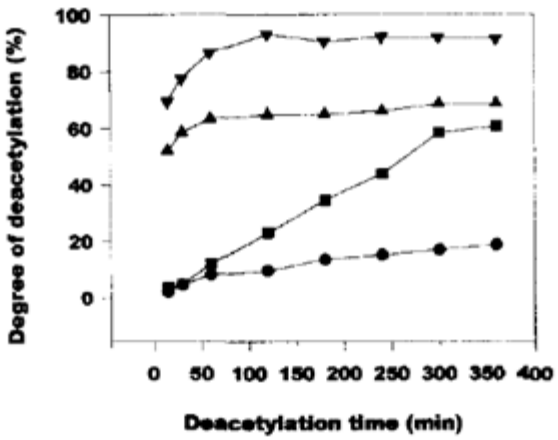


Fig. 1. Time-course of alkaline *N*-deacetylation of shrimp shell chitin under different conditions. ● 25 mL solution/g chitin, 35% NaOH, 70 °C; ■ 25 mL solution/g chitin, 35% NaOH, 110 °C; ▲ 36.89 mL solution/g chitin, 51.89% NaOH, 133.78 °C; ▼ 36.89 mL solution/g commercial chitin, 51.89% NaOH, 133.78 °C.

A third approach to deacetylating chitin is known as the Broussignac method. It uses a reagent a mixture of solid potassium hydroxide (50%, w/w), 96° ethanol (25%, w/w) and monoethyleneglycol (25%, w/w) (Tolaimate et al., 2000). Chitin is added and the mixture is heated. After 2 hours at 120°C the degree of acetylation is 4% (Tolaimate et al., 2000).

References:

Chang, K. L. B., Tsai, G., Lee, J., & Fu, W.-R. (1997). Heterogeneous N-deacetylation of chitin in alkaline solution. *Carbohydrate Research*, *303*(3), 327–332. https://doi.org/10.1016/S0008-6215(97)00179-1

Geoghegan, I. A., & Gurr, S. J. (2017). Investigating chitin deacetylation and chitosan hydrolysis during vegetative growth in Magnaporthe oryzae. *Cellular Microbiology*, *19*(9). https://doi.org/10.1111/cmi.12743

Min, B.-M., Lee, S. W., Lim, J. N., You, Y., Lee, T. S., Kang, P. H., & Park, W. H. (2004). Chitin and chitosan nanofibers: electrospinning of chitin and deacetylation of chitin nanofibers. *Polymer*, *45*(21), 7137–7142. https://doi.org/10.1016/j.polymer.2004.08.048

Pires, C. T. G. V. M. T., Vilela, J. A. P., & Airoldi, C. (2014). The Effect of Chitin Alkaline Deacetylation at Different Condition on Particle Properties. *Procedia Chemistry*, *9*(Supplement C), 220–225. https://doi.org/10.1016/j.proche.2014.05.026

Tolaimate, A., Desbrières, J., Rhazi, M., Alagui, A., Vincendon, M., & Vottero, P. (2000). On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer*, *41*(7), 2463–2469. https://doi.org/10.1016/S0032-3861(99)00400-0

Appendix E: Additional Potential Strategies for R&D

Enzymatic cross-linking

Carbohydrate-binding modules

Reference: Malho J-M, Arola S, Laaksonen P, Szilvay GR, Ikkala O, Linder MB. Modular Architecture of Protein Binding Units for Designing Properties of Cellulose Nanomaterials. *Angew Chem Int Ed*. 2015;54(41):12025-12028. doi:10.1002/anie.201505980.

Annotation: This manuscript explores the use of carbohydrate-binding modules (CBMs) for crosslinking and tuning the mechanical properties of cellulosic materials. This strategy is inspired by the hierarchical structures found in biological materials, where the mutliple length scales imbue the dual properties of high strength and flexibility. The key insight from the authors is examining the relationship between –mer linker length in the CBMs and its effect on the mechanical properties of the wet and dry cellulose films.

Transglutaminase

Reference: http://www.cookingissues.com/transglutaminase-aka-meat-glue/index.html

Reference: Chen, T., Embree, H. D., Brown, E. M., Taylor, M. M., & Payne, G. F. (2003). Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. *Biomaterials*, *24*(17), 2831-2841.

Annotation: Transglutaminase is an enzyme that has found popularity recently in the molecular gastronomy community. Colloquially termed “meat glue,” transglutaminase is a naturally occurring enzyme that modern chefs used to bind meat proteins together. The key question with this strategy is whether chitosan can be cross-linking to itself or chitin and cellulose. Given its prevalence in the food industry, most work has looked at transglutaminase cross-linking for creating edible films (e.g. chitosan + gelatain) rather than mechanically strong materials.

Appendix F: Team Member Biosketches

Katie Deeg is a 5th-year PhD candidate in Physical Chemistry at UC Berkeley. In her doctoral research, she uses computational chemistry methods to investigate nanoporous materials for the storage of clean fuels and removal of atmospheric pollutants. She holds a BA in Chemistry from Carleton College.

Zach Gima is a 4th-year PhD student in Mechanical Engineering at UC Berkeley. His doctoral research focuses on control and modeling applications for Li-ion batteries. His undergraduate degree from University of Southern California is also in Mechanical Engineering.

Audrey Smith is a 2nd-year MPH student in Environmental Health Sciences at UC Berkeley. Her recent projects include research on regulation of toxic chemicals in consumer products and on methods to integrate cumulative health impacts from multiple environmental hazards into toxicological risk assessments. She earned her BA in Ecology and Evolutionary Biology from Rice University.

Oana Stoica is a 2nd-year MPH student in Environmental Health Sciences at UC Berkeley in the Industrial Hygiene concentration. She has 11 years of experience working in the pharmaceutical and biotechnology industries, where she gained broad experience ranging from working in the cleanroom with human stem cells to evaluating the performance and stability of bacterial enzymes in household cleaning products. Oana has a BS in Biological Sciences and a BA in Comparative Literature from Binghamton University in New York.

Kathy Tran is a 4th-year PhD candidate in Environmental Health Sciences at UC Berkeley examining the health hazards associated with the energy sector. She possesses a BS in Molecular, Cell and Developmental Biology from UCLA and an MPH from Emory University with a concentration in Global Environmental Health. She has conducted lab-based research aimed towards identifying the cancer cell-of-origin in order to develop treatments to prevent or treat skin cancer. While pursuing her MPH, she conducted a health vulnerability assessment to heat among slum dwellers in India.