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[The following are protocols[1] designed for the polymerization, expression, and coagulation of recombinant silk-like protein threads after monomeric ICA assembly and film formation.]

Procedure 1: Construction of the synthetic spider silk multimeric gene.

[**This procedure employs a head-to-tail assembly-like method of ligating monomeric silk protein cassettes to form a multimeric product for silk protein expression. We will employ GGC/ICA to construct these silk monomers, then employ the following procedures in the cloning vectors. Refer to the following figure for an abstract analysis of the methodology].**

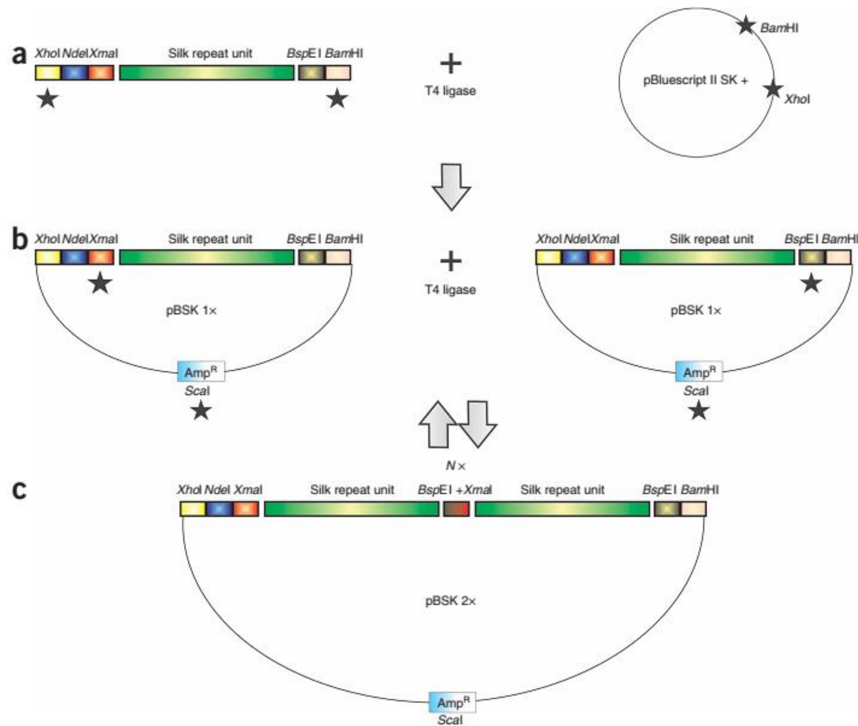

Figure 2 | Strategy to build large synthetic spider silk-like tandem repeat sequences from small double-stranded monomer DNAs flanked by compatible but nonregenerable restriction sites. (**a**) The engineered silk-like module with appropriate flanking restriction sites is cloned in the plasmid vector. (**b**) The recombinant plasmid is subjected to two separate restriction digestions and, in both cases, fragments containing the insert are isolated and ligated to each other. (**c**) The resulting plasmid contains an insert that was doubled in size and has a nonfunctional internal *Xma*I/*Bsp*EI hybrid site. The black stars (★) indicate the restriction digestion of DNA and *N*× means that the strategy can be repeated as many times as needed.

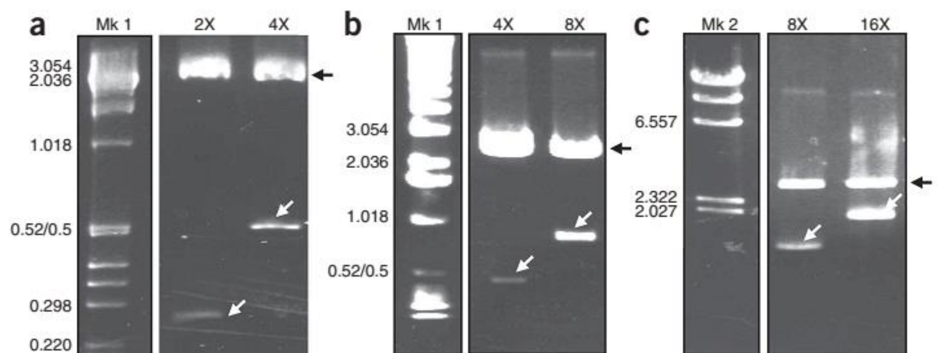

Figure 4 | Agarose gel analyses showing the synthetic Flag/MaSp 2 silk DNA multimers at different doubling stages. The sequential recombinant plasmids containing the different silk-like insert fragments[43] were subjected to restriction digestion with *Xma*I and *Bsp*EI to release the silk insert. The restriction digestion products were separated on (**a**) Nusieve/agarose 3:1 and (**b**,**c**) 0.8–1% agarose gels. After staining with ethidium bromide, the DNA fragments were visualized using UV light. In **a**–**c**, Mk: molecular marker; Mk 1:1 kbp DNA Ladder; Mk 2: Lambda DNA-*Hind*III; 2×–16×: repetitive synthetic silk sequences after sequential insert doubling (i.e., in **a**, 4× is twice the size of 2×). The size of the silk inserts are 236, 472, 944 and 1,888 bp for 2×, 4×, 8× and 16×, respectively. The black arrows show the linearized pBluescript plasmid and the white arrows show the silk inserts. The molecular weights in kbp are indicated.

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Reagents Needed:

10x NEB Buffer 2
10x NEB Buffer 3
5x ligase reaction buffer
1x running buffer
T4 DNA ligase (1 U/µl)
BSA (1mg/ml)
Gel Extraction Kit
pBluescript/monomer silk DNA (1 µg/µl)
***Sca*I (10,000 U/ml)**
***Xma*I (10,000 U/ml)**
BspEI (10,000 U/ml)
Nuclease-free sterile water
electrocompetent *E. coli* XL1-Blue colonies
LB agar/ LB media
Ampicillin
X-gal
IPTG
Plasmid Miniprep kit
ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit

Equipment Needed:

Centrifuge/Microfuge Tubes
Incubator
Agarose
Ethidium Bromide
Clean excision blades
Gel rig

PROCEDURE

Construction of the synthetic spider silk gene ● TIMING 5–7 d for one insert doubling

1| Digest one aliquot of the pBluescriptII SK (+) vector containing the synthetic DNA monomer (see Experimental design and **Fig. 2b**) with *Sca*I and *Xma*I as detailed in the table below.

Component	Amount (µl)	Final
10× NEB buffer 2	5	1×
BSA (1 mg ml^{-1})	5	100 µg ml^{-1}
pBluescript/monomer DNA (1 µg µl^{-1})	2	2 µg
*Sca*I (10,000 U ml^{-1})	0.4	4 U
*Xma*I (10,000 U ml^{-1})	0.4	4 U
Nuclease-free water	37.2	

2| Digest one aliquot of the pBluescriptII SK (+) vector containing the synthetic DNA monomer with *Sca*I and *Bsp*EI restriction enzymes (**Fig. 2b**) as described in the table below.

Component	Amount (µl)	Final
10× NEB buffer 3	5	1×
pBluescript/monomer DNA (1 µg µl^{-1})	2	2 µg
*Sca*I (10,000 U ml^{-1})	0.4	4 U
*Bsp*EI (10,000 U ml^{-1})	0.4	4 U
Nuclease-free water	42.2	

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3| Pulse the tubes from Steps 1 and 2 in a microfuge tube and incubate for 1 h at 37 °C.
▲ CRITICAL STEP To save time, perform both restriction digestion reactions at the same time.

4| Check 5 µl of each restriction digestion by agarose gel electrophoresis using a 0.8% (wt/vol) agarose gel made in 1× running buffer (see REAGENT SETUP).
? TROUBLESHOOTING

5| If both restriction digestions are complete, load the remainder of each reaction separately onto a new gel with bigger wells (2 cm wide) and repeat the electrophoresis.

6| Using a clean blade, excise the gel bands corresponding to the vector containing the synthetic monomer (from Step 1: *Sca*I → *Xma*I; from Step 2 *Bsp*EI → *Sca*I). Purify the DNA fragments using a Gel Extraction Kit as instructed by the manufacturer or by standard electroelution procedures[52].
▲ CRITICAL STEP Using agarose gel electrophoresis (as in Step 4), verify the concentration of the recovered incomplete plasmid/monomer DNA fragments for higher ligation efficiency.

7| Follow the manufacturer's instructions for the T4 DNA ligase. Set up a 20-µl ligation reaction, using a 1:1 ratio of the restriction fragment DNA (*Sca*I → *Xma*I fragment: *Bsp*EI → *Sca*I fragment) as detailed in the table below. The DNA should not exceed a total of 1.0 µg.

Component	Amount	Final
5× ligase reaction buffer	4 µl	1×
*Sca*I → *Xma*I DNA fragment ends	30–120 fmol	30–120 fmol
*Bsp*EI → *Sca*I DNA fragment ends	30–120 fmol	30–120 fmol
T4 DNA ligase (1 U μl^{-1})	1 µl	1 U
Nuclease-free water	Up to 20 µl	

8| Incubate the reaction for 16–24 h at 4 °C.

9| Transform the electrocompetent *E. coli* XL1-Blue cells with 2–4 µl of the ligation mix using the standard electroporation method[52].

10| Plate the transformed cells onto LB agar plates supplemented with ampicillin, X-gal and IPTG for blue-white screening (see REAGENT SETUP). Incubate overnight (16 h) at 37 °C.
? TROUBLESHOOTING

11| Pick ten white recombinant colonies containing the plasmid with the double monomer insert and use to inoculate 10 ml of LB media containing ampicillin.

12| Grow the recombinant colonies overnight at 37 °C in a shaking incubator[52].

13| Purify the recombinant plasmid DNA using a Plasmid Miniprep kit or the standard alkaline lysis method[56].

14| Confirm the presence of the appropriate insert by restriction digestion analysis with *Xma*I/*Bsp*EI enzymes followed by DNA sequencing using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit and vector specific primers.
▲ CRITICAL STEP The sequence verification of each selected plasmid clone using vector-specific primers will confirm the doubling of the insert and avoid the selection of point mutations during the successive cloning steps.
? TROUBLESHOOTING

15| To further increase the size of the insert, repeat Steps 1 through 14 as many times as necessary, always using the recovered recombinant plasmid from Step 14 as a template for the next cloning round starting in Step 1 (**Fig. 2c**).

▲ CRITICAL STEP Multimerized inserts larger than 2.5 kbp are still fairly stable in *E. coli*, but generating highly repetitive inserts longer than 2.5 kbp may lead to internal recombination/deletion as well as lower recombinant gene expression levels in bacteria.

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Procedure 2: Cloning of the spider silk multimer into the expression vector.

[This procedure is designed as a method to clone the final gene product produced above into the expression vector that will modulate the expression of the silk protein in *E. coli*. Below is an abstract figure detailing this procedure.]

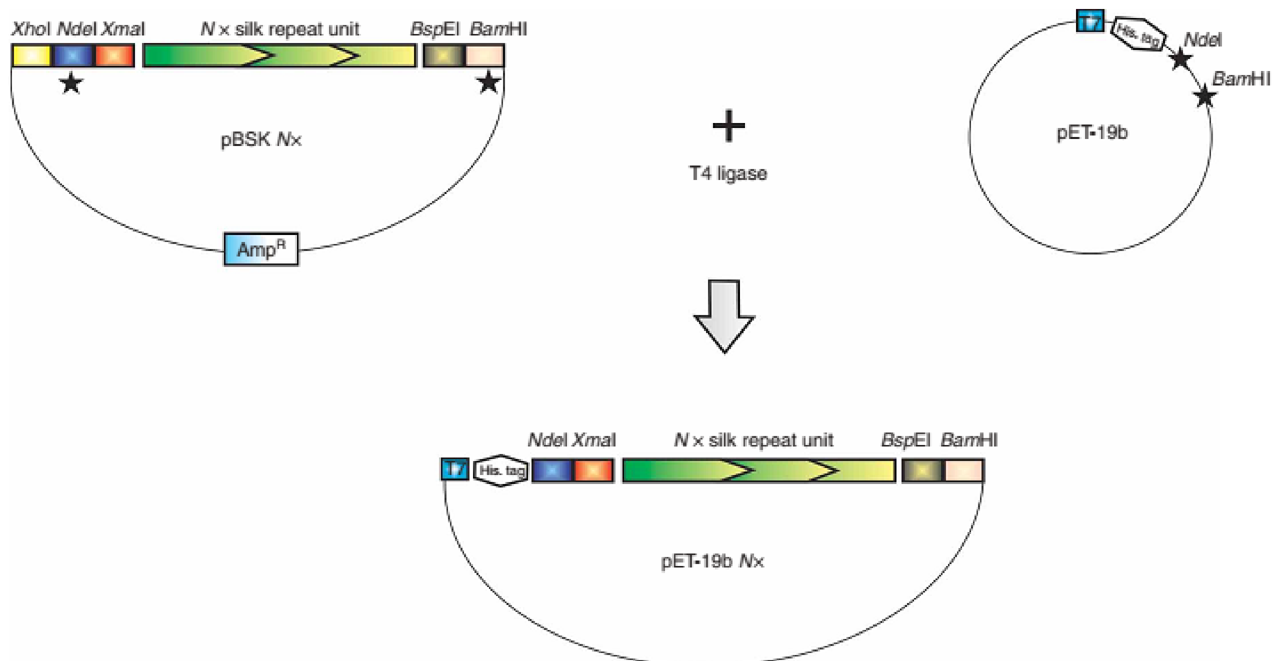

Figure 3 | Strategy to clone the engineered synthetic silk-like sequences in the pET-19b expression vector. The black stars (★) indicate the restriction digestion of DNA.

Reagents Needed:

10x NEB Buffer 2
BSA
pBluescript/multimer or pET-19b
***Bam*HI**
***Nde*I**
Nuclease free water
5x ligation reaction buffer
T4 DNA ligase
Agarose
Gel Extraction Kit
Competent *E. coli* XLI-Blue cells.
LB agar/media
Ampicillin
Plasmid Maxiprep Kit
TE Buffer/ Glycerol Stock

Equipment Needed:

Gel rig / Pipets, microfuge tubes.
Agar plates
Clean blades
PCR rig

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Cloning of the synthetic spider silk gene into the expression vector ● TIMING 4–6 d

16| To excise the inserts, digest the recombinant pBluescriptII SK (+) vector DNA containing the multimerized synthetic sequence with *Bam*HI (5′) and *Nde*I (3′) as described in the table below. At the same time, also digest the pET-19b expression vector DNA with the same restriction enzymes (**Fig. 3**).

Component	Amount (µl)	Final
10× NEB buffer 2	5	1×
BSA (1 mg ml^{-1})	5	100 µg ml^{-1}
pBluescript/multimer or pET-19b (1 µg µl^{-1})	2	2 µg
*Bam*HI (20,000 U ml^{-1})	0.2	4 U
*Nde*I (20,000 U ml^{-1})	0.2	4 U
Nuclease-free water	37.6	

17| Mix the components in a microfuge tube and incubate for 1 h at 37 °C.

18| Check 5 µl of both restriction digestions as described in Step 4.

19| If both reactions are complete, separate individually the remainder of each reaction by agarose gel electrophoresis on a 0.8% (wt/vol) agarose gel as described in Step 5.

20| Using a clean blade, excise the appropriate fragments separately in each case, i.e., the linearized pET-19b vector and the multimer insert, and purify both DNA fragments from the gel using a Gel Extraction Kit as instructed by the manufacturer or standard electroelution protocol[52].

▲ CRITICAL STEP Verify the concentration of the recovered plasmid and insert DNA fragments for higher ligation efficiency as described in Step 4.

? TROUBLESHOOTING

21| Set up a 20 µl ligation reaction using the pET-19b *Bam*HI/*Nde*I digested vector and the synthetic multimer insert, as detailed in the table below. Use a 1:3 (vector:insert) ratio, not exceeding a total of 0.1 µg of DNA.

Component	Amount	Final
5× ligase reaction buffer	4 µl	1×
*Nde*I–*Bam*HI pET-19b vector ends	3–30 fmol	3–30 fmol
*Nde*I–*Bam*HI multimer insert ends	9–90 fmol	9–90 fmol
T4 DNA ligase (1 U µl^{-1})	1 µl	1 U
Nuclease-free water	Up to 20 µl	

22| Incubate the ligation reaction at room temperature for 5 min or overnight at 4 °C.

23| Transform competent *E. coli* XL1-Blue cells with 2 µl of the ligation mix using the standard electroporation method[52].

24| Plate the transformed cells onto LB agar supplemented with 50 µg ml^{-1} ampicillin. Incubate overnight at 37 °C.

? TROUBLESHOOTING

25| Screen for recombinant plasmid clones using PCR to directly amplify the insert from each bacterial recombinant colony[57]. Alternatively, isolate the plasmid DNA from each bacterial clone[56] and verify the presence of the insert by restriction digestion with the two cloning enzymes (see restriction digestion setup in Step 16).

? TROUBLESHOOTING

26| Grow the selected recombinant clone colonies overnight in 10 ml of LB liquid containing 50 µg ml^{-1} of ampicillin in a shaking incubator at 37 °C. Purify the recombinant pET-19b/multimer vector using a Plasmid Miniprep kit or a standard alkaline lysis protocol[56].

27| Confirm the presence of the appropriate insert by restriction digestion analysis with the two cloning enzymes, i.e., *Bam*HI *and Nde*I (see restriction digestion setup in Step 16).

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28| Set up a large-scale culture (100–250 ml) of the selected positive recombinant clones from Step 27 in LB with 50 μg ml^{-1} ampicillin. Incubate overnight in a shaking incubator at 37 °C.

29| Purify the recombinant plasmid using a Plasmid Maxiprep Kit, as instructed by the manufacturer or following standard Plasmid Maxiprep protocols[52]. The expression vector containing the synthetic spider silk gene (pET*silk*) is now ready.
■ **PAUSE POINT** The pET*silk* expression vector DNA can be kept for several years in TE buffer at −20 °C and the corresponding bacterial silk clone should be preserved as a 25% glycerol stock[58] at −80 °C.

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Procedure 3: Gene expression of pET*silk*

[This details the procedure used to generate the silk proteins in transformed *E. coli.* cell cultures]

Reagents Needed:

***E. coli* BL21 (DE3) cell colonies**
pET/silk plasmid
LB agar/medium
Ampicillin
Plasmid miniprep kit
Glycerol stocks
IPTG
1x lysis buffer
Distilled water

Equipment Needed:

PCR rig
Centrifuge/ microfuge tubes
Incubator/shaker
Analytical balance
Beakers/ agar plates

Gene expression ● TIMING 6–7 d

30| Transform the electrocompetent *E. coli* BL21 (DE3) cells with 2–4 µl (5 pg to 0.5 µg) of the recombinant pET/silk plasmid following standard electroporation methods[52].

31| Plate the transformed cells onto an LB agar supplemented with 50 µg ml^{-1} ampicillin and incubate overnight at 37 °C.
? TROUBLESHOOTING

32| Screen for recombinant clones by performing colony-PCR[57]. Alternatively, isolate the recombinant plasmid DNA following a standard alkaline lysis protocol[56] and confirm the presence of the insert by restriction digestion of the 5′- and 3′-flanking restriction enzyme sites.
▲ **CRITICAL STEP** The positive clone colonies must be grown in LB containing the selective antibiotic to an optical density at 600 nm (OD_{600}) of 0.8. Check the OD_{600} using a UV-visible light spectrophotometer (see ref. 52 for details).

33| Make frozen glycerol stocks[58] of the bacterial clones and store at –80 °C for future use.

34| Inoculate 10 ml of LB medium containing ampicillin with one of the recombinant clones from Step 32. Incubate the culture overnight in a shaking incubator at 37 °C.
▲ **CRITICAL STEP** It may be necessary to let the starter culture grow to an OD_{600} of 0.6–0.8 before inoculating the large culture, as some constructs show lower expression if the culture is grown in the stationary phase.

35| Use the 10 ml overnight culture to inoculate 1 liter of LB medium containing 50 µg ml^{-1} of ampicillin. Grow the culture in a shaking incubator at 37 °C until it reaches an OD_{600} of 0.6–0.8 and induce recombinant gene expression by addition of 0.5 mM of IPTG (see REAGENT SETUP).

36| To monitor gene expression, remove 1 ml of culture before adding IPTG and at 1-h intervals after addition of IPTG (see Steps 55–71 for analyses of these expression time points).
▲ **CRITICAL STEP** Maximum protein accumulation generally occurs between 2 and 4 h after gene expression induction[29,37].
? TROUBLESHOOTING

37| Harvest the cells from Step 35 by centrifugation for 15 min at 5,300*g* and 4 °C and discard the culture medium. Wash the cell pellet once with 8 ml of distilled water. Centrifuge for 15 min at 3,300*g* and 4 °C, remove the supernatant and weigh the mass of cell pellets using an analytical balance.

38| Resuspend the cell pellet at a 1:3 ratio (wt/vol) with 1× lysis buffer (see REAGENT SETUP). Store at –80 °C.
■ **PAUSE POINT** The cell pellets can be stored for up to 6 months at –80 °C.

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Procedure 4: *E. coli* cell lysis

[This procedure is necessary for the recovery of the expressed recombinant silk proteins from the cell colonies]

Reagents Needed:

Ice
Lysozyme
PMSF
Deoxycholic Acid
DNase I

Equipment Needed:

Incubator/shaker
Waterbath setup
Pipets / Tubes
Centrifuge/ Microfuge

***E. coli* cell lysis** ● TIMING **0.5 d**

39| Thaw the cell solution on ice and add lysozyme to a final concentration of 0.2 mg ml^{-1}. Incubate the sample on ice for 30 min, swirling periodically.
▲ CRITICAL STEP Occasional stirring allows endogenous lysozyme from BL21 (DE3) cells to act on unlysed cells.

40| Add PMSF (see REAGENT SETUP) to a final concentration of 1 mM to prevent protein degradation.

41| While stirring continuously, slowly add 1.5 g of deoxycholic acid per gram of cells (as determined in Step 37). Incubate the lysate for 20 min at 37 °C.
▲ CRITICAL STEP As the cell membranes are lysed, the sample will become extremely thick and viscous when the bacterial genomic DNA is released.

42| Add 20 µg of DNase I per gram of cells (as determined in Step 37) using a 2 mg ml^{-1} stock to the cell lysate, and incubate for 30 min at room temperature on a shaking platform.
▲ CRITICAL STEP The addition of DNase I reduces the viscosity of the sample by degrading the bacterial DNA.

43| Centrifuge the sample for 15 min at 3,300*g* and 4 °C to pellet the cellular debris. Transfer the supernatant to a 50-ml tube and heat-treat the cell extracts in a waterbath for 10 min at 80 °C.
▲ CRITICAL STEP Spider silk-like proteins are fairly stable upon heat treatment[29,46]. Most of the *E. coli* native proteins are denaturated at this temperature and eliminated in the successive centrifugation step.

44| Centrifuge the sample as described in Step 43 to pellet the denaturated proteins. Store the cleared protein extract at −80 °C.
■ PAUSE POINT The heat-treated protein extracts can be stored at −80 °C for up to 6 months.

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Procedure 5: Silk protein purification using IMAC

[This procedure defines the immobilized-metal ion chromatography (IMAC) technique that will be used to purify the silk protein from the cell lysate].

Reagents needed:

Sterile deionized water
Charge buffer
Strip buffer
1x Binding buffer
1x wash buffer
Elution buffer
Ice
Imidazole

Equipment needed:

His-Bind resin
Polyprep chromatography column
Tubes for fraction collection
Conical tubes

Protein purification using IMAC ● TIMING 0.5 d

45| Add 2 ml (resin bed volume, BV) of His–Bind resin to a 10-ml polyprep chromatography column. Wash, charge and equilibrate the resin using the following sequence of buffers: 10 BV of sterile deionized water, 5 BV of charge buffer (see REAGENT SETUP), 10 BV of sterile deionized water and 5 BV of binding buffer (see REAGENT SETUP).
▲ CRITICAL STEP Add each successive buffer when the previous buffer has just covered the top of the resin bed. Be careful not to disturb the surface of the resin bed when adding the first few drops of the buffer, and prevent the resin from drying.
■ PAUSE POINT When 3 ml of the binding buffer remains, close the bottom of the column and wait until the sample is ready to load into the column. At this point, the prepared columns can be kept at 4 °C for up to 2 months. Drain the buffer before loading the protein sample.

46| Dilute the protein extract in 1× binding buffer in a 1:1 ratio (vol/vol), and pass the sample through the column prepared in Step 45.

47| Collect the flow through in a 15-ml conical tube (fraction 1: 'unbound proteins' or 'flow through') for later analysis (see Steps 60–71) and store on ice.

48| When the sample reaches the top of the resin bed, add 1 ml of 1× binding buffer. Collect the flow through and combine with fraction 1.

49| Wash the resin with 10 ml of 1× wash buffer (containing 20 mM imidazole; see REAGENT SETUP) and discard it into a waste beaker. Retain 100 µl for analysis (fraction 2) and keep on ice.

50| Repeat Step 49 using the second wash buffer containing 40 or 50 mM imidazole (see REAGENT SETUP) and retain 100 µl on ice for further analysis (fraction 3).

51| Elute the bound proteins using 2 ml of elution buffer containing 100 mM or 250 mM imidazole (see REAGENT SETUP). Repeat twice and retain the three eluates (fractions 4–6) on ice.
▲ CRITICAL STEP Proteins can be eluted using an imidazole step gradient. For protein purification, the concentration of imidazole in the buffer is higher in each successive buffer (washes: 20–50 mM; elutions: 60–250 mM). His-tagged silk proteins usually elute using imidazole concentrations equal to or greater than 60 mM induction[29,37]. Collect samples from each step of the protein purification procedure to check for the optimum elution process (see Steps 55 and 60–72 for analysis and characterization).

52| Strip off the nickel ions from the resin using 4 ml of strip buffer (see REAGENT SETUP). Collect and save this sample (fraction 7) on ice for analysis, as it may still contain residual amounts of silk proteins.
? TROUBLESHOOTING

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Procedure 6: Dialysis

[This procedure is necessary for the final purification and isolation of the silk protein]

Reagents Needed:

Running DI water
Ammonium Bicarbonate

Equipment Needed:

Dialysis tubing
Tubing clips
Beakers
Stirring plate
Conical tubes
Speed vac/freeze dryer

Dialysis ● TIMING **2.5 d**

53| Take a piece of prepared dialysis tubing[54] and wash it under running deionized water. Clip one end of the tubing to make a bag. Transfer 4 ml of the eluted fractions into the dialysis bag (fractions 4–7 from Steps 51 and 52) and close the bag with another clip. Place the closed bag in a large beaker filled with deionized water (4 liters) over a stirring plate to dialyze the samples at room temperature. Change the dialysis buffer (water) at least 10 times, at 2 h intervals over a period of 24 h.
▲ CRITICAL STEP The dialysis is important to remove the components of the elution buffer. For freeze-drying[58], dialyze the sample extensively against 5 mM ammonium bicarbonate.

54| Recover the sample from the dialysis bag and place in a 15-ml conical tube. Estimate the concentration of the purified recombinant proteins using the Bradford methodology[59]. Lyophilize the proteins in a speed vac or by freeze-drying[58].
■ PAUSE POINT The pure lyophilized proteins can be stored at −20 °C for up to 1 year.

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Procedure 7: Protein Analyses/ Western Blot Analyses

[This procedure is designed to prepare the silk proteins for analyses under staining & western blotting techniques. Below is a diagram indicating the optimal result]

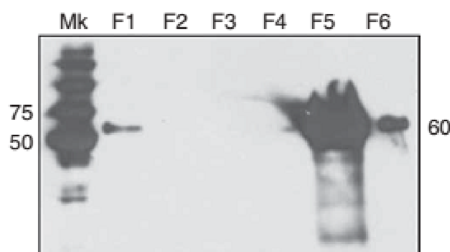

Figure 6 | Western blot analysis showing the IMAC purification steps of a chimeric Flag/MaSp 2 recombinant protein. The 60-kDa His-tagged $A4S8_8$ recombinant protein was detected using the 6× His mAb–HRP conjugate. Mk: molecular weight marker Precision Plus Protein Standard Dual color; F1–6: IMAC collected fractions—F1: unbound proteins; F2–4: wash 1 (20 mM imidazole), wash 2 (40 mM imidazole), wash 3 (50 mM imidazole), respectively; F5: elution fraction (250 mM imidazole); F6: strip fraction. The recombinant protein is highly concentrated in the elution fraction (F5) and residual in the strip fraction (F6). The numbers indicate the molecular weights in kDa.

Reagents needed:

SDS/ Polyacrylamide
GTE Buffer
RNAse A
Alkaline lysis buffer
Potassium Acetate Buffer
2x sample buffer
Coomassie Brilliant Blue
Blocking buffer
6x His mAb-HRP conjugate
1x PBST
ECL substrate

Equipment needed:

SDS Gel Rig
Centrifuge
Centrifuge/Microfuge tubes
Pipets
Beakers
PVDF Membrane
Electroblotting apparatus
Plastic Wrap
Light-proof cassette
X-ray film developer

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Protein analyses ● TIMING **2 d**

55| Prepare two 10% (wt/vol) SDS-PAGE gels[52] for protein analysis: one for staining and a duplicate gel for western blot analysis.

56| For analyses of the silk clone expression time points, centrifuge the collected culture samples from Step 36 for 5 min at 14,000*g* at 4 °C in a microcentrifuge and perform the first three steps of a mini alkaline lysis protocol[56] to obtain a raw protein extract.

57| Resuspend the cells in 100 µl of cold GTE buffer. Add RNAse A to a final concentration of 20 µg ml^{-1} before adding 100 µl of alkaline lysis buffer.

58| Incubate for 5 min and then add 100 µl of cold potassium acetate buffer and incubate for a further 5 min.

59| Centrifuge the sample for 15 min at 14,000*g* at room temperature (20–22 °C) to pellet the cell debris. At this point, transfer the supernatant, which contains DNA and total proteins, to a fresh 1.5-ml tube.

60| Mix 40 µl of the raw protein extract obtained from Step 59 with an equal volume of the 2× sample buffer (see REAGENT SETUP). Additionally, prepare the samples collected throughout the IMAC purification (Steps 47–52, fractions 1–7) using the 2× sample buffer for SDS-PAGE analyses.

61| Once mixed with sample buffer, heat-treat the samples at 95 °C for 5 min.

62| Perform SDS-PAGE analyses on the prepared samples (two duplicate gels) using 1× electrode buffer (see REAGENT SETUP) at a constant voltage of 80 V. Use protein molecular markers ranging from 25 to 250 kDa according to the manufacturer's instructions.

63| After SDS-PAGE analysis, stain one of the gels with Coomassie Brilliant Blue (R-250) according to the published method[60].

64| For imaging, predry the Coomassie-stained SDS-PAGE gel in 10% (vol/vol) glycerol for 1 h and finish drying the gel in a plexiglass frame between two sheets of ultra-clear cellophane.

65| Using an electroblotting apparatus, transfer the separated proteins from the second SDS-PAGE gel onto a PVDF membrane at a constant current of 25 mA overnight at room temperature. Blots are set up as specified by the manufacturer.

66| After transfer, fix the proteins following the manufacturer's instructions.
■ **PAUSE POINT** After transfer and protein fixation, the membranes can be wrapped in plastic cling wrap and stored at −20 °C for several days to months.

67| To block nonspecific binding, completely cover the membrane with blocking buffer (see REAGENT SETUP) and incubate the membrane for 1 h with gentle rocking at room temperature (or alternatively overnight at 4 °C).

68| To detect the silk recombinant proteins, fully cover the membrane with blocking buffer containing the conjugated antibody (6× His mAb–HRP conjugate) diluted according to the manufacturer's specifications. Incubate the membrane for 1 h with gentle rocking at room temperature.

69| Wash the membrane twice with sufficient 1× PBST to cover the membrane (5 min each time) at room temperature (see REAGENT SETUP).

70| Drain the excess 1× PBST from the membrane and wrap the membrane protein-side up in plastic wrap. Cover with the freshly mixed ECL substrate (0.125 ml of 1:1 ECL mix per cm^2 of membrane) and incubate for 1 min at room temperature.

71| Drain the membrane, wrap it in plastic wrap and place in a light-proof cassette. Expose the membrane to an X-ray film for chemiluminescent detection. Develop the film manually or automatically. The initial exposure time should be 30 s to 1 min and should be adjusted depending on the strength of the signal obtained (**Fig. 6**).
? TROUBLESHOOTING

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Procedure 8: Amino Acid Analyses

[Outlines several procedures that we can take to analyze the amino acid composition of the purified silk proteins.]

Amino-acid analysis ● TIMING 2 d

72| For amino-acid analysis, dialyze the IMAC-purified protein samples against 5 mM ammonium bicarbonate and freeze-dry to lyophilize. Proceed to standard amino-acid analysis (hydrolysis, derivatization and separation by HPLC).

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Procedure 9: Production of synthetic silk-like fibers.

[This procedure outlines the final step in the collection of the silk-like proteins and their synthetic into synthetic silk threads. This employs a protein-alcohol coagulation bath for thread extrusion. Included are pictures of the resulting threads, and at the end is a picture of the apparatus and image of silk thread production.]

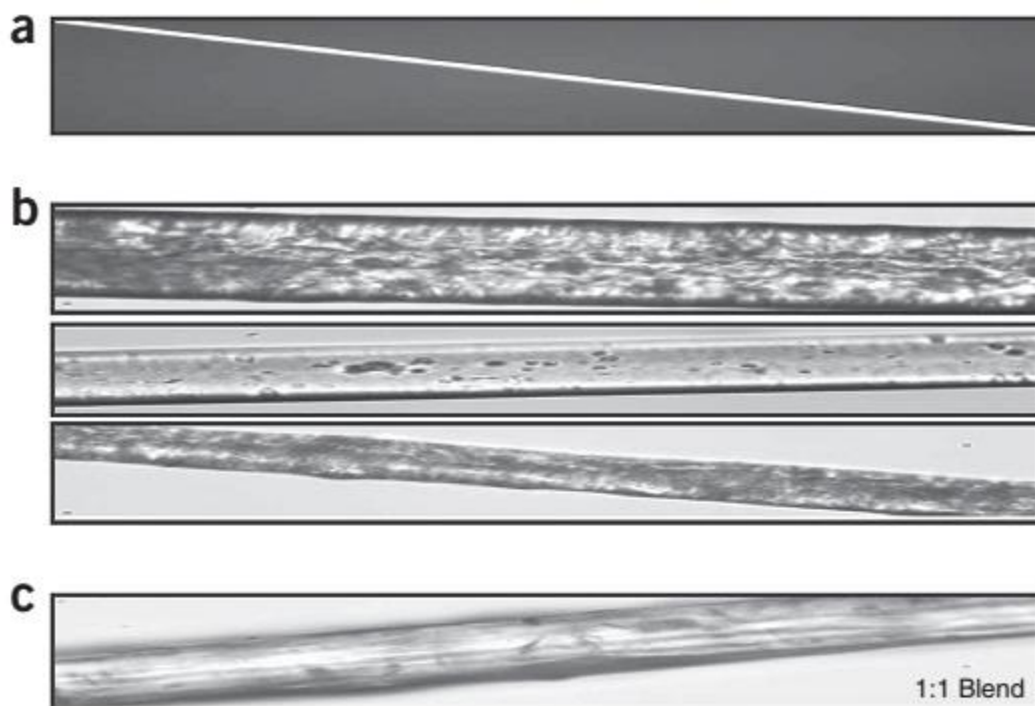

Figure 7 | Native and synthetic spider silk fibers. (**a**) Native *N. clavipes* major ampullate fiber has a diameter of 4 µm. (**b**) MaSp 2-like synthetic spider silk fibers. Notice the highly variable appearance and much larger diameter compared with native fibers. (**c**) Synthetic spider silk blend produced by extrusion of a 15% (wt/vol) MaSp 1/MaSp 2 spinning dope. These photos were taken using a Nikon Eclipse E200 microscope at ×40 original magnification.

Reagents Needed:

HFIP
90% Isopropyl Alcohol

Equipment Needed:

Glass vials
Vortex
Hamilton-Gastight Syringe
Peek Tubing
Spinning Apparatus
Syringe Pump
Forceps

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Production of synthetic fibers ● TIMING **2–3 d**

73| Dissolve the pure lyophilized protein obtained in Step 54 in HFIP in a 1.5-ml sample glass vial to make a 25–30% (wt/vol) silk spinning dope. Vortex if necessary.

! CAUTION HFIP is highly toxic and volatile. Avoid inhalation. Wear appropriate apparel (gloves, lab coat and face protection) and operate in a properly ventilated room.

▲ CRITICAL STEP The estimated silk protein concentration in the spider major ampullate silk gland is 30–40 mg ml^{-1}. Make sure that the protein concentration in the spinning dope is high enough to generate a continuous fiber. The protein sample must be completely solubilized in HFIP before spinning; otherwise, the presence of insoluble aggregates in the dope will clog the needle/PEEK tubing of the spinning apparatus.

? TROUBLESHOOTING

74| Load 250 μl of the spinning dope in a 1 ml Hamilton Gastight syringe mounted with a 10-cm-long PEEK tubing with an internal diameter of 0.127 mm (spinning apparatus, see EQUIPMENT SETUP).

? TROUBLESHOOTING

75| Extrude the silk dope using a syringe pump at a plunger speed of 0.6–1 mm min^{-1} into a 90% isopropyl alcohol coagulation bath (see REAGENT SETUP) to generate a fiber (**Fig. 5**).

▲ CRITICAL STEP The spinning apparatus will align the protein molecules by shearing, thus allowing for higher intermolecular interactions. Fiber formation will occur as the proteins coagulate when the spun dope penetrates the dehydrating alcohol bath.

? TROUBLESHOOTING

76| Collect the extruded fibers from the bath using forceps, and save them for further observation (**Fig. 7**) and analyses.

▲ CRITICAL STEP Carefully collect the fibers spun making sure that they are not stretched inadvertently if post-spin modifications (stretching/treatment) or investigations need to be performed.

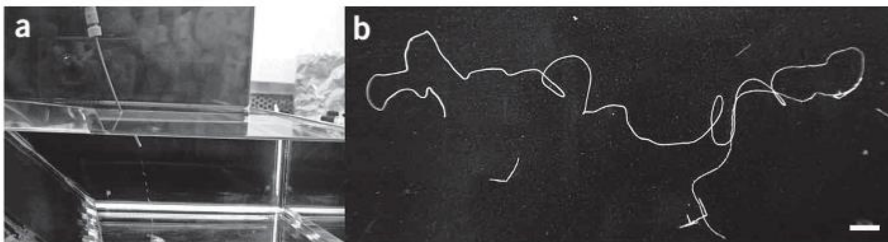

Figure 5 | Synthetic silk fiber formation by extrusion. The pure lyophilized silk recombinant protein was solubilized in 100% HFIP. The silk spinning dope was loaded in the spinneret constituted of a glass syringe attached to PEEK tubing. Manual extrusion of the dope into a 90% isopropanol coagulation bath (**a**) generated a uniform silk fiber (**b**). The photograph in **b** was taken using a Nikon Coolpix 950 digital camera. The white bar in **b** represents 1 cm.

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Overall: Timing/Length of Procedure

● **TIMING**

This protocol takes at least 40 d to complete from vector engineering to fiber spinning if generating a 2,000-bp synthetic repetitive gene. It will take less than 25 d if cloning and expressing complete or partial native (cDNA) sequences.

Steps 1–15, construction of the synthetic spider silk gene: 5–7 d for one insert doubling, thus about 4 weeks to obtain a 2,000-bp repetitive synthetic sequence (4 doublings) from small double-stranded oligonucleotides (60 bp).
Steps 16–29, cloning of the synthetic spider silk gene into the expression vector: 4–6 d
Steps 30–38, gene expression: 6–7 d
Steps 39–44, *E. coli* cell lysis: 0.5 d
Steps 45–52, protein purification using IMAC: 0.5 d
Steps 53 and 54, dialysis: 2.5 d
Steps 55–71, protein analyses: 2 d
Step 72, amino-acid analysis: 2 d
Steps 73–76, production of synthetic fibers: 2–3 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

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TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4, 20 and 25	Incomplete restriction digestion: 3 fragments are seen instead of 2	Too much DNA used for the restriction digestion	Dilute the plasmid DNA and reset the digestion reaction or incubate longer times until the digestion is complete
		The purified plasmid DNA contains residual cellular proteins and needs to be cleaned up	Perform additional standard phenol/ chloroform extractions followed by ethanol precipitation using salts[52] before resetting the digestion
		Enzyme(s) are old and less efficient or inactive	Add more enzymes and incubate for longer duration. Purchase new enzymes if inactive
10, 24 and 31	Few or no colonies	Cells used for transformation have lost competence	Change the method of preparation of competent cells and use the original plasmid vector to check the efficiency of the competent cells
		Low transformation efficiency	The time constant must be 4–5 for maximum efficiency
14	The recombinant plasmid contains no insert	A contaminant colony was selected	Discard the negative clone and screen more colonies. If the problem persists, step back to the previous valid clone and restart the experiment from there
	The recombinant plasmid contains an insert with errors in its sequence	Possible mutations occurred during cloning	Discard the clone and restart the cloning from the previous step
	The recombinant plasmid contains an insert of the wrong size	Possible recombination/deletion occurred during cloning due to the repetitive nature of the insert	Discard the clone and restart the cloning from the previous step
36	Low expression efficiency	mRNA secondary structure formation	Optimize spider silk codon usage for *E. coli* in design avoiding G- and C-rich codons
		Bacterial clone with low expression level	Screen colonies in the presence of IPTG in culture medium. The smallest colonies usually give highest expression level
52	Total protein not binding to the resin	Either the resin is old or the binding buffer may not have been added to the protein extract. The buffer composition and/or pH are wrong	The binding buffer should be at least in 1:1 ratio with the protein extract. Check the buffer composition. Replace the resin if the problem persists
	Protein of interest not binding to the resin	Possible change in the conformation of the protein concealing the His-tag or loading too much of the total silk protein	Analyze the flow-through and wash fractions to see if the protein of interest is lost during binding or washing. To avoid conformation problems, engineer a C-terminal His-tag
	No protein elution peak	The concentration of imidazole in the elution buffer is too low	Increase the concentration of imidazole and use gradient elution
	Too many proteins are present in the eluted fraction	Nonspecific elution of proteins	Increase the washing time and/or decrease the elution buffer concentration
	Degradation of products after purification	Possible proteolysis	Maintain low temperature during the entire extraction and purification processes. Add protease inhibitors to the purification buffers

(continued)

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TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
71	No signal on the X-ray film	Poor transfer or no transfer	Stain the gel with Coomassie stain following transfer to determine if transfer is complete. Increase or decrease the transfer time. The duration may vary depending on the protein size. Higher molecular weight proteins require longer transfers and low molecular weight proteins require shorter transfer
		No expression	If the protein marker gives a positive signal but the recombinant proteins are not detectable, verify the identity of the recombinant plasmid clone. The frozen stock may have been contaminated
		Antibody or substrate not working	Some of the proteins in the marker are also His-tagged and serve as a positive control. Replace the antibody or substrate if there is no signal from the protein marker
		Insufficient chemiluminescent substrate or incubation time too short	Add more substrate and increase the time of incubation
	High background and low signal	Inadequate blocking or insufficient washing	Change the blocking agent to bovine serum albumin. Block with 5% (wt/vol) milk and wash stringently by increasing the amount of Tween-20 in the 1× PBST
73–75	Clog of PEEK tubing	Presence of insoluble protein in spinning dope	Dissolve the protein in HFIP and vortex for a longer time. You can also crush the protein agglomerate with a pipette tip
		Presence of residual salts in the lyophilized protein due to inefficient dialysis	Increase dialysis time and number of buffer changes
	No silk formation	Low protein concentration in the spinning dope	Make sure to have a 25–30% protein solution for the spinning dope
		Inadequate solvent for spinning dope preparation or for the coagulation bath	Increase or decrease the amount of water added to the spinning dope. If fibers still do not form, try different alcohols or organic solvent (acetone) as coagulants

ANTICIPATED RESULTS

The cloning procedure is very straightforward and allows rapid construction of highly repetitive silk-like sequences. Restriction digestions of the intermediate plasmids are critical to confirm the successive doubling of the silk-like inserts (**Fig. 4**). Typically, both double restriction digestions (*Xma*I/*Sca*I and *Bsp*EI/*Sca*I) when complete should generate only two fragments: one empty vector arm and a second containing vector and doubled insert fragments. Throughout the doublings, only the second fragment increases in size and thus can be easily identified. Spider silk-like proteins can be produced and purified from recombinant *E. coli* cells. The yield of purified protein (7–10 mg liter^{-1} of pure protein) depends on the nature of the primary sequence of the protein and the equipment used to produce and purify the recombinant protein. The transformation vector yield is approximately 80–90 µg liter^{-1} of culture. The average time to complete the entire protocol is at least 40 d. The timeline will vary depending upon the extent of manipulation of the silk modular sequences and the amount of synthetic silk protein necessary to prepare a spinning dope that can be extruded into fibers. For one 30% silk-spinning dope of 250 µl, it is necessary to grow 8–10 liters of silk clone cultures to get enough pure recombinant silk proteins.

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[1]This protocol is adapted from the following source:

Teule, F., Cooper, A.R., Furin, W.A., Bittencourt, D., Rech, E.L., Brooks, A., & Lewis, R.V. "A protocol for the production of recombinant spider silk-like proteins for artificial fiber spinning." Nature Protocols, Vol. 4, No. 3, 19 February 2009 (online). Pages 341-355. Nature Publishing Group. doi:10.1038/nprot.2008.250