

Research Idea

Chimeric spider silk production in microalgae: a modular bionanomaterial

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Abstract

Background

In this project, we propose to explore the modular characteristic of spider silk proteins, through synthetic biology techniques, by combining and directing its properties to the desired application. The aim of this project is to generate a modular bionanomaterial able to immobilize proteins. This bionanomaterial will be composed of modular recombinant proteins from spider silk, which will be the immobilization support to other proteins, in this project an antimicrobial protein (enzymatic). By combining these proteins and their properties, the primary focus will be the use of this technology for the development of artificial skin for burn victims.

New information

The recombinant proteins, spider silk proteins and enzybiotics, will be expressed in *Chlamydomonas reinhardtii* strains by nuclear transformation. Each recombinant strain will express a different protein, which will contain the N- and C-terminal polymerization domains from native spider silk proteins. These domains are essential to the polymerization step and, subsequently, for production of a material very similar to silk. This material will be evaluated regarding its antimicrobial and mechanical properties, as well as the system productivity. These results may shed some light on spider silk-based immobilization support effectiveness, even for other biotechnological applications, such as the one idealized here.

Keywords

Bionanomaterial, Silk protein, Recombinant protein, Microalgae, Enzybiotic

Overview and background

Immobilization techniques are applied to a wide range of treatments and processes, from medical applications to biotransformations in industrial plants. The industrial/commercial application of biomolecules such as proteins depends on the stability and functionality of the process employed. This process often differs from the natural environment of proteins in terms such as temperature, presence of organic solvents and pH values. Consequently, techniques such as immobilization can promote stabilization and add functionality even when these biomolecules are under different environmental conditions. This stabilization is normally achieved by protein binding to a scaffold (Liese and Hilterhaus 2013). Recent studies explored spider silks as a possible immobilization support (Blüm et al. 2013, Monier 2013). This biomaterial has exceptional properties such as tensile strength and tension. Furthermore, medical applications are possible due to its biocompatibility and biodegradability, as coating for implants and transplanted organs, drug delivery and scaffolding for cell lines (Lewis 2006, Hardy et al. 2008, Kluge et al. 2008).

The aim of this project is to use this biomaterial for protein immobilization. Initially, we will immobilize enzybiotics for application to burn wounds, as a model to test this support. However, there are other possible applications with economic and academic interest. Fig. 1 presents an overview of the project.

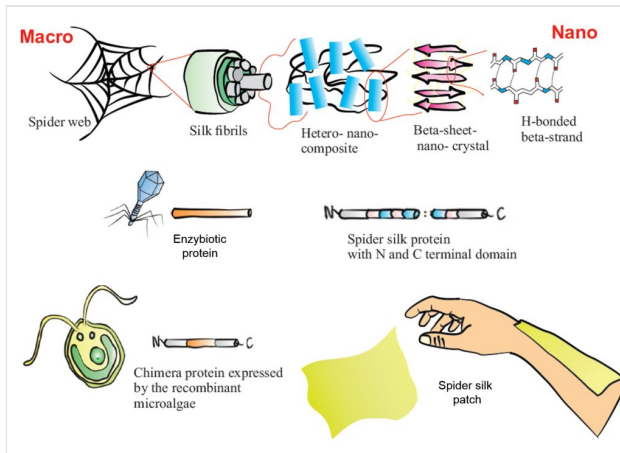


Figure 1.

Project overview. Schematic representation of spider web structure from macro to nano scale. A representation of: enzybiotic protein from a bacteriophage; a spider silk protein with repetitive domains and N and C terminals; host expression system *Chlamydomonas reinhardtii* and a chimeric protein envisioned in this project; and the final product, a biopatch produced from recombinant silk proteins and chimeric proteins.

Immobilization Systems

The retention of molecules inside a reactor, or an analytical system, is described as immobilization, whose purpose is to improve protein stability, selectivity, and particularly for the enzymes, increase catalytic activity (Tomotani et al. 2005). Aside from enzymes, other protein types can be immobilized, for example peptide domains for protein purification or immobilization such as: albumin binding domain (ABD), avidin (AvBD) and immunoglobulin G (ImGBD) (Luisi et al. 2013).

Enzymes are widely used in our society, and their applications range from industrial processes - such as in food production, biofuels and tissue - to more complex therapeutic applications, such as biopharmaceuticals (e.g., asparaginase use for acute lymphoblastic leukemia treatment (Pieters et al. 2010).

A medical application that benefits from enzyme immobilization is biosensor development (Sassolas et al. 2012). These devices display additional advantages such as reliability, speed, ease of handling and low cost compared to traditional diagnostic methods (Khan and Alzohairy 2010). Immobilized therapeutic enzymes are also being exploited due to their stability and reusability which enhance the targeting of specific tissues and cells (Bosio et al. 2015).

Enzybiotics

Enzybiotics comprise a class of enzymes with antibiotic activity. These enzymes are able to fight resistant bacteria, such as MRSA, VRSA and VISA (Rashel et al. 2007), antibiotic resistant strains of *Staphylococcus aureus*, which is a serious and recurring problem in hospitals. Enzybiotics refers mainly to the antibacterial potential of bacteriophage lytic enzymes endowed with the capacity to degrade bacterial cell wall (Rashel et al. 2007). This set of enzymes exhibits potential application on burn wounds otherwise susceptible to opportunistic colonization by microorganisms (Merabishvili et al. 2009). Thus, an artificial skin graft with antimicrobial properties may present a therapeutic option. Furthermore, an immobilized enzyme may not easily penetrate the skin and would thereby exhibit low or no allergenicity (Sheldon and van Pelt 2013). Considering this application and the different materials used as support matrix, enzybiotic functionalized spider silk proteins become an attractive alternative.

Spider webs

The polymer constituting the spider silk bears interesting properties for various applications, including immobilization of molecules such as proteins. Spider silk is known mainly for its tensile strength and fracture resistance, but also exhibits elasticity, adhesion, biocompatibility and low degradation. Its strength can be compared to Kevlar synthetic polymer, which is composed of aramid and is used in for manufacturing body armor (Lewis 2006).

It is known that certain repetitive sequences of amino acids confer specific properties to these structures and proteins in tissue, allowing one to obtain materials with desired characteristics through genetic manipulation of these structural domains. The poly-alanine domains (poly(A/GA) (Glycine-Alanine) in MaSp1 proteins, MaSp2 and MISp are associated with formation of beta-sheets and the production of strong fibers, while repeating sequences "GPGGx" and "GGX" as in Flag protein, preferably generates an elastic beta-spiral region, which provides elasticity (Tokareva et al. 2014).

In addition, terminal domains (N-terminal NT and C-terminal CT) are highly conserved both among species and different types of silk (Garb et al. 2010), which suggests they play important roles in the formation of silk and not in the generation of its mechanical properties *per se*. The change in CO₂ and proton concentrations controlled by the glandular duct ensures that polymerization occurs in precise time and space, at high speeds, reaching more than 1 m / s (Andersson et al. 2014).

There is some evidence of dependence on N- and C-terminal domains for polymerization, which can lead to interesting technological possibilities. The adaptation of these domains flanking a protein of interest opens the possibility of its immobilization if spun alongside "native" spider silk proteins. Moreover, core structural domains' ("poly(A/GA" and "GPGGx" and "GGX") customization influence the physical properties of the silk (Tokareva et al. 2014). Fig. 2 represents a scheme of the spider silk proteins and the chimeric protein proposed in this project.

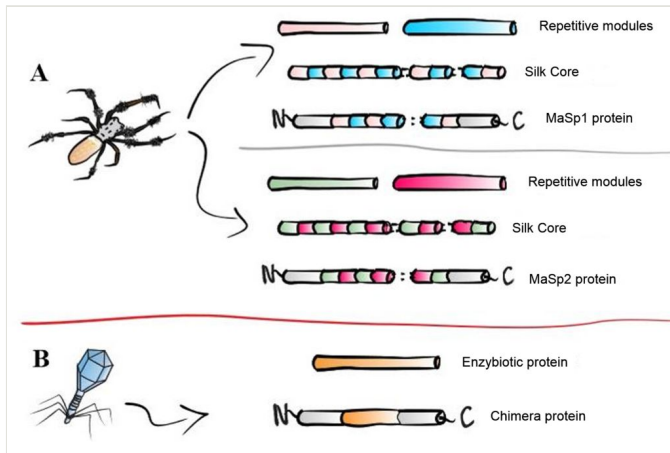


Figure 2.

Schematic representation of spider silk proteins and chimeric protein. **A:** MaSp1 - Major ampullate spidroin 1, MaSp2 - Major ampullate spidroin 2 **B:** Chimeric protein of an enzymatic protein with N and C terminal domains of spider silk proteins.

Expression System

Heterologous proteins are produced in several established expression systems, such as *Escherichia coli*, mammalian cell lines and yeast, but several systems are in the developing pipeline. Appropriate expression system choice should be based on priorities regarding performance and requirements of each recombinant protein (Walsh 2014). Currently, mammalian "CHO" cells (Chinese Hamster Ovary) and *E. coli* are the most used systems, which correspond to 35.5% and 19%, respectively, of the total products approved by the FDA in biopharmaceuticals (Walsh 2014). *E. coli* cells have rapid growth, productivity and low production cost, but it is a system unable to produce complex proteins and some post-translational modifications. Moreover, the production of endotoxins and inclusion bodies requires more purification process steps (Petsch 2000).

Therapeutic proteins are preferably produced in transgenic mammalian cell systems, because of their ability to express and correctly fold proteins. However, its production cost is high, especially when compared to plants as expression systems. Molecules such as monoclonal antibodies (mAbs) are mainly produced in mammalian cells and their average production cost in this system is estimated to be \$ 150.00 per gram of raw materials, whereas production in plant systems costs approximately US\$0.05 per gram (Dove 2002, Mayfield et al. 2003). But the estimated value for algae reaches US \$ 0.002 per liter, making them potential competitors for land plants (Mayfield et al. 2003). Microalgae present several desirable features in an expression system, such as: rapid growth (characteristic for microbial growth), swift and stable transgenic lineage generation, scalability and low-cost production (Wijffels et al. 2013, Rosenberg et al. 2008). They are also able to produce and secrete complex proteins and perform post-transcriptional

modifications. They are Generally Regarded As Safe (GRAS), with low risk of virus contamination, prions or bacterial endotoxins, and establish no gene flow with the flora around through pollens as in transgenic plants (Mayfield et al. 2007).

However, the studies on genetic engineering using microalgae are incipient and present challenges. The main challenge is the low productivity of recombinant proteins expressed in the nuclear genome, hindering commercial applications to date (Rasala and Mayfield 2011). Strategies to increase nuclear expression levels in *Chlamydomonas reinhardtii* are being developed. These strategies may result in productivity gains, enabling commercial-scale production. Such strategies include codon optimization (León-Bañares et al. 2004), development of alternative vectors (Heitzer and Zschoernig 2007, Lauersen et al. 2013) and modifications to the design expressed constructs. Changes in constructs include the addition of autocleavage peptide (Rasala et al. 2012), use of fused promoters (Eichler-Stahlberg et al. 2009, Schroda et al. 2000) and addition of intronic sequences (Eichler-Stahlberg et al. 2009, Lumbreras et al. 1998). Alongside these improvements, *Chlamydomonas reinhardtii* presents a GC-rich genome, which may play an important role in spider silk protein expression due to its high GC content. It is hypothesized that high GC content clogs the heterologous expression of this kind of protein in non-GC-rich systems (Yang et al. 2016).

Objectives

- Evaluate production capacity of synthetic spider silk proteins (based on MaSp1 and MaSp2) and protein chimeras with NT and CT domains flanking enzymatic domains in *Chlamydomonas reinhardtii* by transforming the nuclear genome;
- Set methods for polymerization of silk produced by microalgae;
- Test biopolymer and antibiotic properties of spun spider silk, pure and in combination with enzymatic chimeric proteins;
- Assess the project development in an iGEM competition context regarding its scientific achievement and the real-time openness in all process steps: idealization, laboratory procedures, results and discussion

Impact

Many obstacles need to be overcome for the effective production of a biomaterial such as a recombinant spider silk capable of immobilizing proteins in its matrix. With this purpose, this project offers solutions, as of yet untested, for example: the use of microalgae as a production platform for the expression of spider silk proteins, as well as chimeras with NT and CT domains, and flanking enzymatic domains. Research on transgenic microalgae are driven by the global demand for recombinant proteins and other bioproducts. This biotechnological market is growing exponentially, it has reached 140 billion dollars in sales as of 2013, and it continues to grow (Walsh 2014) with the potential for commercial application.

More than developing a product that could help thousands of patients, the development of an antibiotic chimeric biopolymer in *C. reinhardtii* can result in many scientific outcomes. Spider silk is a challenging material to work with, and accessing its production with chimeric and functional materials can pave the way for many applications in the biomaterial field. Moreover, the microalgae community is still incipient, in comparison to the *E. coli* system, and integrative projects like this one can expand the synthetic biology tools applied to this model microorganism, which will benefit the scientific community.

It is important to highlight the fact that this project will be carried out at an international competition dedicated to the development of high-level research in Synthetic Biology (iGEM - International Genetically Engineered Machine) with an open and integrative approach. This competition takes place annually in Boston, USA, and it stimulates interdisciplinary groups to problem solving through genetically modified organisms. In line with this proposal, the team responsible for this project consists of undergraduate and graduate students from various institutes of the University of São Paulo. The blending of open approaches and such interdisciplinary groups contributes to the development of the research *per se*, ultimately impacting its quality and depth for the better. The project is also integrated with the SynBio Brasil community, actively engaged in promoting synthetic biology education, leading to a powerful impact on scientific awareness in Brazil.

Implementation

Plasmid Construction

The vector pBluescript II (Thermo Fisher Scientific Inc.) will be used. The constructed cassette will be flanked by the restriction sites *Kpn* I at one end, and *Xba* I at the other. There are also two different restriction sites, *Xho* I and *Bam* HI, flanking the coding sequence of the desired protein. The codons of the proteins will be optimized for the expression in *C. reinhardtii* nucleus (Fuhrmann et al. 1999) and the restriction sites *Kpn* I and *Xba* I will be removed. Rubisco introns will be inserted in the promoter hsp70A/rbcs2 sequence, in the Sh-ble sequence and in the terminal region RbcS2 3' UTR, aiming to increase the expression of the protein of interest (Eichler-Stahlberg et al. 2009, Lumbreras et al. 1998). Primers will be designed to confirm (by PCR) the insertion of the whole sequence in the plasmidial vector. Fig. 3 shows the generic cassette for expression.

The native proteins MaSp1 and MaSp2 (*Major Ampullate Silk Protein*) were selected since they are the most studied proteins among the cob constituents. The antimicrobial enzymes were selected from a screening of the phiBIOTICS databank (Hojckova et al. 2013) looking for molecules that are effective against resistant strains of *Staphylococcus aureus*. All these proteins are summarized in Table 1.

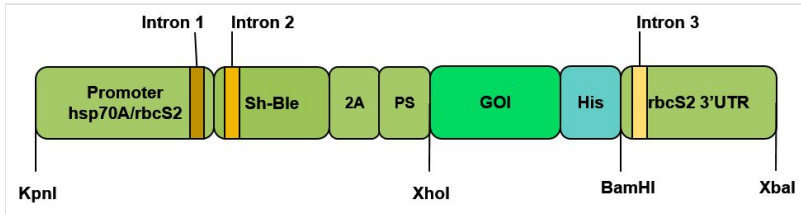


Figure 3.

Cassette construction to be inserted in *C. reinhardtii* nuclear genome for the expression of desired proteins. **Promoter hsp70A/rbcS2**: fusion of the promoters hsp70A and rbcS2 (Eichler-Stahlberg et al. 2009, Schroda et al. 2000). **Sh-Ble**: gene that gives resistance to Zeomycin. **2A**: self-cleavage peptide obtained from Foot and Mouth Disease Virus (FMDV) (Rasala et al. 2012). **PS**: Secretion signal peptide of the gene Ars1. **GOI**: gene of interest coding the proteins to be used in the project. **His**: coding sequence of six histidines (histidine tag). **RbcS2 3'UTR**: terminal sequence (untranslated region) of the gene RbcS2 (Fuhrmann et al. 1999)

Table 1.

Desired proteins to be expressed in *C. reinhardtii*.

NTD: N-terminal domain of MaSp 1. **CTD**: C-terminal domain of MaSp1. **MRSA**: Methicillin-resistant *S. aureus*. **VRSA**: Vancomycin-resistant *S. aureus*. **VISA**: Vancomycin-intermediate *S. aureus*. **ORSA**: Oxacillin-resistant *S. aureus*. **CDS**: Coding DNA sequence. **MaSp1**: Major Ampullate Silk Protein 1. **MaSp2**: Major Ampullate Silk Protein 2.

Protein	Source	Information	Ref.
MaSp1	<i>Latrodectus hesperus</i> (Black Widow)	CDS fully sequenced	Ayoub et al. 2007
MaSp2	<i>Latrodectus hesperus</i> (Black Widow)	CDS fully sequenced	Ayoub et al. 2007
NTD + MV-L + CTD	Bacteriophage phiMR11	Effective against MRSA, VRSA e VISA	Rashel et al. 2007
NTD + LysK + CTD	Bacteriophage K	Effective against MRSA e VRSA	O'Flaherty et al. 2005
NTD + Lysostaphin + CTD	<i>Staphylococcus simulans</i>	Effective against MRSA, ORSA e VISA	Yang et al. 2007

C. reinhardtii cultivation

The *C. reinhardtii* strain cc1690 will be obtained at the Chlamydomonas Resource Center. After going through the process of transformation, the ones identified to produce the protein of interest will be grown for protein production. The strains will be cultivated in TAP (Tris-Acetate-Phosphate) medium (Gorman and Levine 1965) in 250 mL Erlenmeyer flasks, containing 50 mL of the medium, at temperatures between 20-25°C, under constant agitation at 100-150 rpm and constant illumination of $50 \pm 10 \mu\text{E/m}^2 \text{ s}$. The growth will be

evaluated by cell counting with Neubauer chambers and by reading the absorbance at 750 nm wavelength every 12 h (Eichler-Stahlberg et al. 2009). Aliquots will be taken during the growth process for further analysis and for identification of the proteins through Western Blot.

***C. reinhardtii* transformation**

C. reinhardtii cells will be grown in TAP medium until reaching the cell density of $3-6 \times 10^6$ cells/mL. The cells will be collected by centrifugation and resuspended in TAP enriched with 40 mM of sucrose reaching a cell density of $3-6 \times 10^6$ cells/mL. Then, 250 μ L of the culture will be incubated with 300-1000 ng of plasmidial DNA, previously linearized through the digestion with *Xba* I and *Kpn* I for 5-10 min in cuvettes kept in an ice bath. An exponential electrical pulse of 2000 V/cm will be applied to the sample with an electroporation device, GenePulser XCell™ (BioRad, Hercules, CA). Capacitance will be adjusted to 25 mF and the resistance will not be regulated. After that, the cells will be incubated for 18 h in 10 mL of TAP/40 mM sucrose and plated in TAP/Zeocin solid medium (Rasala et al. 2012).

Selection of mutants and identification of recombinant proteins

Recovered cells will be plated on TAP agar medium with increasing antibiotic concentrations (0.1, 2 and 5 μ g/mL Zeocin). Candidate transformed colonies, displaying high Zeocin resistance, will be analyzed through PCR screening, and PCR positives colonies will be tested for protein of interest production by Western blot. Basically, the mutant cells are cultured as described above and fractions of the supernatant and cell lysate will be tested for the presence of the protein of interest. Cell lysis will be accomplished by sonication as described in the literature (Lumbreras et al. 1998).

Western Blot

Samples of supernatant and total soluble proteins will be denatured by adding SDS-PAGE loading buffer (Laemmli) with β -mercaptoethanol, followed by incubation at 95 °C for 5 min. Proteins will be separated on 12% polyacrylamide gels at 120-150 V and transferred to nitrocellulose membrane at 200mA for 1h. Then they will be blocked in 5% solution of skimmed milk and the protein of interest will be probed with monoclonal mouse anti-His antibody. The membrane will then be washed 3 times with TBS-T (Tris-buffered saline with Tween 20 detergent) for 10 min and incubated with anti-mouse antibody conjugated with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) substrates which forms an insoluble dark blue diformazan precipitate, allowing protein identification.

Recombinant proteins purification

Purification of the proteins of interest will be carried out by Nickel resin (Ni Superflow His60, Clontech®) following the manufacturer's instructions. The method is based on the

affinity interaction with the hexa histidine tail, present in recombinant protein with the already mentioned resin. Basically, the sample is added to the column with the resin precharged with nickel ions, in which the proteins of interest containing histidine residues on its surface will be attached. Proteins not bound to the column will be washed out with the wash buffer, while the protein of interest is eluted with buffer containing 500 mM Imidazole.

Quantification of recombinant proteins

Quantification of purified proteins will be obtained via the Enzyme Linked Immunosorbent Assay (ELISA). Thus 200 μ L of sample are incubated in 96-well plates at 37 °C for 30 min, then the solutions are removed, blocked in 5% solution of skimmed milk and the wells are washed 3 times with TBS-T (Tris-buffered saline with Tween 20 detergent) . Then, 200 μ L/well of TBS-T solution of monoclonal mouse anti-His is added and incubated at room temperature for 2 h and washed as described above. A new TBS-T solution with anti-mouse monoclonal antibody conjugated with alkaline phosphatase is added, incubated at room temperature for 2 h and washed 3 times with TBS-T for 10 min. For the development, a freshly prepared solution of p-nitrophenyl phosphate is added and incubated in the wells for 30 min, and the plate is subsequently read in a plate reader at 405 nm.

Data analysis

Results will be evaluated by analysis of variation (ANOVA) performed in Statistica software 10. Statistical significance will be evaluated by estimating the descriptive level (p) and the results will be considered statistically significant at $p < 0.05$ (confidence level of 95%) .The methods described above are shown in the flowchart in Fig. 4 and the execution chronogram in Table 2.

Table 2.

Chronogram of execution

Month	1 st	2 nd	3 rd	4 th	5 th
Plasmid construction	X				
<i>Chlamydomonas reinhardtii</i> transformation		X	X		
Transformed strains evaluation		X	X	X	X
Cob polymerization evaluation				X	X
Results analysis	X	X	X	X	X
Wiki development					X

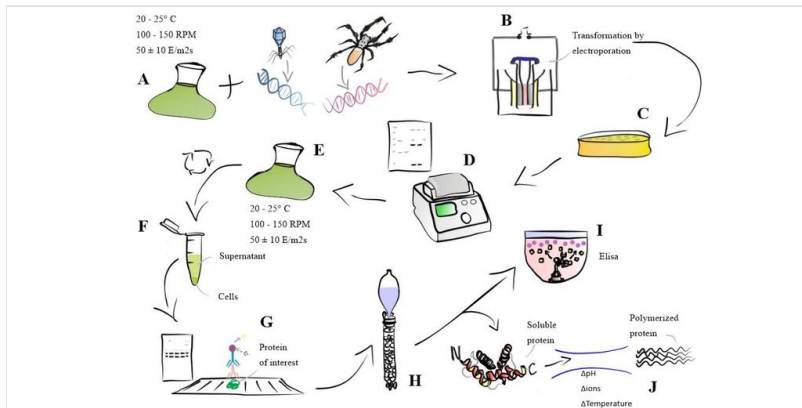


Figure 4.

Experimental Flowchart. (A) Wild Cells incubated with built vectors. (B) Wild-cell transformation by electroporation. (C) Selection of mutants resistant to Zeocin. (D) Screening of antibiotic resistant cells by PCR. (E) Cultivation of PCR positive cells. (F) Fractions to be tested for the presence of recombinant proteins. (G) Detection of recombinant proteins present in the fractions by Western Blot. (H) Protein Purification. (I) Quantification via ELISA. (J) Spider silk polymerization reaction.

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Hosting institution

University of São Paulo.

Author contributions

All the authors contributed to this manuscript by reviewing and editing the final text, as well as either writing, doing background research or making figures.

Conflicts of interest

The authors report no conflict of interest.

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