Bioengineering Brochosomes as the future of Superhydrophobic coatings

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Abstract

Superhydrophobic coatings are nanoscopic surface layers that repel water with a high degree of effectiveness and efficiency. Water droplets hitting treated superhydrophobic surfaces can fully rebound in the shape of a column or a pancake. However, despite the many potential applications of such coatings, they are not used industrially on a large scale as they are too expensive to process and need to be constantly retreated due to the low mechanical and chemical stability of the coating bond. Furthermore, the cost of manufacturing and treating substrates with superhydrophobic coatings is prohibitively high. Through the Central Dogma of Molecular Biology, this project has created a novel procedure for superhydrophobic treatments by harvesting lab-produced brochosomes, a protein that is unique to leafhoppers (*O. Hemiptera, F. Cicadellidae*), through bacterial transformation that was both relatively inexpensive and the resulting superhydrophobic surface retaining its mechanical stability. The effectiveness of proteins as viable superhydrophobic coatings has been proven through small-scale model ship resistance testing. From the results, the proposed alternative would be effective as a replacement for the high-cost treatments of the maritime and other industries.

1. Introduction and Literature Review

Superhydrophobic surfaces are described by static contact angles (of water) higher than 150° and sliding angle of less than 10°. They have many potential applications and one of the major applications of these superhydrophobic surfaces is to increase the corrosion resistance of surfaces exposed to corrosive environments as well as be applied for water-rich environments (Arabian Journal of Chemistry, 2018).

Due to their superhydrophobic properties, these coatings are mainly used by the maritime industry as well as other industries, as they can yield skin friction drag reduction, which will increase fuel efficiency. However, current superhydrophobic treatments are non-viable as they are easily destabilized mechanically by wear and tear, causing the performance efficiency to be

lost. Recoating is also prohibitively expensive (Aculon, 2015). While there are many potential uses of superhydrophobic coatings, including but not limited to surgical tools, medical equipment and stain-proof or self-cleaning textiles, it is ironic as demand for superhydrophobic coatings is highest in industries with conditions where coatings are subjected to constant abrasion and friction. Unless any further advancement can resolve this weakness of commercial superhydrophobic treatments, they are limited in their usefulness despite their great potential. This is ironic because the coatings are mostly used in industries such as the aforementioned maritime industry where they will be subject to constant wear and tear, which means that the maritime industry will have to spend a considerable amount of money and resources to maintain the effectiveness of the coatings. They are also an environmental hazard, most being non-biodegradable due to their chemical composition, with others being potentially toxic to humans.

2. Proposed Solution and Solution Design

Solution Background

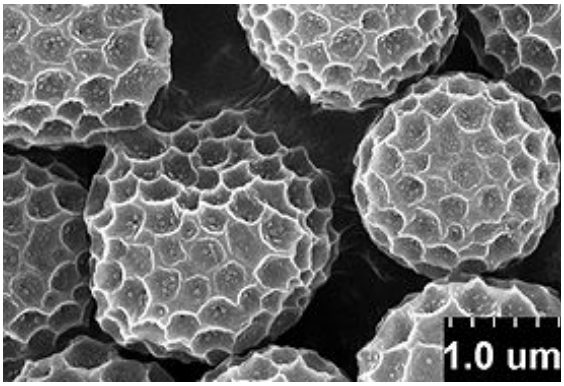

Figure 2.1 Scanning Electron Micrograph of brochosomes

Brochosomes are secretory granules resembling buckyballs (Fig. 2.1) ranging between 0.2-0.6 micrometers, produced intracellularly in specialized glandular segments of the hind Malpighian

tubules and used to form superhydrophobic coatings on the integuments of leafhoppers (of *Hemiptera, Cicadellidae*). The major (60-70%) structural component of brochosomes is a novel family of 21-40-kDa secretory proteins (Fig. 2.2), referred to in literature as brochosomins, cross-linked by disulfide bonds. Brochosomins and other brochosome-associated proteins have no homologs among all known proteins, thus representing taxonomically restricted gene families (ORFans). Brochosomes are effective superhydrophobic proteins (R, Rakitov, N. Gorb) as they are capable of trapping air pockets, repelling moisture, in addition to being resistant to mechanical damage due to their small size. Leafhoppers actively apply anal secretions containing brochosomes on their wings with their integuments and to their egg nests. The small size and intricate surface structure of brochosomes (Fig. 2.3) apparently render layers of these particles unwettable by water and phloem. Another possible function of such coatings is direct or indirect protection against the attachment and germination of fungal spores. Brochosomes were also reported to have antimicrobial properties (R Rakitov, 2015), thus enhancing applications in various fields.

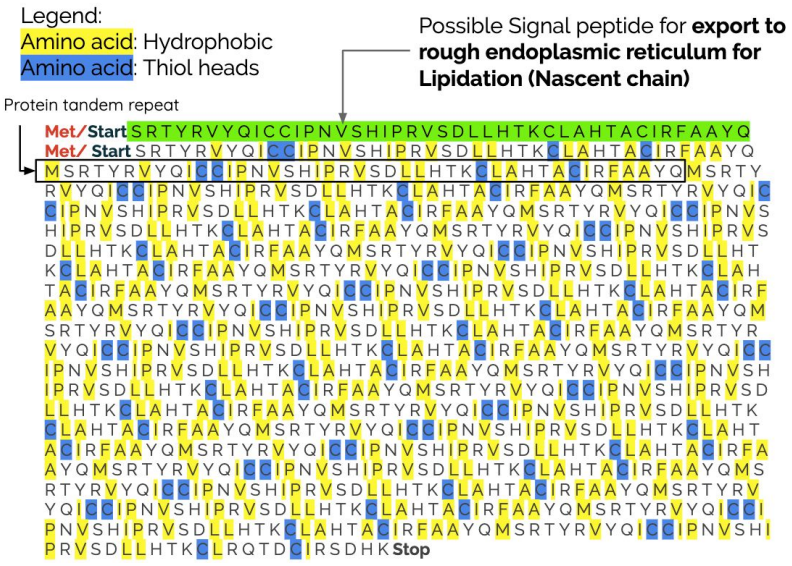

Figure 2.2 DNA sequence coding for brochosome

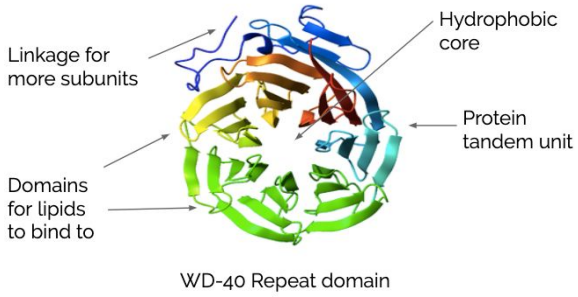

Figure 2.3 Visual illustration of brochosome unit folding

While leafhoppers are the only taxonomic family that are able to produce brochosomes, there is no need to harvest brochosomes from leafhoppers. Following the Central Dogma of Molecular Biology, we are able to produce brochosomes from *E. coli*, a bacterium, which will increase the efficiency of protein harvesting. The Central Dogma of Molecular Biology (Fig. 2.4) was first proposed in 1958 by the English physicist Francis Crick, co-discoverer of the structure of DNA with American biologist James Watson. The central dogma explains the flow of genetic information from DNA to RNA to create a functional product, the protein, which is the brochosome in the context of this project. The central dogma suggests that DNA contains the information needed to make all of our proteins, and that RNA is a messenger that carries this information to the ribosomes of a cell. The ribosomes serve as factories in the cell where the information is translated from a base sequence code into the functional product. The process by which the DNA instructions are converted into the functional product is called gene expression. Gene expression has two key stages - transcription and translation. In transcription, the information in the DNA of every cell is converted into small, portable RNA messages. During translation, these messages travel from where the DNA is in the cell nucleus to the ribosomes where they are 'read' to make specific proteins, or brochosomes as the ultimate aim of this project. Following the Central Dogma of Molecular Biology, by inserting the DNA sequence coding for brochosomes into *E. coli* will enable *E. coli* to produce these superhydrophobic coatings, eliminating the need for harvesting these proteins from leafhoppers. This is possible as *E. coli* possesses cellular machinery such as ribosomes similar to the leafhoppers and operate based on the same principles, thus allowing them to produce exactly identical proteins.

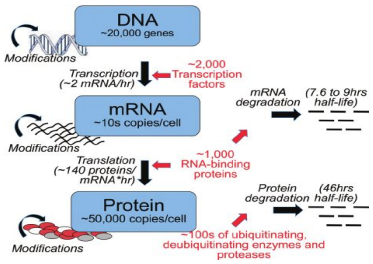

Figure 2.4 Central dogma of molecular biology, illustrated

Aims and Objectives

In this project, we aimed to do the following:

- Produce viable brochosomes in a cheap and controlled manner, without having to harvest them from live leafhoppers which is difficult and cannot guarantee the purity of harvested brochosomes;
- Apply brochosomes to a variety of potential surfaces to test the superhydrophobic qualities of these surfaces;
- Upload sequenced data to K1TE to contribute to the small body of knowledge on the sequenced genomes of insects;

Procedures and Materials

This project was divided into three phases of action.

Firstly, the focus of Phase 1 was to derive the cDNA (complementary DNA) sequence coding for brochosomes to be inserted into a suitable bacterium that would carry the vector (designated steps 1 to 3). Secondly, the aim of Phase 2 was to transform the bacteria to produce brochosomes which this project would then harvest for the purpose of testing (designated steps 4 to 5). Thirdly and lastly, the focus of Phase 3 was to test the effectiveness of brochosomes as a viable superhydrophobic coating (designated step 6).

Phase 1

Sequencing cDNA with Next Generation sequencing

Step 1 - Obtaining samples for RNA sequencing

1. Application for a research permit from NParks, allowing the capture of specimens from nature reserves via non-destructive, sustainable means;
2. Capture of adult leafhoppers (*Bothrogonia addita*) of both sexes from Sungei Buloh Wetland Reserve with light traps;
3. Establishment of laboratory colony of leafhoppers from captured specimens;
4. Other insects and species inadvertently captured were set free.

Step 2 - RNA sequencing to identify DNA sequence coding for brochosomes

1. Adult specimen was dissected and secretory glands were obtained from Malpighian tubules;
2. Obtained cells fixed with Triton X-100 to prevent lysosomes from lysing and releasing RNAse that would degrade RNA present in samples;
3. Sample placed in a microcentrifuge and lysate collected, DNAse added to the sample to remove genomic DNA;
4. Resulting mRNA was hybridised with polyT tails to filter out other mRNAs;
5. Gel electrophoresis was performed to determine the purity and integrity of the RNA;
6. 1.20kB band of the formaldehyde gel was cut out (as the sample contains only mature mRNA with the introns having already been spliced) and RNA electrophoresis buffer was then added to the slice and the gel was melted at 65°C for 5min;
7. Phenol was added to the solution and melted again at 65°C for 5min before repeating again;
8. RNA was extracted by adding 0.1 volumes of 3M sodium acetate at pH4.7 and 2.5 volumes of ethanol;

9. Reverse transcriptase and free NTPs were then added to the mixture incubated at room temperature for 25 mins;
10. Gel electrophoresis was performed again and the 1.20kB band was then cut out and extracted with GeneJET Gel Extraction Kit;
11. cDNA was sent for sequencing at NUS and loaded into next-generation sequencer and the sequence for the gene of interest was then obtained.

Step 3 - Restriction enzyme based plasmid cloning

3A - Restriction digest of pGreen 0000 of plasmid

1. Added 1 µg DNA to the microcentrifuge tube;
2. Added 5 µl SmartCut® Buffer to the microcentrifuge tube;
3. Added 1 µl CviAII to the microcentrifuge tube;
4. Diluted with 44 µl deionised water to reach a 50 µl reaction volume;
5. Mixed gently by flicking the microcentrifuge tube;
6. Incubated at 25 °C for 15 mins;
7. Gel purification carried out.

3B - Restriction digest of DNA insert

1. Added 1 µg DNA to the microcentrifuge tube;
2. Added 5 µl SmartCut® Buffer to the microcentrifuge tube;
3. Added 1 µl CviAII to the microcentrifuge tube;
4. Diluted with 44 µl deionised water to reach a 50 µl reaction volume;
5. Mixed gently by flicking the microcentrifuge tube;
6. Incubated at 25 °C for 15 mins;
7. Gel purification carried out.

3C - Ligation of vector and insert

1. Added 3 µl of DNA insert and 1 µl of plasmid to the microcentrifuge tube;

2. Added 1 µl of deionised water to the microcentrifuge tube;
3. Added 5 µl of DNA Ligase to the solution;
4. Incubated at 25 °C for 15 mins;
5. Gel purification carried out;
6. Added 0.7g of Agarose powder to the 100ml 1xTAE solution;
7. Microwaved solution for 2 mins;
8. Solution left to stand for 5 mins;
9. Added 2 µl of ETDA to solution;
10. Poured the solution into gel tray with well comb;
11. Solution left to stand for 30 mins;
12. Added loading buffer to the plasmid DNA;
13. Added 1x TAE buffer with 2 µl ETDA until the gel was covered;
14. Loaded a molecular weight ladder onto the first lane of the gel;
15. Loaded plasmid DNA onto other wells of the gel;
16. Ran the gel at 80-150 V for 1.5 hours;
17. Turned OFF power, disconnected the electrodes from the power source, and removed the gel from the gel box;
18. Using UV light imaging, 7kb band of the gel was cut out;
19. Excised band placed into a microcentrifuge tube;
20. Cut a 5cm length of dialysis tubing and rinsed it inside and out with distilled water, then rinsed it with 1xTAE and left it submerged in a small beaker of the same buffer, before one end was sealed with a clip;
21. Inserted the frozen gel-slice into the tubing and added 200–400µL of 1xTBE, before the other end of the tubing was sealed with a second clip;
22. Immersed the sealed tubing in an electrophoresis tank so that the DNA band is parallel to the electrodes and applied 5V for 15 mins;
23. Removed buffer from tubing and placed sample inside the microcentrifuge tube;
24. Mixed the sample with (equal volumes) 1 volume of Tris-saturated phenol and 1 volume of chloroform;

25. Centrifuged at 10,000 rpm for 5 min at room temperature;
26. Transferred the upper aqueous phase to a fresh microcentrifuge tube;
27. Added an equal volume of chloroform and mixed;
28. Centrifuged at 10,000 rpm for 5 min at room temperature twice;
29. Transferred the upper aqueous phase to a fresh tube;
30. Added 1/10 the volume of 2M sodium chloride;
31. Added 2.5 volumes of ethanol to precipitate DNA from sample;
32. Incubated the mixture for 30 min at -20°C;
33. Centrifuged for 10 min at 10,000 rpm;
34. Discarded the supernatant and rinse the pellet with 70% cold ethanol;
35. Air-dried the pellet and dissolved the pellet in water.

Phase 2

Transformation of E. coli bacteria

Step 4 - Bacterial transformation

1. Mixed 0.5 µL of DNA into 40 µL of competent cells in a microcentrifuge tube;
2. Incubated E. coli ATCC 49979/DNA mixture on ice for 20-30 mins;
3. Heat shocked each transformation tube by placing the tube into a 42°C water bath for 45s and removing afterwards
4. Put the tubes back on ice for 2 min;
5. Added 250µl LB media to the bacteria and grew them in 37°C shaking incubator for 45 min;
6. Plated all of the transformation onto a 10 cm LB agar plate containing ampicillin;
7. Incubated plates at 37°C overnight.

Step 5 - Protein purification

1. Scraped a single bacteria colony into a conical flask and incubated the mixture at 37°C overnight;

2. Pipetted solution into microcentrifuge tubes;
3. Added 10 µl of deionised water into microcentrifuge tubes;
4. Centrifuged at 6000 RPM for 30 mins;
5. Filtered solution;
6. Collected brochosomes pellet deposited on the paper.

Phase 3

Testing the effectiveness of brochosomes as a viable superhydrophobic coating

Step 6 - Testing the effectiveness of brochosomes

6A - Contact angle analysis with CAST3.0

1. Coated cloth with one layer of brochosomes;
2. Filled syringe with 5 ml water;
3. Syringed 5 ml of water on cloth;
4. Using a goniometer, a photo of the water droplet was taken at eye level;
5. Droplet contact angle analysis with CAST3.0

6B - Small-scale model ship resistance testing

1. Made model ship out of aluminium foil;
2. Attached dynamometer and pulled ship by 1m;
3. Recorded how much force was required to pull ship;
4. Coated 1mm layer of brochosomes on surface of ship;
5. Measured how much force was required to pull ship forwards by 1m.

3. Results and Discussions

From Phase 1, we could observe in the derived DNA sequence that brochosomes were composed of 24 protein tandem repeats. Fig. 3.1 shows a peptide sequence which is repeated 24 times to fold into a brochosome translated from a section of our DNA sequence we obtained.

M S R T Y R V Y Q I C C I P N V S H I P R V S D L L H T K C L A H T A C I R F A A Y Q

Legend

Amino acid : Hydrophobic

Amino acid : Thiol heads

Figure 3.1 Repeated tandem peptide sequence

As seen from the above amino acid sequence, brochosomes are mainly composed of hydrophobic amino acids which contribute to the hydrophobicity of the protein. Thiol heads are present on the multiple cysteines on the peptide sequence which aid in binding to the substrate through ionic bonds. This mitigates the effect of abrasion on brochosomes and makes it resistant to mechanical damage as a superhydrophobic coating.

Robetta is a protein structure prediction server developed by the Baker laboratory at the University of Washington. At its core is the Rosetta macromolecular modeling suite developed by the Rosetta Commons, a multi-institutional collaborative research and software development group. Robetta's primary service is to predict the 3-dimensional structure of a protein given the amino acid sequence. Three main options are provided for structure prediction: (1) Rosetta Comparative Modeling (RosettaCM), (2) Rosetta Ab Initio (RosettaAB), and (3) a fully automated pipeline that first predicts domains as independent folding units, models each unit with (1) or (2), and then assembles them into full chain models.

Using the RosettaAB algorithm, we inputted the amino acid sequence of the brochosome into the server, which generated a computer-modelled prediction of the brochosome. RosettaAB also

identified the structural motif of the brochosome which was revealed to be a WD-40 repeat domain. The model of the brochosome subunit is shown in Figure 3.2. The primary structure is a peptide chain mainly composed of hydrophobic amino acids to increase hydrophobicity, the secondary structure has numerous Beta-pleated sheets to increase hydrogen bonding for stability. 6 subunits assemble and fold into the WD-40 repeat domain shown in Figure 2.3 to form the tertiary structure, which 4 of these repeat domains then assemble and fold into the buckyball-shaped structure, which is the quaternary structure. The tertiary structure, WD-40 repeat domain, allows for hydrophobic interactions at the centre to make the brochosome protein insoluble in water, the quaternary structure is a buckyball-shaped structure that is hollow and traps air, increasing hydrophobicity, which is shown in Figure 2.1.

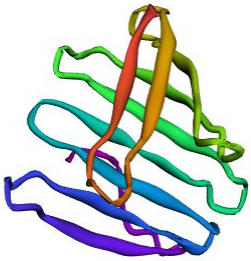

Figure 3.2 Rosetta Ab Initio computer-generated model of a brochosome subunit

It can be seen from Fig. 2.3 that brochosomes trap air pockets in between the assembled quaternary structure. This increases the ability for brochosomes to repel water. Thus, on every level of protein structure, the peptide sequence of brochosomes allow it to increase its hydrophobicity which allows it to perform its function.

Furthermore, the DNA sequence in Fig. 2.2 contains a signal peptide that the Golgi complexes recognise as the lipidation signal. This results in several hydrophobic lipid heads attached to the structure of the brochosomes during post-translational modifications. This post-translational modification increases hydrophobicity, allowing the brochosomes to attain a higher degree of water repellency.

Thus, from the sequence derived, we can deduce the superhydrophobic nature of brochosomes and evaluate its effectiveness.

From Phase 2, brochosomes are known to be proteins that are produced intracellularly in leafhoppers, they can be produced by regular bacterial cellular bio machinery which are similar to every aspect of eukaryotic cellular machinery. The biotechnology scheme for the production of proteins by the *Escherichia coli* (E. coli) bacterium is currently in use worldwide for basic research as well as industrial applications, the most well-known of which is insulin and its derivatives. Thus, E. coli was selected to be the host for the improvised lab-cultured brochosome production line for the purposes of this project. As brochosomes are superhydrophobic proteins, they were secreted by E. coli in solid form instead of dissolving into the solution. Thus, this allowed us to reduce the effort needed to purify brochosomes.

From Phase 3, the picture taken by the goniometer allowed us to run a droplet shape analysis using CAST3.0, an advanced software that provides contact angle analysis. CAST3.0 allowed us to measure the contact angle of the water droplet against a flat surface provided by the goniometer, which we then coated with brochosomes. Through CAST3.0 droplet shape analysis, it concluded that the contact angle of the water droplet as shown in Fig. 3.3 was 168.4°, having met the minimum criteria of superhydrophobic coatings which was a contact angle of at least 150°. In comparison, polyurethane composites, another viable alternative as a superhydrophobic coating, only has a contact angle of 160.8°.

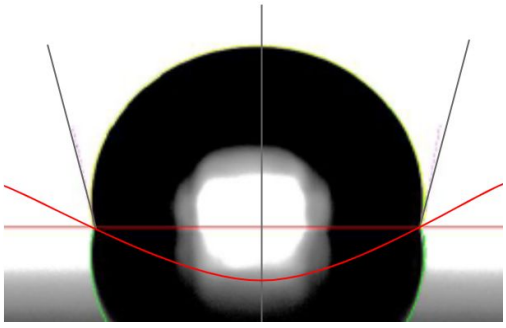

Figure 3.3 CAST3.0 droplet shape analysis

From the model resistance ship testing procedure (Purnamasari, Dian & Utama, I Ketut & Suastika, Ketut.) the resistance coefficient of the model ship greatly decreased when brochosomes were applied onto the ship's surface which was in contact with the water. Given that $R=F_I/0.5\rho V^2S$, the resistance coefficient of the model without brochosomes was $5.00Nm^{-1}$ but after applying a brochosome coating to the model, the resistance coefficient decreased to $3.80\ Nm^{-1}$. More details can be found in Appendix I. According to this formula, the resistance coefficient would still remain the same, even if the model is scaled up to actual size. Thus, since there was a significant decrease in the resistance coefficient in the model, this shows that brochosomes are viable alternatives as superhydrophobic coatings.

4. Conclusion and Further Work

Based on our results, this project has proven that brochosomes can be manufactured in a new process inexpensively through bacterial transformation. However, this takes into consideration that the initial budget was stretched in order to sequence the DNA coding for brochosome production. This step can be completely avoided in the future as there is no longer any need to sequence a novel DNA sequence to produce brochosomes as one can instead search a genomic database for the sequence (after we have uploaded our sequenced data).

From our testing, we can conclude that brochosomes are a viable alternative to current superhydrophobic coatings as they outperform other superhydrophobic coatings such as polyurethane and polymethyl methacrylate in areas such as contact angle and resistance coefficient respectively while maintaining a relatively inexpensive cost of manufacturing.

It was encountered during experimentation that the E. coli bacteria can only produce small amounts of brochosomes. Thus, potential usage may be limited to only small products, such as handheld electronics or common portable tools. This limitation can be overcome by increasing the colony size of E. coli in order to produce more proteins.

As this project did not allow access to appropriate resources, including proper reagents, it was noted that cloning efficiency was drastically reduced. However, this problem could have been solved by using a phosphatase, which would allow cloning to reach the threshold of 90% efficiency.

Lastly, to increase brochosome production of recombinant E. coli, additional enhancers and customised activators (Fig. 4.1) can be bioengineered onto the plasmid. By taking appropriate steps to prevent the production of drug-resistant strains of bacteria, production efficiency can be increased by many folds. However, this would require specialised equipment unavailable on campus and carries certain ethical implications.

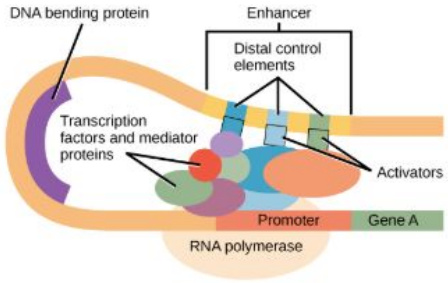

Figure 4.1 Diagram of enhancers and distal control elements

5. Acknowledgements

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Appendix I

Where F_I is the initial force required to move the ship by 1m, ρ is the density of water, V is the model ship velocity and S is the surface area of the model ship in contact with water.

Figure A.1 Model ship resistance coefficient testing