Electronic Supplementary Material (ESI) for ChemComm.

Electrical Supporting Information

Self-assembled adhesive biomaterials formed by a genetically designed fusion protein

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Experimental Design

General remarks

All the reagents and solvents employed were commercially available and used as supplied without further purification. The genes used for recombinant protein generation were synthesized by Genscript, USA. NMR spectra were recorded on Ascend 850 MHz spectrometer (Bruker). Tyrosinase enzyme for transformation of tyrosine to DOPA, was purchased from Sigma-Aldrich. Anti-histidine tag (mouse) and anit-mouse IgG horseradish peroxidase conjugate antibodies were purchased from Thermofisher. SDS-PAGE was performed in a Mini-PROTEAN Tetra cell electrophoresis chamber (Bio-Rad). Western blot transfer was performed in a mini-trans blot cell (Bio-Rad). The enhanced chemiluminescence (ECL) kit was purchased from Bio-Rad. Gels and Western blot membrane images were documented by ImageQuant LAS500 (GE).

Gene construction and gene synthesis

The recombined genes were cloned into pET 30-a+ vector between EcoRI-HindIII restriction sites (Genscript, USA). The details of the individual sequence are described in Table S1. The Mussel protein part (Mfp3) was linked to the spidroin part (MaSp1) with a flexible linker 'GGGGS'. *N*-terminal 6X His tag of the vector was used for protein purification using affinity chromatography. The cloned vector was transformed into a host *E.coli* BL21(DE3) by following the instruction manual supplied from Genscript. The bacteria were grown up to OD_{600} ~ 0.8 in 300 mL of LB media containing 50 µg/mL of kanamycin at 37ºC before the protein induction by adding a solution (300 µL) of isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.5 M) at 30 ºC strictly for 4 hr.

Table S1: (green: MaSp1, red: MfP3, blue: linker)

'MaSp1' in pET 30-a+ Theoretical MW: 22051 Da
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSEFGGGGSSGRGGLG GQGAGAAAAAGGAGQGGYGGLGSQGTSGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGTSGR GGLGGQGAGAAAAAGGAGQGGYGGLGSQGTSGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQG TSGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGTSGRGGLGGQGAGAAAAAGGAGQGGYGGL GSQGTGGGGS-
'MS' in pET 30-a+ Theoretical MW: 27937.54 Da
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIGSEFADYYGPKYGPPR RYGGGNYNRYGRRYGGYKGWNNGWKRGRWGRKYYGGGGSGGGGSSGRGGLGGQGAGAAAAA GGAGQGGYGGLGSQGTSGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGTSGRGGLGGQGAGA AAAAGGAGQGGYGGLGSQGTSGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGTSGRGGLGGQ GAGAAAAAGGAGQGGYGGLGSQGTSGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGTGGGGS-

MaSp1 (originated from dragline proteins of Mytilus galloprovincialis): Protein induction and purification

The induced bacteria (transformed with MaSp1 in pET30-a+) were collected by centrifugation as a pellet and subsequently washed with PBS containing 1X protease inhibitor cocktail (Thermo Fisher). The bacteria pellet was resuspended in a solution (10 mL) of a lysis buffer (50 mM potassium phosphate pH 7.2, 300 mM NaCl, 10% sucrose, 1X protease inhibitor cocktail, 10 mM imidazole and 1% Triton X-100). The re-suspended bacteria were treated with a solution (20 µL) of lysozyme (50 mg/mL in water) and incubated in an ice bath for 30 min. The incubated cells in an ice bath were sonicated to lyse the bacteria walls for 20 cycles of on (30 sec) and off (60 sec) to cool down between each time. A supernatant was collected by centrifugation of the lysed solution for 30 min at 4 ºC and incubated with 10 mL of Ni-NTA resin (Qiagen) overnight at 4 ºC. After the incubation, the resins were loaded on a disposable gravity column and washed with a solution (50 mL) of a washing buffer (50 mM potassium

phosphate pH 7.2, 300 mM NaCl and 50 mM imidazole). A solution (35 mL) of an elution buffer (50 mM potassium phosphate pH 7.2, 300 mM NaCl and 250 mM imidazole) passed through the washed column. The eluted solution was dialyzed using a dialysis tube (SnakeSkin^{TM} dialysis tube, ThermoFisher, MWCO: 10 kDa) against acetic acid (10 mM, in water) and finally concentrated through a centrifugal protein concentrator filtration unit (Amicon Ultra, Millipore, MWCO: 10 kDa).

MS: Protein induction, transformation of tyrosine to DOPA and purification

The induced bacteria (transformed with MS in pET30-a+) were collected by centrifugation as a pellet and subsequently washed with PBS containing 1X protease inhibitor cocktail (Thermo Fisher). The bacteria pellet was resuspended in a solution (10 mL) of a lysis buffer (50 mM potassium phosphate pH 7.2, 300 mM NaCl, 10% sucrose, 1X protease inhibitor cocktail, 10 mM imidazole and 1% Triton X-100). The re-suspended bacteria were treated with a solution (20 µL) of lysozyme (50 mg/mL in water) and incubated in ice bath for 30 min. The incubated cells in ice bath were sonicated to lyse the bacteria walls for 20 cycles of on (30 sec) and off (60 sec). A supernatant was collected by centrifugation of the lysed solution for 30 min at 4 ºC and incubated with 6 mL of Ni-NTA resin (Qiagen) overnight at 4 ºC. After the incubation, the resins were loaded on disposable gravity column and washed with a solution (50 mL) of a washing buffer (50 mM potassium phosphate pH 7.2, 300 mM NaCl and 50 mM imidazole). For transformation of tyrosine to DOPA, the washed column was equilibrated with a solution (50 mL) of a treatment buffer (100 mM Tris pH 8.0, 300 mM NaCl, 20 mM Na-borate and 100 mM ascorbic acid). After equilibration, a freshly prepared solution (4 mL) of tyrosinase (0.5 mg/mL) in the treatment buffer was added to the equilibrated resin. The column with the reaction mixture was closed and kept in a mild shaking condition at 25ºC for 2 hr. The resin in the column was washed with a solution (50 mL) of the washing buffer without imidazole to remove the enzyme. The target protein was eluted with a solution (35 mL) of the elution buffer and the eluted solution was dialyzed using a dialysis tube (SnakeSkin^{TM} dialysis tube, ThermoFisher, MWCO: 10 kDa) against acetic acid (10 mM, in water) at 4 ºC for 2 d. The dialysed samples were concentrated with a protein concentrator unit (Amicon Ultra, Millipore, MWCO: 10 kDa) to give MS. The protein was stored in a freezer (- 80 ºC) before used for experiments. Protein precipitation is confirmed by drop in protein concentration in supernatant phase after a high speed centrifugation (13000 rpm, 10 min).

General characterization of the purified proteins

Comassie brilliant blue staining:

A solution (16 µL) of the purified protein as above was mixed with a solution (4 µL) of a sample buffer (5X, 10% w/v SDS, 10 mM 2-mercaptoethanol, 20% v/v glycerol, 0.2 M Tris-HCl, pH 6.8 and 0.05% w/v bromophenolblue) and the mixture was boiled at 95 ºC for 5 min. The sample was run through 12% SDS-PAGE and dipped in a staining solution (0.1% w/v Coomassie Blue R-250, 50% v/v methanol and 10% v/v glacial acetic acid) for 1 hr. The stained gel was washed with a destaining solution (25 % v/v methanol and 15 % v/v glacial acetic acid)

Western blot analysis for Anti-his-tag antibody:

The boiled sample was run through 12% SDS-PAGE and the separated proteins in the gel were transferred into a nitrocellulose membrane following the instruction manual supplied from Bio-Rad. The membrane in a solution (15 mL) of tris-buffered saline with tween 20 (TBST, 25 mM Tris, 150 mM NaCl and 0.1% tween 20) was washed for 5 min. The washing was repeated 3

times. The washed membrane was blocked by addition of a solution (20 mL) of 5% skimmed milk in TBST for 1 hour at room temperature, followed by washing with TBST 3 times for 5 min. The primary antibody (anti-His tag, mouse) was prepared in 1:1000 dilution, in 5% skimmed milk in TBST and a solution (10 mL) of the prepared antibody incubated with the membrane at room temperature for 1 hr. The membrane was then washed with TBST 3 times for 5 min. The secondary antibody (anti-mouse IgG-HRP conjugate) was diluted to 1:2000 in 5% skimmed milk in TBST, and a solution (2 mL) of the antibody was incubated with the membrane for 2 hr. The membrane was developed using a commercial enhanced chemiluminescence (ECL) kit (Bio-rad) as per the manufacturer's instructions.

MALDI-ToF MS of the protein was performed with α-Cyano-4-hydroxycinnamic acid (CCA) as a matrix. The sample mixture was air dried on a metallic plate and analysed by Autoflex Speed LRF MALDI-TOF instrument.

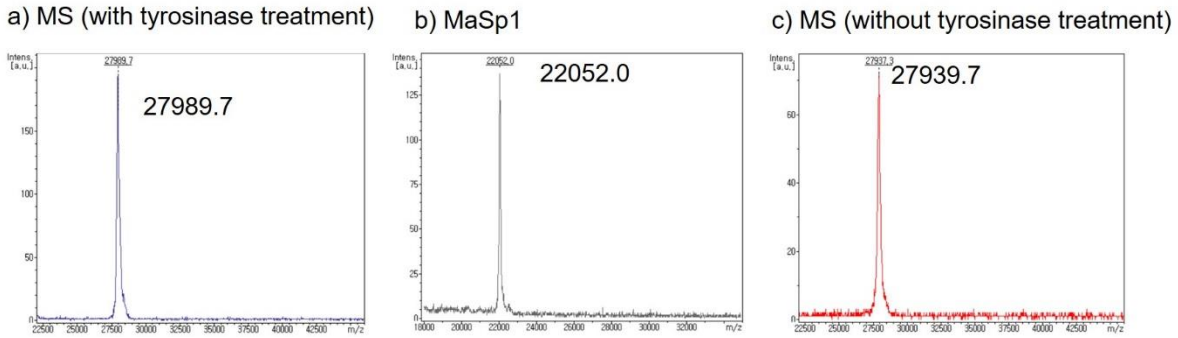

Figure S1. MALDI-TOF mass spectra of a) MS with tyrosinase treatment, b) MaSp1 and c) MS without tyrosinase treatment

Congo red staining for amyloid fibril detection and nitrobluetetrazolium chloride (NBT) staining for DOPA identification

Congo red staining: A drop (50 µL) of the purified protein (50 µg/mL) was spotted on a nitrocellulose membrane using dot blot manifold. The membrane was immersed in a solution (20 mL) of Congo red in water (0.0025 %) and slowly shaken for 1 hr. Then the membrane was thoroughly washed with deionized water and kept rocking with sufficient water overnight.

NBT staining: Similarly, blotted membranes as above stated were incubated in a solution (20 mL) of a reaction solution (0.6 mg/mL NBT in 2 M potassium glycinate, pH 10) at room temperature in a dark condition for 45 min. After washing with a solution (25 mL) of sodium borate in water (0.16 M) 3 times for 5 min per each, the membranes were incubated in the borate solution (25 mL) overnight with mild shaking at room temperature.

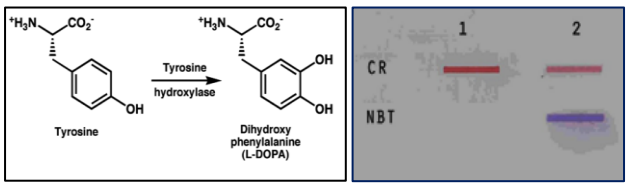

Figure S2: Congo red staining and NBT staining of the protein 1: before and 2: after treatment with tyrosinase

DOPA quantification by NBT assay

The content of DOPA in the protein was estimated by a colorimetric method by a reaction with NBT in slightly basic condition.[S1] The protein samples were initially prepared in acetic acid solution (10 mM) and diluted in the reaction buffer. Each protein (450 pmol) was taken in a solution (570 µL) of a potassium glycinate in water (2.0 M, pH 10) and then a solution (30 µL) of NBT in methanol (3 mg/mL) was added. The mixed solution was incubated in dark at 37 ^{o}C for 1 hr and the UV spectrum was recorded by Cary6000i UV-Vis spectrometer and read at 530 nm.

CD spectroscopy

The proteins for CD spectroscopy were prepared by diluting the purified protein with acetic acid solution (10 mM) to reach to the concentration of 4.5 µM. A solution (600 µL) of the diluted protein was incubated with addition of hexafluoroisoproanol (HFIP, 2% v/v final concentration) at 4 ^{o}C for 2 d. Circular dichroism spectroscopy of the sample was measured by a CD spectrometer (Jasco, J-815) within a range of 190 nm - 260 nm in an inert environment of nitrogen.

Scanning electron microscopy

A glass cover slips (1.0 cm × 0.5 cm) were thoroughly cleaned by NaOH (1.0 N) and HCl (1.0 N) sequentially and rinsed by deionized water. Then the rinsed cover slips were dried within a hot air oven (50 ^{o}C) for 4 hr. A solution (5 µL) of the protein (230 pmol) was dropped on the coverslip and covered by another coverslip in an eclipsed manner. Cover slips were dried at room temperature for 2 hr and then detached from each other. The surface was treated with platinum at 20 mA for 45 sec and observed under High Resolution FE-SEM-I (JSM 7401F).

Dynamic mechanical analysis of proteins

Dynamic mechanical analysis was performed by a rheometer (Anton Parr, MCR302) at a constant temperature (24 ^{o}C). We kept the same molar amount of the protein samples for direct comparison and analysis of the data among the proteins. 450 pmol of each protein was prepared within a solution (2 µL) of acetic acid (10 mM) in each case. Cleaved mica discs (Tedpella, 1.2 mm diameter) were attached to each (upper and lower) flat sample holder of the rheometer. After the sample was placed on a disc, the discs were contacted to each other and waited for one hour. (Distance between two plates was kept constant at 0.05 mm). We applied a strain sweeping from 0 - 30% with a constant frequency at 10 rad/s. Storage modulus and loss modulus of the sample were recorded against the strain applied in the x-axis.

Measurement of surface adhesive force using a surface force apparatus (SFA)

Underwater surface adhesive forces of MS and MaSp1 were measured using a surface forces apparatus (SFA; SurForce LLC, Goleta, CA, USA) as described in the previous report.[S2] For surface adhesion between a bare mica surface and asymmetric MS film, a Tyrosinase-modified MS solution (0.02 g/L in 0.1 M acetic acid, pH 3) was deposited onto a freshly cleaved mica surfaces for 10 min for adsorption of MS. The MS coated mica surface was rinsed washed with a solution of acetic acid (0.1 M, pH 3). The interaction force and the separation distance between MS coated mica and bare mica surfaces were determined after 5 min of compression and 10 min of contact time. The measured force (F) was correlated to the energy per unit area (W) by $W = F/1.5\pi R$ for a soft, deformable surface.[S2] Measurement of surface adhesive force of MaSp1 was carried out in the same manner.

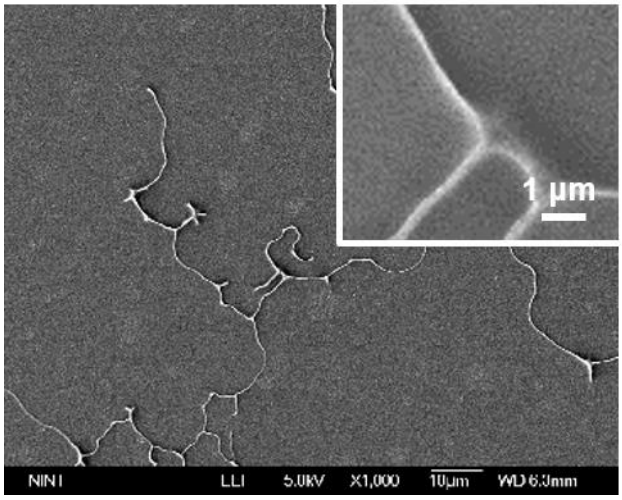

Figure S3: SEM image of MS before tyrosinase treatment

Diffusion-ordered spectroscopy by Pulsed-field gradient (PFG) NMR experiments

The diffusion coefficient measurements were carried out using a 5 mm Bruker QNP probe with an actively shielded z gradient coil. Diffusion coefficients were extracted from a series of ^{1}H NMR spectra measured at 25 °C by the bipolar pulse longitudinal encode-decode (BPPLED) pulse sequence as a function of gradient amplitude. In each experiment, the gradient duration time was 2.0 or 2.5 ms and the amplitudes of gradient pulses ranged from 1 to 40 G cm^{-1}. The diffusion time was 50 to 100 ms. Diffusion coefficients were calculated from the data obtained by 2D diffusion-ordered spectroscopy (DOSY).[S3] The diffusion coefficient of Mfp3 and MS in D_2O was measured to be 1.0×10^{-10} and 5.6×10^{-11} m^2 s^{-1}, respectively. By applying the diffusion coefficient to the Stokes-Einstein equation,[3] the hydrodynamic radius of MS (3.3 nm) is calculated to be approximately 1.8-fold larger than that of Mfp3 (1.9 nm) in D_2O.

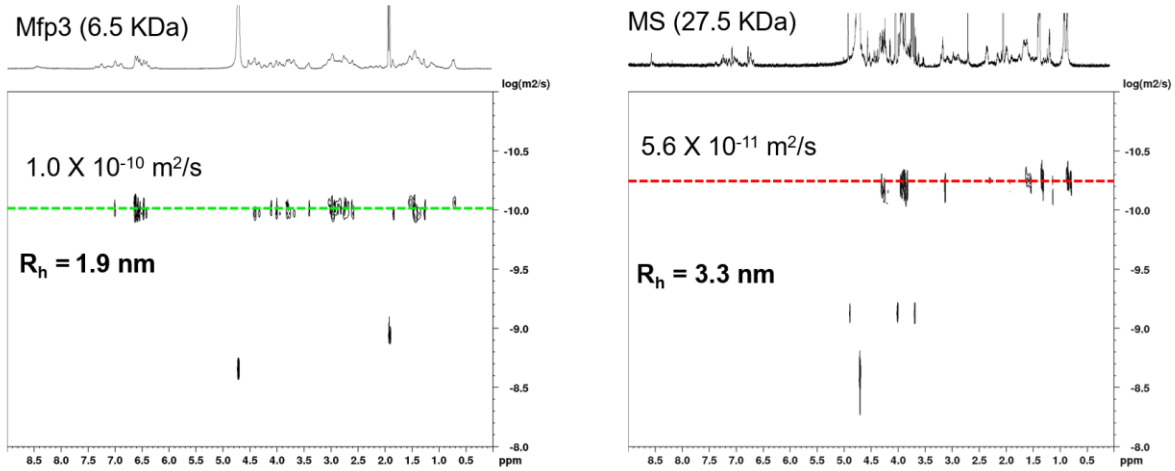

Figure S4. DOSY spectra of Mfp3 and MS

References:

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S2 B. Yang, C. Lim, D. S. Hwang, H. J. Cha *Chem. Mater.* 2016, **28**, 7982-7989

S3 D. K. Wilkins, S. B. Grimshaw, V. Receveur, C. M. Dobson, J. A. Jones and L. J. Smith, Biochemistry, 1999, 38, 16424-16431.