Spider silk-producing recombinant yeast for enhanced wound healing

Monica Chen, Tulasi Cherukuri, and Sophia Hopper

BioBuilderClub, Green Hope High School, Cary, North Carolina, USA; BioBuilderClub, Ravenscroft School, Raleigh, North Carolina, USA

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Chronic wounds and wound infections can be challenging for the body to heal independently; consequently, millions of people suffer from unhealed chronic wounds and infections that can lead to elevated rates of disease and death. These risks are intensified in the half of the world which lacks access to basic medical care. To assist the body in healing and mitigate occurrences of infection, we designed a portable ointment containing modified yeast cells that produce a spider silk bandage, enhancing the natural wound healing process. Spider silk is known for its superior mechanical properties, but is also biocompatible and nurtures wound healing. The combination of yeast, pyriform silk, and antimicrobial human beta-defensin 3 creates a self-assembling, strong, flexible, biodegradable antimicrobial bandage that is customized to the wound shape and responsive to the wound environment. The ability to add chosen properties to the silk bandage creates enormous potential for enhanced wound healing. The current design features antimicrobial properties which prevent dangerous infections. The excretion of pyriform silk from the avirulent *Saccharomyces cerevisiae* Y55 strain will be controlled by the modified yeast-specific ANB1 promoter. This promoter is activated by sufficiently high levels of reactive oxygen species (ROS) and low levels of oxygen in the environment. The genes for silk production and microbial protection will be placed in separate plasmids, but both under the control of the same modified ANB1 promoter.

Keywords: Wound healing, spider silk, antimicrobial, medical innovation, bandage, synthetic biology, recombinant yeast

Mentors: Patricia Silveyra

Direct correspondence to ptrcslvr@gmail.com; shopper2024@ravenscroft.org

Watch a video introduction by the authors at https://youtu.be/QYjk3OO5V0Y

Background

Need for improved wound healing technology

Most wounds heal independently and only require little medical assistance, if any. However, unhealed wound infections, *surgical site infections* (SSIs), and chronic wounds impact millions of people worldwide. Wound infections can lead to osteomyelitis, necrotizing fasciitis, sepsis, and cellulitis (Leonard, 2019). Sepsis annually kills approximately 270,000 people in the US alone (Leonard, 2019), whereas cellulitis is contracted by 14 million people and results in 650,000 hospitalizations per year (Brown, 2021). Between 2% and 4% of people undergoing surgery contract an SSI, and 3% of SSIs are fatal (*Surgical Site Infections*, 2019). The rising number of chronic wounds - such as recurring wounds or wounds which are slow to heal - around the world (6.5 million in the US alone) is known as "the silent epidemic." Between 1–2% of the world's population will obtain at least one chronic wound in their lifetime (Nelson, 2017). These wounds can lead to death or amputation.

The spider silk bandage's properties have potential to enhance the natural wound healing process and facilitate healing for challenging wounds (Chouhan & Mandal, 2020). These wounds – which the body requires assistance in healing (Cuffari, 2020) – include burns, ulcers, diabetic wounds, deep gashes requiring stitches, other chronic wounds, post-surgical sutures, and wounds at risk for infection (Nelson, 2017).

Accessibility to medical equipment and services is another global issue. Chronic wounds and severe wound infections require treatment by a medical professional (Leonard, 2019; Nelson, 2017). Treatment often utilizes antibiotics, and sometimes stitches or a debridement procedure. If wound infections go untreated, they can spread to other areas of the body (Leonard, 2019). The annual treatment cost of chronic wounds in the US is approximately $25 billion (George, 2018), and $3.7 billion is spent just on ambulatory costs relating to cellulitis; therefore, lack of financial resources for treatment is problematic for a large part of the population. Since half of the world lacks access to basic healthcare (World Health Organization, 2017), wound infections heighten the risk of fatality in these areas. Our modified yeast cream will provide an accessible and affordable solution for areas which do not have access to medical professionals or supplies.

Pyriform silk

Each type of spider produces up to seven different kinds of spider silk (Rogers, 2017), and each of these contains a different protein. Pyriform silk, created by the expression of pyriform spidroin-1 (encoded by the *PySp1* gene), is a sticky and extremely strong silk used by spiders to catch victims in their webs and attach to substrates. Pyriform silk is relatively unexplored compared to the commonly utilized dragline silk (derived from spidroin-1 and spidroin-2, encoded by *MaSp1* and *MaSp2*). Having evolved to adhere to anti-adhesive plants, it can attach to substrates with low surface energy (Wolff et al., 2015). This property will ensure attachment to the body in our design, including skin and tissue (Kent, 2020). The cohesion of pyriform spider silk will allow it to form a tight and protective material. This cohesion arises due to the nanofibrils' interfibrillar friction (Kiseleva et al., 2020) and the presence of a layer of sericin, a hydrophilic protein (Cao & Wang, 2009). This cohesion is in turn responsible for the material's superior strength and elasticity. Only a small amount of pyriform silk is required to ensure attachment, making the strength-to-weight ratio extremely high. This strength and attachment can persist for years (Wolff et al., 2015). However, the silk naturally biodegrades. Proteases – such as protease XIV, α-chymotrypsin, and collagenases – degrade the silk proteins into amino acids through proteolysis. First, the proteases bind to the surfaces of the silk biomaterials and absorb into them. Then, the silk is digested by proteases through hydrolysis; the silk proteins' respective amino acids are the byproducts (Cao & Wang, 2009). These amino acids can either be absorbed by the body or easily removed, as they are nontoxic and can be recognized by the immune system (Salehi et al., 2020). This means the degradation process brings no harm to the body. The rate of biodegradation is highly variable and dependent on the progression of healing, but is naturally slow enough that the wound will be protected until new tissue is fully developed (Scheibel, 2020).

Most spider silks contain only one component; however, pyriform silk is a two-component material made up of protein fibers and a fluid glue that contains acidic proteins, hydrocarbons, nanofibrils, lipid enclosures, and an isotropic boundary layer. This glue has the potential to be a natural superglue. When pyriform silk is excreted, the glue can create a smooth surface for attachment. This glue also gives the silk its concrete-like properties. Fluid pyriform silk becomes usable after less than a second and then creates an immovable monobloc. Therefore, a bandage made from this substance will have cast-like properties with regard to support, stability, and protection from disturbance; while still being light-weight and allowing air flow.

Due to its complex structure, composed of many different materials, pyriform silk has many additional properties that make it highly desirable for a bandage: stress distribution, toughness, hardness, and elasticity. This two-compound material behaves like a viscoelastic

fluid: It must be immediately in contact with the substrate or will form beads-on-a-string (BOAS; Wolff et al., 2015). The means the glue, a viscoelastic solid, must be excreted directly onto the wound in order to adopt the correct shape. The glue component's glycoprotein's viscoelastic structure is also responsible for the flexibility and stickiness (Sahni et al., 2010). Because viscoelasticity allows the material to deform slightly, but then resume the prior position, the adhesive ligands will attach effectively instead of ricocheting off like solids (*Viscoelasticity and hysteresis*, n.d.). The elasticity means this connection will not break under pressure (*What Exactly*, 1997). The silk's structure also inherently results in defense against cracking (Wolff et al., 2015). When cracks appear in the layers of microscopic fibers, the multi-layered structure of the silk prevents the cracks from spreading. In addition, the adhesion and toughness are controllable variables, creating a highly adaptable material.

Spider silk for medical applications

Spider silk is known to be stronger than steel, tougher than Kevlar, and extremely elastic. This elasticity, toughness, and strength make it far superior to most other fibers, and its biodegradability and biocompatibility with the human body have made it useful for many medical applications (Cuffari, 2020). The body does not have allergic or inflammatory reactions to spider silk (Scheibel, 2020). As a result, it has a multitude of medical applications including: support during nerve regeneration (Schacht & Schiebel, 2014); tissue glue (Kent, 2020); vaccine carriers; protective medicinal carriers (University of Bayreuth, 2018); tissue engineering; implant coatings to inhibit immune response (Scheibel, 2020); and surgical sutures. However, harvesting silk from spiders is not a scalable option as they are cannibalists (Scheibel, 2020), so recombinant organisms capable of secreting long proteins are required.

Spider silk bandages

Since spider silk is biocompatible, biodegradable, non-immunogenic (Cuffari, 2021), non-antigenic, and anti-inflammatory (Jagatia, 2017), it is well-suited for biomedical applications. Its superior mechanical properties, including being lightweight, capable of absorbing energy (Jagatia, 2017), being highly elastic, and having a high tensile strength (Cuffari, 2021) – combined with its natural antimicrobial properties – have led to the exploration of spider silk bandages to enhance the wound healing process (Rogers, 2017). Research has shown silk may naturally possess some antimicrobial properties (Jagatia, 2017), and stronger antimicrobial activity can be introduced relatively easily to the spidroins through click chemistry (Rogers, 2017), or through enhancement of the body's natural processes, for example, by the production of beta-defensin 3 (hBD-3). Silk also naturally exhibits hemostatic properties (Cao & Wang, 2009) and increases basic fibroblast growth factor (bFGF) production (Salehi et al., 2020). Cell migration, cell proliferation, angiogenesis, and re-epithelization are all enhanced (Chouhan & Mandal, 2020). In vitro experiments have shown that spider silk promotes the development of epidermal layers and keratinization (Cuffari, 2020).

Using antimicrobial pyriform silk in combination with a living system will allow the bandage to have many properties that go beyond current bandages. By using a promoter that is sensitive to ROS and oxygen levels, the bandage will adapt to its environment and be customized to the exact wound shape. Since the concentration of ROS increases at the site of a wound, their levels can be used as a readout of which areas are affected by a wound and which are not, such that the bandage will develop to encompass only the regions characterized by the wound. The silk could also evolve with the wound shape and conditions, as the wound microenvironment progresses. In addition, silk could be more specialized for wound healing than existing bandages, through its many different and selective material, physical, and chemical properties (Schacht & Scheibel, 2014). These properties allow the silk to further enhance the naturally-occurring wound healing functions. Variables like temperature, moisture, nutrient levels, pH, and bioburden (Galloway, 2018) could all be controlled by the bandage to ensure an improved healing process. Combined with the properties of spider silk – which already create an immensely strong, flexible, and tough bandage – these added properties would create a "super-bandage" (Figure 1). Eventually, these bandages could be adapted to release pharmaceuticals directly into the bloodstream, or to renew their antimicrobial properties based on the status of the wound (Rogers, 2018; Jagatia, 2017). For now, sustained antimicrobial properties were chosen through the production of hBD-3. In the future, adding other beneficial functions and properties will be explored.

Instead of requiring a trained medical professional, this antimicrobial bandage would self-assemble once placed on the wound. Biodegradability or bioresorbability would prevent the painful and sometimes harmful process of removing bandages or stitches, as well as eliminating the need to periodically change bandages (Cuffari, 2020). This uninterrupted protection makes the bandage well-suited for chronic wounds and wounds likely to be infected. This work will therefore provide an accessible, affordable option to treat wounds as large quantities will be self-manufactured through yeast reproduction. Since only a small amount of yeast would be required (enough to cover the wound surface with a thin layer), due to its

ability to reproduce, this would make for an extremely portable and lightweight first-aid resource. This yeast cream would also be much easier to transport and completely biodegradable, avoiding paper waste.

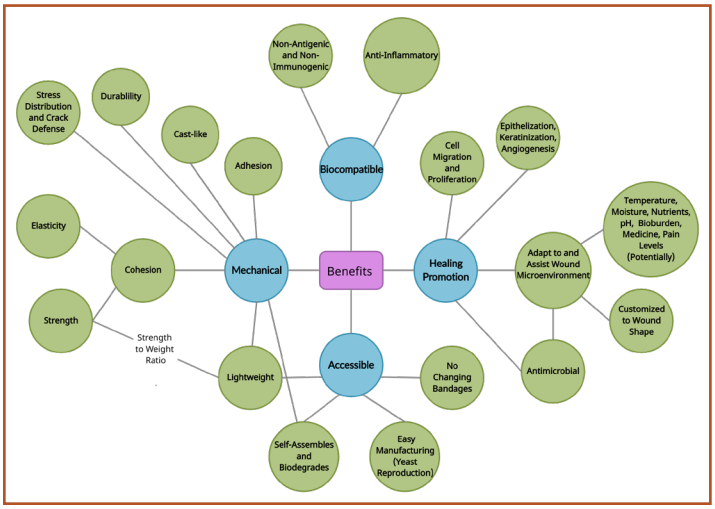


Figure 1. *A conceptual diagram showing the benefits of the spider silk bandage produced by yeast, relating to mechanics, accessibility, enhanced healing, and biocompatibility.*

Wound healing

Reactive oxygen species (ROS) are radical and non-radical derivatives of oxygen that are the byproducts of the reduction of oxygen during cellular respiration (Reactive Oxygen Species, n.d.). Different levels of ROS can be either beneficial or harmful. Low levels of ROS can result in cell cycle arrest. Basal levels of ROS result in cells functioning normally and, therefore, allow for homeostasis to be maintained. Increased levels of ROS induce wound healing through the activation of many transcription factors, and excessive ROS result in inflammation, cell apoptosis, and senescence, which refers to a cell's inability to grow and divide. Elevated levels of ROS for long periods of time are associated with chronic wounds (Dunnill et al., 2015).

Acute wounds go through the stages of hemostasis, inflammation, proliferation, and remodeling, each of which relies on ROS signaling. These stages often overlap. Acute wounds normally heal within a time frame of 30 days or less, but chronic wounds persist in one of these stages, resulting in a prolonged time frame for recovery. During hemostasis, ROS signaling activates platelets and induces vasoconstriction, the narrowing of blood vessels; and coagulation, the process of adding fibrin to reinforce the thrombus (Rodrigues et al., 2019). During inflammation, defense mechanisms to combat pathogens are activated. First, neutrophils, and later, macrophages, secrete growth factors and cytokines that aid in antimicrobial defense. Next, during proliferation, tissue repair occurs. Endothelial cell division; blood vessel reformation; fibroblast division; new extracellular matrix formation; and keratinocyte proliferation and migration, all occur during this phase. During the last phase, remodeling, fibroblast-rich granulation tissue is replaced and a scar develops. Cross-linked collagen molecules confer tensile strength to the scar, allowing it to be nearly as strong as intact skin (Dunnill et al., 2015).

Oxygen fuels the repair processes and also helps to disinfect wounds (Cano Sanchez et al.,2018). As the final electron acceptor in the aerobic metabolism of glucose, the body uses oxygen to generate ATP for powering cellular processes. This process, oxidative phosphorylation, occurs at a higher rate to produce more ATP when there is a wound, so more oxygen is also needed (Kimmel et al., 2016). Therefore, oxygen is essential for wound healing to occur. A lack of enough oxygen at a wound can inhibit healing, which is why a promoter that responds to inadequate oxygen levels makes the proposed system more effective.

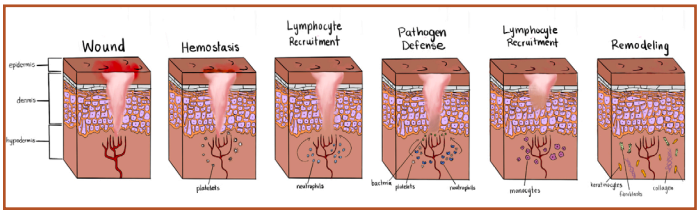


Figure 2. *Diagram showing cross-sections of a wound at different stages of healing (elements not to scale). During hemostasis, ROS signaling initiates platelet activity to induce vasoconstriction and coagulation. In the next stage, inflammation, local blood vessel-bound neutrophils are recruited. They offer bacterial protection. Next, ROS gives off more signals to recruit farther neutrophils and platelets to combat bacteria at the wound site. Other white blood cells, like monocytes, continue to arrive at the wound site to fight pathogens as the wound heals. During proliferation, the general release of ROS contributes to tissue repair. During remodeling, a scar forms, completing the wound healing process (Dunnill et al., 2015).*

Systems level

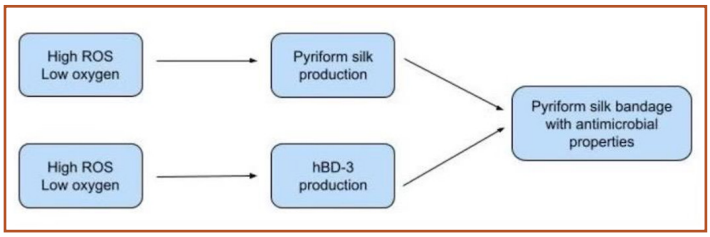
Since eukaryotic transcription is usually monocistronic, i.e., will only express one gene downstream of the promoter, we have designed two systems (Figure 3). Each system will contain the same modified ANB1 promoter, which is regulated in yeast by oxygen supply. One system will be responsible for pyriform silk production, and the other for hBD-3 production to confer increased antimicrobial properties. There are

two independent signaling pathways that respond to the same stimuli.

Upon identification of a wound, the topical ointment is to be applied to the wound site. The first system in these cells responds to two different stimuli: high levels of ROS and low oxygen. Once either or both of these conditions are satisfied, the production of pyriform silk will begin, self-assembling to form a bandage to assist wound healing.

The second system that will activate also responds to the same stimuli: high levels of ROS and low oxygen. The difference lies in the fact that, when either or both of these conditions are met, a human beta-defensin will be secreted to protect against pathogens. These two systems will be operating simultaneously, so they will act jointly to result in a wound healing mechanism consisting of a silk bandage with antimicrobial properties.

Figure 3. *The proposed design contains two systems that both respond to the same conditions: high ROS and low oxygen. One system produces pyriform silk, whereas the other produces hBD-3. The products of the two separate systems combine to form a pyriform bandage with antimicrobial properties. Because both systems respond to the same stimuli, their products, pyriform silk and hBD-3, will act synergistically.*



Device level

Chassis

The yeast *Saccharomyces cerevisiae* is an ideal chassis for several reasons. First, this organism has been studied intensively as a model eukaryote in synthetic and molecular biology. The depth of knowledge gathered from this research ensures that its structures and functions are clear. It also has a short doubling time and can be easily cultured, which is fundamental in producing silk spidroins quickly. Thirdly, it can be easily transformed, allowing for deletion or addition of new plasmids or genes for protein production (Help:Yeast, n.d.). Since several genes will be added to the yeast in this design, they must be put into separate open reading frames (ORFs) due to the yeast's eukaryotic nature (Brown, 2002). The yeast also does not have as many introns as higher eukaryotes, so its genome is easier to examine and alter. In addition, compared to bacteria, yeast are better at producing large proteins such as spider silk spidroins in elevated quantities. Lastly, the Y55 strain of S. cerevisiae was chosen because it has been identified as avirulent, unlike other strains that have been known to cause infection in humans (Byron et al., 1995). Not only is it avirulent: this yeast has also shown antibacterial activity through producing bioactive peptides, and it has been classified as a generally regarded as safe (GRAS) organism (Al-sahlany et al., 2020). Since our design is for medical purposes, the fact that the Y55 strain does not produce harmful toxins to humans and also is not known to infect wounds makes it a feasible chassis.

Wound sensing

This device allows for the system to respond to the two important stimuli: high ROS and low oxygen. Firstly, the Keap1–Nrf2 pathway is crucial to responding to ROS. This pathway can be manipulated to achieve the goal of transcription of the pyriform silk gene only in the region of the wound. Keap1 is an adaptor protein of Cullin 3-based E3 ligase. Under unstressed conditions, it interacts with specific regions of the Nrf2 transcription factor: the DLGex and ETGE sites. Through the process of ubiquitination, the Keap1 protein tags the Nrf2 (attaches ubiquitin proteins to the Nrf2), marking it for proteasomal degradation. Therefore, Nrf2 is unable to translocate to the nucleus or initiate any transcription. Conversely, under stressed conditions caused by ROS, the redox-sensitive cysteine residues of Keap1 proteins are modified by oxidation. This results in inactivation of the ubiquitin E3 ligase activity of Keap1. Therefore, the Nrf2 proteins are no longer tagged for degradation. The levels of Nrf2 increase, and it translocates to the nucleus to initiate transcription (Suzuki et al., 2016).

Nrf2 binds to the antioxidant response element (ARE) of promoters that lie upstream of several cytoprotective genes, initiating transcription of detoxification enzymes including GSTA2 and NQO1. These detoxification enzymes then function to deactivate the ROS (Johnson et al., 2008). Though the transcription of the detoxification enzymes is the natural course of action to combat the ROS, this mechanism can be manipulated to transcribe the pyriform silk-producing gene only when the ROS levels are high enough to induce damage to the Keap1 protein. The ARE enhancer sequence can be added to the ANB1 promoter of the pyriform silk-producing gene, *PySp1*, in order to be recognized by the Nrf2 once it arrives in the nucleus.

While this is occurring, the ANB1 promoter is also responding to the levels of oxygen in the wound microenvironment. Oxygen levels are sensed by whether or not heme synthesis occurs (Matthes, 2012). Heme is a molecule that has a crucial role in oxygen transport,

storage, catalysis, and sensing. Aerobic conditions are marked by heme synthesis. Heme induces the ROX1 gene, which encodes a repressor preventing the transcription of ANB1 (Matthes & Rudolph, 2012). When conditions are anaerobic, and heme synthesis does not occur, the repressor encoded by the ROX1 gene is not expressed, and therefore, ANB1 is transcribed. We have used the ANB1 gene promoter as a regulatory part of our systems. Under anaerobic conditions in System 1, pyriform silk will be excreted, and in System 2, human beta-defensin 3 will be excreted. Under aerobic conditions, these two systems are repressed.

Silk creation

The second device is responsible for the production of silk through the *PySp1* gene. Once the ANB1 promoter, with the added ARE and CACG motifs (see below), is positioned upstream of the *PySp1* gene, *PySp1* expression will be induced under the specifically-designed conditions, producing pyriform silk that will be excreted by the yeast. Since spider silk self-assembles due to electrostatic interactions (Wolff et al., 2015), the wound will thicken itself into an immovable, woven structure that securely fits the shape of the wound. Silk is naturally biodegradable, so the proteases from the wound healing process will gradually degrade the bandage away (Cao & Wang, 2009).

Antimicrobial properties

To ensure a bandage that is not only durable and protective but also increases the healing rate of wounds, while preventing infection, the device containing the DEFB103B gene is inserted. This gene activates the body's immune response by producing hBD-3, a human beta-defensin. Normally, beta-defensins are expressed in infected or diseased tissue as an immune response. When induced, they prevent infection by acting as antibacterial peptides. For example, hBD-3 is found at sites of skin disease, like psoriatic lesions. Infections are rare due to the high presence of beta-defensins in these lesions (Dhople et al., 2006). hBD-3 has in particular been found to inhibit Gram-positive bacteria with greater capacity than other human beta-defensins, and is also effective against drug-resistant bacteria (Dhople et al., 2006). This reason, among others, is why hBD-3 is being investigated for pharmaceutical applications. It is expressed in lung, gut, tongue, and skin, as well as other epithelial surfaces. Whenever it is present in higher concentrations it has been shown to have antibacterial and anti-inflammatory properties (Mangoni et al., 2016), so inserting the DEFB103B gene to increase the production of hBD-3 at wound sites would improve the strength of the bactericidal properties. A higher concentration of this beta-defensin would also allow it to overcome obstacles that would otherwise inhibit its activity, such as certain serums and saliva. hBD-3 also does not inhibit eukaryotic cell growth, which is ideal when the yeast S. cerevisiae is being used as our chassis (Dhople et al., 2006).

When integrated into the silk, the hBD-3 will induce secretion of the pro-inflammatory cytokine interleukin-17 (IL-17), a signaling protein. This cytokine is normally found in human skin but the beta-defensin would increase its secretion (Mangoni et al., 2016). However, this process would have to be regulated somehow to prevent a "cytokine storm", or an over-presence of cytokines (Zenobia & Hajishengallis, 2015). When IL-17 is secreted it induces antimicrobial peptides (AMPs), that are already present in epithelial cells, but increases their concentration and effectiveness. These AMPs are able to inhibit bacterial and microbial growth, without causing resistance in the microbes, while promoting wound healing in the skin through modulation of cytokine production, and in some cases, angiogenesis (Mangoni et al., 2016). The AMPs inhibit microbes, at a molecular level, through electrostatic interactions with the cell membranes or cell walls of the microorganisms, thereby killing them (Zhang et al., 2016). Other functions of AMPs include the synthesis of collagen in the skin and cell migration, which aids in wound healing (Mangoni et al., 2016). Therefore, the combination of the activity of hBD-3 and antimicrobial peptides with the pyriform silk will greatly reduce the chances of infection in skin wounds while making wound healing more effective.

Parts level

The stimulus of high ROS initiates the Keap1–Nrf2 pathway, eventually resulting in binding of the Nrf2 transcription factor to the modified ANB1 promoter. When heme synthesis is not occurring due to the lack of oxygen, the ROX1 gene does not express a repressor, and the modified ANB1 promoter is then activated.

The ANB1 promoter is modified by two added components: The ARE enhancer and CACG motif. The addition of the ARE enhancer is important because without it, the Nrf2 transcription factor would not bind to the promoter (Johnson et al., 2008). The major sequence of the ARE enhancer is as follows: 5'-TGACNNNGC-3' (Bhakkiyalakshmi et al., 2018).

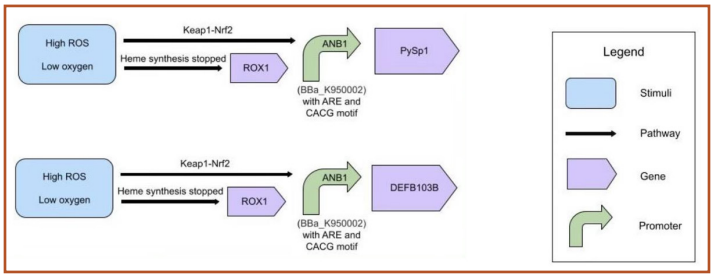
The CACG motif was found 72 bp upstream of the A. argentata *PySp1* start codon (Chaw et al., 2017). This is a putative transcription promoter motif that enhances the expression of the silk gene, and has been found upstream of at least six other spider species' silk genes. It is not specific to pyriform silk as it has been found upstream of the *MaSp1*, *MaSp2*, and Flag silk genes as well. Although research is still ongoing, the CACG motif seems to be a necessary part of the transcriptional

promoters for many silk genes.

The *PySp1* gene produces pyriform silk. A. argentata's *PySp1* gene has been sequenced, so that is the chosen spider (Chaw et al., 2017). The gene already contains a signal peptide, ensuring excretion.

For the silk bandage to have antimicrobial and wound healing properties, the gene DEFB103B will be inserted to code for the production of human beta-defensin 3, also called hBD-3 (National Center for Biotechnology Information, 2021). This gene would be placed in a separate system than the *PySp1* gene, but under control of the same promoter assembly (Brown, 1970), as shown in Figure 4.

Figure 4. *The current design is divided into two systems. In System 1, high ROS initiate the Keap1–Nrf2 pathway, and*



the Nrf2 transcription factor binds to the ARE element of the modified ANB1 (Johnson et al., 2008). The second stimulus, low oxygen, suppresses the heme synthesis pathway, therefore, the ROX1 gene will no longer repress ANB1 promoter activity, and the modified ANB1 promoter will work in concert with the CACG motif to activate expression of the PySp1 gene. Pyriform silk is then produced. System 2 involves the same stimuli, same pathways, ROX1 gene, and modified ANB1 promoter. The difference lies in the activation of another gene, DEFB103B, which produces hBD-3.

Safety

The skin microbiome is the vast collection of microorganisms which live on the skin, including bacteria and fungi. The microbiome is essential in assisting the body in healing, especially from chronic wounds and wound infections. These microorganisms decrease cutaneous inflammation and signal for keratinocyte proliferation, epithelial differentiation, and epidermal blood vessel growth (Johnson et al., 2018). Though the microbiome is very effective, technologies have been developed that supplement its workings, such as probiotics, which are live microorganisms that benefit the health of a host when given in controlled amounts. The strains of bacteria that have predominantly been used in current topical probiotics are Streptococcus thermophilus, Vitreoscilla filiformis, Enterococcus faecalis, Lactobacillus plantarum, Bifidobacterium longum, Staphylococcus hominis, and Lactobacillus johnsonii. They have been used to treat the following skin conditions: atopic dermatitis, seborrhoeic dermatitis, acne, and reactive skin (Knackstedt et al., 2020). These topical probiotics are not taken orally; they are applied directly to the skin. Similarly, the technology designed in this work will enhance the microbiome with the addition of the silk-producing S. cerevisiae yeast.

There are several crucial safety concerns involved with our design since it is closely tied to the internal health of the user. Firstly, putting yeast, which is potentially pathogenic, on a wound to heal it, might seem counterproductive. However, the strain of yeast chosen for this design's chassis (the Y55 strain of S. cerevisiae) is avirulent and does not cause infection or harm to the human body (Byron et al., 1995).

Another concern with the bandage is the material that it is made of: pyriform silk. Fortunately, spider silk is anti-inflammatory, non-antigenic, and non-immunogenic (Jagatia, 2017). It does not have adverse effects on wounds (Scheibel, 2020). Instead, the silk has been shown to have positive effects that speed up the healing of wounds (Setooni, 2018). It has been used as a material for wound dressing and scaffolds, and the silk has shown no unwanted side effects when placed on open wounds. The pyriform silk bandage is also biodegradable (Scheibel, 2020), so it would not stay on the wound for an unnecessarily long amount of time.

With regards to the antimicrobial properties of the bandage, there are safety concerns over the aspect of cytokine production downstream of DEFB103B gene induction. When cytokines are overproduced, there is the potential for a cytokine storm to take place (Zenobia & Hajishengallis, 2015). Although unlikely, given the amount and location of the cytokines that would be induced with the bandage, the cytokine storm is still a possibility that must be investigated further to determine the health risks. The cytokine IL-17, in particular, is pro-inflammatory, which in excess could cause the wound site to become irritated or inflamed. A potential solution is to balance out the effects of IL-17 with other cytokines that have counteracting effects.

The health of the chassis itself when coming into contact with antimicrobial peptides is also something that must be considered. Although the hBD-3 produced by the DEFB103B gene would not harm the yeast, the effect of AMPs on the Y55 strain of S. cerevisiae is unclear. There is a strain of S. cerevisiae, CCMI 885, that produces AMPs itself (Branco et al., 2016). This may indicate that other strains have resistance to AMPs, but more research needs to be done before a conclusion is reached.

For potential lab experimentation with our design, safety will be maintained by using proper safety equipment, such as gloves and safety glasses, and following lab safety protocols under biosafety level 1 guidelines. All students involved in this design will strictly follow laboratory safety guidelines in the potential testing of this design. During the early stages of testing, where the ability of the yeast to optimally produce silk and antimicrobial compounds will be assessed, this product would not be tested on human skin until its safety is confirmed.

Discussion

Using synthetic biology tools, we designed a system that creates an antimicrobial bandage made of spider silk when detecting a wound. Several alternative approaches were explored regarding the wound sensing portion, before making the decision to choose high ROS and low oxygen as the stimuli. One approach involved sensing the extracellular matrix (ECM). The ECM is an integral part of the dermis. It contributes to the structural support of cells, and lubrication, and acts as a transport system for waste and nutrients. Therefore, one approach centered on identifying a wound by detecting damage to the ECM. Under normal conditions, the ECM would not be directly exposed to the outside environment, but if a wound was deep enough, it would be. In order to design a system to detect ECM damage, we explored its composition. Proteoglycans are hydrated molecules that make up a key portion of the ECM. They assist with the cushioning and lubrication of cells. Their ability to hold water allows them to create a gel-like substance that forms a lubricated path for ions, nutrients, and hormones to move through. Proteoglycans also help with cell signaling by interacting with many growth factors and assisting them with binding to their respective cell-surface receptors (Schultz et al., 2005). Therefore, the possibility of a system responding to proteoglycans was investigated.

Ideally, when the yeast in the ointment would come into contact with proteoglycans from the ECM, the two systems that produce pyriform silk and hBD-3 would be activated. However, there were several challenges with this idea. It was relatively difficult to find a promoter that responded to levels of proteoglycans, when compared to promoters involving more explored alternatives relating to subjects like ROS or oxygen. In addition, even if such a promoter were found, the bandage responding to detection of the ECM would only function appropriately if the wound was deep enough. Though this type of system would allow the shape of the bandage to be customized to the shape of the wound by detecting the region where the ECM was exposed, it would not be effective for varying degrees of wounds, which was an important priority for this project. The shape customization aspect of the bandage could be realized through ROS-mediated detection of the wound region. The ANB1 promoter served as an operative base to build on due to its functionality in responding to low levels of oxygen, which indicate that a wound is in an emergency state. Adding the ARE enhancer so that it responds to ROS made it more effective.

Next steps

All aspects of this design must be repeatedly tested in order to determine the practicality and feasibility of our project. One such experiment would be to test the speed at which the bandage is assembled and biodegraded. The speed of assembly will inform design modifications. If assembly is too slow, leading to an extended time with little protection, we may need to design a temporary covering that would degrade away once the long-term bandage is fully assembled. We could also consider applying a partially formed bandage that could provide some initial protection and then grow to fit the wound.

The effect of wound enzymes on the bandage's biodegradability must also be considered. Fortunately, the degradation rate is controllable (Cao & Wang, 2009) and can be adjusted to coincide with the timescale of wound healing. Silk is classified by the United States Pharmacopeia as non-degradable since it does not lose its tensile strength in vivo. However, the literature has established that silk degrades, just at a low rate. The process is catalyzed by surrounding enzymes, which hence determine the degradation rate. The body's response to the wound healing technology will also impact the rate of degradation. Enzyme production or inhibition, naturally produced along with the silk to control degradation timing, could be regulated in order to change the rate of degradation. Degradation is also controlled by other factors including silk fibroin crystallinity, pore size, porosity, and molecular weight distribution (MWD) as well as the chemical, mechanical, biological, and immune conditions of the microenvironment (Vepari & Kaplan, 2009; Cao & Wang, 2009). We would investigate how these factors could be controlled if the rate of biodegradability needs to be altered.

The effects of how the structure of pyriform silk will be impacted by being excreted by yeast, as opposed to the pyriform glands, are not clear. The distal half of the gland produces the spidroins while the proximal half creates the glue fluid (Wolff et al., 2015). The consequences of mixing together the two compounds before excretion and their being excreted together must be evaluated. In addition, the woven structure, adhesion, and toughness are all controlled by microscopic spinneret movements. Experiments must be conducted to determine the extent to which these properties will be replicated when the silk

is produced by the yeast, or see whether it is somehow possible to imitate the pyriform glands. The attachment discs, elaborate patterns spun by the glands, are responsible for natural silk's strong adhesive properties. We would need to conduct experiments to determine if these attachment discs can be created without pyriform glands, or what adhesive properties could be sustained without them. Adhesion and toughness are also variables controlled by spinneret movements. While other methods of control would be utilized, we could work out how to make adhesion and toughness into adjustable variables.

There are a multitude of ways to functionalize spider silk to further assist the body in healing. In addition to spinneret movements, the properties of spider silk are controlled by the amino acid sequence; therefore, amino acid changes or other sequence modifications can result in different properties or growth factor production (Cuffari, 2020). Additionally, functional peptides can be added to assist with aspects such as cell growth and attachment. Examples of these peptides include the RGD motif (consisting of the 3 amino acids arginine, glycine, and aspartate), growth factors like the basic fibroblast growth factor (bFGF), and the antimicrobial cationic peptides. Beneficial peptides can be taken from proteins already found in the body, such as fibronectin (Salehi et al., 2020). We need to look into additional peptides and sequence modifications, as well as the necessary genetic alterations in order to implement them.

We need to identify genetic parts capable of sensing abnormal pH, moisture, nutrients, pain, pharmaceutical, and bioburden levels, as well as genetic parts that could respond in order to keep the wound microenvironment in an ideal state. We would then need to test how changing the levels of each affects wound healing.

It is possible that the extreme repeat homogenization found in the A. argentata pyriform silk sequence will make it challenging for the yeast to produce. In this case, we may need to use a mix of spider silk proteins besides only pyriform silk.

Another aspect that must be tested is the productivity of the hBD-3 produced by the DEFB103B gene and its effects on microbes. This can be done by exposing different pathogens to the hBD-3 produced by the altered yeast and seeing how effective the antimicrobial is against them. Similarly, the effects of the hBD-3 and the antimicrobial peptides induced by the beta-defensin on the yeast chassis must be observed. If there are negative effects on the chassis, a self-protection method must be researched and added into the design. Regarding the IL-17 cytokines induced through the DEFB103B gene, the amount of IL-17 that is needed to cause a potentially dangerous cytokine storm must be established.

Author contributions

M.C. was responsible for research and writing regarding the chassis, antimicrobial device, DEFB103B gene, hBD-3, next step, and safety sections. T.C. was responsible for the systems level section, stages of healing and design figures, wound sensing device, ANB1 promoter, ARE enhancer, discussions, and background on wound healing. S.H. was responsible for the benefits figure, and material on the CACG motif, *PySp1* gene, silk creation device, next steps, background on the need for improved wound healing technology, pyriform silk, and spider silk for wound healing and bandages.

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