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论文题目：A Fusion of Artificial Spidroin and Mussel Foot Protein That Retains High Adhesion and Natural Glue Formation Ability

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李沛霖，1981 年生，中国共产党员。2003 本科年毕业于四川大学生命科学学院，同年考取四川大学与中国科学院联合培养硕士研究生，硕士在读期间 2005 年考入中央党校四川分院法律专业（2007 年毕业），2006 年硕士毕业后进入深圳外国语学校工作至今。在外国语学校工作期间 2007 年~2010 年辅导生物奥林匹克竞赛，每年都有学生获得广东省生物竞赛一二三等奖。2007 年参与编写《城市突发公共事件政府、部门、企业应急演练策划与实战》一书（已出版）。2012 年与本校生物科组老师一起进行深圳高中生物校本课程开发与实施研究课题，并顺利结题。2013 年参加教育部主办创新学校 VCT 大赛，个人获得国家级一等奖。2013 年获得国际微软精英教师称号。2013~2019 年每年组织外国语学校学生参加 iGEM 竞赛（国际基因工程机器大赛），期间 2015 年所带团队项目获国际基因工程机器大赛银奖，个人获得国际基因工程大赛银牌教练荣誉，2016 年所带团队项目获得国际基因工程机器大赛金奖（中国赛区首金），个人获得国际基因工程大赛金牌教练荣誉。2015 年参与编著《高中生物创新实验与实践活动》一书（已出版）。

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A fusion of artificial spidroin and mussel foot protein that retains high adhesion and natural glue formation ability

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Keywords Artificial spidroin, Mussel foot protein, Adhesion, Natural glue, Synthetic biology

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Abstract

Recently, artificial bioadhesive and bioglue have become increasingly prospective and widely applicable. Artificial spidroin and mussel adhesive protein are two types of molecules with high biocompatibility. Artificial spidroin has strong cohesive force and unique ability of pH sensing that controls glue formation. Mussel foot protein, comparatively, has strong adhesive force. Our project designed and expressed a recombinant fuse protein of spidorin and mussel adhesive protein. The N-terminal and C-terminal of the spidroin is preserved in our design so that this protein can sense pH and environmental change, then transform itself into glue accordingly. We successfully expressed and purified the fused protein and validated that this protein retains the ability of pH sensing and subsequent glue formation. We verified that compared to the original spidorin, the fused protein has around 20 folds increment in adhesive force. This research produces a recombinant adhesive protein with new characteristics that are potentially applicable in adhesion conditions where biocompatibility and biodegradability are required, thereby provide new method for the development of a new bioadhesive.

Introduction

Bioadhesive is a kind of biocompatible, degradable adhesive material that is widely applicable medically in wound repair, epidermal grafting, tissue culture, organ repair, etc (Shi et

al., 2020; Zhu et al., 2018). An ideal bioadhesive should have both powerful adhesive and cohesive force. The former one contains the bonding between the adhesive material and the surface of attachment. The latter one aggregates molecules of bioadhesive to resist the disruption pressed by external forces (Zhu et al., 2018).

Over years, Spider silk protein (Spidroin) is identified as a promising material in a variety of fields, most notably in military and medications. Starting from the twentieth century, silk were used to make bullet proof vest and recently the US and UK militaries have recognized the spidroin silk as an appropriate material (Roberts et al., 2019). Due to its light weight and high tenacity, spider silk protein is also applied in medication. Mucus adhesion is an important focus area, in which spider silk has the potential to be adapted to meshes, coatings, and foams and served as medical dressings (Petrou et al., 2018).

Besides its impressive toughness, natural spider is capable of pH sensing. The structure of typical spider silk encompasses a repetitive middle core (Rep) and non-repetitive N-terminal (NT), C-terminal (CT) domains. The middle repetitive region contains repeating polypeptide chains forming tenacious fibre structure, hence, it is the source of the high cohesive force and toughness of spider silk protein (Brown et al., 2011). The NT and CT regions, being capable of sensing pH in the environment and changes in pH, would lead to conformational change in these pH sensitive regions, impacting the solubility of the protein. While spidroin is stored in silk glands of spiders, NT remains monomeric and water soluble, which likely contributes to the solubility of the whole structure. When the spidroin needs to be secreted and spinned into fibres, the low pH in the spinning duct promotes NT to form dimer structure and consolidate network structure of interconnecting fibres (Andersson et al., 2017; Kronqvist et al., 2014). CT responds differently to a lowered pH. Then it destabilizes and transforms into Beta sheet structure, leading to the transition of the repetitive region into Beta sheet and form the tenacious spider web. This property allows glue formation at low pH or high salt concentration while keeping the protein water soluble at high pH or low salt concentration, which largely facilitates the storage and application of spider silk protein-based adhesives (Gauthier et al., 2014; Kronqvist et al., 2014).

However, spider silk protein is deficient in adhesive force, another important property of an ideal bioadhesive, which limits its application in rough surfaces such as clothes and wounds healing. To solve this problem, recent research tried to fuse the mussel foot protein (Mfp) with

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spider silk protein (Aich et al., 2018). Mussel foot protein is secreted by Marine mussels to anchor themselves on rock surface underwater, and experiments have shown decent adhesion force in recombinant Mfp proteins due to the interaction between chemical groups (dihydroxyphenylalanine, or DOPA, in particular) and various surfaces of attachment (Stewart et al., 2011). However, this fusion design disposes of the pH sensing function of natural spider silk as N, C terminals of natural spider silk is excluded from the protein sequence and the resultant protein needs to be denatured before purification.

Here we report an innovative design that produces spider silk-Mfp fusion protein with adhesive and cohesive properties while preserving the pH sensing ability of natural spider silk, thereby provide a solution to the current problems in bioadhesives.

Methods

1. DNA Construction

1. 1 SOE Assembly of MFP5

SOE PCR is a method to assembly short DNA sequence by using oligo DNAs. We designed oligo DNAs (Table 1) contain 20 bp overlap with each others to assemble the MFP5 gene, genes were ordered from RuiBiotech Co., Ltd. Mix oligo 001 to 008 with 5μl each to form primer mix. Then prepare FastPfu(Ap221-11, TransStart® FastPfu DNA Polymerase) PCR mix followed to Table 2, and run the process in Table 3.

Table 1 Primers and Oligo DNA

Name	Sequence
SFLS001	GGCCATCATCATCATCATCATATGTCATCAGAGGAATACAAGGGGGGATATT
SFLS002	CAGAATGATAATGGTACGTGTTACCAGGGTAATATCCCCCCTTGTATTCCTC
SFLS003	GTAACACGTACCATTATCATTCTGGGGGGTCCTACCACGGTAGTGGCTACCA
SFLS004	CCTTCCCATAATACTTGCCCTTGTAACCACCGTGGTAGCCACTACCGTGG
SFLS005	GGGCAAGTATTATGGGAAGGCAAAGAAATACTACTATAAGTATAAGAATAG

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SFLS006	CTTTCTTCAAGTATTTGTACTTACCACTATTCTTATACTTATAGTAGTATTTC
SFLS007	GTAAGTACAAATACTTGAAGAAAGCACGCAAGTATCATCGTAAGGGATACAA
SFLS008	CGCCACTGCTGCCCCCACCATAATACTTTTTGTATCCCTTACGATGATACTT
SFLS009	ACTACCACCGCCACCGCTACCACCACCGCCACTGCTGCCCCCAC
SFLS010	CGGTGGCGGTGGTAGTATGAGCCATACCACCCCGTG
SFLS011	CATATGATGATGATGATGATGGCCCATG
SFLS012	GGCAGCAGTTAAGCTTCACCACCACCACCA
SFLS013	GGTGAAGCTTAACTGCTGCCCCCACCATAAT
SFLS018	GGTGGTAGCGGTGGCGGTGGTAGTATGTCATCAGAGGAATACAAGGGG
SFLS019	ACCGCCACCGCTACCACCACCGCCGCCCACATACTGACCCACGC
SFLS054	CCCAAGGGGTTATGCTAGTTATTGC
SFLS055	CCATCGGTGATGTCGGCGATATA

Table 2 SOE Assembly Mix

Reagent	Volume
2x pfu	15 µl
Primer mix	5 µl
ddH2O	10 µl

Table 3 PCR process

Steps	Temperature	Time	Cycles
1	95℃	5 min	
2	95℃	30 s	30 cycles
3	55℃	30 s	
4	72℃	30 s	
5	72℃	3 min	
6	10℃	∞	

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1. 2 Amplify MFP5 Fragment

After assembling the MFP 5 gene, we need to amplify linear Mfp 5 fragment for Gibson assembly method by PCR. Template is the product from SOE assembly, and we use protocols in Table 4 to prepare the PCR mix. In order to amplify MFP-N, we use primer SFLS001 and SFLS009, and for MFP-C, we use primer SFLS018 and SFLS013. The process were all followed to Table 3.

Table 4 Pfu PCR mix

Reagent	Volume
2x pfu	15 µl
SFLS01/ SFLS018	1 µl
SFLS09/SFLS013	1 µl
Product from SOE assembly	1 µl
ddH2O	12 µl

1. 3 Amplify pET28a-NTRepCT

To amplify the fragment from the plasmid named pET28a-NT2RepCT provided by LINK SPIDER Co.,Ltd. Shenzhen., we use pET28a-BT2RepCT as template, and make the following mix in Table 5. To amplify pET28a-N, we use primer SFLS010 and SFLS011, and for pET28a-C, we use primer SFLS012 and SFLS019. The processes were all followed to Table 6.

Table 5 PCR mix of pET28a-NT2RepCT

Reagent	Volume
2x pfu	15 µl
SFLS010/ SFLS011	1 µl
SFLS012/SFLS019	1 µl
pET28a-NT2RepCT	1 µl
ddH2O	12 µl

Table 6 PCR process of pET28a-NT2RepCT

Steps	Temperature	Time	Cycles

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1	95 ℃	5 min	
2	95 ℃	30 s	30 cycles
3	55 ℃	30 s	
4	72 ℃	3 min 20 s	
5	72 ℃	3 min	
6	10 ℃	∞	

1.4 Agarose gel electrophoresis

Add 0.3 g of agarose (RB008-100 from RuiBiotech) into 30 ml of TAE (RB007-500, RuiBiotech) solution, and use microwave oven to melt agarose. Add 1 μl Gel-red (70021000, biosharp) DNA dye after the solution is not thorny and gently mix the gel solution. Waiting for 20 minutes after pouring the gel solution, and run the electrophoresis with 140 V for 20 minutes. DNA marker (RB-MKS, RuiBiotech) was used to confirm the size of DNA.

1.5 Gel extraction of DNA fragments

We use TIANGEN Midi Purification Kit (DP209-02, TIANGEN) to extract DNA.

In short, we do the following operation. To balance the absorption column CA2, add 500 μl balance buffer BL and centrifuge the tube for 1 minute with 12000 rpm, and pour out the waste liquid. Then, cut the gel, and move the rest part of gel as much as possible. Put the gel into a centrifugal tube and weigh it. (0.1 g of gel is considered as 100 μl) add same volume of PN solution as volume of gel, and melted the gel at 50 degrees Celsius. Add the solution into the CA2 column. After placing 2 minutes, centrifuge the tube for 1 minute with 12000 rpm, and pour out the waste solution. Add 600 μl PW and centrifuge the tube after placing for 2 minutes for 1 minute with 12000 rpm. Add 600 μl PW and centrifuge the tube under the same condition. After charge the CA2 column back to the collecting tube, centrifuge the tube for 2 minutes with 12000 rpm, and evaporate ethyl alcohol at 55 degrees Celsius. Change to clean EP tube, add 30 ul ddH2O, place the tube for 2 minutes, and centrifuge the tube for 1 minute with 12000 rpm. Collect DNA solution, and throw the CA2 column (notice: do not pour out the solution!)

1.6 Gibson Assembly

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After extract fragments from DNA gel, add 5 μl extracted plasmid into 5 μl Gibson assembly mix (E5520S NEB), and then use PCR amplifier to run the assembly with the process in Table 8.

Table 7 Gibson mix

Reagent	Volume
Gibson mix	5 μl
Insert DNA	50 ng
Vector DNA	50 ng
ddH2O	up to 10 μl

Table 8 Gibson Process

Step	Temperature	Time
1	50℃	60 min
2	10℃	∞

1.7 Transformation (DH5a)

Transformation is prepared with 5μl Gibson mix (or 1μl purified plasmid) and 50μl DH5a competent cell (O1330806, *TransStart*®). After 30 minutes incubation on ice, 80 seconds heat shock at 42 degrees Celsius, 5 minutes incubation on ice again and one-hour resurrection with 300 μl liquid LB at 37 degrees Celsius, the bacteria are ready to cultivate. Add 100 bacteria mixture on to LB agar plate with 50 ug/mL kanamycin and spread it evenly.

1.8 Colony PCR

Pick the DH5a colony, and prepare the PCR mix followed to the Table 9. Add 1μl PCR mix on a new agar plate to keep the strain. Run the process is in Table 10.

Table 9 Bacteria PCR mix

Reagent	Volume
2x taq	15 μl
SFLS054	1 μl
SFLS055	1 μl
ddH2O	13 μl
Template	one colony

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Table 10 PCR Process of Bacteria

Steps	Temperature	Time	Cycles
1	95 ℃	10 min	
2	95 ℃	30 s	30 cycles
3	55 ℃	30 s	
4	72 ℃	1 min 40 s	
5	72 ℃	5 min	
6	10 ℃	∞	

To examine the sequence of our DNA, we sent to Guangzhou Rui Biotech.

1.9 Plasmid Extraction

To extract plasmid, we use TIANGEN Genomic DNA Kit from TIANGEN.

In short, to entrifuge bacteria for 1 minute with 12000 rpm, and pour out the supernatant. Add 150 μl P1 and use vortex to mix the bacteria evenly. Add 150 μl P2, and turn upside down gradually for about 8 times in order to mix the solution evenly (the solution should become purple). Add 350 μl P5, and turn upside town immediately for about 20 times (don't stop until the purple disappear and precipitate appear). Centrifuge the tube for 10 minutes with 12000 rpm. Move 650 μl supernatant to the absorption column, and put the column into the collecting tube. Centrifuge the tube for 2 minutes with 12000 rpm, and pour out the solution. Add 300 ul ethyl alcohol, place for 2 minutes, and centrifuge 1 minutes with 12000 rpm. Pour out the solution and centrifuge the tube emptily for 2 minutes with 12000 rpm. Pour out the solution in the tube. Put the column into a new EP tube, and evaporate the ethyl alcohol at 42 degrees Celsius. Finally, add 50 ul water into the column, and centrifuge 1 minute with 12000 rpm to wash the plasmid into the EP tube.

1.10 Transformation (BL21)

After extract plasmid, transformation can start. Transformation is prepared with 1 μl plasmid 50 μl BL21(DE3) competent cell (O150620, *TransStart*®). After 30 minutes incubation on ice, 80 seconds heat shock at 42 degrees Celsius, 5 minutes incubation on ice again and one-hour

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resurrection with 300 μl LB in shaker at 37 degrees Celsius, the bacteria are ready to cultivate. Add 100μl bacteria mixture on to LB agar plate with kanamycin and spread it evenly.

2. Protein Expression and Purification

2.1 Induction of recombinant protein

Pick up one colony on the plate, add it to 15 ml shaker tube with 5 ml LB medium and 5 μl kanamycin, and shake it overnight at 200 rpm, 37 degrees Celsius. Seed the culture according to the ratio of 1:100 in flask bottle. In our experiment, we use 400 ml LB for each conical flask, so we add 4ml bacteria solution into LB with 400 μl kanamycin. Culture the solution at 200 rpm, 37 degrees Celsius. After OD reaches to 0.8, add 300 μM IPTG to induce the protein expression under 20 degrees Celsius overnight.

2.2 Protein expression, purification and concentration

To extract bacteria, add 45 ml-50 ml solution into 50 ml centrifugal tube and centrifuge for 5 minutes with 6,000 to 8,000 rpm. Then, add 20 mM Tris (67086740, biosharp) and use vortex to mix bacteria with tris. Break the cell with ultrasound with 25,000 Hz for 15 minutes. To separate cytoderm and protein, put tubes into centrifugal machine and centrifuge for 30 minutes with 11,000 rpm. Then, separate precipitate and supernatant. SDS-PAGE assessment is performed to validate the incorporation of target protein in the lysate. Then the supernatant is collected and purified with high His-tag affinity Ni-nitrilotriacetic acid (Ni-NTA) resin (P2218, Beyotime Biotechnology) in vertical purification column. 20 mM Tris buffer is used to balance and resuspend the resin. Afterward, the resin is incubated in the supernatant under 4 degrees for 2-3 hours to allow for the combination between His-tag and Ni^{2+} ions in the resin. Then, the protein is washed with 10ml 20mM Tris and eluted by 3 ml 20 mM Tris with a rising concentration of 20-100 mM imidazole for two repeated rounds. The protein in the elute solution is concentrated in protein concentration tube under 12,000 rpm, 4 degrees Celsius centrifugation. The product is stored under 4 degrees Celsius.

To combine with tyrosinase, after incubation with resin, add 50 ml buffer, which is the mixture 300 mM NaCl, 100 mM Tris, and 20 mM Na-borate. Then, add 0.2 ml tyrosinase (356356, Sigma) with concentration of 10 mg/ml into the column with supernatant and resin, and incubate

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for 2-3 hours. After discharge tyrosinase with buffer, use 20 mM Tris to elute the rest of tyrosinase and buffer. Finally, repeat the procedure starting from washing protein with a rising concentration of 20-100 mM imidazole.

2.3 SDS-PAGE

Prepare samples with SDS-PAGE loading buffer (RB005-001, RuiBiotech). We use a kit (P0012AC, Beyotime Biotechnology) to make SDS-PAGE gel, and normally we use 10% or 12% of separation gel. Our electrophoretic buffer solution is Tris-Gly, 10X (P0014D, Beyotime Biotechnology), and add protein marker (RB26616-250, RuiBiotech) after SDS-PAGE, use protein staining buffer (RB010-500, RuiBiotech) to color the gel.

3. Examination of protein's performance

3.1 BCA Protein Assay

To assay the concentration of our proteins, we use BCA Protein Assay Kit (biosharp).

Before use microplate reader, we need to do the following preparation. To make BCA working fluid, mix reagent A and B in a ratio of 50:1. Then use PBS solution to dilute 20 μl BSA standard solution with concentration of 5 mg/ml to 100 μl. the final concentration should be 1.0 mg/ml. After that, make up standard solution by using the following information in the table.

Table 11 Standard Solution

Number	0	1	2	3	4	5	6	7	8
	1 mg/ml BSA standard solution μl							5 mg/ml BSA standard solution μl	
BSA standard solution μl	0	0.5	2.5	5.0	10	15	20	6	8
PBS solution μl	20	19.5	17.5	15	10	5	0	14	12
BSA final concentration μg/ml	0	25	125	250	500	750	1000	1500	2000
Total volume μl	20 μl								

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Add samples into microwell plate, and use PBS to make up to 20μl. Then, add 200μl BCA working fluid into the plate, mix with samples, and place in 37 degrees Celsius for 30 minutes. Afterward, measure the light absorb value at 562 nm, and record numbers. Use samples that do not contain BSA as blank control. To graph the cure, A562 is the y-axis, and BSA content is x-axis. Then calculate the concentration of protein in samples.

3.2 pH sensing and Sensitivity detection of sodium chloride ion concentration

To examine the performance of the protein in different pH level, we prepare reagents from pH 1 to pH 8 (Table 12). To examine the sensitivity of protein towards NaCl concentration, we prepare NaCl solution with different concentration from 20 mM to 1 M (Table 13).

Table 12 Solution with Different pH Value

pH value	Recipe
1	1M HCl dilute 10 times
2	0.1 mol/L CH_3COOH
3	4.11 ml K_2HPO_4 + 15.89 ml 0.1 mol/L citric acid
4	23.07 ml 0.2 mol/L Na_2HPO_4+ 26.96 ml 0.1 mol/L citric acid
5	21.64 ml 0.2 mol/L Na_2HPO_4 + 28.36 ml 0.1 mol/L citric acid
6	43.85 ml 0.2 M KH_2PO_4 + 6.15 ml K_2HPO_4
7	19 ml 0.2 M KH_2PO_4 + 31 ml K_2HPO_4
8	2.65 ml 0.2 M KH_2PO_4 + 47.35 ml K_2HPO_4

Table 13 Solution with Different Concentration of NaCl

Final Concentration	Tris (20 mM)	NaCl (1 M)
20 mM NaCl	49 ml	1 ml
50 mM NaCl	47.5 ml	2.5 ml

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100 mM NaCl	45 ml	5 ml
200 mM NaCl	40 ml	10 ml
500 mM NaCl	25 ml	25 ml
1 M NaCl	stock solution	

We first add 3ml solution (either pH solution or NaCl solution) into a quartz cell, and add 1μl protein into the solution. Observe whether the protein gelatinize in the solution, and record the size of the gel. Then, pour out the solution, use ddH2O to wash the cell with 1-2 times, rinse the cell with next pH solution or NaCl solution for one time, and repeat the above steps.

3.3 Examination of Adhesion

Prepare pieces of glass, plastic and different kinds of clothes in the shape of circle, whose diameters are 5 mm. Use 502 glue to attach these materials with mini hooks, made by UV sensitive resin (Creality) and a 3D printer (LD-002R, Creality), to form bases, and weight them. Use 30 mg protein (1 μl) to attach a base and the corresponding material (if the base is made by glass and hook, use protein to attach the base with another piece of glass). Each kind of material has three groups. In the first group, add 20 mM Tris after attaching materials with protein. For the second group, add acid with pH level that form most gel in the previous pH sensing test. To form control group, we do not add any other solution in the third group. Dry the gel for 1 hour. Then, turn the base upside down, and add weights, made by UV sensitive resin by 3D printer. Record the total weight of weights before and after the base drop down. Each kind of material should repeat the procedure at least 3 times in order to get accurate result.

Results

4. Protein Design

To preserve optimal pH sensing ability, we adopted a classic simulation of spider silk protein: NT2RepCT, with NT from *E. australis*, a species of spider whose NT is highly sensitive to pH and salt concentration change; CT from *Araneus ventricosus*, another species of spider whose CT is highly sensitive; and a shortened repetitive region from *E. australis.* The repetitive region, if

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overlength, can disrupt the expression of the protein and lower the final yield, so only two repetitive units are included.（Fig. 1 A)

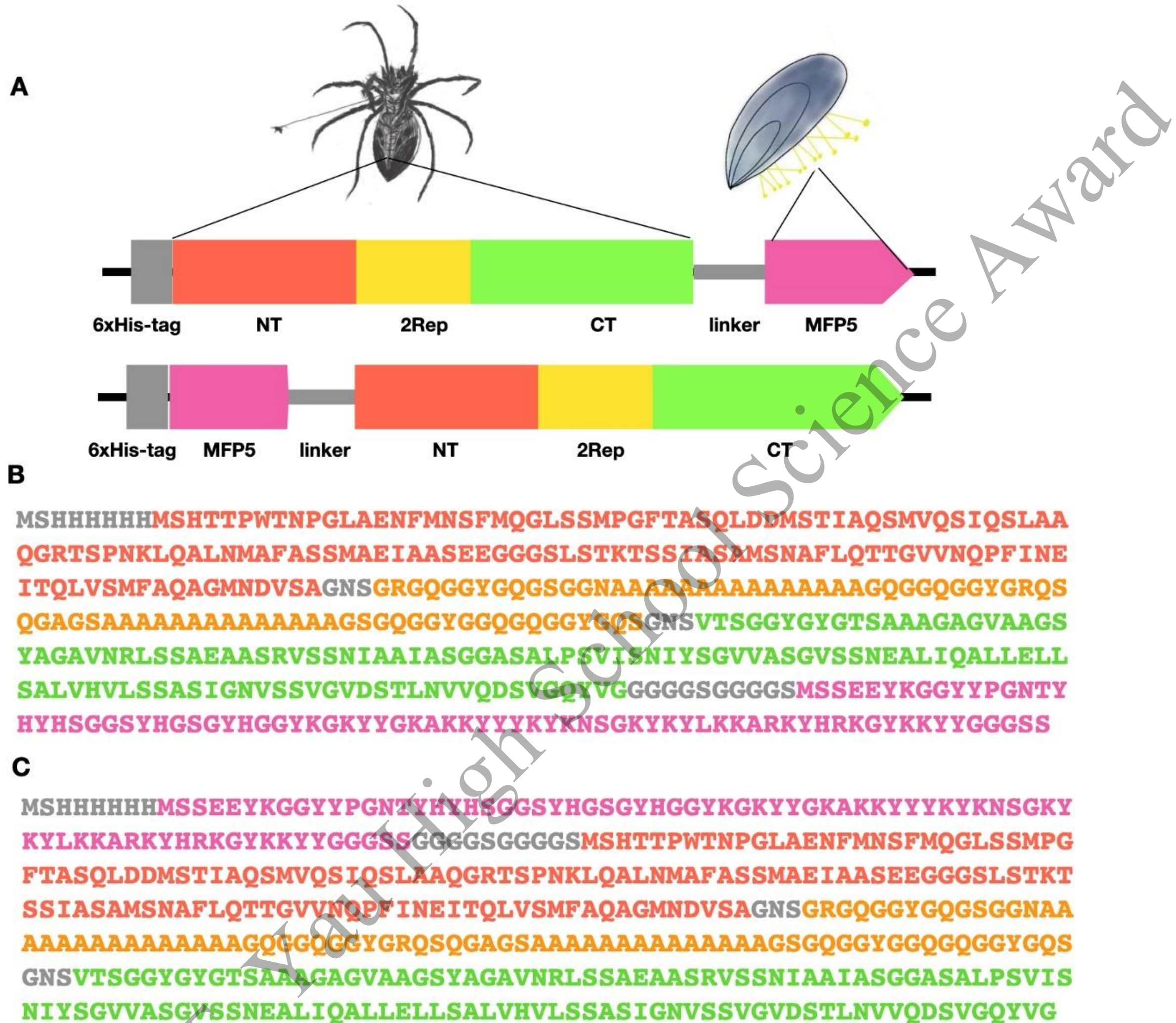

Figure 1 The design of fusion protein. Spidroin NT2RepCT and mussel adhesive protein MFP5 are connected with a flexible linker (A). The complete amino acid sequence of this fused protein is shown in B and C, pET28a vector based prokaryotic expression is adopted to express this protein.

To retain optimal adhesion property, we sought the most adhesive Mfp subtype in the huge Mfp family proteins. Among these proteins, Mfp5 is proven to be most efficient at adhesion because of its tyrosine rich amino acid sequence (Hwang et al., 2004). The tyrosine residue, after catalyzed by tyrosinase, can be transformed into DOPA and facilitates surface attachment (Yu et al., 1999).

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Therefore, we fuse NT2RepCT with Mfp5 and construct two designs, one fusing Mfp5 before NT and the other fusing Mfp5 after CT. To insulate these two proteins and prevent functional interruption, we add a flexible linker comprised of ten amino acids GGGGSGGGGS to separate them. A His-tag is attached to the N terminal of each design. (Fig 1.B)

5. Expression vector design and construction

We acquired the DNA sequence of Mfp5 by assembling oligo DNAs with SOE PCR. (Fig 2. A) The plasmid containing DNA sequence of NT2RepCT is donated by Shenzhen LINK SPIDER Co.,Ltd. Shenzhen, with a backbone of pET28a, a specialized expression vector that carries a strong T7 promoter. Four sets of primers are used to amplify product of SOE assembly and pET28a-NT2RepCT respectively, resulting in Mfp-N, Mfp-C, (Fig 2. B) pET28a-N, pET28a-C. (Fig 2. C). Agarose gel electrophoresis results show that these DNA fragments corresponds to the theoretical values (231 bp and 6271 bp). (Fig 2. D)

Gibson assembly is then performed to assemble Mfp-N with pET28a-N and Mfp-C with pET28a-C, resulting in pMfp-N, pMfp-C. (Fig 3. A, B) After purification of DNA, we transformed E. coli DH5 with the two plasmids and performed colony PCR with a set of primers 054 and 055. pMfp-N and pMfp-C both have a base pair length that corresponds to our theoretical design of 1602bp. (Fig 3. C) We sequence sample DNA with 054 and 055 to further validate that we've obtained the correct DNA sequences. (Fig 3. E, F)

6. Protein expression and purification

We transformed the plasmids into E. coli BL21 (DE3) and performed IPTG induction to induce protein expression. SDS-PAGE is performed to analyze whole cell, supernatant, and precipitation of the cell lysate. Mfp-C and NT2RepCT showed clear induction bands that match our theoretical design (33 kDa and 48.2 kDa). (Fig 4. B, D)

However, the Mfp-N design showed no significant induction band. (Fig 4. A) We postulate that this can be caused by the order of translation and folding of polypeptide chains. In Mfp-C, NT is translated and folded first whereas in Mfp-N, Mfp5 is translated and folded first and this might interfere with the further expression of NT2RepCT. The cell lysate is purified with high His-tag affinity Ni-NTA resin to capture the target protein. Purification result of Mfp-C showed

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approximately 48kDa protein band at different concentration of elution sample, indicating the successful purification of our target protein, and that there is no observable degrading in the process. (Fig 4. C)

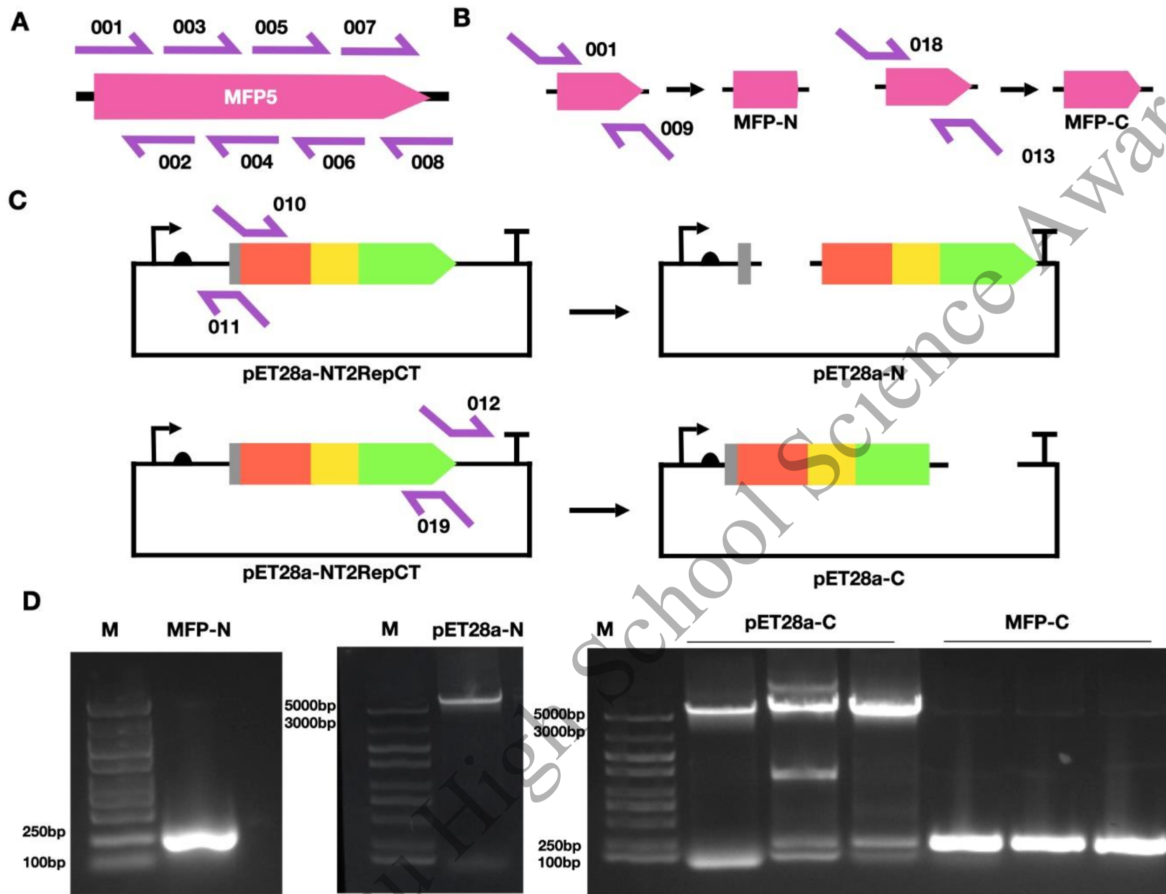

Figure 2 Design of primers and amplification of assembly fragments. Eight primers are designed and ordered, and Gibson Assembly is performed to assebly they into DNA sequence of MFP5. (A) MFP-C Based on different assembly strategy, DNA fragment assembled by SOE PCR amplified by primers 001 and 009, resulted in MFP-NT, which is later assembled into the N-terminal of pET28a-NT2RepCT. In the same manner, primers 013 and 018 resulted in MFP-CT, which is later assembled into the C-terminal of pET28a-NT2RepCT. (B) pET28a-NT2RepCT amplified by primers 010 and 011 resulted in pET28a-N, which is later assembled with MFP-NT. In the same manner, primers 012 and 019 resulted in pET28a-C, which is later assembled with MFP-CT. (C) After PCR amplification, Agarose gel electrophoresis is performed to recycle the four DNA fragments above. (D)

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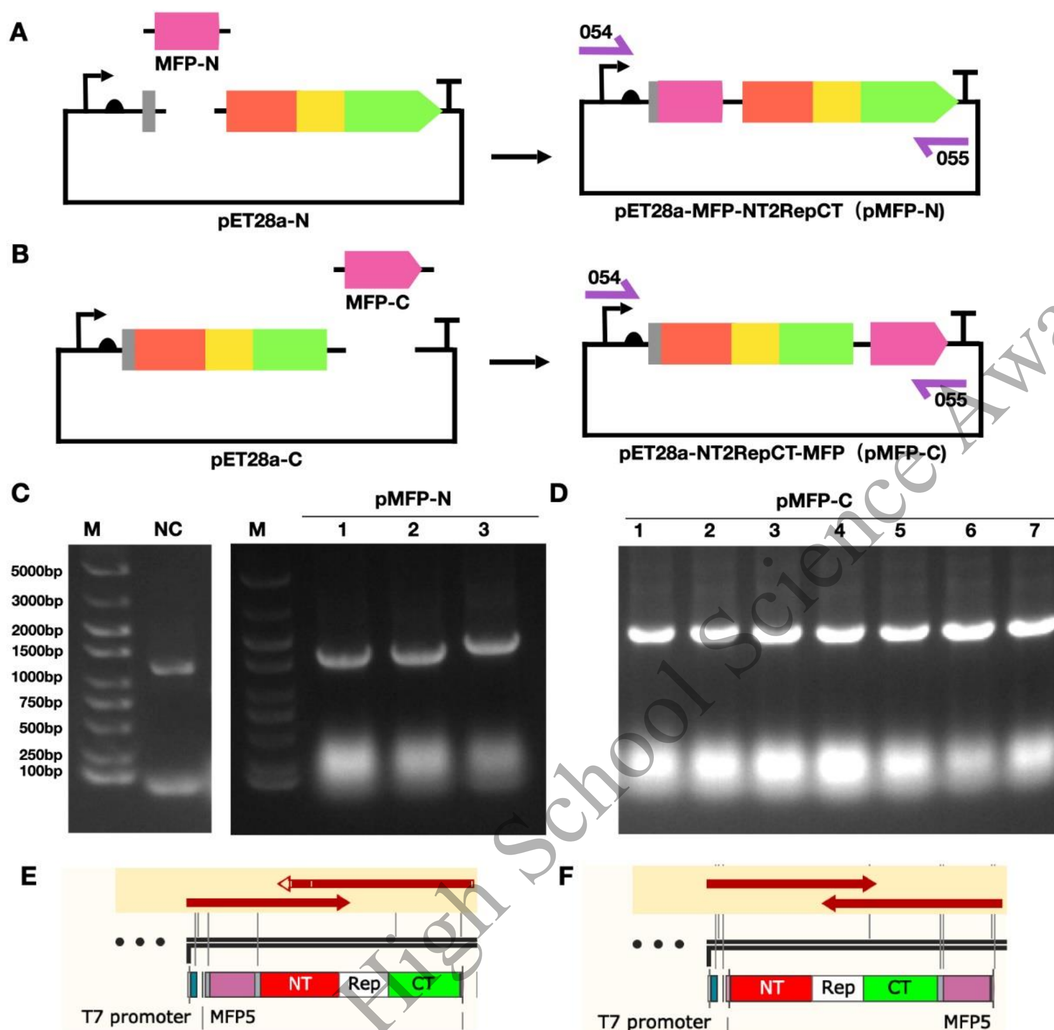

Figure 3 Construction of recombinant plasmids. Gibson assembly is performed to asssemble pET28a-N and MFP-N into pMFP-N, (A) as well as pET28a-C and MFP-C into pMFP-C. (B) After transforming E. Coli DH5a with products of assembly, we performed colony PCR and validated that pMFP-N (C) and pMFP-C (D) both have a positive transforming of base pair length 1603bp. DNA sequencing is performed to further validate the correctness of these colony (E) (F) so that further experiments can be continued.

FT sample of Mfp-C contains a large portion of target protein, which implies an inefficient combination between His-tag and Ni-NTA resin. Comparatively, FT sample NT2RepCT contains little target protein, indicating an efficient combination between His-tag and Ni-NTA resin, resulting in a much more robust purification band in elution samples. (Fig 4. D) Here, again, adhesive Mfp protein might interfere with the combination between His-tag and resin. The complex interaction between proteins might also cover some of the His-tags and impede the combination process.

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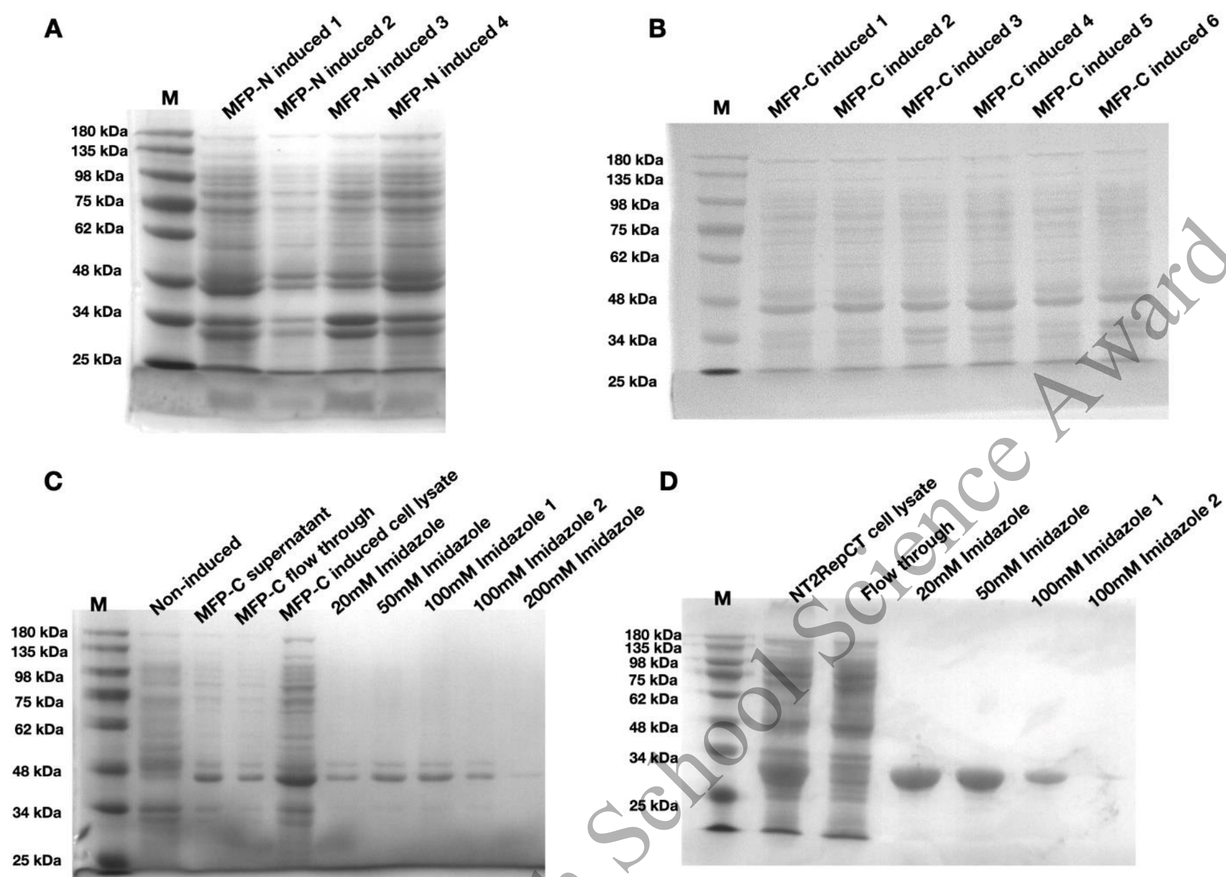

Figure 4 Expression and purification of recombinant protein. MFP-N(A) and MFP-C (B) have induction bands as shown in the figure. MFP-N induction sample show blurred induction band at target position, and further purification failed to extract this target protein. Clear induction bands can be observed in the induction sample of MFP-C. MFP-C is then purified with Ni-NTA resin, clear purification bands showed at elution sample of 20-100mM imidazole (C). As comparison, NT2RepCT is also purified and analyzed with SDS-PAGE (D).

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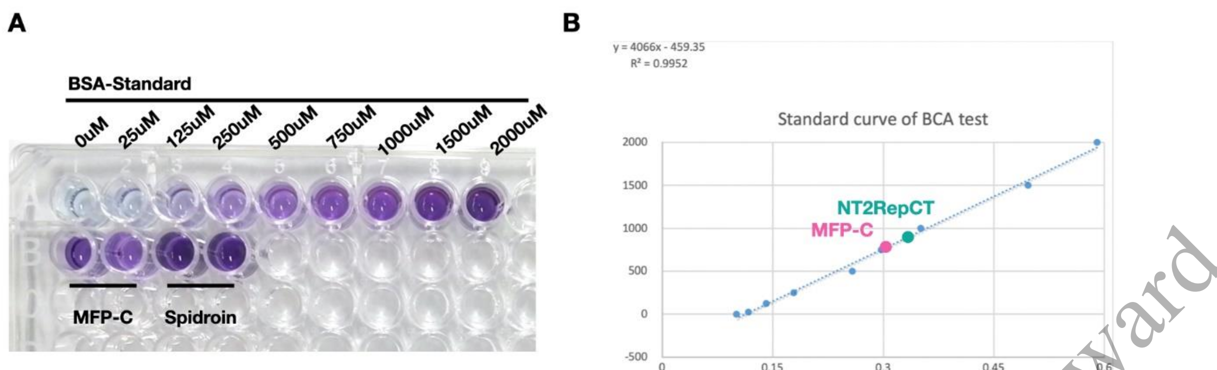

Figure 5 Measurement of recombinant protein's concentration. (A) Illustration of BCA protein concentration measurement (B) Compareing the absorbance of MFP-C and NT2RepCT with the standard BSA curve shows the concentration of MFP to be 770.2ug/ml and the concentration of NT2RepCT to be 884.8ug/ml.

7. Validation of pH sensing

Prior to pH sensing tests, we measured the concentration of each protein sample to eliminate the effect of concentration on pH. BCA protein assay is performed to analyze the concentration. (Fig 5. A, B)

We test the fused protein with buffers of different pH levels. A gradient of pH 1-8 is established (see the ingredient table in methods) and tested with NT2RepCT and Mfp-C. Between pH=1 to pH=5, observable solidification occurs to both proteins. Starting from pH=6, the protein's solubility starts to increase (Fig 6. A, C) This corresponds to the behavior of nature spider silk, which denatures in the acidic spinning duct and remains water soluble in basic storing glands. The fused protein, therefore, has a stable and rapid glue formation ability, which will be crucial to its application as bioadhesive.

We further tested the protein's sensitivity to salt solution. A gradient of 20 mM, 50 mM, 100 mM, 200 mM, 500 mM, and 1000 mM NaCl solution with 20 mM Tris-HCl buffer is established and tested with NT2RepCT and Mfp-C. At 20 mM, 50 mM and 100 mM, both protein exhibits different extent of salting-out. As concentration of salt increases, both proteins becomes increasing water soluble. (Fig 6. C, D) This property to sense environmental salt concentration is also in accord with spider silk.

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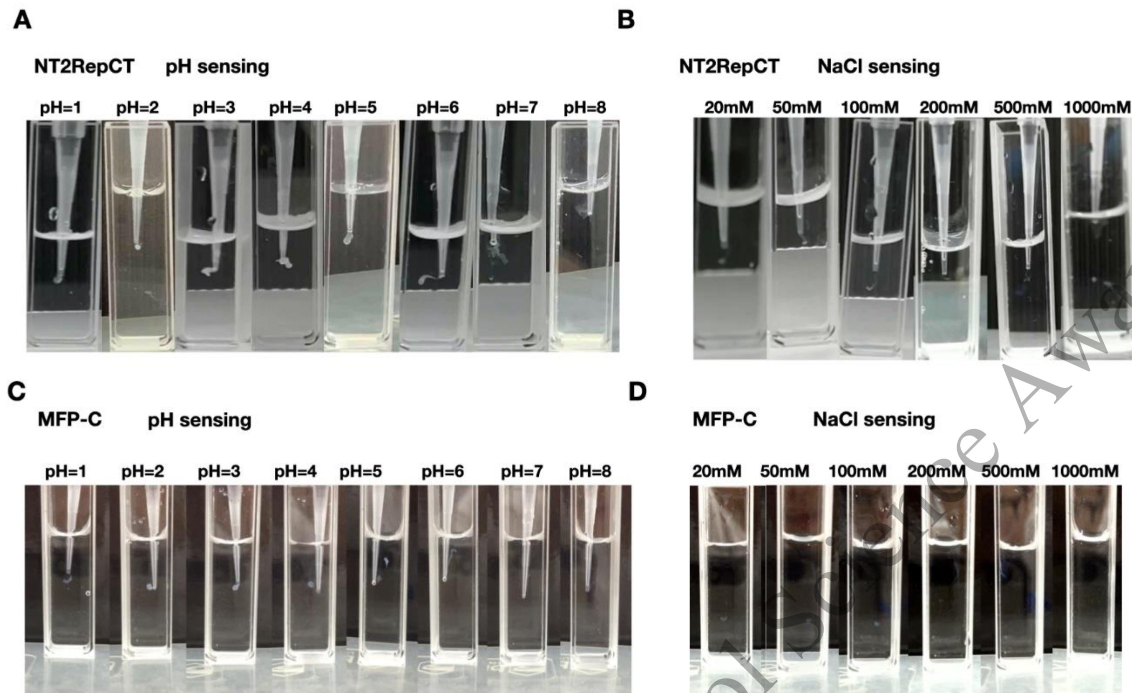

Figure 6 PH and salt concentration sensitivity test of spidroin and recombinant adhesive protein. Clear white precipitate is formed by NT2RepCT in solutions of pH=1 to pH=6, and little precipitate is formed in solutions of pH >= 7 (A). White precipitate is also formed by spider silk protein in solutions of 20mM NaCl to 1000mM NaCL (B). Recombinant adhesive protein MFP-C also preserves the property of pH sensing, forming clear precipitation in solutions of pH=1 to pH=6. C) In lower salt concentration, MFP-C can form precipitation as well (D).

8. Assessment of adhesion

The adhesion property can be readily measured by sticking standardized glass plates with diameters of 5mm onto different adhesion surfaces. A small hook is mounted onto each plates and weights are added gradually to test for the maximum capacity. To simplify the testing process, we choose glass slide as the adhesion substrate. For less adhesive material, 3D-printed weights are hung onto the hook until the glass plates detach. The adhesive fused protein is tested with plasticine, and 0.05g of plasticine is added each time until the maximum capacity is reached. Three types of bioadhesive is tested: the spider silk protein NT2RepCT, the Mfp-C, and Mfp-C with a special treatment in the purification process that combines it with tyrosinase to promote DOPA transformation and increase adhesion force. (Fig 7. A)

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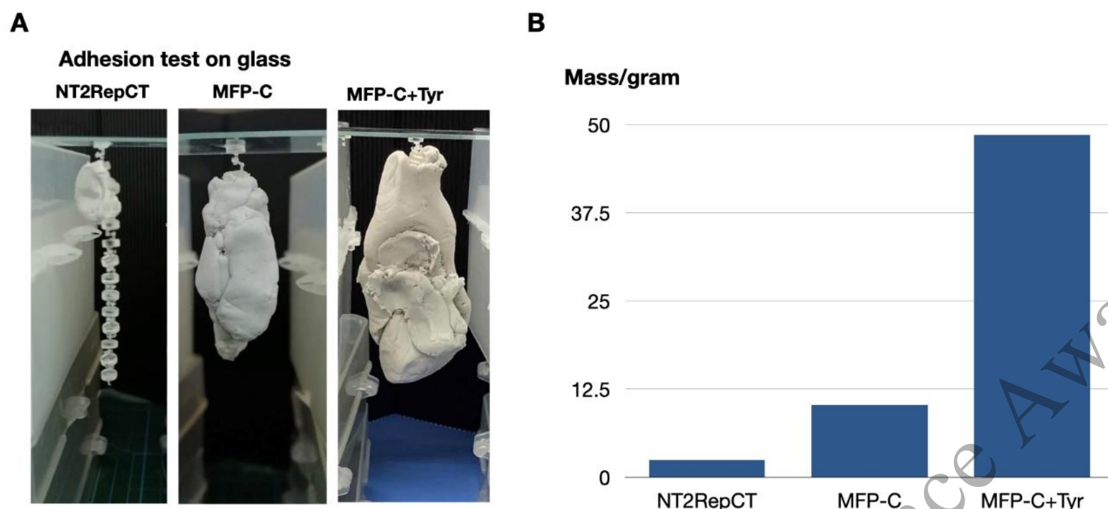

Figure 7 Qualitative adhesion test of spidroin and recombinant adhesive protein. Prepare glass plates 5mm in diameter and adhere hooks to those plates with super glue. Apply the three proteins (artificial spidroin, fusion protein without tyrosinase treatment, fusion protein with tyrosinase treatment), each around 50 ng, to glass substrates and leave them to dry overnight. Hang plastic weights and plasticine, each 0.05 grams in weight, on the hooks and record the total weight before the hooks fall (A). Results indicate that artificial spidroin, Mfp-C (without tyrosinase treatment), and Mfp-C (after tyrosinase treatment) can withstand a total weight of approximately 2.43 grams, 10.24 grams, and 48.51 grams, respectively (B).

As expected, the Mfp-C showed a significantly higher maximum capacity that NT2RepCT, with a roughly 4 folds increment. Tyrosinase treatment further increase the mass capacity to around five times than Mfp-C, which adds up to 20 folds incremental to NT2RepCT. Therefore, we have successfully improved the adhesion property of NT2RepCT to allow it to be applied in more complicated conditions.

Discussion

We attempted to improve performances of artificial spidroin by fusing the highly adhesive Mfp5 on either the N terminal (Mfp5-NT2RepCT) or C terminal (NT2RepCT-Mfp5) of NT2RepCT. It was later proven that the latter design allowed for protein expression while the former did not. More importantly, this fusion protein (NT2RepCT-Mfp5) preserved key characteristics of natural spiker silk protein, namely NaCl and pH sensing, while exhibiting significantly increased adhesiveness.

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However, this fusion protein does have the following limitations and possible improvements. a) Its purification efficiency is significantly lower than that of NT2RepCT probably because that Mfp5 affected the protein folding, making His-tag unable to be exposed on the outside of the N terminal. A possible improvement would be separating the N terminal and His-tag with a GGGGSGGGGS linker to make His-tag more readily exposed. Alternatively, we can place the His-tag on the C terminal, though it is not clear whether this will affect the protein performance. If neither of the aforementioned methods works, we can consider using other tags, such as an HA tag, for purification. b) Though tyrosinase treatment can significantly improve the adhesiveness of the fusion protein, it would also increase protein loss and thereby increase the cost of production. Therefore, it is important to find a balance between blue performance and cost. Notably, the recommended buffer of commercialized tyrosinase includes 300 mM of NaCl, a concentration at which Mfp-C protein will aggregate and precipitate, as we have proven. This will affect the final protein yield to some extent. In the future, we may need to test whether commercialized tyrosinase can function in low pH solutions or find the lowest concentration of NaCl that allows tyrosinase to work normally in order to minimize the amount of protein loss due to precipitation. c) Even after treatments with tyrosinase, this protein is still not viscous enough compared to many types of industrial glues, such as cyanoacrylate, and cannot be applied to settings where strong cohesiveness is required. A possible solution is to add additional repeats of Mfp5 on the C terminal of NT2RepCT, though this will potentially harm protein yields, which makes it important to find a balance between viscidity and yield. Similarly, the cohesiveness of this fusion protein can be improved by adding more repetition units of spidroin, though this will lead to decreased protein yield can cause the protein to degrade due to self-cross-linking (Bowen et al., 2018).

In this project, we proposed a new approach to bioadhesive formation. Our recombinant adhesive protein can be stored in 20 mM Tris buffer (pH=8.0) and be denatured with the addition of acidic solution (pH=3.0) to glue together two surfaces. This protein can be stored in 4˚C for two months or longer. Notably, the storage and use of this bioadhesive do not involve any heavy metals, toxic materials, or organic solvents, which best retains the biocompatibility of this protein and causes no harm to the environment. Additionally, the cohesiveness of this protein in acidic solutions makes it a potential glue for underwater usage—for instance, underwater repair of damaged ships or tissue repair in liquid environments in human bodies. In the future, this

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bioadhesive has many promising applications in fields that emphasize biocompatibility and biodecomposability.

Conflict of interest

The authors have no conflict of interest to declare.

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Yifei Wang: female, a student in Shenzhen Foreign Languages School. She attended iGEM 2019 and iGEM 2020, served as Human Practices Leader.

Ruoming Li: male, a student in Shenzhen Foreign Languages School. He attended iGEM 2019, helped designed experiments.

Yiyang Jiang: male, a student in Shenzhen Foreign Languages School. He attended iGEM 2019 and iGEM 2020, helped designed experiments, and was the team leader in 2019.

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