NEW APPROACHES IN COTTON CROSSLINKING

GREEN TEAM FINAL REPORT TO LEVI STRAUSS & CO.

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*These descriptions were prepared by the Biomimicry 3.8 Institute

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Green Team Final Report

FINAL REPORT

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GOAL

The goal of our collaboration with Levi Strauss & Company [LS&Co.] is to identify biologically inspired opportunities to modify crosslinking technologies currently used in their wrinkle-resistant and water repellent finishes. The new crosslinking technologies must comply with current manufacturing processes, produce cost-competitive products that meet consumer expectations, and reduce occupational and environmental health hazards.

CONTEXT + APPROACH

The Current Problem

The garment and textile industries have made great strides in meeting consumer demands for high-performance and low-maintenance 100% cotton apparel. Two of these performance features, wrinkle-resistance and water-repellency, are conferred using crosslinking chemicals. Unfortunately, there are significant health hazards surrounding the application of these features. Formaldehyde, released from crosslinking chemicals that are used to impart wrinkle resistance, is a known carcinogen and can cause nasopharyngeal irritation and sensitization (Figure 1a).[1] While LS&Co. regulates maximum formaldehyde concentrations in its final distributed products, the use of formaldehyde-based resins does pose significant occupational hazard prior to product distribution and consumer use. Diisocyanates, used to bind durable water repellent (DWR) coatings, pose hazards of similar concern. These chemicals can cause respiratory tract irritation, occupational asthma, and possibly cancer (Figure 1b).[2] There is an identified need for LS&Co., and the textile industry as a whole, to develop an alternative approach to crosslinking chemistry.

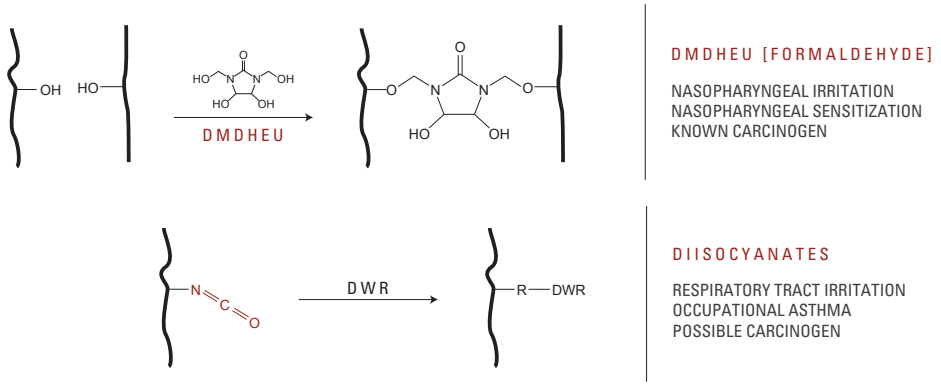

Figure 1a. DMDHEU, the most common formaldehyde-based resin used for cotton crosslinking, contributes to free formaldehyde in fabric and poses significant occupational health hazard.
Figure 1b. Diisocyanates, which bind water repellant coatings to cotton, pose potential respiratory hazard.

Inspiration from Nature

Nature has been evolving for over three billion years. The animals, plants, and microbes found in nature have used this time to engineer solutions to problems within their environment in a manner that is appropriate and sustainable to life. Biomimicry is a design discipline that draws upon nature's abilities and seeks sustainable solutions to humans' problems through the emulation of its patterns and strategies.[3] This discipline has procured innovative solutions to many challenges across a wide variety of industries, and we have used it to identify sustainable crosslinking technologies for the textile industry.

In partnership with the Biomimicry 3.8 Institute, we identified 12 examples of crosslinking within nature (Appendix A). Upon analysis, we noticed that nature employs a wide variety of bond types to achieve crosslinking. We distilled these bond types into four themes: covalent, non-covalent, metal-containing, and structural (Table 1).

Table 1. Examples of crosslinking within each of the four themes

Bond Type	**Strategy in Nature**	**Place of Use**
Covalent Bonds	Disulfide bonds	- Chinese soft shelled turtle - Human joint - Malaysian tree frog - Pearl Oyster
	Imine bonds	- Slug
	Peroxide-mediated bonds	- Flax stem
Non-Covalent Bonds	Hydrogen bonds	- Chinese soft shelled turtle - Chiton tooth - Deep sea sponge - Flax stem - Malaysian tree frog - Woody plants
Metal-Containing Bonds	Coordination complexes	- Blue sea mussel - Malaysian tree frog
	Direct metal crosslinking	- Slug
	Metal ion interfaces	- Chiton tooth - Human joint
Structural Bonds	Anti-parallel sheets	- Chinese soft shelled turtle
	Checkerboard reinforcement bonds	- Deep sea sponge
	Flexible coils	- Blue sea mussel - Vineyard snail
	Large, branched structures	- Flax stem
	Micelles	- Chinese soft shelled turtle

Additionally, we noticed that none of these aforementioned bonds work in isolation. Instead, several different bonding themes come together in a single organism to impart a stable and robust crosslink (Appendix B). We decided to emulate these two ideas in our biologically inspired crosslinking technologies.

Translation to Textile Crosslinking

Biological inspiration led us to consider textile crosslinking in a new way. Traditional textile crosslinking relies on a covalent bond, but we consider other bonding types. While all four bonding strategies demonstrate potential, metal-containing bonds are outside the scope of this report (Appendix C). Our suggestions focus on covalent bonds, non-covalent bonds, and structural bonds.

Traditional textile crosslinking also relies on one bonding step alone to impart the desired crosslink: in one step, the cellulose and crosslinking chemicals are joined (Figure 2a). We look at crosslinking as a two-step process. First, a bond is employed that attaches the crosslinking chemical to cellulose (Figure 2b). Second, a bond is employed that imparts the desired crosslinking property. In the case of wrinkle resistance, the crosslinking chemical will employ a second bond and attach to itself, thereby connecting the strands of cellulose (Figure 2c). In the case of water repellency, the crosslinking chemical will employ a second bond and attach to a durable water-repellent, thereby connecting the strand of cellulose to the durable water-repellent (Figure 2d).

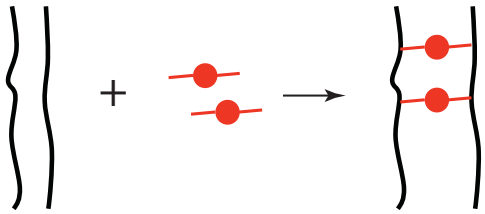

Figure 2a. Traditional Crosslinking imparts desired attributes in one step (Top, wrinkle resistance. Bottom, water repellency).

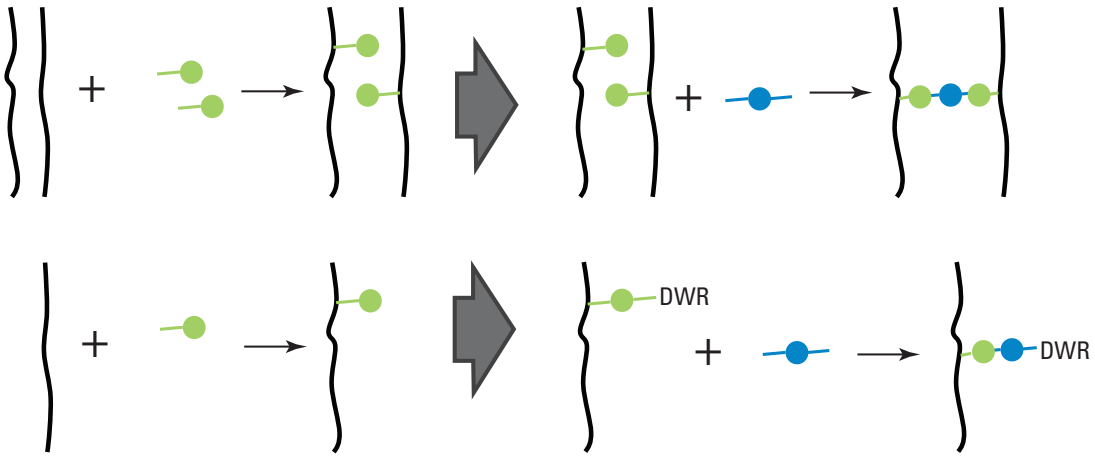

Figure 2b,c. Biologically Inspired Crosslinking imparts desired attributes in two steps (Top, wrinkle resistance. Bottom, water repellency).

STRATEGIES

Process Considerations

Addressing cellulose bonds and crosslinking bonds separately provides us freedom to perform the crosslinking step any time after the initial bond with cellulose has been formed. We propose cellulose bond treatments that could be applied in the yarn, fabric, or garment form (Figure 3, following page). The crosslinking bond treatment could then occur during the fabric finishing stage or in garment form.

The large span of steps between the treatment's initial and secondary applications forces us to consider potential interference from intermediate processes on the crosslinking chemicals. The most unavoidable chemical conditions include the presence of water and air, which may trigger premature hydrolysis or oxidation of chemicals bound to the cellulose. Depending on the point of application, these chemicals must also withstand high temperatures, strong bases, and strongly oxidizing bleaches. The anticipated performance of these chemicals during the manufacture process is assessed in the Technical Evaluation.

	STEP	SUBPROCESS OVERVIEW	CHEMICAL INPUTS
YARN FORMATION	RAW COTTON		
	FIBER PREPARATION	**WAX REMOVAL**_ hydrophobic coating is removed to allow dyes to penetrate into the cellulose	
	SPINNING		

	STEP	SUBPROCESS OVERVIEW	CHEMICAL INPUTS
FABRIC FORMATION	WARPING	Lengthwise yarns are held in tension and weft yarns are later woven through in the weaving step.	
	SIZING	Formulations added to warp yarns to reduce friction and fraying; adds at least 20% by weight.	polyvinyl alcohol polyacrylic acid carboxymethyl cellulose
	WEAVING		**For Levi's**_ indigo dye applied prior to weaving

KNITTING

	STEP	SUBPROCESS OVERVIEW	CHEMICAL INPUTS
WET PROCESSING	PREPARATION	may include: desizing, scouring, bleaching [for pale fabrics], and/or mercerizing	
	DYEING/PRINTING		**For Levi's**_ indigo dye applied prior to weaving
	FINISHING	May include DWR or permanent press application; traditionally spray or dip process, though more recently CVD. LS&Co. uses a dip process.	DMDHEU; Diisocyanates; perfluorinated acids; fluoropolymers; paraffin-based DWRs

STEP	SUBPROCESS OVERVIEW	CHEMICAL INPUTS
DESIZING	Removal of starches and other water-soluble compounds in sizing formulations	amylase other enzymes
SCOURING	Remove fats, waxes, tannins, pectins, proteins, and any dirt or husk residues.	boiling conc. NaOH 1 hr
BLEACHING	Remove naturally occurring pigments	100+°C H_2O_2
MERCERIZING	Swells fibers in diameter and shrinks them longitudinally, making them more receptive to dyes	25-30% caustic soda

	STEP	SUBPROCESS OVERVIEW	CHEMICAL INPUTS
FINISHING	CUTTING		
	SEWING		**For Levi's**_ DWR properties imparted here [garment form]
	CURING		
	FINISHED GOODS		**Lifecycle performance**_ 10-30 washes, depending on line

Figure 3. Textile manufacturing process, with potential application points of crosslinking treatment highlighted.

Linkages to Cellulose: Non-Covalent and Structural Bonds

A major obstacle to crosslinking cellulose is the low reactivity of hydroxyls, cellulose's most available functional group. By inserting functional groups that are more reactive than hydroxyls onto the cellulose, better "handles" can be made for crosslinking. A non-covalently bound or blended polymer that is more easily cross-linked could become these "handles." Polymers could be blended into the threads of the fabric or applied to the textile at a variety of points its manufacture. Such an addition is the most radical alternative that our group proposes in this report; these treatments are upstream of LS&Co.'s typical crosslinking work space.

Polymer Blending

Polyester polyethylene terephthalate (PET) is frequently incorporated into LS&Co. fabric (Figure 4).[4] Certain styles of Dockers incorporate PET blends in proportions upwards of 40%, while Levi's WasteLess jeans include polyester in proportions up to 29%.[5] Within LS&Co., PET is sourced from recycled drink bottles and is an excellent source of sustainability for the company. Additionally, PET has phthalate ester handles, an attractive target for crosslinking. The expanded use of PET within LS&Co. is very attractive; it could potentially achieve wrinkle repellency and water resistance.

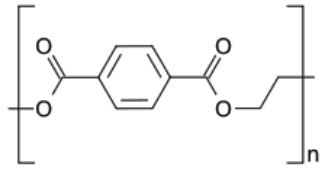

Figure 4: PET, the most common polyester in textiles.[6]

PET is almost exclusively used for the weft fibers.[4] It is unclear how much crosslinking to only the weft fibers of a fabric could solve either the permanent press or DWR crosslinking challenge. Crosslinking between synthetic and cotton fibers could work well in permanent press applications. It is possible that the weft fibers alone of jeans may provide enough surface area for the EcoRepel DWR treatment to adhere, but there may be complications as most weft fibers are located on the inside of the garment. If LS&Co. investigates this alternative, they should consider treatments on a variety of blends of fabric to understand its full potential.

Polymer Application

Applying a polymer to the cellulose rather than blending it is an attractive alternative, as this technique could be integrated into existing manufacturing processes. However, several obstacles must be overcome for this strategy to become viable. First, the polymer must provide more reactive functional groups than are available on cotton for crosslinking. Secondly, the polymer must be rendered sufficiently insoluble in water for it to hold fast to both the cotton and a cross-linker under the conditions of manufacture and use. This is primarily a concern for alternatives in a sizing-type coating. It is essential that the polymer coating not be removed in the desizing process, as this would

eliminate the crosslinking functionality. This leads to a third challenge: the treated garments must have a pleasing feel to the hand. Some polymers may interfere with the softness of 100% cotton clothing. North Korea manufactures a high-performance clothing called vinylon entirely out of poly(vinyl alcohol) (PVA) that is described as feeling like wallpaper.[7] This level of discomfort would outweigh any benefit in functionality. Finally, the added polymer must not have interactions harmful either to itself or the fabric in subsequent steps in the manufacture. Research reveals numerous possibilities for overcoming these obstacles (described below), and their expected performance is codified in the Technical Performance Evaluation.

Most unconventional would be the application of polymers for crosslinking in the fiber phase. The concept of coating fibers with polymers, however, is not foreign to textile manufacture. Analogous procedures take place during the sizing process, where chemicals including poly(vinyl alcohol) (PVA) (Figure 5), carboxymethyl cellulose (CMC), acrylates, starch, and wax coat warp fibers to impart tensile strength and facilitate weaving. We investigated alternatives using fiber procedures similar to the sizing already used by LS&Co. that could impart easier crosslinking functionality. While these coatings would be held onto the cellulose by numerous hydrogen bonds – a crosslinking strategy common in nature – the systems of physical application are derived directly from procedures already employed by the industry. EcoRepel DWR is able to coat individual fibers, thus imparting water resistance without losing breathability. This strategy complements the DWR crosslinking application.

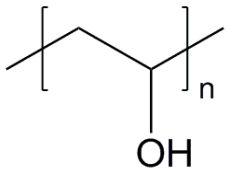

Figure 5. PVA, a water-soluble polymer frequently used in the textile industry.

Of the chemicals used in sizing, PVA and starch only offer more hydroxyls to crosslink, while CMC, acrylates, and wax might offer more reactive carboxylic acid, ester, and vinyl functional handles. But a variety of functional groups can be added to PVA, including thiols, esters, and urethanes.[8,9] These functional groups complement methods we propose for crosslinking applications. For instance, thiolated PVA (Figure 6) would lend itself to forming disulfide bonds (discussed below). While the variety of functional groups that can be added to polymers is promising, LS&Co. should take a cautious approach while considering modifying polymers. Functionalizing treatments often occur under intense physical or chemical conditions, many of which do not advance the goal of reducing health hazard. For instance, PVA is typically made insoluble by treatment with heat and formaldehyde, which is unacceptable from a health hazard standpoint.

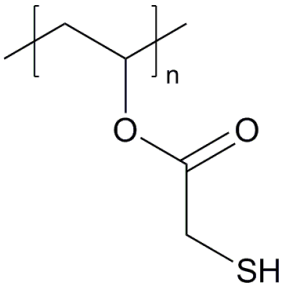

Figure 6. Thiolated PVA could be used with disulfide bond crosslinking.

Rather than limiting itself to coatings derived from currently used sizes, LS&Co. should also consider polymers that will coat fibers with better crosslinking potential than sizes. Poly(vinyl acetate), poly(lactic acid), poly(methacrylic acid) and polyimides (Figures 7a-d) all offer strong possibilities for noncovalent crosslinking applications and should coat fibers well. These polymers are also more resistant to desizing and subsequent processes than PVA is. Nonetheless, some of these polymers do raise concerns. PLA, for instance, may hydrolyze if exposed to water before being crosslinked, and the chemical precursors of polyimides have many of the same health concerns as the di-isocyanates used in crosslinking DWR finishes. While limited research has been done on the applications of these polymers to a cellulose substrate, other polymer-coated textiles show good performance in hand and feel.[10] Teams have succeeded in coating natural and synthetic fibers with polymers to achieve enhanced functionality with similar physical characteristics and handling as the virgin material.[11,12] Others have modified PLA to decrease its brittleness and increase its tensile strength.[13] This modification could even allow PLA to be used as a sizing agent that, rather than being desized, is retained for functionality later in the manufacture process. PET fibers are also sized, so this alternative could also be applied to cotton-poly blends.

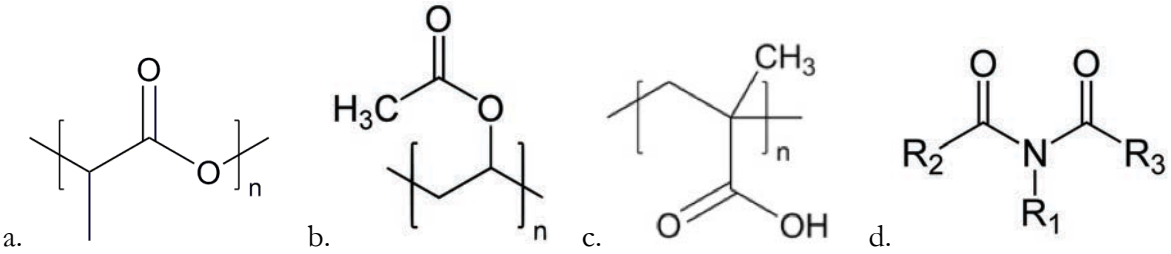

Figure 7: a. poly(lactic acid), b. poly(vinyl acetate), c. poly(methacrylic acid), d.polyimide.

Polymers could also be applied in either the textile or the garment phase, which are much more conventional points in the process to impart crosslink functionality. Polymer coatings are often applied in the fabric form. This is currently the case for DWR finishes like EcoRepel and the shape-holding features of Levi's Revel line.[4] The polymers could be screen printed onto the garment, as in the Revel line, or be polymerized on the surface of the fabric *in situ* as is done with electrically conductive fabrics often used in technology like "smart braces".[14] These polymers, however, would serve to enable later crosslinking functionality. This could be a viable pathway for coating textiles, as the chemical intermediates of the polymerization can penetrate into the fibers themselves, making

the polymer an integral part of the fabric.[11] Such polymers have demonstrated physical and chemical stability under many relevant conditions (though they may require the fabric to be dry-clean only) and can resist shearing away from the fabric at stress upwards of 6MPa.[11]

In conclusion, to augment the reactivity of cellulose in cotton clothing and enable easier crosslinking in either the permanent press or DWR crosslinking application, polymeric handles could be non-covalently bound to the cellulose. These handles could be coated on in fiber form like the sizes currently used in textile manufacture, woven into the fabric in cotton-poly blends, or formed *in situ* on the fabric. These alternatives offer radically different approaches than those presently used by the textile industry but this new perspective may yield functional and nontoxic results.

Linkages to Cellulose: Covalent Bonds

Polycarboxylic acids have been studied for nearly 50 years as possible formaldehyde-free wrinkle-release finishes for cellulose. They are also biologically-inspired, since many of them are found in nature (Figure 8), and recent review stated that they are "the most promising non-formaldehyde durable press finishes".[15] Despite this, they have not proven to be a fully effective substitute for DMDHEU (dimethylol dihydroxyethyleneurea, the current technology) due to problems with loss of tensile strength,[15] the catalyst,[16] cost,[17] and fabric yellowing.[18]

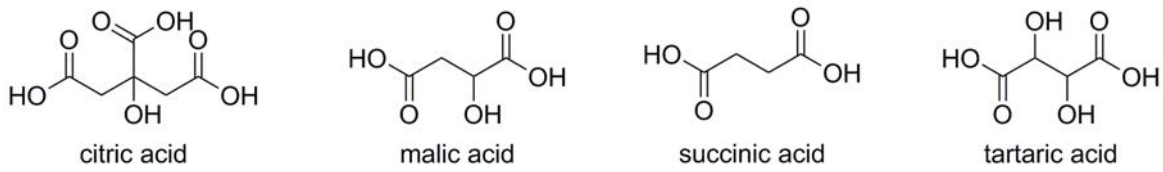

Figure 8. Some polycarboxylic acids commonly found in nature.

Polycarboxylic acids work as cotton crosslinkers by covalently bonding to cellulose fibers. When heated, they form cyclic anhydride intermediates that react with the hydroxyl groups on cellulose to form ester linkages (Figure 9). Thus, the acids that have been studied typically have at least three carboxylic acid groups, as two are required to form each cyclic anhydride. Examples include citric acid, 1,2,3,4-butanetetracarboxylic acid (BTCA), and 1,2,3,4-cylopentanetetracarboxylic acid.[16–23] Of these, BTCA has proven the most effective but is cost-prohibitive.[24] The cost of citric acid is comparable to that of DMDHEU, but it causes fabric yellowing.[16–18] Unsaturated dicarboxylic acids have also been studied, such as maleic and itaconic acids: the double bonds found in these compounds enable further crosslinking activity without cyclic anhydride formation.[16,20,25–27]

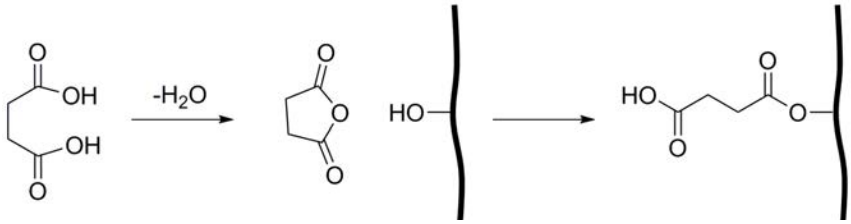

Figure 9. Formation of a cyclic anhydride enables polycarboxylic acids to react with cellulose.

The loss of tensile strength seen with polycarboxylic acid crosslinking is attributed to the stiffness of the crosslinking itself, as well as the uneven heating in the curing step.[22] To overcome this, longer-chain acids are proposed as a way of making the crosslinker more flexible.[15] One example of in situ oligomerization of maleic and itaconic acids resulted in improved tensile strength over DMDHEU, with lower cost than BTCA and no fabric yellowing.[25] Microwave heating has also been proposed as a way of curing the fabric more evenly.[22] Finally, pre-cationization of the fabric with an ammonium reagent, followed by ionic crosslinking, has also been shown to improve tensile strength.[28]

The most common catalyst used to perform crosslinking with polycarboxylic acids is sodium hypophosphite (SHP). Unfortunately, it causes changes in dye shade and can degrade cellulose fibers.[16,24] It is also an environmental hazard, leading to eutrophication.[16] Other phosphorus-based catalysts[18] and non-phosphorus-based catalysts[16,24] have been studied, however, SHP remains the standard.[23] The yellowing that occurs when α-hydroxy acids such as citric acid are used has been attributed to the formation of unsaturated acids at high temperatures, which can be reduced by addition of boric acid[16] or triethanolamine.[18] Esterification of citric acid with a polyol can also reduce unsaturated acid formation.[23]

In our case, we propose to use a difunctional carboxylic acid, such as succinic acid, which can form a single linkage with cellulose. The acid would be applied and linked to the fabric in the finishing step. At this point, an extra carboxylic acid group would be available for further reactivity. We then propose using common bioconjugation reagents to react with this carboxylic acid in situ to attach a thiol group (Figure 10).[29] Alternatively, we propose using a diamine to crosslink with the carboxylic acid groups, either through reductive amination or amidation.[29,30] As this two-pronged approach has not yet been tried with polycarboxylic acid crosslinking, we think it may open up new avenues of research that could help mitigate some of the current problems.

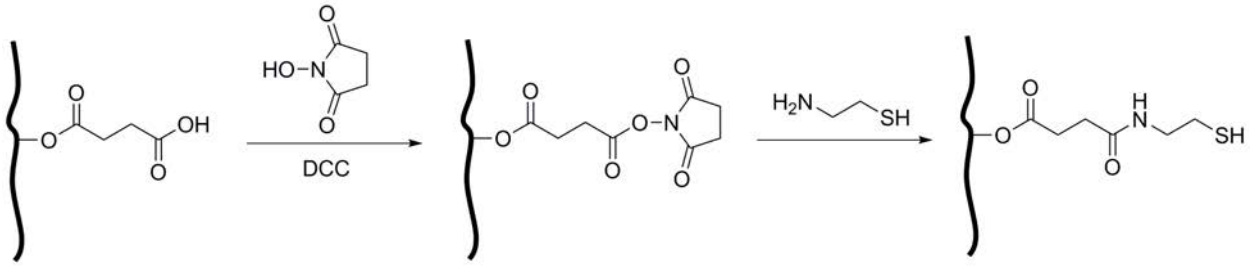

Figure 10. A polycarboxylic acid bound to the cellulose could react with *N*-hydroxysuccinimide (NHS) and DCC (*N*,*N*'-dicyclohexylcarbodiimide) to form an NHS-ester that can then react with an amine to attach a thiol group.

Crosslinking Strategies: Covalent Bonds

Disulfide Bonds

Disulfide bonds are ubiquitous in nature, and are therefore an obvious choice for a biologically-inspired crosslinking strategy. In biological systems, disulfide bonds are formed by the oxidative reaction of two cysteine residues (Figure 11) and stabilize secondary and tertiary protein structure. Formation of the disulfide linkage requires an oxidizing agent (electron acceptor), which is usually oxygen, a redox-active cofactor, or another thiol.[31]

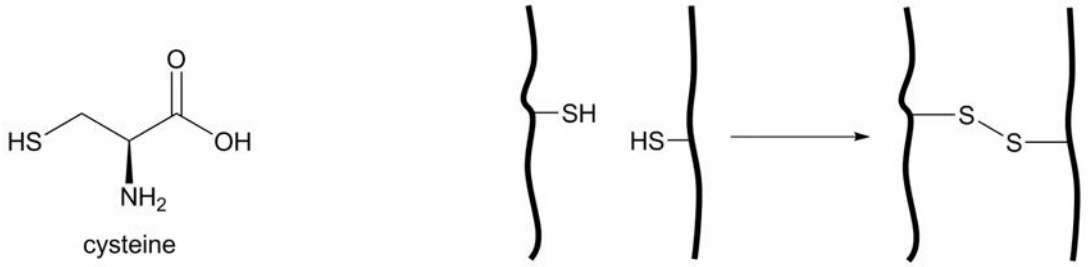

Figure 11. The thiol (-SH) groups on cysteine can react to form disulfide linkages.

Disulfide bonds have also been extensively explored for synthetic applications,[32] and a wide variety of oxidants and catalysts can be used. (The term "oxidant" will be used here to denote the reagent that accepts electrons from the thiol groups, meaning that it must be used in amounts equivalent to the amount of thiol groups. "Catalyst" denotes an additional, optional chemical that is unchanged by the reaction and is likely used in small amounts.) The simplest option is to simply allow the thiol groups to oxidize in air, although this can take days,[33] and more often stronger oxidants, catalysts, or heat are used to accelerate the reaction process. Hydrogen peroxide is a common oxidant and can be made even more effective by acidic pH or elevated temperature.[34–36] Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid), is also often used, usually at alkaline pH.[37] Sulfoxides are effective oxidants at elevated temperatures, or in either acidic or alkaline media, and are often used with an additional catalyst.[32,38] Other common oxidants used include sodium periodate,[36] tetranitromethane,[36] and potassium permanganate.[39]

Recently some "greener" options for disulfide bond formation have been reported. Some use a pre-oxidized catalyst such as graphite oxide, although in that case the reagents used to oxidize the graphite are not especially "green".[40] Others use air as an oxidant with an additional catalyst, such as potassium fluoride on alumina,[41] potassium phosphate,[42] metal salts on silica,[32] iron metal-organic frameworks,[43] or nickel[44] or gold nanoparticles.[45] One additional advantage of some of these systems is that they work in solvent-free conditions, which could be an advantage for setting a permanent crease.

Although disulfide bonds have not, to our knowledge, been used in textile applications before, there are examples of disulfide bond crosslinking to form biodegradable polymers,[34] hydrogels,[35] or bioconjugated systems.[46] For our application, we propose that thiol groups be attached to cellulose either through a thiolated polymer coating[46–49,8] or by reaction with a poly(carboxylic acid). This application would likely occur in the finishing step, at which point the crosslinking could also occur (for example, attaching a DWR finish). Alternatively, the crosslinking step could be performed in the garment form, as would be required to set a permanent crease. Either way, the crosslinking step would occur upon addition of the necessary oxidant or catalyst.

Imine and Amine Bonds

Imine bonds are formed reversibly in the slug, *Arion subfuscus*, to harden the mucus secreted by the animal and form a protective layer (Figure 12). Iron and copper ions catalyze carbonyl formation on protein side chains; the carbonyls subsequently react with the amine group on lysine to form the imine bonds.[50] Imine bond formation is a dehydration reaction, and imine bonds are easily hydrolyzed back to the starting amine and carbonyl groups. While this reversibility is useful for the slug, we were concerned about an easily hydrolyzable crosslinking bond.

Figure 12. Imine bond formation, resulting in a release of water.

To impart added stability to our proposed crosslinking bond, we suggest taking the imine bond formation one step further and performing a *reductive amination* (Figure 13). The starting materials are still an amine and a carbonyl, which react to form an imine intermediate. Upon addition of a catalyst and a reducing agent (electron donator), this intermediate is then reduced to an amine, which is more stable and resistant to hydrolysis than the imine.[51]

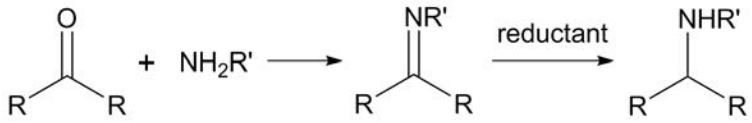

Figure 13. Amine bonds can be formed by the reduction of an imine.

Reductive amination is most commonly performed using catalytic hydrogenation or borohydride reagents. Catalysts for hydrogenation are usually nickel, platinum, or palladium.[52] Sodium cyanoborohydride is the most extensively studied borohydride reagent, but it is extremely toxic, in part because it can form hydrogen cyanide during the reaction workup. [52–54] More recent results report that sodium borohydride and sodium triacetoxyborohydride are also effective reducing agents for reductive amination, with or without catalysts. Catalysts for sodium borohydride reduction include titanium (IV) isopropoxide[53], iron triflate,[54] and acetic acid.[55] Although reductive amination is usually performed using aldehydes or ketones, carboxylic acid-amine reactions may work,[30] and analogous alcohol-amine reactions have also been reported.[56]

Reductive amination has been explored for crosslinking in other applications, with varying success. In one case, polyamines were successfully used to crosslink polysaccharides using sodium borohydride.[57] In another, a poly(caprolactone)-based polymer was reacted with a diamine and sodium cyanoborohydride, which resulted in polymer chain cleavage instead of crosslinking.[58] This suggests that success depends greatly on both the nature of the polymer and the reductant. For textile applications, we propose to use a diamine crosslinker with a carbonyl-containing cellulose treatment. The cellulose treatment could be polyethyelene terephthalate woven with the cotton, a

polymeric fabric coating such as poly(lactic acid), or a poly(carboxylic acid) that can covalently bind to cellulose. The diamine would be applied during the finishing step, and crosslinking would be effected upon addition of the reducing agent and catalyst.

FRAMEWORKS + EVALUATIONS

Boundary Conditions

We limited our proposed crosslinking and cellulose binding strategies to ones that are minimally disruptive to the overall garment manufacturing process. Radical overhauls are not feasible solutions, as there are currently no regulatory drivers forcing significant changes to crosslinking technologies. While we did develop and implement an approach to evaluating the technical performance of our proposed strategies beyond manufacturing feasibility, we did not evaluate these strategies on the basis of either cost or measured performance. These are beyond our purview as we have neither the data nor the laboratory equipment and expertise to evaluate cost and performance parameters. Some of the considerations in the Technical Evaluation, nonetheless, are excellent initial approximations for cost and measured performance considerations.

We also limited our proposals to those that showed promising health evaluations. Reducing human health and environmental impacts were top priorities as we researched numerous approaches to both crosslinking and cellulose binding; therefore, we neither pursued in depth, nor included in this report, those strategies that required input chemicals whose impacts offered little or no improvement over current technologies.

Technical Framework

For our technical evaluation, we chose eight factors that we felt we could qualitatively evaluate using the time and resources available to us. The first four factors deal with innovation and disruption to the current system, and are in some ways good proxies for cost as they represent the possible research investment required before a strategy could be fully implemented. The remaining four factors deal with specific problems associated with binding to cellulose, crosslinking, and other effects on the fabric, and are thus reasonable proxies for performance.

In all eight categories, we gave each strategy a score from "red" to "green" (Table 2). "Red" scores indicate that a technology is a pretty big change or that we foresee numerous technical and performance hurdles. "Green" scores are given for technologies that fit well into the existing fabric treatment framework or do not have a lot of anticipated hurdles. "Yellow" scores fall somewhere in the middle.

INNOVATION	ADDITIONAL RESEARCH NEEDED	MAJOR HURDLES ANTICIPATED	MINOR HURDLES ANTICIPATED	OPTIMIZATION ONLY
DISRUPTION OF INFRASTRUCTURE	CHEMICAL SUPPLY	SPECIAL MANUFACTURE	LIMITED AVAILABILITY	WIDE AVAILABILITY
	FABRIC APPLICATION	NEW PROCESS	MODIFY EXISTING PROCESS	USES EXISTING PROCESS
	CROSSLINKING STEP	NEW EQUIPMENT	NEW CHEMICALS, SOLVENTS	HEAT OR AIR CURED
ROBUSTNESS	CONTROLLABLE CROSSLINKING	TOO REACTIVE OR UNREACTIVE	SPECIAL CONDITIONS OR EXTRA CHEMICALS	ADD CATALYST, REAGENT, HEAT
	RESILIENCE DURING MANUFACTURING	LIKELY PROBLEMS	POSSIBLE PROBLEMS	NO FORESEEABLE PROBLEMS
	RESILIENCE DURING CONSUMER USE	LIKELY PROBLEMS	POSSIBLE PROBLEMS	NO FORESEEABLE PROBLEMS
SIDE EFFECTS	EFFECTS ON FABRIC	REQUIRES PROBLEM CHEMICALS	POSSIBLE NEED FOR PROBLEM CHEMICALS	NO FORESEEABLE PROBLEMS

Table 2. Eight technical evaluation factors, with scoring requirements from red to green.

It is important to emphasize that a "red" rating does not necessarily make a strategy bad, any more than a "green" rating makes it good. We want to be clear that "red" ratings merely reflect technology disruptiveness and our ability to predict potential problems. "Green" ratings, meanwhile, may disguise problems that we are unable to predict, or simply indicate that a technology is well-researched. However, at this point, no rating corresponds to the eventual success or failure of strategy.

Detailed descriptions of the eight technical evaluation factors are given in Appendix D.

Combined Strategies Yielding Solutions

In order to get a technical evaluation of a complete cellulose binding-and-crosslinking solution, the technical results for one cellulose binding strategy and one crosslinking strategy can simply be averaged. In other words, if the final proposed solution was to use a diamine crosslinker with the PET weave, the results for PET weave would be averaged with the results for imine & amine crosslinking. Since our health framework depends on specific chemical evaluations, we chose some specific strategy combinations to evaluate using both our technical framework and our health framework (Figures 14 and 15).

Based on our combinations of strategies, we can see that imine bond crosslinking with poly(ethylene terephthalate) weaving is the most promising solution according to our current information, although any of the combinations may be ultimately viable. More detailed descriptions of how these classifications were determined are provided in Appendix E.

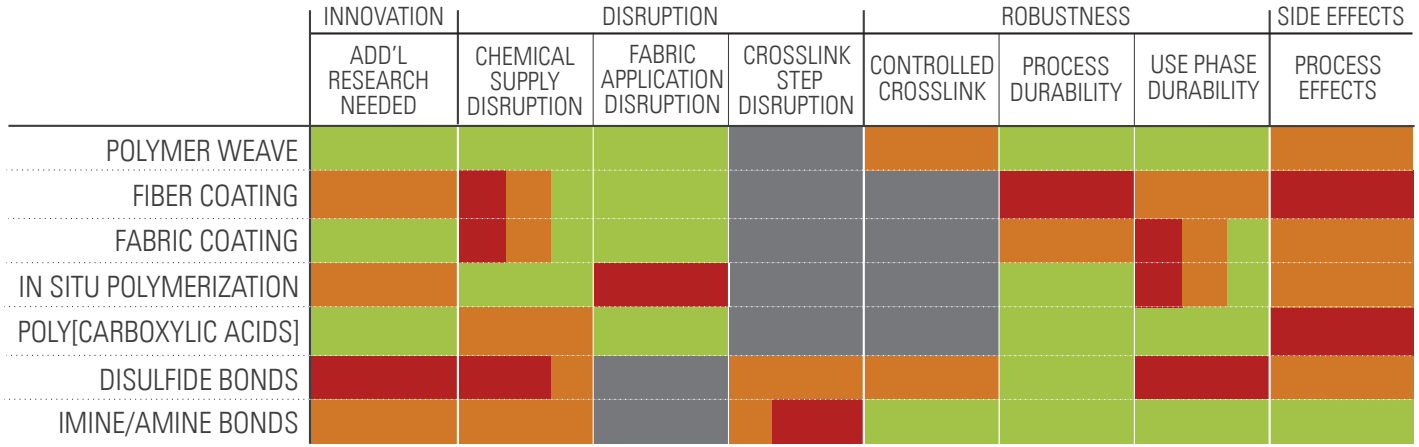

Figure 14: Technical evaluation of each strategy.

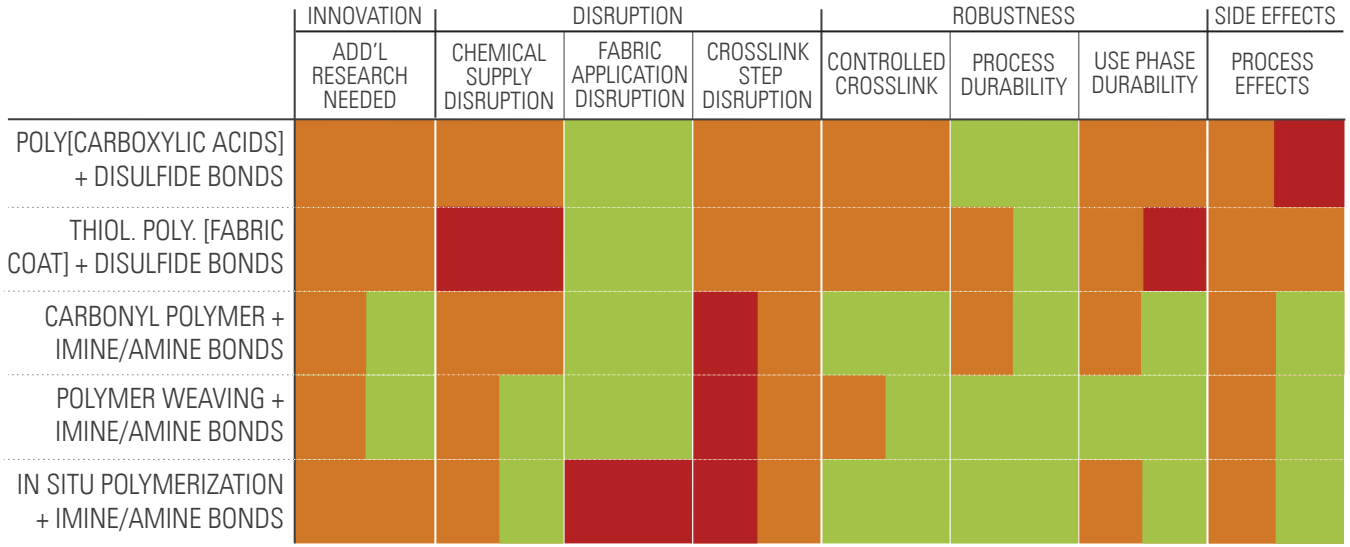

Figure 15: Technical evaluation of combined strategies.

Health Framework

GreenScreen Precedent

We used a hazard-based approach to evaluate the health impacts of the chemicals used in our proposed crosslinking alternatives. While there are numerous hazard- and exposure-based methods for assessing health risk, LS & Co., and other major stakeholders in the textile industry, have embraced a hazard-based approach. To align as closely as possible with this current paradigm, our health evaluation framework was derived from the GreenScreen Chemical Hazard Assessment Procedure, which LS&Co. already implements.[62] The GreenScreen framework is a comparative hazard assessment tool that builds upon nationally and internationally recognized hazard evaluation frameworks to assess and classify hazards and to apply benchmarks to chemicals.[63] Each chemical evaluation includes a hazard assessment of the parent chemical as well as potential environmental transformation products. Individual chemical evaluations draw upon measured data gathered from the literature, modeled data, data for suitable chemical analogs, and hazard lists published by governmental agencies, academic researchers, and non-governmental agencies.[64] Chemicals are evaluated for 18 different endpoints sorted into five hazard groups. Each endpoint is assigned a hazard classification [vH, H, M, L, vL] and confidence level. These hazard classifications are aggregated into a benchmark score based on the GreenScreen's weighting system.[65]

Framework scope and goals

The context of our research posed two key challenges that required us to develop and implement an abbreviated version of the GreenScreen framework: First, limited time and scope do not allow a comprehensive evaluation of the scientific literature; second, many of the proposed chemicals are not well studied and therefore have very limited toxicological and epidemiological data. As a result, our proposed framework will not result in GreenScreen benchmarks for individual chemicals. Rather, our framework aims to do the following:

1. Identify [and subsequently eliminate] chemicals meeting any Benchmark 1 criteria [Table 3].
2. Compare, on a relative basis, chemicals for which Benchmark 1 designation does not apply using a weighting system derived from GreenScreen[12].
3. Compare hazard of individual chemicals and the lifecycles in which they operate, including the inputs and conditions for their synthesis, subsequent crosslinking syntheses in which they participate, and use phase and end of life hazards.
4. Compare hazard of each of the four crosslinking strategies on a system level.

Table 3: GreenScreen Benchmark 1 Criteria

Hazard Classification	Criteria
PBT	High P + High B + [very high T (ecotoxicity or Group II Human) or high T (Group I or II* Human)]
vPvB	Very high P or very high B
vPT	Very high P + [very high T (ecotoxicity or Group II Human) or high T (Group I or II* Human)]
vBT	Very high B + [very high T (ecotoxicity or Group II Human) or high T (Group I or II* Human)]
High T	Group I Human

See GreenScreen Guidance documents[10] for definitions of the five GreenScreen endpoint groupings: Group I Human, Group II Human, Group II* Human, Ecotoxicity and Fate, and Physical Hazards.

Evaluation

We researched five strategies, including two crosslinking and three cellulose-binding strategies. Lifecycle hazard evaluation for each of these strategies included:

1. Chemical inputs and conditions required to make crosslinking or binding chemical
2. Chemical inputs and conditions required to crosslink or bind
3. Probable environmental transformation products occurring in use or end of life phases

This scope of evaluation was expanded from that of the GreenScreen to include of the parent crosslinking chemical both to be more complete and because these chemicals can serve as surrogates for some proposed chemicals whose health hazards are not well understood.

For each chemical, we first gathered data from authoritative lists designated by GreenScreen[11]. Unlike GreenScreen, we did not weight some lists more heavily than others. Where list data is unavailable, inconclusive, or conflicting, we turned to the primary literature to gather supplementary hazard information. Where toxicological and epidemiological data were limiting or inconclusive, we will use modeled data or data from suitable analogs. Modeled data was used almost exclusively for estimations of persistence and bioaccumulativity.

We completed the GreenScreen hazard table to the extent that was possible given time and data constraints. Where possible, we identified and eliminated Benchmark 1 chemicals. Our framework assessed remaining chemicals relative to other chemicals serving comparable roles. Our assessment then prioritized the evaluation of those endpoints weighted most

heavily by the GreenScreen weighting system. We performed this data collection and relative ranking process for each chemical within each of the proposed crosslinking and binding strategies. The process cumulatively enableed comparison both within and among the four strategies [Figure 16], though due to data and time limitations, we believe that the more useful comparison is among chemicals performing similar roles within single strategies. Results from these health evaluations can be used to guide further research, either through prioritization or elimination of a candidate chemical for the strategy of interest.

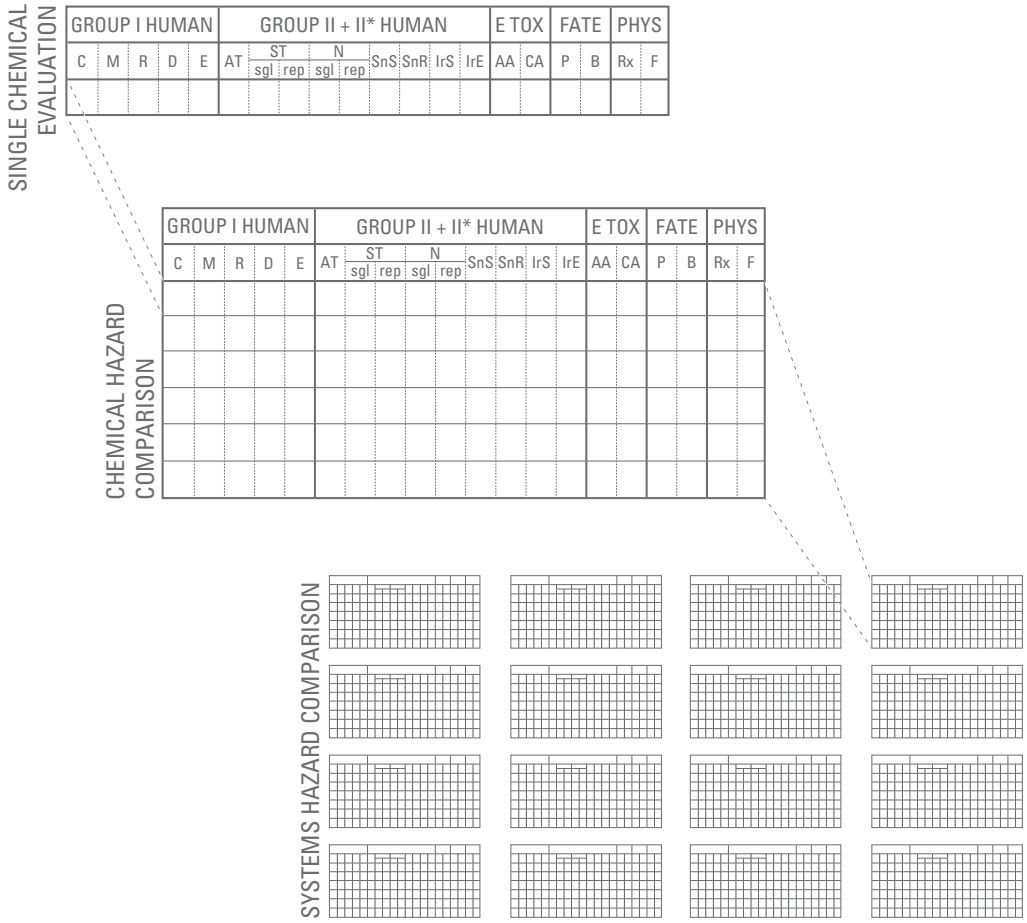

Figure 16: Health evaluation process.

Alignment with GreenScreen

While our evaluation framework is an abbreviated version of the full GreenScreen, the underlying structure of the assessment is the same, allowing the data collected for this initial screening to feed directly into a full GreenScreen, in the future, should one be performed. Figure 17 shows the compatibility of our adapted evaluation with the GreenScreen framework.

	SCOPE			DATA COLLECTION + PRIORITIZATION				OUTCOME
	INPUTS	USE	DEGREDATION	LISTS	PRIMARY LIT	ANALOGS	MODELED DATA	
GREENSCREEN				2	1 [comprehensive]	3	3	benchmark score
ADAPTED EVAL				1	2 [as needed]	3	3	relative rank

Figure 17: A comparison of scope, data collection and prioritization, and outcomes of the GreenScreen with our proposed adapted evaluation framework.

Health Evaluations

The following health evaluations rely on graphics that have been created from data gathered from authoritative lists and the primary literature using the approach described above. The raw data from which these graphics have been developed, as well as the corresponding references, can be found in the accompanying Excel File: *HealthEvaluationData.xlsx*. Figure 18, below, describes the color coding system that was used to translate from the raw data to the tables shown in this section.

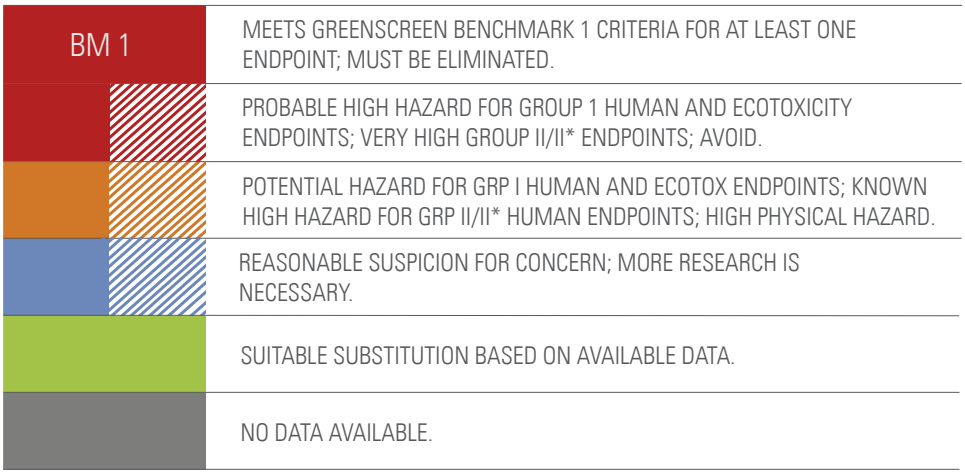

Figure 18. Health evaluation framework weighting scheme.

While we tried to be comprehensive in our evaluations, we could not study everything. It is possible that a proposed strategy that appears to offer limited hazard reduction may actually be a strong alternative, but the right candidate chemicals may have yet to be identified and evaluated.

Baseline evaluation

Health evaluations for the baseline chemicals are shown in Figure 19 below. While we evaluated many chemicals currently used in textile processing, we targeted the last four chemicals for substitution, which are currently used in LS&Co.'s cotton crosslinking systems.

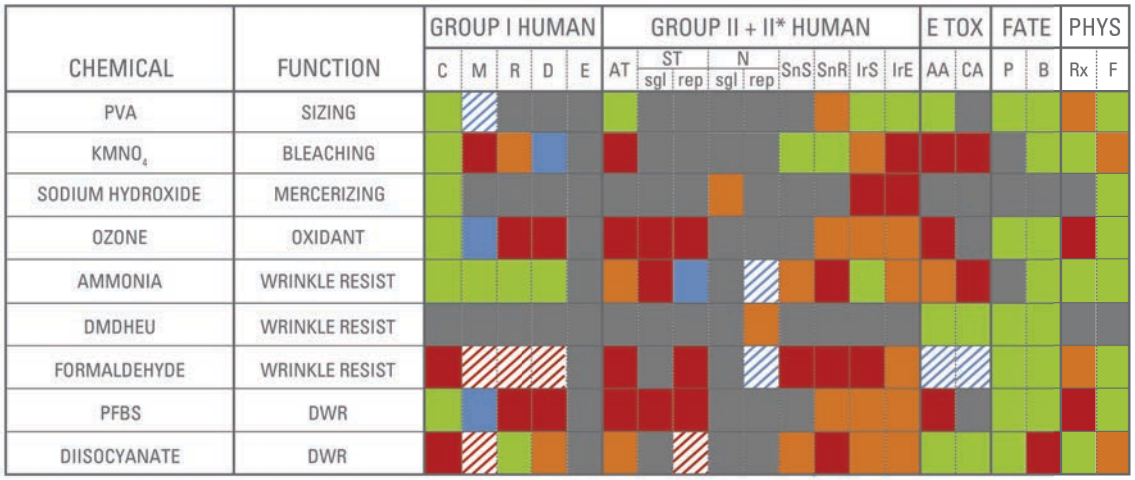

Figure 19. Health evaluation data for the baseline wet processing technologies.

Formaldehyde is most notable among the chemicals listed above; it is both well studied and highly toxic. While formaldehyde is cheap, versatile, and high-performing, it is also a known carcinogen, acutely toxic, and a known skin and respiratory sensitizer. PFBS, a common DWR, is another chemical of concern. Most notably from a health perspective, the PFBS DWR has demonstrated reproductive and developmental toxicity, and is highly acutely toxic to aquatic life. LS&Co. has partially phased out PFBS and has substituted it with a paraffin-based alternative in many of its products. Finally, diisocyanates, which are also widely used in water repellency technologies, are respiratory sensitizers and are possibly carcinogenic, among other hazard concerns.

Each of these chemicals is integral to textile crosslinking, both for LS&Co. and across the textile industry. As the demand and expectation for hazard transparency and reduction gains prevalence, however, safer alternatives for these chemicals will become increasingly necessary.

Comparing within strategies: disulfide bonds evaluation

As Figure 20 shows, the disulfide bond strategy integrates chemicals that appear, overall, to pose reduced hazard relative to the baseline technology, particularly for the Group I Human endpoints and acute mammalian toxicity. Additionally, unlike many of the baseline chemicals, many of the hazards for which these chemicals have been listed are more easily mitigated [i.e. skin and eye irritation]. That said, most of these compounds are not as well studied as the baseline chemicals, which significantly limits the conclusions that can be drawn from this comparison.

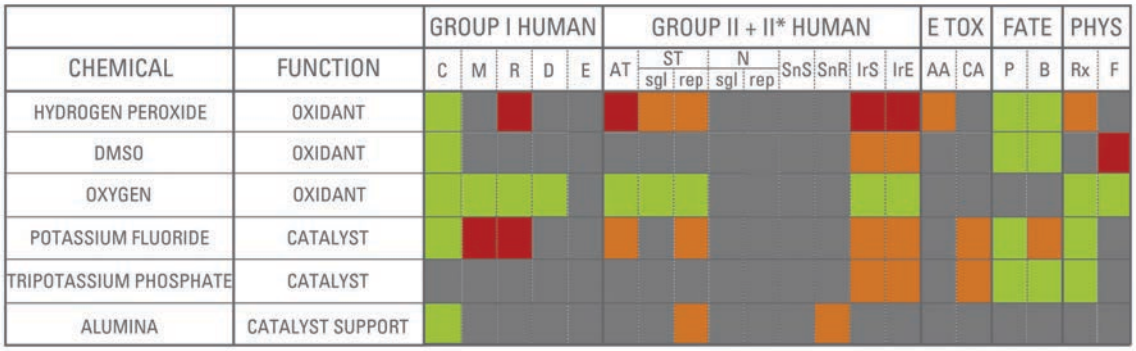

Figure 20. Health evaluation data for the proposed disulfide bond crosslinking strategy.

The table does indicate that some candidates are more favorable than others of comparable function, both on the basis of data availability and on based on the hazard levels those data indicate. For example, the evaluation clearly favors further research on oxygen's feasibility as a thiol oxidant in textiles.

Comparing within strategies: amine/imine bonds evaluation

Like the previous evaluation, the amine/imine bond health evaluation is plagued by data gaps [Figure 21]. The table can, however, be used to create a relative hazard ranking, both among candidate chemicals within the strategy, and, to some extent, relative to the other crosslinking and binding strategies.

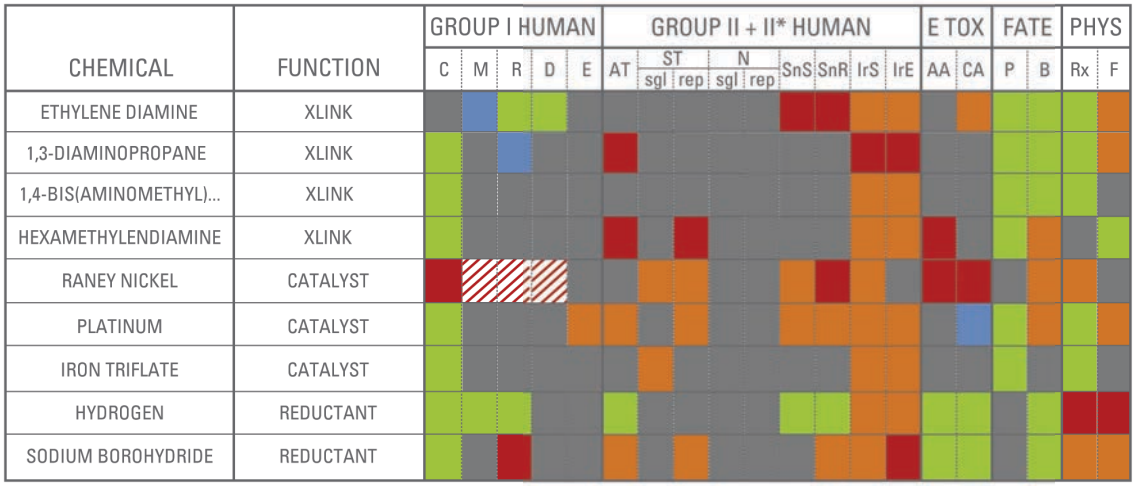

Figure 21. Health evaluation data for the proposed imine/amine bond crosslinking strategy.

For example, the results differentiate and prioritize among the proposed catalysts and the reductants. While the data gaps cannot be ignored, hydrogen emerges as a promising starting point for an imine reductant relative to sodium borohydride; similarly, Raney nickel is quickly singled out as a low priority catalyst for further feasibility and performance research, while platinum may be a reasonable candidate, as it has shown reduced hazard for more easily mitigated hazard endpoints.

Comparing within strategies: poly[carboxylic acids]

The data gaps for the poly[carboxylic acid] strategy make hazard nearly impossible to interpret, even on a relative basis [Figure 22]. That said, more so than any of the other strategies, poly[carboxylic acid] crosslinking in cellulose is well studied within the textile industry; therefore, it is possible that some proprietary hazard data may already exist, or data may become available as poly[carboxylic acid]-based systems are developed and more widely used.

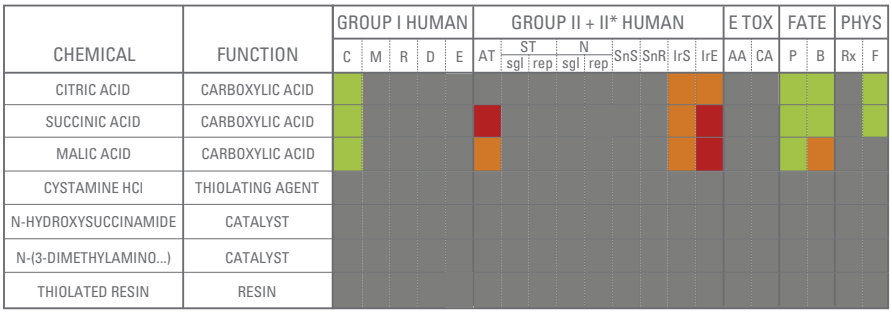

Figure 22. Health evaluation data for the proposed poly(carboxylic acid) crosslinking strategy.

Comparing within strategies: polymer thiolation evaluation

More so than many of the other strategies, the chemicals required of the polymer thiolation strategy show great, well-supported improvement relative to the baseline chemicals for Group I Human endpoints [Figure 23]. There are many promising compounds in several of the functional categories, such as the cysteine-based thiolating agents.

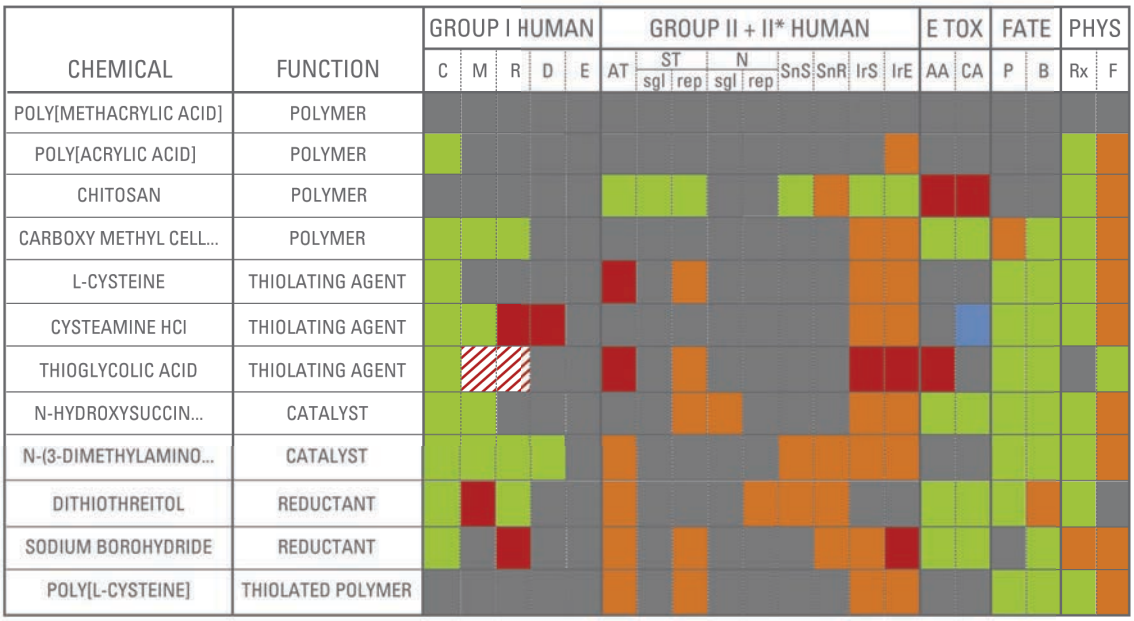

Figure 23. Health evaluation data for the proposed polymer thiolation strategy.

Furthermore, we were able to find some data describing hazard impacts for the thiolated polymer; thus, we can begin to understand not only the inputs and processing chemicals required to implement the technology, but also the impacts of the resulting chemical products.

Comparing within strategies: polymer coating evaluation

Figure 24 shows the health evaluation data summary for the polymer coating strategy. Based on the health evaluation shown, this strategy appears less promising than the others, as few of the chemicals demonstrate clear improvement over existing technologies at LS&Co. Even comparing candidate chemicals of similar function, it is difficult to identify chemicals that do not pose significant hazard in at least one of the Group I or Group II Human endpoints.

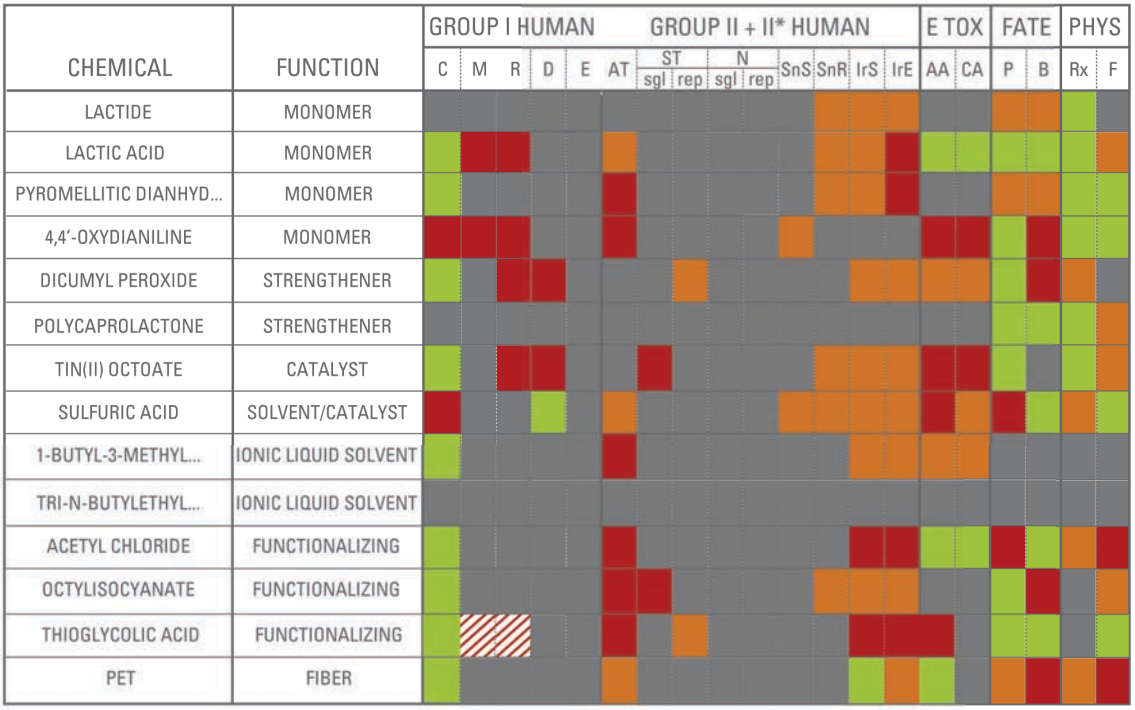

Figure 24. Health evaluation data for the proposed polymer coating strategy.

RECOMMENDATIONS

Based on our assessment of biologically-inspired crosslinking strategies for cellulose, we have several recommendations for Levi Strauss & Co. First is that we recommend that non-traditional crosslinking strategies be considered. Nature employs various bonds in conjunction with one another to achieve crosslinking, and there is room for the textile industry to emulate this through the adoption of non-traditional bonds and piece-wise crosslinking techniques.

This report identifies several non-traditional bonding alternatives that are inspired by nature. We have additionally outlined two evaluation tools – a technical evaluation framework and a health evaluation framework – to frame the validity of each bonding strategy.

Of the five strategy combinations we considered, imine/amine bond crosslinking with poly(ethylene terephthalate) weaving is the most promising solution from a technical standpoint, but any of the combinations are potentially worth pursuing. From a health standpoint, the most promising solution is the thiolated polymer coating, thus highlighting the complex and nuanced interactions and tradeoffs between technical considerations and health considerations.

Moving forward, we recommend that Levi Strauss & Co. use these two frameworks and the information contained in this report to further pursue one of the alternatives suggested, or to pursue another proposed solution.

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APPENDICES

Appendix A: 12 Examples of Crosslinking Within Nature*

*These descriptions were prepared by the Biomimicry 3.8 Institute

1. Blue Sea Mussel (Mytilus) – Mussel Foot Protein 1, Fe^{3+}

Overall Composite Material: Mussel byssus thread cuticle

Main Characteristic of Composite: Hard, extensible, resists abrasion, resists biodegradation

Material Being Crosslinked: Mussel foot protein 1 (mfp-1)

Crosslinker/Binder: Catecholato-Fe^{3+} coordinate complexes

Figure 1. Mussel with numerous byssal threads anchoring it to a rock at low tide.

Introduction

Blue sea mussels live in near-shore coastal waters and feed on suspended plankton. Given the constant onslaught of waves and current in their turbulent environment, these mollusks produce numerous threads that securely anchor them to large rocks and other stable objects. To produce these threads, the mollusk foot secretes a fiber with a collagen-based core and coats it in a 2-5 micrometer protective cuticle made predominately of the muscle foot protein (mfp)-1. In some cases, the thread as a whole is able to withstand >100% extension and return nearly to its original strength. The cuticle can be up to 5 times harder than the fibrous core yet retains nearly the same breaking strain.

Crosslinking Activity

The architecture of the cuticle is one of large granules dispersed in a matrix (fig 2). Both the granules and the surrounding matrix are rich in inorganic ions, notably iron III (Fe^{3+}), and mfp-1 proteins characterized by the amino acid 3,4-dihydroxyphenylalanine (dopa); dopa molecules are decorated with catechol side rings. The dopa catechol rings from two or more mfp-1s form a coordinate bond crosslink when each hydroxyl oxygen on the catechol ring donates a nonbonding electron pair to an iron (III) atom (fig 3). This dipolar type of bonding is much more forgiving of being hyperextended and reformed than a traditional covalent bond in which both atoms in the bond donate electron pairs. Though not even half as strong as ordinary covalent bonds, the coordinate bonds that link multiple mfp-1 molecules to one Fe^{3+} atom can be broken and reversibly repaired hundreds of times. The matrix is composed of loosely packed crosslinked mfp-1 proteins sparsely scattered throughout; in contrast, the granules contain dense clusters of the crosslinked mfp-1s.

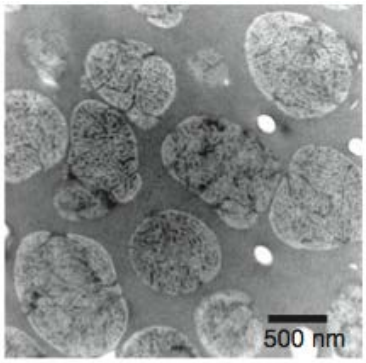

Figure 2: TEM micrograph of byssal cuticle granules. Dense cross-linking in the granules provides hardness, whereas the less cross-linked matrix provides extensibility. (Harrington 2010)

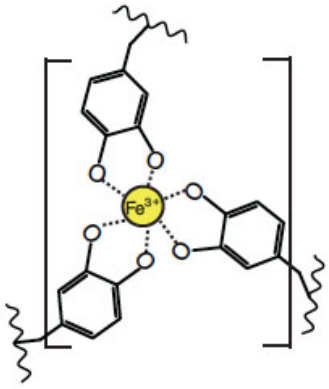

Figure 3: Catechol-iron complex

At rest, the mfp-1 proteins exhibit a random coil structure with few short bent helices throughout. These random coils and the dopa-iron (III) complexes complement each other's performance characteristics to achieve impressive qualities.

During strain up to 30%, the randomly coiled mfp-1s of the cuticle (both the matrix and granules) unravel and stretch out taught. Past 30% strain, stress is absorbed by the sacrificial breaking of catecholato-Fe^{3+} complexes. Due to the much higher density of the iron complexes in the granules, they are able to retain bond integrity even when strain has started to create microtears in the more iron-poor matrix. When the strain is relaxed, the granules have maintained their structural strength. Microtears are channeled through gaps between the granules and don't threaten the cuticle as a whole.

This model of sequential energy absorption and sacrificial yet self-repairing breakages explains the incredible resilience of the mussel byssus cuticle. This evolved strategy has helped propel mussels of the *Mytilus* genus into highly successful organisms capable of colonizing environments in even the toughest environments throughout earth's oceans.

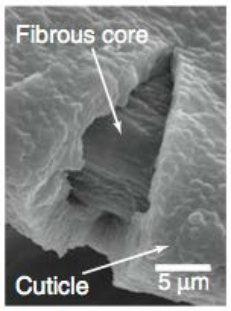

Figure 4: SEM micrograph of byssal thread torn by extreme strain (Harrington 2010)

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2. Chinese Soft-Shelled Turtle (*P. sinensis*) - Pelovaterin

Overall Composite Material: Turtle eggshell

Main Characteristic of Composite: Soft, pliable, macro-porous, antimicrobial

Materials Being Crosslinked: Calcium carbonate crystals (aragonite and vaterite)

Crosslinker/Binder: Pelovaterin protein

Figure 5: Chinese Soft-Shelled Turtle

Introduction

Chinese soft-shelled turtles (*Pelodiscus sinensis*) lay their eggs in the loose soil and sandy shores of many common bodies of water. Unlike bird eggs with their hard shells of dense calcium carbonate ($CaCO_3$) crystals, *P. sinensis* eggs are soft and more sparsely mineralized; they also lack a cuticular layer and so depend entirely on the mineral shell and thin underlying membrane to carry out all of the necessary functions of an eggshell. A small protein called pelovaterin serves both to mediate the nucleation of the carbonate mineral and to regulate the form of the crystal structure. It also interacts with certain bacterial cell membranes in an antimicrobial capacity.

Crosslinking Activity

Pelovaterin is a 42 amino acid peptide with a unique sequence not found in any other organism. Three internal disulfide bridges and hydrogen bonding between three anti-[illegible] -defensin 3 (fig 2). Although these two compounds have a similar 3D shape, a key difference is pelovaterin's extremely hydrophobic core compared to defensin's hydrophilic core.

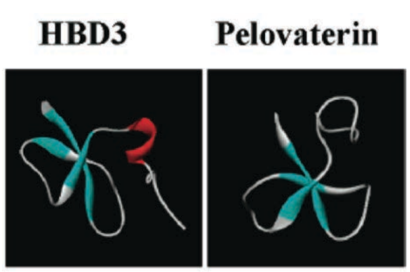

Figure 6. Structure similarities to [illegible]-defensin 3 (HBD3 at left), and pelovaterin (right). (Lakshminarayanan, 2008)

Entropic driving forces cause dozens to hundreds of individual pelovaterin molecules to coalesce into micelles (detergent-like spherical structures with a hydrophilic exterior and an oily, hydrophobic interior). The small hydrophilic N-terminal tails of the proteins extend out into the solution while the larger hydrophobic regions collect together into a sphere.

The two aspartic acid residues at the N-terminus permit the nucleation of calcium carbonate crystals and provide mineral binding potential owing to their flexible location. The micelle provides a much more stable nucleation site for calcium carbonate crystals than free-floating individual proteins could. As the concentration of pelovaterin in solution increases, micelles grow and the number of nucleation sites increases.

[illegible]
Pelovaterin regulates the phase of the nascent carbonate minerals to produce either aragonite or vaterite crystals as opposed to calcite, the form found in avian egg shells. This is thought to be the result of thermodynamic stability inversions mediated by the hydrophobic-hydrophillic interface of the micellar surface.

When crystals from many neighboring micelles meet, they form a mineral film that becomes the eggshell (fig. 3). While the pelovaterin peptide is the major intracrystalline component in the fully-formed eggshell, it's not clear if there is an actual chemical bond, even a weak one, linking the organic and mineral faces or if the peptide stays in place because it's now wedged between the crystals.

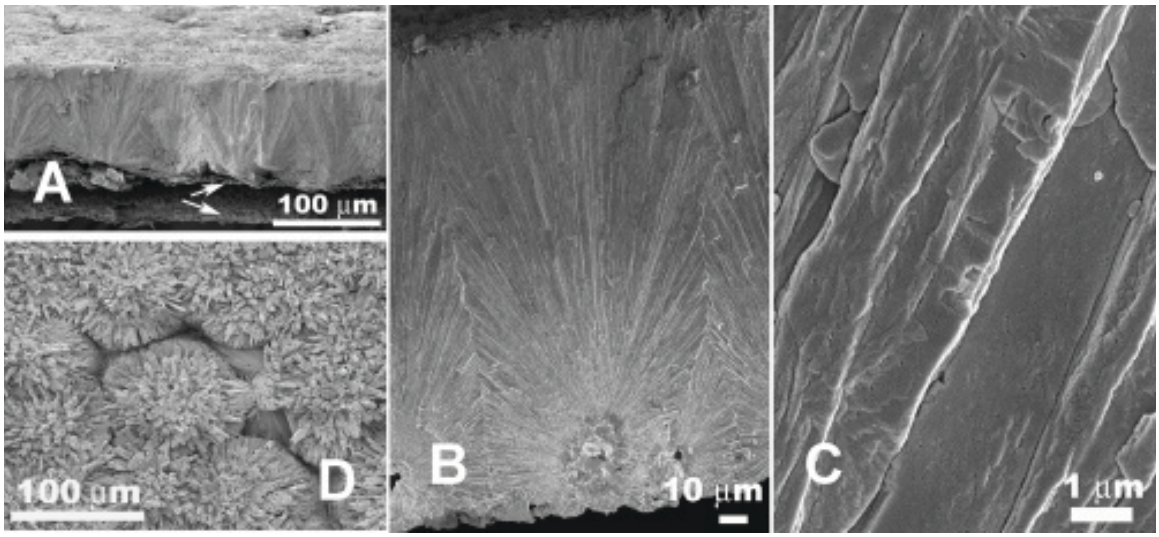

Figure 7. Ultrastructure of turtle eggshell. (A) Cross section of the whole turtle eggshell. The white arrow indicates the shell membrane. (B) Cross section of the bleach-treated eggshell. Note that the membrane is completely removed after bleach treatment. (C) Magnified image of central portion of crystalline layer. (D) Crystalline layer that is attached to the shell membrane. Note the acicular needles of aragonite radiate from the center. (Lakshminarayanan, Fig 1, 2008)

Antimicrobial activity: Defensin is a cationic membrane-active peptide that uses electrostatic charge differential to interact with, and eventually destroy, the integrity of lipid bilayer membranes of many microbes in a non-specific manner. Pelovaterin interacts with membranes as well, but, having a net charge of only -1, does so for very different reasons; its core is extremely hydrophobic (in stark contrast to defensin's hydrophilic core), which enables it to embed in the hydrophobic microenvironment of lipid bilayers rather than on the surface. Although pelovaterin shows nowhere near the antimicrobial spectrum breadth of defensin, it's a fascinating quirk of evolution that it adopted such a similar 3D structure and function to defensin despite using a totally different mechanism. Pelovaterin's interactions extend well beyond lipid membranes, however.

References

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3. Chiton Tooth – Mg^{2+} and Na^{+}

Overall Composite Material: Chiton tooth

Main Characteristic of Composite: Hard, crack resistant, wear resistant

Materials Being Crosslinked: Semi-crystalline α-chitin polysaccharide (poly-ß-1,4-N-acetylglucosamine), aspartate-rich proteins, and magnetite (Fe_3O_4) crystals

Crosslinker/Binder: Mg^{2+} and Na^{+} ions

Figure 8: Chiton

Introduction

Chitons are small marine mollusks that graze on algae and other microbes present on hard surfaces in their environment. They scrape these surfaces with a mouth organ called a radula (rasping tongue), which contains two rows of extremely hard teeth (fig. 2). The radula acts as a conveyer belt on which teeth are present in progressively more developed forms. Fully mature teeth at the tip of the radula are used and lose integrity until they are shed off. At the other end of the conveyer belt, an organic scaffold mediates the biomineralization of new teeth. This progressive system allows the chiton to wield 'fresh' teeth at all times.

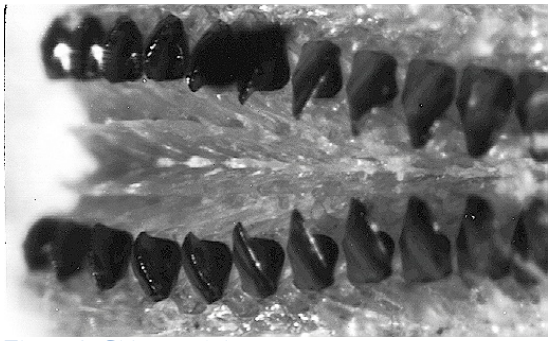

Figure 9: Chiton radula

Crosslinking Activity

The organic scaffold is made of α-chitin polysaccharide (poly-ß-1,4-N-acetylglucosamine) strands arranged in fiber bundles of 5-10 nm diameter. Chitin is present in both crystalline and amorphous states. The core of the

fiber is primarily composed of crystalline chitin while amorphous chitin loops extend from its surface to give the structure a rough appearance.

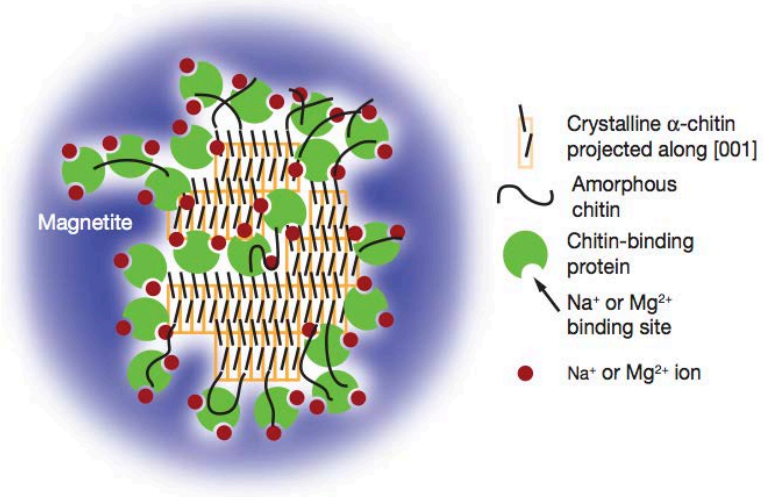

Figure 10: Chitin fiber (Gordon, 2011)

Bound to the surface and embedded in the core of the chitin fiber are numerous proteins. These probably feature chitin-binding domains that hydrogen bond with the polysaccharide. Most appear to be aspartate-rich proteins that have ion binding sites specific primarily for either Mg^{2+} or Na^{+} (metal binding sites may be of the DEAD box motif variety). Aspartate-rich proteins that selectively bind the different ions segregate to different regions of the fiber; sometimes only a few micrometers separate the different clusters. This probably relates to how the different protein-ion complexes regulate biomineralization and accounts for an additional level of the structural hierarchy.

Mg^{2+} and Na^{+} act as the main interface between the organic scaffold and the magnetite mineral. Carboxyl, amine, and hydroxyl side chains of the aspartate-rich proteins also probably form electrostatic bonds with the iron cations and oxygen anions of the mineral. In addition, free Mg^{2+} probably increases the stability of the magnetite-precursor ferrihydrite in solution and prevents biomineralization. Binding the Mg^{2+} would destabilize the soluble ferrihydrite and cause biomineralization of the magnetite on the scaffold.

After complete biomineralization, the magnetite crystal lattice completely occludes the organic scaffold. The rough interface gradient between the scaffold and the mineral probably increases adhesion, toughness, and the number of sacrificial bonds between the structures.

References

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4. Deep Sea Sponge (*Euplectella sp.*) – Silicatein

Overall Composite Material: Sea sponge skeleton

Main Characteristic of Composite: Hard, flexible, crack resistant fibers in rigid construction

Materials Being Crosslinked: Amorphous hydrated silica

Crosslinker/Binder: silicatein, silintaphin, and galectin proteins

Figure 11: Euplectella sp. Deep sea sponge

Introduction

Euplectella sp. sponges live on the deep sea floor. Exposed to constant currents, they've evolved the capacity to produce extremely rigid skeletons to maintain their structural integrity. Despite belonging to the *Porifera* phylum, often regarded as the simplest animals, these sponges produce a remarkable biosilica (i.e., glass) composite skeleton that features complementary, hierarchically layered organization (fig. 1).

Glass, though very hard, is a poor structural material due to its brittleness. Applied stresses collect at any point of surface defect and the material fails catastrophically once a crack forms. The bottom-level architecture of the skeleton, like bullet-proof glass, consists of concentric sheets of hydrated silica nanospheres (50 to 200 nm in diameter) glued together by an organic matrix forming a spicule about 50 microns in diameter. This strategy prevents cracks from propagating deep into the spicula and provides multiple, energy absorbing failure points before the entire structure yields (fig. 2).

This laminated construction of alternating organic/silica layers underlies the strength of the entire material as a whole. Successively higher tiers of organization require the fundamental integrity of this most basic building block. The sponge has therefore evolved extremely efficient mechanisms for forming and gluing together the biosilica sheets.

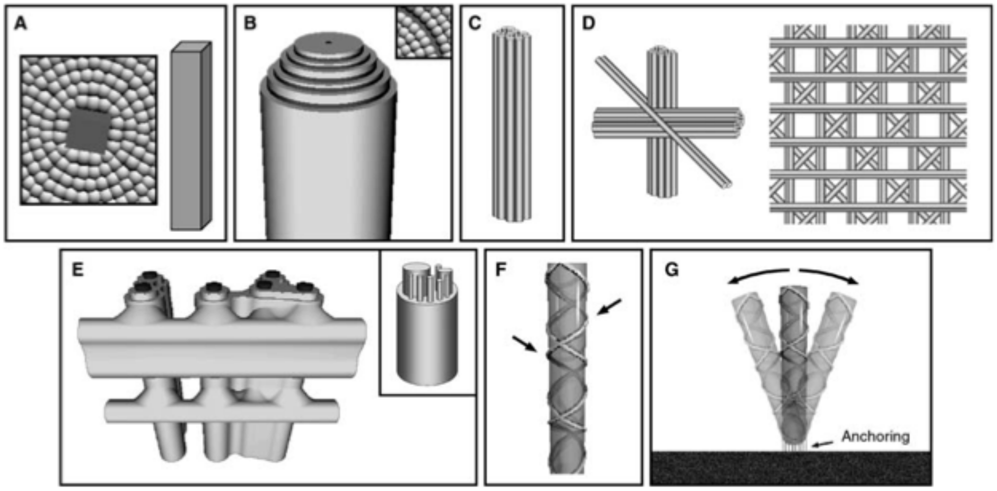

Figure 12. Hierarchical construction of the Euplectella sp. skeleton. A) silica nanoparticles deposited on organic axial filament B) alternating layers of organic and silica lamina to form spicule C) many spicula bundled into a fiber D) horizontal, vertical, and diagonal bundles form checkerboard cage of skeleton E) architecture of node between bundles F) ridges surrounding anchor structure G) flexible anchor attached to inflexible silica rod (Aizenberg, 2005)

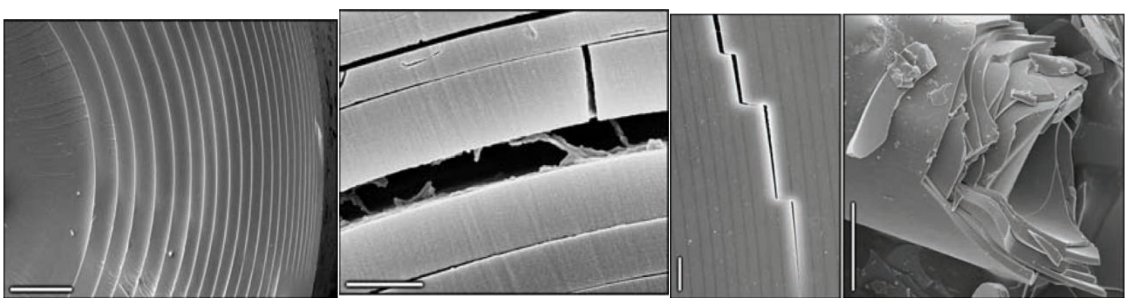

Figure 13. SEM micrographs of the lamina of the spicula. From left to right, the images depict: an undamaged spicule; a damaged spicule with organic matrix visible between separated biosilica sheets; a severely damaged spicule with a crack propagating through successive layers of silica; a severely damaged spicule. (Aizenberg, 2005)

Crosslinking Activity

Silica is produced by the enzyme silicatein in sponge cells called sclerocytes. The mechanism depicted in figure 4 shows how the active site histidine and serine side chains, bound by a hydrogen bridge to increase nucleophilicity, interact with a free silicate ion to yield a trisiloxane ring. This compound is extremely reactive and readily self-assembles to produce amorphous silica nanoparticles. Silicateins have a tendency to form flat oligomer complexes comprised of multiple isoforms. These complexes direct the spatial assembly of the nascent silica nanoparticle into solid pieces of basically uniform structure; the complexes remain attached to the silica due to the clusters of serine hydroxyls present on silicatein-[illegible] material through a sclerocyte during production of biosilica.

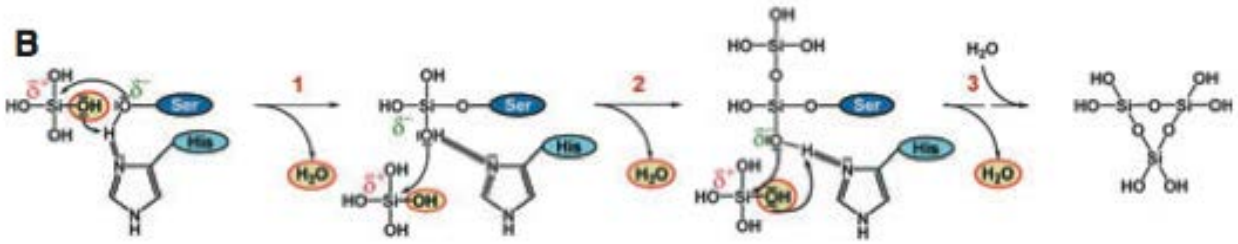

Figure 14. Cartoon of the mechanism by which the active site serine and histidine of silicatein produce a reactive trisiloxin ring (Wang, 2012)

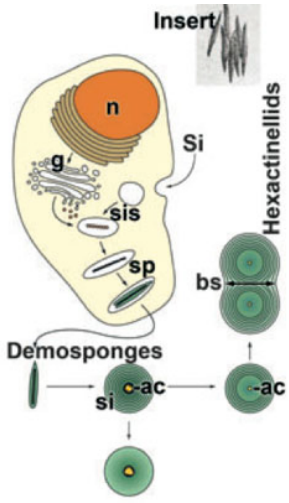

Figure 15. Cartoon of a sclerocyte absorbing silicate, transporting it into the silicasome vesicles, forming the basic silica nanoparticle, and transporting the nanoparticle to the extracellular space for incorporation into the nascent biosilica layer on the spicule. (Wang, 2012)

Proteins released from the sclerocytes called silintaphins deliver calcium ions to proteins called galectins in the extracellular space. In the presence of Ca^{2+}, galectins aggregate into glue like sheets that are capable of adhering to silicatein-coated silica nanoparticles (fig. 5). Sequential release of silica nanoparticles and silintaphins allows the sclerocytes to regulate the formation of the concentric lamina in the growing spicula.

According to certain structural engineering theory, evolution has optimized the architecture of the reinforced cage structure seen in these *Euplectella* sponges. Each spicula-bundle node has six connections (2x horizontal, 2x vertical, 2x diagonal); this produces a "checkerboard" reinforcement pattern in which alternating square sectors are diagonally reinforces instead of every one (8 connections/node) (fig. 6). Six connections is considered sufficient for a 2-dimensionally reinforced structure to be maximally stable. Eight connections would confer no additional strength to the skeleton. In the sea, silicate is a scarce compound and this architecture helps reduce waste without loss of structural integrity.

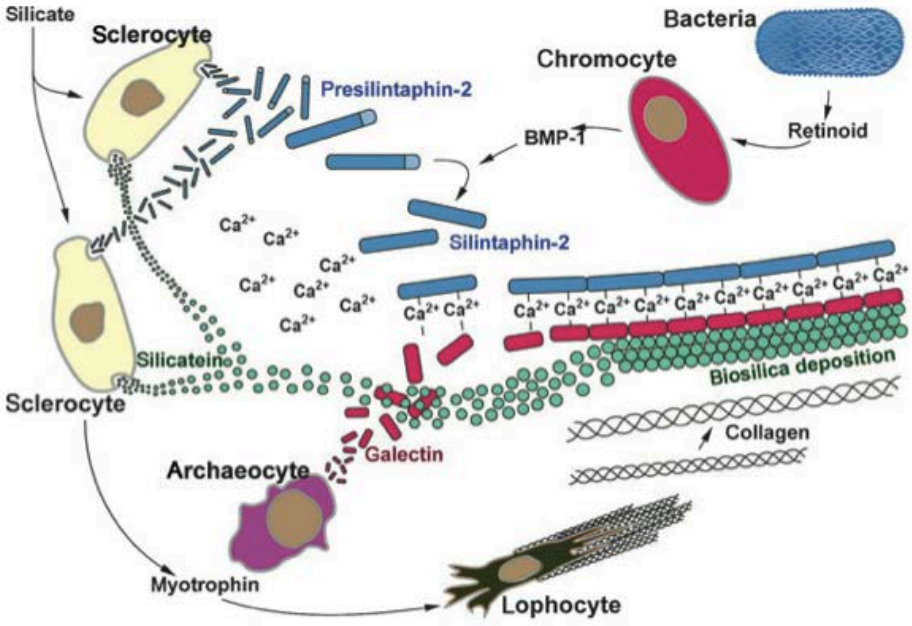

Figure 16. Cartoon of the major actors and components in the lamina forming process (Wang, 2012)

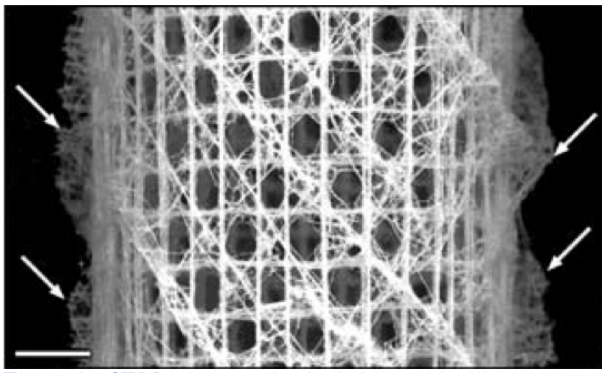

Figure 17. SEM micrograph of reinforced biosilica skeleton

References

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Wang, Xiaohong, Ute Schloßmacher, Matthias Wiens, et al. "Silicateins, Silicatein Interactors and Cellular Interplay in Sponge Skeletogenesis: Formation of Glass Fiber-like Spicules." *FEBS Journal* 279.10 (2012): 1721–1736.

5. Flax Stem – Arabinogalactan protein (AGP)

Overall Composite Material: Flax stem fiber

Main Characteristic of Composite: Tough, resists compression and tension, water resistant, and antimicrobial

Materials Being Crosslinked: Cell wall polysaccharides (cellulose, hemicelluloses, and pectic galactans)

Crosslinker/Binder: Arabinogalactan protein AGP

Figure 18: Flax plant (left); flax fibers (right)

Introduction

Plant fibers are highly specialized cells that serve primarily mechanical functions. To fulfill that function, fiber cells tend to be relatively long (up to several centimeters) and have relatively thick cell walls. Fiber cells can be categorized according to the polyphenolic (i.e. lignin) content of their cell walls. Wood fibers are lignin-rich while crop stems such as flax, hemp, ramie, and nettle are considered non-lignified fibers. The elementary fibers (single cells) of flax and hemp consist of layers with a central lumen (fig 2). In flax stems, fiber cells present in the phloem layer contribute to their extreme toughness.

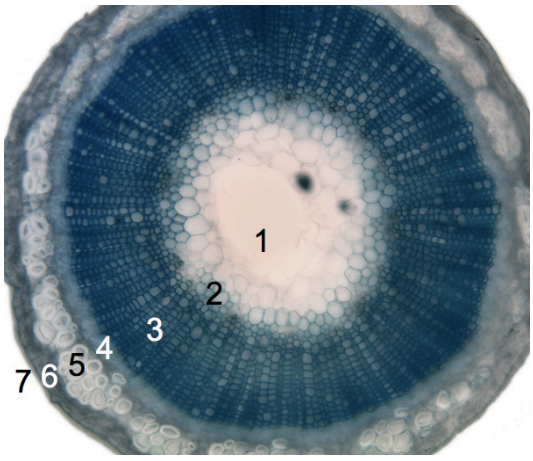

Figure 19: Cross section of a flax stem (1. Pith, 2. Protoxylem, 3. Xylem I, 4. Phloem I, 5. Sclerenchyma (bast fibre), 6. Cortex, and 7. Epidermis.) (source: http://en.wikipedia.org/wiki/Phloem)

Crosslinking Activity

Flax phloem fibers owe their strength primarily to cellulose, a glucose polymer that crystalizes into bundles (cellulose microfibrils) via dense hydrogen-bonding between adjacent strands. These cellulose microfibrils are largely arranged axially with the fiber as a whole and are crosslinked into a network by other polysaccharides, mostly of the pectic and hemicellulosic varieties (fig. 3). Chief among the crosslinking pectins in flax fibers are pectic galactans which consist of an RG-1 (Rhamnogalacturonan I) -like pectin backbone with many galactan branches (fig. 4)

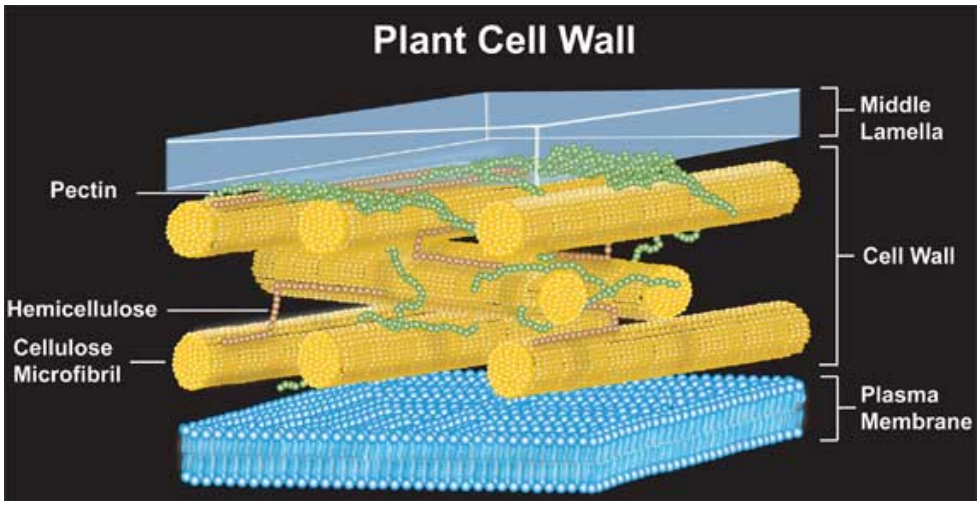

Figure 20. Typical plant cell wall with cellulose microfibrils crosslinked by hemicelluloses and pectins. Although this illustration shows cellulose fibers in alternating orientations, flax stem cellulose fibers run along the longitudinal axis of the plant. (middle lamellae attach cells to each other) (Pingry School)

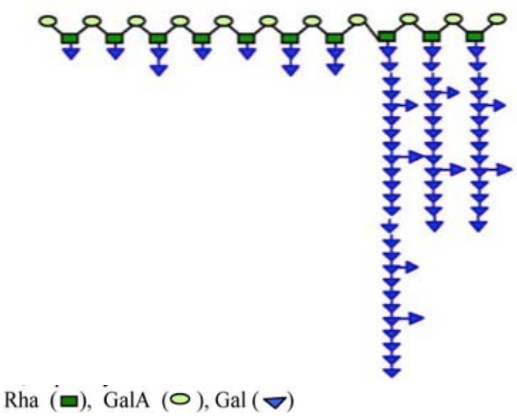

Figure 21: Typical pectic galactan from flax fibers (Rha= Rhamnogalacturonan I, Gal=galactans) (Gorshkova, 2006)

Pectic galactan forms a network of non-covalent bonds with cellulose microfibrils via hydrogens bonds from its numerours hydroxyl groups. Altogether, the carbohydrate component of flax fiber is sufficient to impart the material with most of its structural properties. However, there are certain glycoproteins also implicated as crosslinking agents, particularly a group of proteins with a promiscuous binding repertoire called arabinogalactan proteins (AGPs).

AGPs are membrane surface proteins bound to the lipid bilayer. During cell wall thickening phases, AGPs are transported to key positions on the cell membrane (fig. 5) in concert with cellulose synthase enzymes to reinforce the cell wall. AGP tightly associates with cellulose microfibrils and pectins. It tends to associate with

pectins rich in galacturonic acid (like pectic galactans) and hemicelluloses rich in glucose. Its large, branched glycans probably forms crosslinks with these carbohydrates by hydrogen-bonding between the AGP glycosyl chain hydroxyl groups and those of the structural fibers. It has also been hypothesized that the arabinogalactan side-chains form peroxide-mediated covalent bonds with structural polysaccharides.

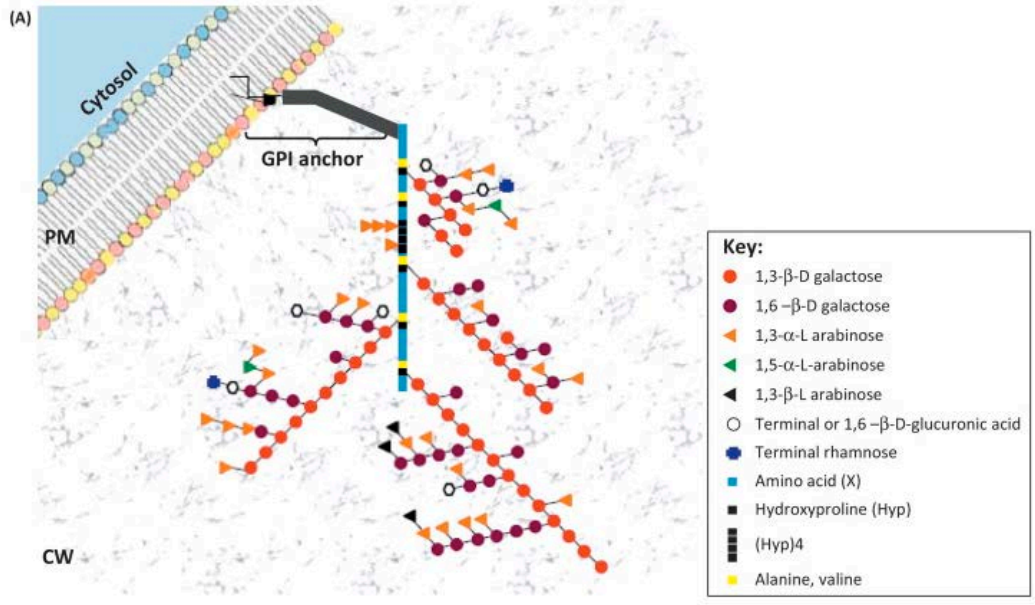

Figure 22. AGP bound to lipid bilayer by GPI anchor. Miscellaneous common glycosylation chains shown O-linked to AGP hydroxylproline residues (Nguema-Ona, 2013)

The lipid anchor is a glycosylphosphatidylinositol (GPI) in which the C-terminal is linked by a phosphoethanolamine to a short oligosacharride that is finally linked to an inositylphosphoceramide lipid (GPI). This lipid tail embeds in the lipid cell membrane and tethers the entire glycoprotein to the surface. Post-translational transformation of proline residues on AGP into hydroxyproline allows for O-glycosylation of the protein. The polysaccharide chains vary greatly in size and composition among different AGPs even within the same tissue; however, they mostly consist of a galactan backbone with various arabinose and galactose branches.

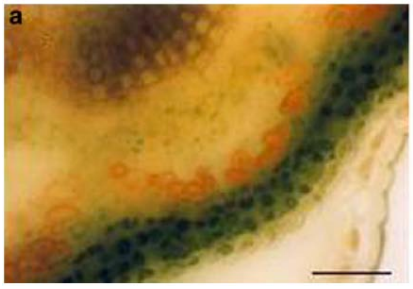

Figure 23: Stained AGP-containing cells in 50-day old flax fiber phloem wall (Gorshkova, 2006)

Though AGPs contribute to less than 0.5% of the total flax fiber mass, their selective high-level release at specific phases of cell wall growth (fig. 6) suggests an important function. Besides forming crosslinks between structurally significant carbohydrates, their potential ability to link the cell wall to the lipid membrane has been hypothesized as possibly significant.

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6. Human Cells – Filamin B

Overall Composite Material: Human cytoskeleton

Main Characteristic of Composite: Rigid, flexible, dynamic

Materials Being Crosslinked: Filamentous actin (F-actin)

Crosslinker/Binder: Filamin B homodimers

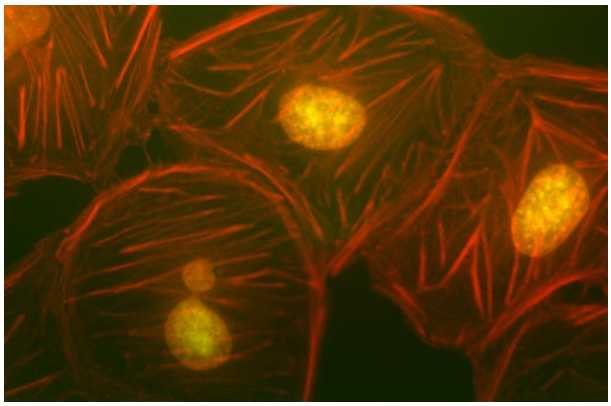


Figure 24: Actin filaments (stained red), and the nucleus (stained yellow). (source: http://itg.beckman.illinois.edu/technology_development/web_atlas/structures/nucleus_actin/)

Introduction

Rather than being amorphous blobs, cells build a cytoskeleton that allows them to tightly regulate their 3-dimensional shape and rigidity depending on their demands and functions (fig 1). Underlying this regulation is a matrix of filaments made of long protein polymers known as filamentous actin (F-actin) (fig. 2). This inter-linking greatly increases rigidity of the cell, supports the cell membrane, regulates key functional molecules, and in some cases, acts as a trackway for cargo transport by motor proteins. The filaments are densely crosslinked by actin binding proteins which direct the formation of different kinds of F-actin composites. A particularly interesting example of an actin binding protein is filamin B.

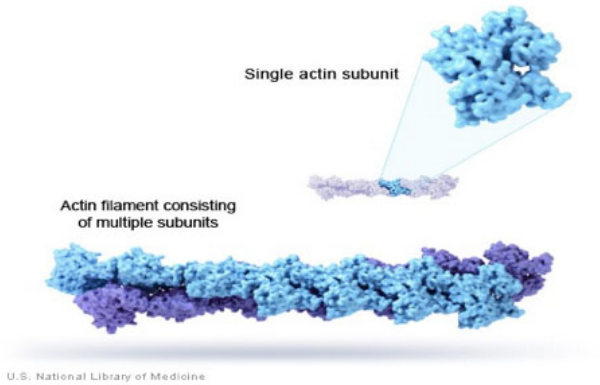


Figure 25. Actin filament made of actin subunits (U.S. National Library of Medicine)

Crosslinking Activity

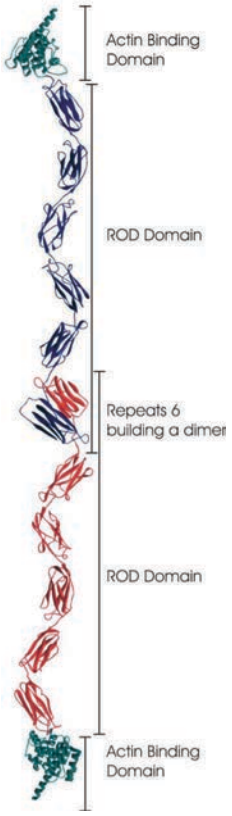


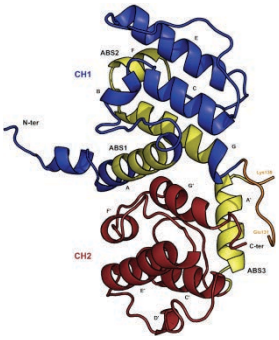
Figure 26. Filamin dimer; note that this illustration depicts a filamin with six immunoglogin-like domain repeats instead of the 24 in human filamin B (Popowicz, 2004)

Two filamin monomers dimerize in an antiparallel fashion (i.e., one laying head to tail, the other tail to head) to produce a long and highly flexible protein link (fig. 3). Separated from each other by a long chain (ROD domain) of 24 repeated immunoglobin-like domains (~95 residues each) are the two N-terminal actin binding domains (ABD). Electrostatic forces between C-terminal 24th repeats of adjacent filamins induce dimerization, which is critical to the functionality of the molecule. A single filamin dimer is able to crosslink F-actin strands up to 100 nanometers away.

The human filamin B ABD is a 242 residue segment containing two calponin-homology domains designated CH1 and CH2 (fig. 4). Both appear to be critical to the functionality of the linker. In consensus with the ABDs of many other actin binding proteins, filamin B's ABD features three actin binding sites (ABS); the first two are located on CH1 while the third is located on CH2. Each calponin-homology domain constitutes four alpha-helices. Interestingly, the ABSs are found on non-aligned surfaces of the ABD. It is likely that the domain folds on contact with actin to induce binding. The residues involved in the crosslink have yet to be precisely defined but probably engage in non-specific electrostatic interactions with the negatively charged actin; a wide variety of binding conformations have been observed experimentally.

Perhaps most fascinating about filamin is the great variety of cytoskeletal structures in can induce depending on its concentration relative to F-actin (fig. 5). At lower concentration, filamin forms sparse crosslinks between F-actin to make a weakly crosslinked matrix. Intermediate concentrations induce formation of bundled F-actin within the matrix. Increasing the concentration further completely transforms the network into a bundled one. Extremely high concentrations cause the bundles to cluster. The precise mechanism underlying these transitions in not yet understood but is obviously related to the increased crosslink density between actin filaments. The diverse array of cytoskeletal conformations directed by filamin B, just one of many dozen different actin binding proteins each with their own characteristics, enables to cell to produce a range of important structures.

Figure 27. Human filamin B actin binding domain featuring two calponin-homology domains (CH1 and CH2) and three actin binding sites: ABS1, ABS2, and ABS3. (Sawyer, 2009)



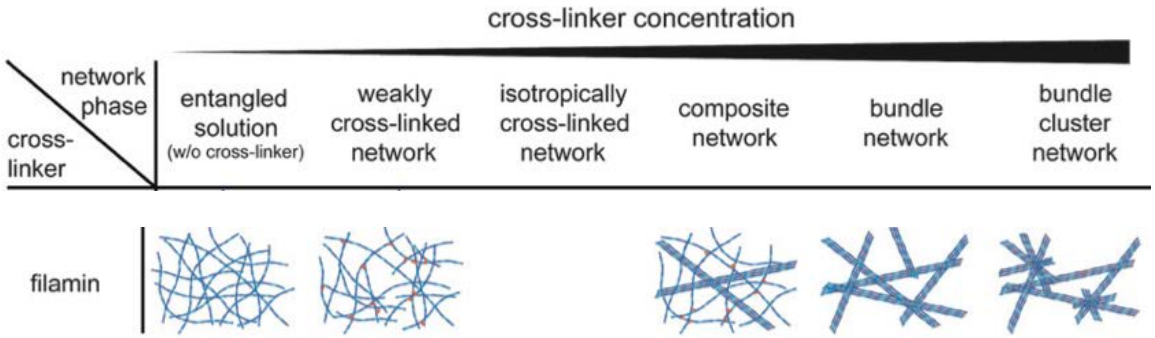


Figure 28. Actin structures formed at different relative filamin concentrations (Lieleg, 2010)

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7. Human Joint – Fibulin-2

Overall Composite Material: Articular Cartilage

Main Characteristic of Composite: Springy, turgid, compression resistant

Material Being Crosslinked: Aggrecan (chondroitin sulfate proteoglycan 1; primary isoform: 2316 residues, >2500 kDa; Core Protein <250 kDa + 100-150 glycosaminoglycan chains)

Crosslinker/Binder: Dimeric Fibulin-2 (X-shaped dimer conformation)

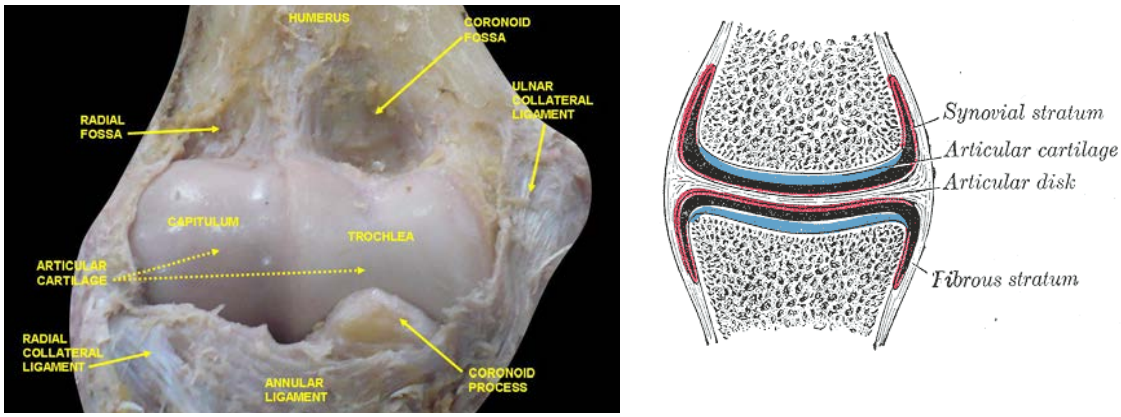

Figure 29: Articular cartilage on human elbow synovial joint

Introduction

Networks consisting of polysaccharide (hyaluronan) and protein (aggrecan) are critical components of articular cartilage; they give the material much of its compressive load resistance. Entrapped within the collagen fibril complex, aggrecan-hyaluronan networks compress to about one seventh of their volume generating high turgor, elasticity, and high compression resistance. The substantial negative charge of the aggrecan-hyaluronan complex is responsible for high water uptake of articular cartilage which can be released upon pressure, serving as a joint lubricant during motion.

Crosslinking Activity

Aggrecan (fig 2) plays an essential role in the formation of the aggrecan-hyaluronan network. Aggrecan proteins are crosslinked to each other by the fibulin-2 dimer forming an aggregan network which, in turn, binds directly to hyaluronan (fig 3).

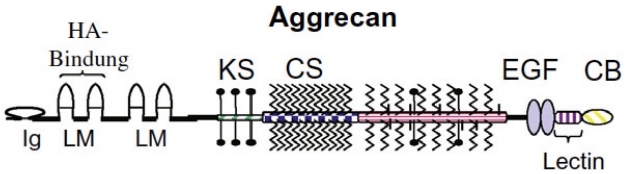

Figure 30: Modular structure of the proteoglycan, aggrecan. The central glycosaminoglycan (GAG) shaft consists of chondroitin/dermatan sulfate (CS), and keratansulfate (KS). To the left of the GAG shaft is the N-terminal domain that binds to hyaluronan (HA). The HA binding site consists of an immunoglobulin-like repeat followed by two proteoglycan tandem repeats. To the right of the central shaft is the C-terminal that binds to Fibulin-2 dimer linking aggrecan molecules together. (sources: von der Mark 2013, and Aspberg 2012)

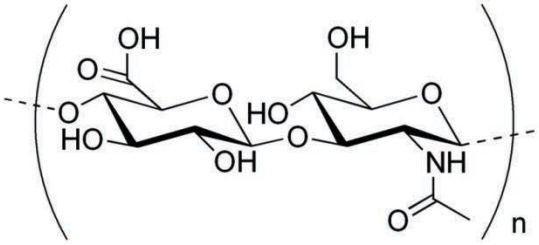

Figure 31: Hyaluronan (formerly called hyaluronic acid, HA), an anionic, non-sulfated glycosaminoglycan

The molecular basis for this compressive load resistance functionality of the aggrecan-hyaluronan network is the extremely high fixed charge density of the many glycosaminoglycans (mostly chondroitin sulfate) that branch out from the long central shaft of aggrecan (fig 2). These create high osmotic swelling pressures for the cartilage as a whole. Flanking either side of aggrecan's central glycosaminoglycan (GAG) shaft are two globular domains responsible for binding the proteoglycans to other matrix elements to direct the supramolecular organization of the tissue. The N-terminal globular domain is responsible for binding to the hyaluronan network of the cartilage while the C-terminal globular domain consists of several binding motifs including a c-type lectin-like domain (CLD); several ligands of this C-terminal have been documented including fibulin-2.

Fibulin-2

Fibulin-2 (fig 5) binds numerous extracellular matrix proteins including several lectican proteoglycans like aggrecan. The binding is mediated through the CLD domain on aggrecan's C-terminal. This binding has been shown to be specific since fibulin-2 will not bind the related CLD appearing on other lectican proteoglycans such as neurocan. Of unknown importance is the observation that only the X-shaped dimer of fibulin-2 (fig 6) is involved in binding with aggrecan.

Fibulin-2 Structure

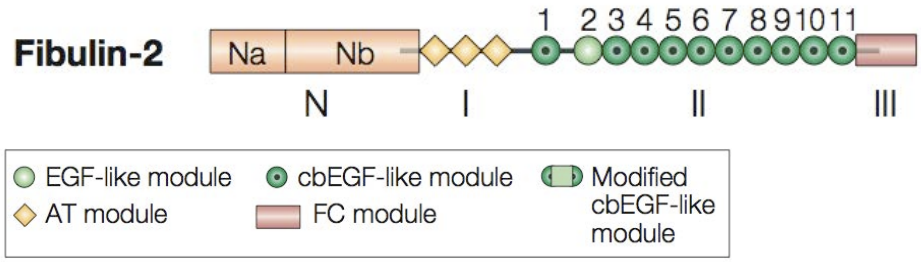

Figure 32: Fibulin-2 monomer

Fibulin-2 consists of 4 domains. The 400 residue N-terminal domain (N) is comprised of a 150 residue cysteine (Cys)-rich region (Na) at its own N-terminal followed by an entirely cysteine-free region (Nb). Following the N domain is domain 1 (I) which consists of three tandem anaphylatoxin-like (AT) modules. Each AT module is ~40 residues in length and contains 4-6 cysteine residues which form 2-3 disulphide bridges amongst themselves which stabilize the compact alpha-helical structure of the modules. The long central shaft domain (II) consists of 11 tandem EGF-like modules (10 of which are of calcium-binding variety; cbEGF). These EGF-like modules are also ~40 residues in length and form 3 internal disulphide bonds to stabilize their structure. Finally, the C-terminal domain (III) is ~140 residues in length and contains only 2 additional cysteines. It is of the eponymous fibulin-type carboxyl terminus (FC) variety of domains.

Fibuliun-2 forms disulphide-linked dimers between un-bridged cysteines in the central AT module of domain I. Mutations which remove/replace that active Cys residue prevent covalent dimerization but do not prevent dimerization completely; there is affinity between domain II and domain N of different fibulin-2 molecules. The covalent dimerization involves anti-parallel (i.e., opposite orientation) fibulin-2 monomers linked together

by their domain Is. The dimer stretches on in both directions from this central junction for a total length of 70-80nm. The non-covalent N-II interactions can form or break and give the dimer one of 3 structures: rod, Y, and X.

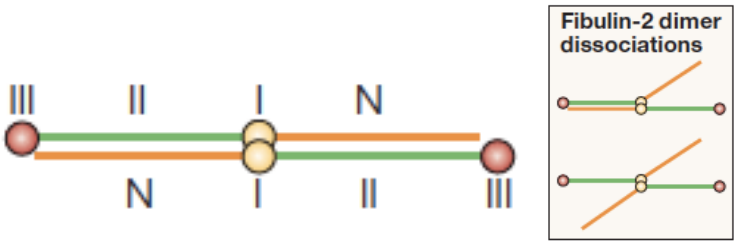

Figure 33: Fibulin 2 dimer (Timpl 2003)

Calcium binding in domain II of Fibrulin-2 is thought to impart this crosslink with rigidity and robustness. The cbEGF-like modules that make up the central shaft of fibulin-2 are probably permanently saturated with calcium due to their binding affinity and the physiological extracellular calcium concentration of ~1 mM. Calcium is ligated by several regions of cbEGF including a D-X-D/N-E region prior to the first cysteine residue (where X is any amino acid), a loop created between Cys2 and Cys4, and in the extremely hydrophobic intermodular contact regions. This binding within the intermodular junctions is thought to impart the long shaft (domain II) with it high rigidity.

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8. Malaysian Tree Frog (*P. leucomystax*) – bis(LTQ), Zn^{2+} complex

Overall Composite Material: Foam nest

Main Characteristic of Composite: Viscous, strong, long-lasting

Materials Being Crosslinked: Ranasmurfin protein

Crosslinker/Binder: bis(lysine tyrosyl quinone) (*N*-linked indophenol bond), Zn^{2+} complex

Figure 34: A mating pair of Malaysian tree frogs and their foam nest

Introduction

The common Malaysian tree frog, *Polypedates leucomystax*, unlike other foam-nest producing frogs, makes its nest on dry surfaces. These nests are positioned so that emerging tadpoles drop into a body of water below. In further contrast with other foam-nest frogs, *P. leucomystax* foam depends on its viscosity for stability rather than the presence of biosurfactancts (i.e. detergents).

Crosslinking Activity

One of the key features of foam nest composite is the crosslink observed when two ranasmurfin protein molecules form a homodimer (fig 2). Ranasmurfin monomers (113 residues) exhibit a novel tertiary structure comprised of several alpha-helices stabilized by three internal disulfide bonds.

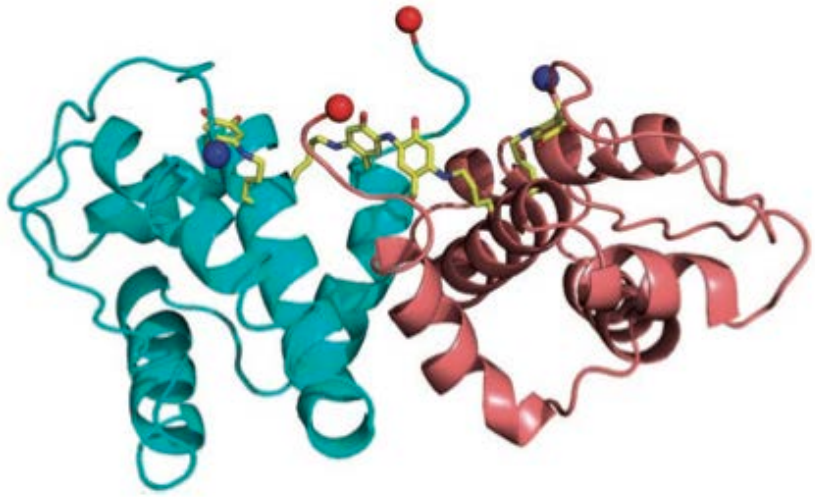

Figure 35. 3D structure of ranasmurfin dimer. Monomers are color-coded cyan and salmon. LTQ and bis(LTQ) groups depicted as yellow stick structures. N-terminal in blue, C-terminal in red (Oke et al., 2008)

The protein features an unusual post-translational modification of lysine-31 and tyrosine-2, dubbed the lysine tyrosyl quinone (LTQ) linkage (fig 3), in which the tyrosine-2 side-chain phenol ring is transformed into a catechol ring (i.e. dopa) and forms an ortho bond with a side-chain nitrogen from lysine-31. The dopa hydroxyl groups of the crosslink ring form a bifurcated hydrogen bond with the hydroxyl side-chain of serine-5 and a water molecule. This unusual internal protein link probably serves to impart strength and stability to the dimerizing crosslink which incorporates the adjacent lysine-30.

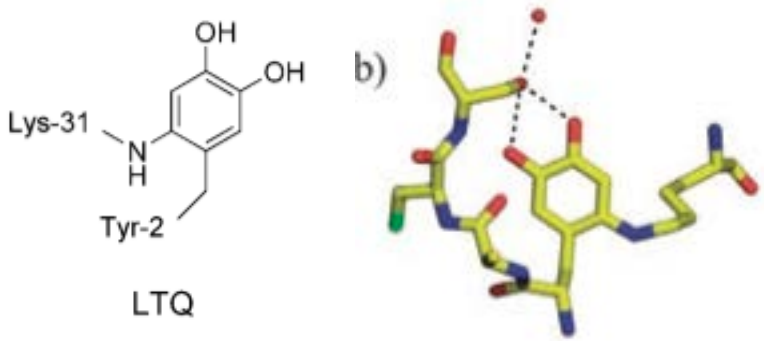

Figure 36. Diagram of the LTQ bond (left) and hydrogen bonds (dashed-lines) formed between the catchol ring hydroxyls, the serine-5 hydroxyl, and water (right) (Oke et al., 2008)

Lysine-30 forms a post-translationally modified bond with tyrosine-108 not unlike that found in the LTQ bond (fig 4). However, unlike the LTQ bond, the Lys-30/Tyr-108 ring crosslinks to that of another ranasmurfin protein via an N-linked indophenol bond (bis-LTQ). This creates an extended aromatic electron system both between disparate points within each monomer (internal crosslink) and between the monomers themselves (external crosslink) that imparts strong covalent stabilization to the dimer. Further strengthening the bond is a single zinc atom coordinated by the oxygen groups of each monomer's Lys-30/Tyr-108 ring and e-nitrogens from imidazole rings of each monomer's His-112. The planes of the rings in the bis-LTQ bond are offset by about 27° from each other to produce a tetrahedryl chelation structure about the metal.

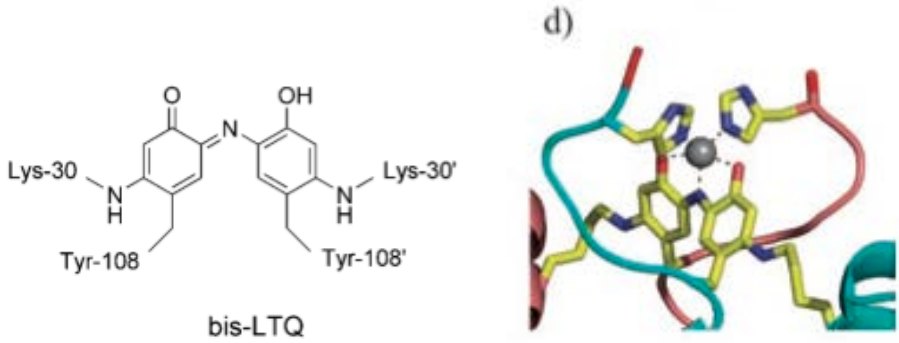

Figure 37. Diagram of bis-LTQ bond (left) and coordination bonds (dashed-lines) between the indophenol's two oxygens and nitrogen, each monomer's His-112, and a zinc atom (Oke et al., 2008)

This crosslink is thought to be part of a large network in the foam that gives the nest long-term strength and stability. Tadpoles take about four days to gestate and the foam gives the embryos a safe microenvironment within which to develop.

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9. Pearl Oyster (*P. fucata*) – Pif Protein Complex

Overall Composite Material: Pearl oyster shell nacre

Main Characteristic of Composite: Hard, tough, smooth, high luster

Materials Being Crosslinked: Aragonite ($CaCO_3$) crystal platelets and chitinous matrix

Crosslinker/Binder: Pif complexes (Pif 80 and Pif 97 dimerized by disulfide bond)

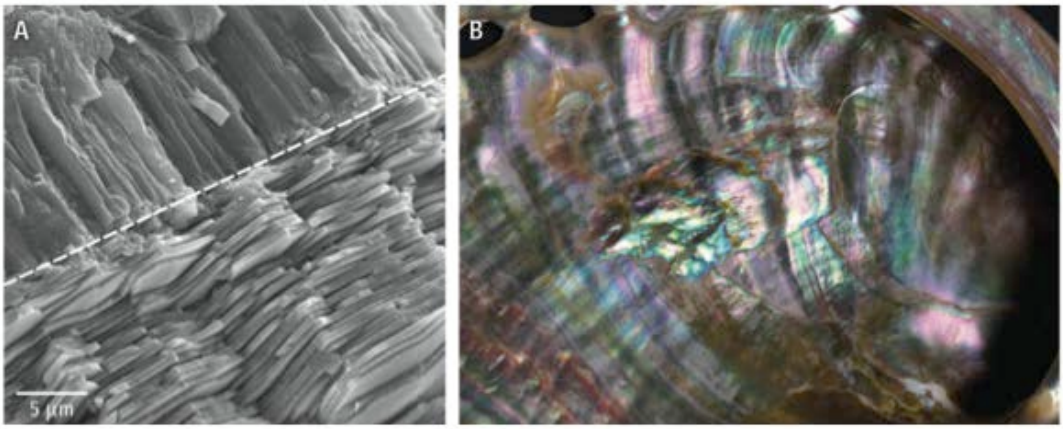

Figure 38: Pearl oyster nacre. A) SEM micrograph of interface between outer, prismatic calcite layer (top) and planar nacre layer (bottom). B) Photograph of nacre (Suzukie 2009)

Introduction

Oysters have extremely soft and delicate bodies. To protect themselves, mollusks produce a hard composite shell capable of withstanding most any insult their environment or predators may deliver. The outer surface, exposed to the oyster's marine environment, is rough and visually dull. But the inner face of the shell, the interface between the oyster's soft body and the shell itself, is composed of an extremely smooth, shiny, iridescent, and hard composite material called nacre.

Crosslinking Activity

While the exterior portion of the shell is composed of tightly stacked elongated prisms of calcite oriented perpendicular to the shell surface (calcite is the most thermodynamically stable crystal polymorph of $CaCO_3$), nacre is composed primarily of flat aragonite plates oriented parallel to the surface (aragonite is the crystal polymorph of $CaCO_3$ stable in marine waters). The mineral crystals are layered with insoluble organic matrices sandwiched between them; they mediate crystal formation and add resilience to the finished composite.

The building blocks of the nacre, nanoparticles of amorphous $CaCO_3$ as well as Ca^{2+} and HCO_3^- are released by mantle epithelial cells and are mineralized onto a chitinous organic scaffold by a protein complex called Pif. The Pif complex not only mediates mineralization of the aragonite plates in the precise orientation required for nacre, it is also responsible for creating a lasting crosslink between the plates and the organic matrix to yield an extraordinarily tough composite.

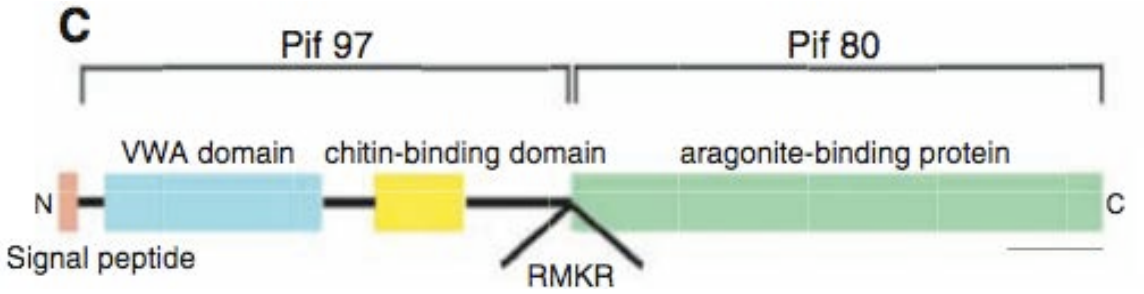

Figure 39. Pif Complex (Suzuki 2009)

The active Pif complex is composed of two proteins (Pif 97 and 80) which are bound together by disulfide bonds. Pif 97 (525 residues) has two primary domains, a von Willebrand type A (VWA) domain for mediating interaction with proteins and a Peritrophin-A type chitin-binding domain. Pif 80 (460 residues) provides the aragonite-binding functionality to the complex. Pif 80 contains a high proportion of charged amino acids (28.5% Aspartic Acid, 18.9% Lysine, 10.9% Arginine). It contains 17 repeats of an Asp-Asp-Arg-Lys motifs scattered throughout its length and an Asp-Glu-Asp cluster at its core. Poly-Asp in particular has been shown to mediate aragonite formation. Aspartic acid is known to regulate crystal polymorphs in a variety of organisms independent of the mineral used. For example, aspartate-rich proteins are involved in the formation of calcium phosphate biominerals of bone and teeth and the amorphous silicate cell walls of diatoms.

During nacre synthesis, the oyster secretes Pif complexes from its mantle epithelium into the extrapallial space. Pif 97, with its chitin-binding domain, binds to chitin microfibrils and then associates with other proteins to form a solid lamellar sheet upon the chitin. The Pif 80 part of the complex then concentrates the calcium carbonate nanoparticles into properly oriented aragonite plates through its many aspartic acid residues. The oyster will then synthesize an additional chitinous network below the finished crystals and the process will be repeated to produce the next layer.

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10. Slug (*Arion subfuscus*) – Ca^{2+}, Fe^{2+}, Mg^{2+}

Overall Composite Material: Defensive glue

Main Characteristic of Composite: Elastic, adhesive, stiff, tough

Materials Being Crosslinked: Slug mucus proteins and polysaccharides

Crosslinker/Binder: Divalent metals such as Ca^{2+}, Fe^{2+}, and Mg^{2+}

Figure 40: The Slug (*Arion subfuscus*)

Introduction

Slugs are extremely vulnerable creatures. To compensate for their lack of conventional defensive assets like teeth, claws, or shells, many have evolved extremely effective chemical defenses. When physically disturbed, the air-breathing terrestrial slug, *A. subfuscus,* secretes specialized mucus packets that rupture releasing a viscous fluid. The fluid could flow off the slug's back, but instead sets within less than a minute into a tough, potent elastic-adhesive gel. This glue acts as a defense against insects and other small predators.

Crosslinking Activity

This tough gel contains more than 95% water; it achieves its defensive properties by forming an interconnected network of proteins and polysaccharides through multiple cross-linking mechanisms.

A. subfuscus adhesive mucus is essentially identical in composition to its relatively non-adhesive locomotive mucus, a complex mixture of polysaccharides and proteins, but differs in that adhesive mucus contains many critical metal ions and specific metal-binding proteins. These divalent metal ions contribute to the formation of two kinds of crosslinks between, and among, proteins and carbohydrates: (1) direct crosslinking with metal atoms, and (2) an oxidative reaction caused by the metals in which proteins link together via imine bridges.

Direct Crosslinking With Metal Atoms

The carbohydrate portion of the adhesive mucus contains relatively high concentrations of sulphate probably due to the presence of heparan sulphate-like glycosaminoglycan carbohydrates, and it likely contains many carboxylated polysaccharides. The protein portion contains a relatively large number of carboxyl and phosphate groups.

Iron, magnesium, and calcium are all strong ligands of these groups. Electrostatic attraction and eventual coordination of these metals by the many instances of these functional groups on the many tangled proteins and carbohydrates in the mucus produces strong crosslinking and accounts for the great part of the adhesive mucus' rapid gelling. In fact, removal of the metals causes a fifteen-fold reduction in the strength of the glue. Experiments designed to remove iron from the glue to test its effects initially concluded that eliminating iron did not have an effect on the material properties. Nonetheless, this is probably due to iron being so tightly chelated in the matrix that removal was impossible. It may be one of the most significant metal crosslinks. The primary cross-links controlling gel stiffness appear to be direct cross-links involving calcium. Calcium likely also plays a roll as an electrostatic crosslink with sulfate (they are the most abundant ions in the gel), but this may be a premature interpretation of data.

Imine Bridge Formation

Metals ions also indirectly contribute to additional crosslinking activity. Iron and copper facilitate the formation of carbonyl groups on protein side chains which readily react with the primary amines of lysine side-chains on other proteins to form imine bonds (fig. 2).

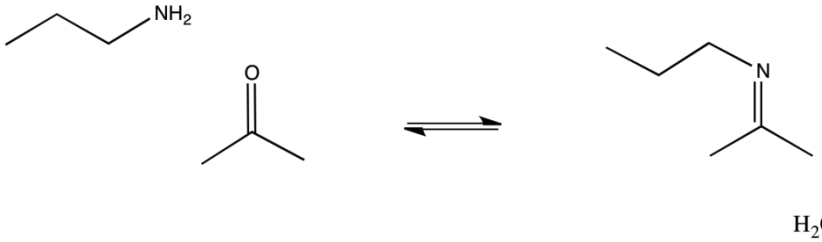

Figure 41 -amino group (i.e. lysine side chain) reacting with a carbonyl group to form an imine bond

Interfering with imine bonds causes a 33% reduction in glue stiffness. Interestingly, the imine bond is one of the few easily reversible covalent bonds. Impeding reversibility actually increased the strength of the glue 40% versus natural control. However, the ready reversibility of this bond and all other crosslinking bonds described here may be of functional significance. After all, the adhesive mucus must rapidly set to deter imminent threats to the slug but long-term adhesion is not needed once the threat is gone and most likely deleterious as dust particles and other debris would accumulate on the surface of the slug. The bonds may also serve as sacrificial shock-absorbers that improve extensibility and self-healing of defensive coating after physical stress.

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11. Vineyard Snail (*C. virgate*) – Epiphragmin hydrophobic effects

Overall Composite Material: Epiphragm

Main Characteristic of Composite: Hard, water resistant, adhesive

Materials Being Crosslinked: Epiphragmin proteins

Crosslinker/Binder: Hydrophobic interactions between coiled-coils of adjacent epiphragmins (forming coiled coils of epiphragmin homo-oligomers)

Figure 42: Snail shell with epiphragm completely sealing the shell opening to prevent water loss and infection

Introduction

The vineyard snail, *Cernuella virgata,* synthesizes a specialized adhesive mucus structure called an epiphragm, which completely seals the shell aperture and prevents water loss during dry weather inactivity. Snails produce two kinds of foot mucus: trail and adhesive. Trail mucus lubricates the animal's locomotion while adhesive mucus helps anchor the animal to substratum during times of inactivity. The two types of mucus are much the same in terms of chemical composition but differ with respect to the addition of certain glue proteins in the adhesive form that are thought to stiffen the material.

Crosslinking Activity

Unlike many other molluscan glue proteins, which rely on covalent crosslinking between glue proteins and carbohydrates in the mucus, the primary glue protein identified from *C. virgata* epiphragm, dubbed epiphragmin, exhibits adhesive/cohesive interactions due to hydrophobic self-assembly.

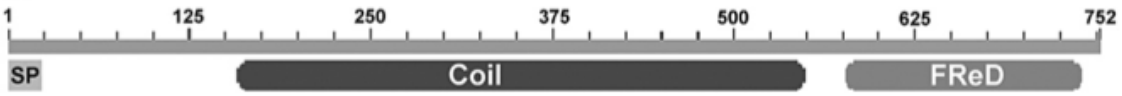

Figure 43: Annotated primary structure of epiphragmin. (Li, 2007)

Epiphragmin (752 residues; ~86 kDa) is the predominant protein in the protein-based epiphragm secretion of *C. virgata.* The N-terminal region begins with a 22 residue signal peptide (SP) domain followed by a ~145 residue section with a sequence homologous only to proteins of unknown function (fig. 2). The central domain shows high sequence similarity to the alpha-helical coiled-coil domains of myosin II and AglZ. A coiled-coil (fig. 3) is a protein structural motif in which multiple alpha-helices closely associate into one "super coil" much like the individual strands of a rope coil around each other to produce the final material. Coiled-coils usually

self-assemble due to an abundance of hydrophobic amino acid side chains exposed on sections of the coil. In water, the individual helices passively aggregate into coils so that as many of their hydrophobic residues are concealed as possible. The C-terminal domain of epiphragmin shows high sequence homology with fibrinogen-related domains (FReDs). It is likely that the epiphragmin FReD binds with other molecules in the mucus, particularly Ca^{2+} ions, to further enhance crosslinking activity, though information on that topic is scarce.

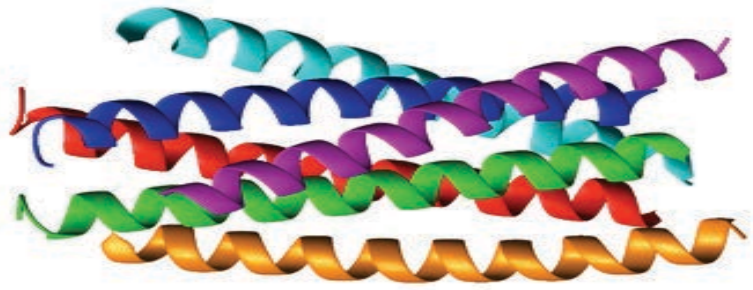

Figure 44: Coiled coils

During synthesis of the epiphragm, it is likely that the presence of high concentrations of epiphragmin causes the rapid formation of huge epiphragmin homo-oligomers that effectively harden the solution. In other words, the coiled-coil domains of many epiphragmin molecules further aggregate with themselves by the same hydrophobic exclusion forces that produced the original coiled-coils themselves. Combined with binding to other molecules mediated by the FReD, the material is sufficiently crosslinked to act as a robust structural material. Though it is tempting to think of this kind of non-specific hydrophobic interaction as inherently weak, this very same kind of aggregation is observed in the coiled-coil assemblies of alpha-keratin in mammalian hair, nails and hooves.

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12. Woody plants – Xyloglucan hemicellulose

Overall Composite Material: Wood

Main Characteristic of Composite: Resists compression and tension; serves as a barrier to temperature fluctuations, water, fire, and microbes.

Materials Being Crosslinked: Cellulose microfibrils

Crosslinker/Binder: Xyloglucan hemicellulose

Figure 45: Wood's functionality at play while living (tree) and dead (house) (NYTimes)

Introduction

Wood – we take it for granted but it's quite a remarkably multifunctional, robust material. It not only supports tall, free standing structures exposed to compressive and tension forces, it also serves as a barrier to temperature fluctuations, water, fire, and microbes.

Crosslinking Activity

Wood's incredible mechanical properties derive from a complex network of polysaccharides (cellulose, hemicellulose, and pectin) and proteins in plant cell walls; about 30 percent of secondary walls surrounding mature cells also contain phenolic compounds, primarily lignin. Cellulose, an enormous homopolymer composed purely of □ (1□ 4) linked D-glucose monomers (Fig. 2), aggregate into extremely sturdy microfibrils due to the incredible number of hydrogen bonds that develop between the hydroxyl groups of tightly packed cellulose chains (Fig. 3a). Tangled layers of cellulose microfibrils are thought to account for the vast majority of wood's structural strength (Fig. 3b). However, similar polymers known as hemicelluloses form important crosslinks between the cellulose microfibrils that increase the performance of the material.

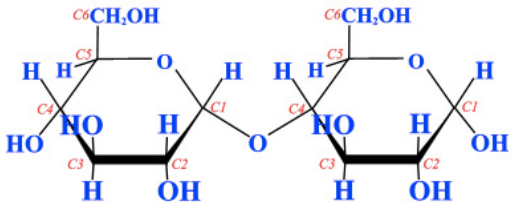

Figure 46. ß1-4 glycosidic linkage between the C1 and C4 carbons of two D-glucose molecules

Hemicelluloses, like cellulose, are based on an equatorial □ (1→4) linked polysaccharide chain. Unlike cellulose, hemicellulose backbones are not necessarily exclusively based on glucose (may include mannose, xylose, etc)

and/or are branched and exhibit numerous substituted sugar side chains. These differences are thought to impart various new functionalities (Fig 4).

Xyloglucan (XyG), a hemicellulose present in wood, features a core backbone of □ (1□ 4) linked D-glucose in an unsubstituted form (monomer code: G), and a more prevalent form with an □ (1□ 6) linked D-xylose side chain (monomer code: X). The most common XyG sequences are XXGG and XXXG depending on the plant's species. The xylose side chain of the X monomer can be further substituted at O-2 with a □ -linked D-galactose (monomer code: L). The galactose side chain of the L monomer can once again be substituted at O-2 with □ -linked L-fucose (monomer code: F). The extremely versatile side chain repertoire of this hemicellulose makes it a powerful crosslinking unit in cell walls. A 'sticky network' model is currently favored as the best explanation for how XyG contributes to cellulose microfibril tethering in cell walls.

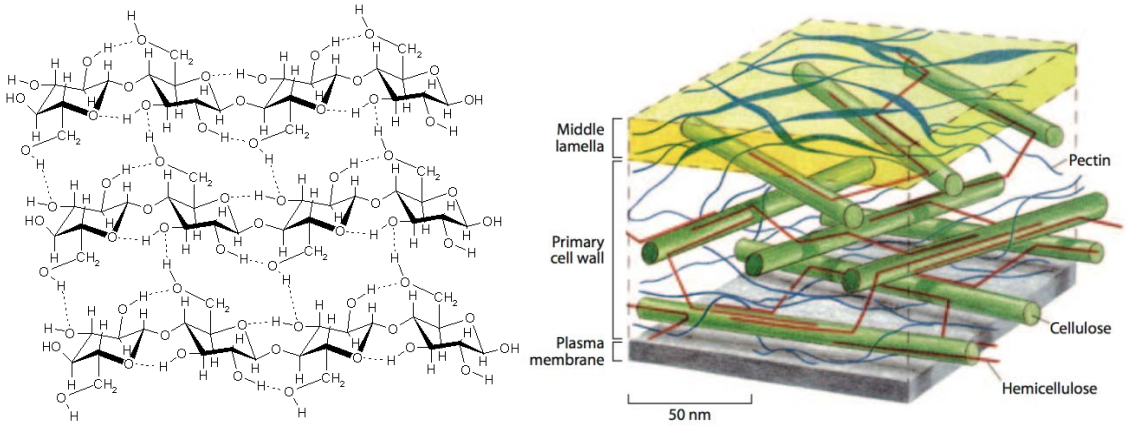

Figure 47a (left). Adjacent cellulose strands forming numerous hydrogen bonds (dashed-lines). Figure 3b (right). Cartoon of major cell wall macromolecules.

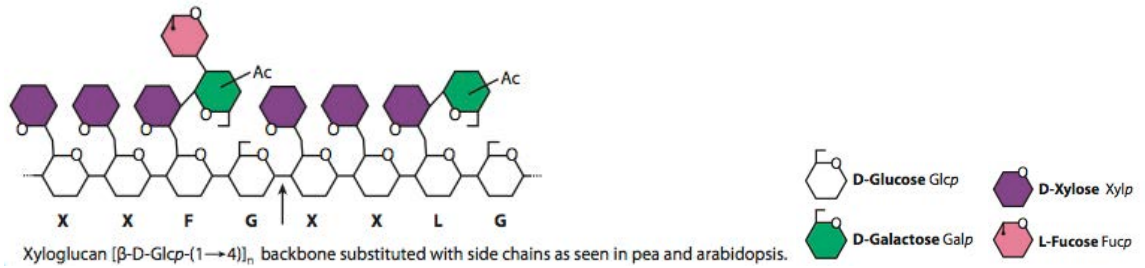

Figure 48. An example of an XyG polymer segment featuring a □ (1□ 4) linked D-glucose backbone with variable substituted (X) and unsubstituted (G) □ (1□ 6) linked D-xylose side chains. Also shown are additional side chain substitutions (F & L) (Scheller 2010)

In the sticky network, tethering glycans like XyG crosslink distal cellulose microfibrils by two means. The first involves parts of XyG strands becoming physically trapped within the cores of cellulose microfibrils during crystallization of the latter. The second type of crosslink involves intense hydrogen-bonding between the side chains of XyG and exposed hydroxyl groups on the surface of the cellulose microfibrils; XyG adopts a flat conformation to increase the available area for hydrogen-bonding.

XyG also forms covalent crosslinks with certain pectins (another type of structural polysaccharide present in cell walls featuring non-equitorial [axial] backbones) like RG-1 in the cell wall (Fig 5). This bond possibly manifests as an RG-1 galactan branch forming from an XyG type L monomer galactin side-chain. The crosslink may form as part of initial synthesis in the Golgi apparatus.

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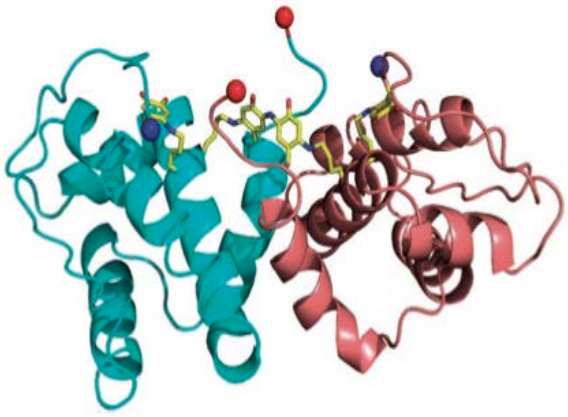
Appendix B: Interaction of Bonding Themes in One Organism

Ranasmurfin, a blue protein from the foaming nest of the Malaysian tree frog, has a unique, chromophoric crosslink. It combines several bonding strategies to create a stable, biocompatible foam environment for developing eggs and embryos:[66]



3D structure of the ranasmurfin protein[67]

Covalent bonding:
3 disulfide bridges stabilize each alpha helix

Non-covalent bonding:
Hydrogen bonds impart strength and stability to the dimerizing crosslink

Ancillary-metal bonds:
Tetrahedral chelation structures around zinc ions stabilize the tertiary structure of the protein

Appendix C: Why Not Metals?

The majority of metals that appear in the biological examples we investigated are Group 1 and 2 cations (Na^{+}, Mg^{+2}, Ca^{+2}) or transition metal cations (Fe^{+2}, Fe^{+3}, Zn^{+2}). They serve to either link proteins or other materials together through binding to a common metal ion, or to enhance already existing structure by providing additional binding.

The main benefit of metal use is that the addition of a metal salt to a resin or fabric could be a straightforward way to "turn on" and control crosslinking. We envisioned application of a resin to fabric in the finishing step, as is done now, and then crosslinking that resin later by adding the appropriate metal, once the fabric has been cut and sewn into a garment.

There are two main drawbacks to using metals, however, that led us to decide not to pursue them further. First, coordinate covalent bonds with metals are weak and susceptible to disruption by EDTA or other water softeners found in detergents. Second, many transition metals, such as iron, produce complexes with vibrant colors that might not be desirable in the finished product.

We did not evaluate metal-containing bonds as a primary strategy, although we are still considering them as a potential supplement to either covalent or non-covalent bonds. Metals are also used in the biological examples to catalyze other reactions; we are considering them for this use.

Appendix D: Technical Evaluation Framework – Detailed Description

Detailed descriptions of the eight technical performance factors are listed below.

1. Degree of additional research necessary
   - Red: There is no precedent in textiles or we anticipate many hurdles
   - Yellow: There is some precedent in textiles or we anticipate few hurdles
   - Green: Ample research has been performed but optimization may be required
2. Disruption of chemical supply infrastructure (i.e., synthesis of new materials)
   - Red: strategy requires special or new manufacture of chemicals
   - Yellow: chemicals have limited availability or can be easily and cleanly made in situ
   - Green: chemicals are widely used industrially (not necessarily in the textile industry)
3. Disruption of fabric application infrastructure (i.e., equipment and materials used in the step where resin is applied)
   - Red: entirely new technology or techniques required, such as anhydrous conditions, airless conditions, high pressure gases, or other specialized equipment
   - Yellow: longer times required; limited or advanced technology (such as lasers); modification of existing technology (such as using washing machines but making sure they're acid-resistant)
   - Green: required infrastructure or techniques are already being used in the textile industry
4. Disruption of crosslinking step infrastructure (i.e., equipment and materials used to perform the crosslinking step)
   - Red: significant change in equipment; time or space intensive
   - Yellow: new reagents or catalysts are required; longer times required; crosslinking requires solvent
   - Green: heat-cured or other minimal curing (such as air-cured)
5. Controllable crosslinking
   - Red: difficult to have "delayed control" or difficult to get reaction to go at all
   - Yellow: may need special conditions or additional chemicals to control (reversing preliminary reactions, using protecting groups, or forcing reaction to go with harsh conditions)
   - Green: crosslinking easy to "turn on" by adding catalyst, reagent or heat
6. Resilience of cellulose linkage or crosslinker during manufacturing process
   - Red: chemicals are likely to get washed off or deactivated using current practices; would need modification of other steps
   - Yellow: chemicals may get washed off or deactivated, but unknown or hard to predict
   - Green: no foreseeable problems
7. Resilience of cellulose linkage or crosslinker during consumer laundry
   - Red: crosslink or linkage to cellulose is likely to be disrupted by water, alkaline pH, turbulence, chelators, surfactants

- Yellow: crosslink or linkage to cellulose may be disrupted but is not likely
- Green: no foreseeable problems

8. Other effects on fabric (fibers, "hand", dyes, other finishes, etc.) during process
   - Red: strategy requires acid catalysis (cellulose degradation), bleaching agents (removal of dye), fiber coatings (may make dye uptake difficult), or excessive use of something that might change "hand" (chemicals or fibers)
   - Yellow: possible need for acid catalysis, bleaching agents, fiber coatings, or excessive use of something that might change "hand", but not required
   - Green: no foreseeable problems

Appendix E: Detailed Technical Evaluation Results

Each strategy was evaluated using these eight parameters, incorporating information from the literature and from textile industry experts (Figure 14). Gray squares in the table indicate parameters that are not applicable to the specific strategy – for example, "disruption of fabric application process" only applies to strategies that deal with binding to cellulose (polymer weaving or coating, polycarboxylic acids). Similarly, "disruption of crosslinking step" and "controllable crosslinking" only apply to the crosslinking strategies (disulfide bonds, imine & amine bonds). In some instances where the specific materials or chemicals chosen will change the ultimate result, we have given multiple ratings.

Poly(Ethylene Terephthalate) Weaving

- Controllable crosslinking and effects on fabric: As PET weaving is already used in the textile industry and involves no additional chemicals, the disruption is anticipated to be low. However, we are unsure of how accessible the carbonyl groups on the PET would be for crosslinking. Additionally, more PET may need to be blended into the fabric in order to make the crosslinking effective, which would affect the hand.

Polymer Fiber Coating

- Degree of additional research: While polymers are commonly applied in the fiber form during sizing, they are also stripped off later in the desizing process. We propose to leave a polymer on the fibers so that it can later be used for crosslinking, which to our knowledge is not currently done.
- Disruption of chemical supply: This varies widely depending on the specific polymer chosen. Some are very common in industry; some may not yet exist.
- Durability during manufacturing process and in consumer laundry: If a polymer is applied to the fibers during the sizing step for later crosslinking, it will need to survive desizing, which includes enzymes, hot water, and potentially concentrated sodium hydroxide (reference?). Depending on the water-solubility of the polymer, this may be possible, but it would be tricky. However, we feel that if the polymer survives manufacturing and manages to be crosslinked, it is more likely to survive consumer laundry.
- Effects on fabric: Applying a polymer coating in the fabric form could inhibit dye uptake later on, although if the fibers are dyed instead of the fabric, as is the case with Levi's jeans, this may be less of an issue.

Polymer Fabric Coating

- Degree of additional research: Polymers are commonly applied in the fabric form, so we anticipate this to be a relatively straightforward strategy to optimize, although it may depend on the specific polymer.
- Disruption of chemical supply: As stated above, this varies widely depending on the specific polymer chosen.
- Durability during manufacturing process and in consumer laundry: It is not clear how well the coating would penetrate the fabric and thus how well it would "stick" to the fabric. The durability of the polymer in consumer laundry will depend on this penetration and also on the water-solubility of the polymer.
- Effects on fabric: Applying a polymer coating could change the hand, depending on the specific polymer and how much is applied.

In Situ Polymerization

- Degree of additional research: In situ polymerization has been demonstrated on textiles for specialty applications such as conductive fabrics, but has not been used on this wide a scale.
- Disruption of fabric application process: Of all the fabric treatments, this is the only one that would likely require new equipment, in order to facilitate the in situ polymerization. In addition, the in situ polymerization may be slower than current fabric treatments.
- Durability during manufacturing process and in consumer laundry: Good fiber penetration has been demonstrated with in situ polymerization, so we anticipate good durability during manufacturing, but as with the polymer coating, the durability in consumer laundry will depend on the specific polymer.
- Effects on fabric: As with the polymer coating, the hand could change depending on the polymer and how much is generated.

Polycarboxylic Acids

- Disruption of chemical supply: As mentioned above, thiolated polycarboxylic acids are almost unknown, but we propose to add a thiol in situ using readily available reagents.[29] The disruption from crosslinking to the carboxylic acid directly with a diamine would depend on the diamine.

- Effects on fabric: As mentioned in detail in the previous section, there are numerous known problems with polycarboxylic acids. The two major effects on the fabric are loss of tensile strength[15] and fabric yellowing.[18] However, here we reiterate again that a "red" ranking can merely represent that we are aware of many of the potential problems, rather than indicating more problems in a world of perfect information. Since this is a well-studied technology, many of the pitfalls have been well-elucidated. However, it still receives a "red" rating here since acids are inherently part of the very nature of the strategy – thus, they are required.

Disulfide Bonds

- Degree of additional research: To our knowledge, disulfide bonds have never been used for textile crosslinking, although there are examples of disulfide bond crosslinking in other industrial applications.[34,35,46]

- Disruption of chemical supply: Thiolated polymers are somewhat developed, with examples of thiolation of poly(methacrylic acid), poly(acrylic acid), carboxymethylcellulose, and chitosan.[48] Nevertheless, they are not widely available. Thiolated polycarboxylic acids are almost unknown; we were only able to find one example of thiolated citric acid.[59] However, the proposed in situ addition of the thiol to the already-linked carboxylic acid would use readily available reagents.[29]

- Crosslinking disruption and controllability: New reagents and catalysts will be required for disulfide bond crosslinking. Depending on the reactivity of the thiol, it is possible that the disulfide linkage will form in air between the time the resin is applied to the fabric and the time the permanent crease is set in the garment. It is possible to use protecting groups[60] or reducing agents[48] to prevent the thiols from reacting prematurely, but this adds additional expense and chemical exposure potential. If disulfide bonds were used to link a DWR to cellulose, on the other hand, this would be a less important consideration.

- Durability in consumer laundry: Disulfide bonds can be cleaved at more alkaline pH, such as those found in commercial detergents.

- Effects on fabric: Some of the oxidants commonly used (hydrogen peroxide, potassium permanganate) are also common bleaching agents already used in the textile industry. Therefore, an oxidant that is not also a bleaching agent would have to be used. In addition, some of the oxidants work better in acidic pH, which could be detrimental to the cotton fabric. The most obvious choice is to use either air or oxygen, potentially at high temperatures, but this would need to be tested along with different possible catalysts. Our recommendation would be to examine some of the "greener" options listed in the previous section, in particular the ones that work solvent-free, as the ability to set the permanent crease without solvent would be quite useful.

Imine & Amine Bonds

- Degree of additional research: Imine bond crosslinking is quite common, and has been explored at least a bit for non-woven fabrics.[61]

- Disruption of chemical supply: This would depend on the specific diamine crosslinker chosen.

- Disruption of crosslinking step: Using hydrogen gas will possibly require new equipment to safely handle the gas, as well as the ability to recover the necessary precious metal catalysts.[52] The technical framework does not specifically evaluate health effects, but the dangers of sodium cyanoborohydride are so great that we cannot recommend using it.[52–54] Sodium borohydride is probably the best choice from a technical standpoint, as it is effective, has fewer health risks than sodium cyanoborohydride, and does not require specialized equipment.

- Other considerations: A reaction that requires an acid catalyst[55] would be problematic for the cotton fabric, but this can almost certainly be avoided. Having a diamine linker means we run the risk of having both amine groups react with one cellulose chain, instead of crosslinking two chains. Using a shorter linker may prevent this. In addition, there is a risk that the crosslinker may cleave the chains in the polymer coating or PET fibers.[58]