PROTEIN- AND METAL-DEPENDENT INTERACTIONS OF A PROMINENT PROTEIN IN MUSSEL ADHESIVE PLAQUES*

Dong Soo Hwang[1], Hongbo Zeng[2], Admir Masic[3], Matthew J. Harrington[3], Jacob N. Israelachvili[4] and J. Herbert Waite[5]

[1]Materials Research Laboratory, University of California, Santa Barbara, CA 93106, USA
[2]Dept Chem. & Mat. Eng., University of Alberta, Edmonton, AB, T6G 2V4, Canada
[3]Dept Biomaterials, Max Planck Institute for Colloids and Interfaces, Potsdam-Golm, Germany
[4]Department of Chemical Engineering, University of California, Santa Barbara, CA 93106
[5]Marine Science Institute, University of California, Santa Barbara, CA 93106

Supporting Data

Scheme S1: Mfps purification
Scheme S2: Raman microscopy data processing
Scheme S3: Surface Forces Apparatus diagram

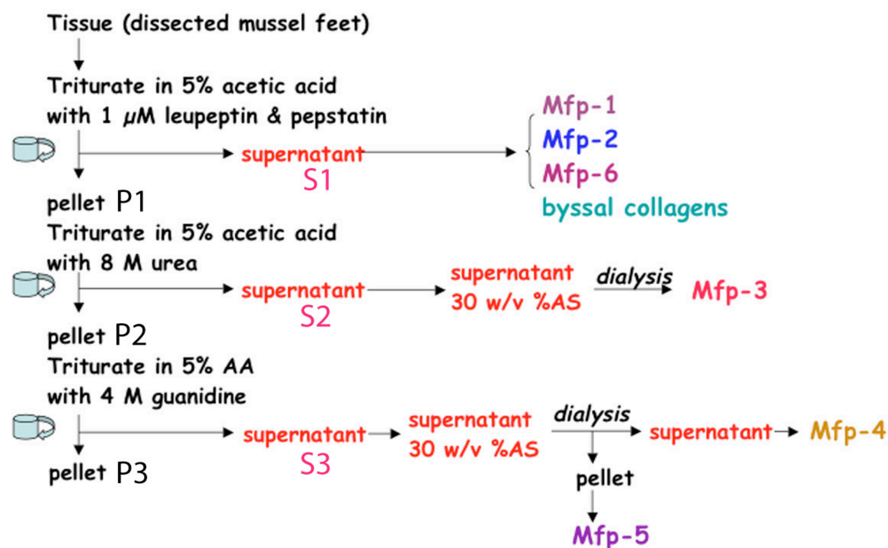
Figure S1: Interaction of mfp-5 and –2 by SFA
Figure S2: Interaction of mfp-3 and –2 by SFA
Figure S3: Confocal Raman microscopy of precipitated mfp-1 and mfp-2 complexed to Fe^{3+}

Mussel Foot Protein Purification. The purification of each Mfp is described in detail elsewhere. Salient steps and solvents are summarized here and in Scheme S1with particular emphasis on innovations. It should be emphasized that these purifications were adapted from earlier procedures used extensively for histones; mussel proteins and histones resemble one another in their high charge density and pI, as well as a dearth of secondary structure in solution. Mfps used for this study were prepared from *Mytilus edulis* feet. Mfp-1 and mfp-2 were extracted from dissected phenol glands using about 100 mL/5g cold 5% (v/v) acetic acid with 0.1 mM leupeptin and 0.1 mM pepstatin. Glands were homogenized with 50 mL tissue grinders from Kontes (Vineland, NJ), and homogenates were spun in a refrigerated (5˚ C) centrifuge 20,000xg for 40 min to produce a supernatant **S1** and pellet **P1**. Mfp-1 and -2 were further purified from **S1** by the addition of 1.5 % (v/v) perchloric acid (resulting insolubles were removed by centrifugation as before). Mfp-1 and -2 were harvested from solution by the dropwise addition of 2 volumes of cold acetone exactly as described in Waite (1995). The concentrate was subjected to gel filtration on a Shodex KW 803 eluted with 5% acetic acid at 0.25 ml/min at room temperature and monitored at 280 nm (Ohkawa et al., 2004). Mfp-1 (108 kDa) typically elutes just after the void volume, whereas mfp-2 (45 kDa), depending on the sample volume injected, elutes as a peak about 10 min after mfp-1. Fractions under the peaks were examined for their mfp content using acid-urea gel electrophoresis (Waite, 1995). Those fractions of mfp-1 without mfp-2, or mfp-2 without mfp-1, respectively, were pooled, freeze-dried, reconstituted in a small volume of 5% acetic acid (~1 mL), and polished by C8 reverse-phase HPLC using an acetonitrile gradient in water with 0.1% trifluoroacetic acid. Both proteins elute at about 22% acetonitrile. Following HPLC, the proteins were freeze-dried and stored at –80 ˚C.

Mfp-3 and –5 were obtained by further extraction of **P1**. Mfp-3 was extracted by homogenization (tissue grinder) of pellets with cold 5% acetic acid with 8M urea (50

mL/5g). Centrifugation (20,000xg @ 40 min) of this resulted in a pellet **P2** and supernatant **S2**. **S2** was collected and 30 % weight/volume ammonium sulfate was added and stirred 1 h at room temperature. Insolubles were removed by centrifugation (as above) and the supernatant was dialyzed against Q-water at 5 ˚C with dialysis tubing MWCO 1,000 (Spectrum Industries). Mfp-3 forms a floc and settles during dialysis. This was harvested by a 5min spin at 15K xg on a microfuge and redissolved in a small volume of 5% acetic acid (Papov et al., 1996).

Mfp-5 was extracted from **P2** by homogenization (tissue grinder) of pellets using cold 5% acetic acid with 6M guanidine·HCl (50 mL/5g). Centrifugation (20,000xg @ 40 min) of this resulted in a pellet **P3** and supernatant **S3**. **S3** was collected and 30 % weight/volume ammonium sulfate was added and stirred 1 h at room temperature. Insolubles were removed by centrifugation (as above), and the supernatant was dialyzed against Q-water at 5 ˚C with dialysis tubing MWCO 1,000. Mfp-5 forms a floc and settles during dialysis. This was harvested by a 5min spin at 15K x g on a microfuge and redissolved in a small volume of 5% acetic acid (Waite & Qin, 2000). Mfp-3 and mfp-5 were polished by C8 HPLC at a flow rate of 1 ml/min using a linear gradient of acetonitrile in water with 0.1 % trifluoroacetic acid. Elution times are well separated.



Scheme S1. Isolation strategy of mfps from *Mytilus* feet. Every mfp precursor can be isolated from a different step of the same starting lot. See supporting text for details on purification to homogeneity.

Waite, J. H. (1995) *Meth. Enzymol.* 258, 1-20.

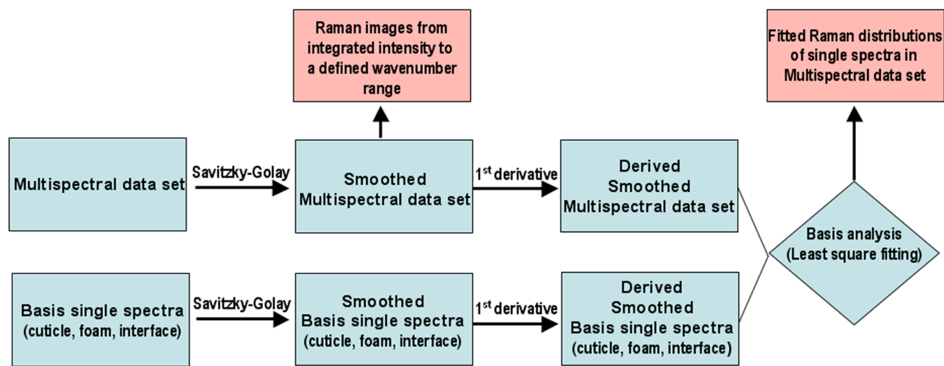
Ohkawa, K., Nishida, A., Yamamoto, H., Waite, J.H. (2004) *Biofouling* 20, 101-115.

***Raman spectroscopic studies* -** Plaques from *Mytilus galloprovincialis* were embedded in PEG-2000 (Carl Roth Gmbh), and 20 μm thick longitudinal sections were microtomed. Plaque sections were washed thoroughly with several changes in distilled

water to remove any remaining PEG, positioned on a quartz slide in distilled water and fixed under a quartz cover slip. For Raman microspectroscopy, a continuous laser beam was focused on the sample through a confocal Raman microscope (model CRM200, WITec, Ulm, Germany) equipped with a piezo-scanner (model P-500, Physik Instrumente, Karlsruhe, Germany). The diode-pumped 785 nm near infrared (NIR) laser excitation (Toptica Photonics AG, Graefelfing, Germany) was used in combination with a 100μm oil immersed (Nikon, NA = 1.25) microscope objective. Laser power ranging between 15-30 mW was used for all measurements. The spectra were acquired using an air-cooled CCD (DU401A-DR-DD, Andor, Belfast, North Ireland) behind a grating (300 g mm^{-1}) spectrograph (Acton, Princeton Instruments Inc., Trenton, NJ, USA) with a 6 cm^{-1} spectral resolution. Software ScanCtrlSpectroscopyPlus (version 1.38, Witec) was used for measurement setup. Raman spectra were processed and analyzed with Witec Project Plus software (Version 2.02).

Single Raman spectra were collected from different regions of the plaque (coating, foam and interface) by averaging 30 acquired spectra, each with a 1 s integration time. Acquired spectra were used as the single spectra basis for further analysis (Scheme 2). A 2-dimensional multi-spectral data set was collected by scanning the surface of the cross section in steps of 0.5-μm, recording spectra with an integration time of 1 s per each scan (pixel). All Raman spectra were background-subtracted and lightly smoothed using the first order polynomial function and 9-point Savitzky-Golay filter (4th order polynomial), respectively.

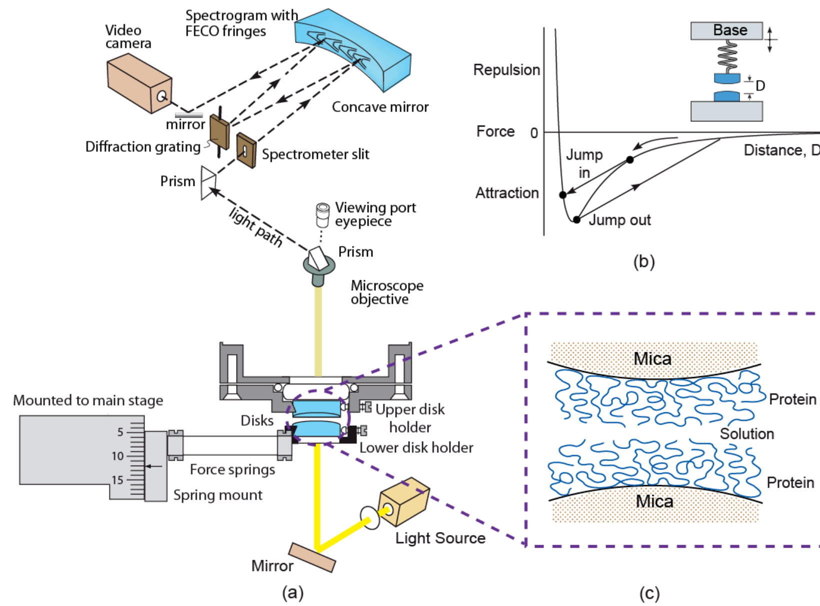
Raman images in Fig.9 B, C and D were generated using a sum filter, which integrates the intensity of the signal for a defined wave number range of interest after subtracting the background as a linear baseline from the upper to lower border. The ranges of interest were the bands for CH stretching (2850-3010cm^{-1}), Fe-dopa (490-696 cm^{-1}), and phenylalanine (980-1020 cm^{-1}). The color images were obtained from Witec Project software using the basis analysis and image color combination functions. In the case of basis analysis, the algorithm fits each spectrum of the multi-spectral data set with a linear combination of the basis single spectra (tree spectra in figure 9E) using the least squares method. To solve the problem of differing fluorescence background in various parts of the sample, the first derivative of both multi-spectral data set and basis single spectra was performed. The weighting factors of various components obtained by fitting were stored in an image and combined in a false color bitmap using the image color combination function. Supplementary Scheme S2 summarizes the Raman data processing procedure.



Scheme S2: Flowchart of data processing in Raman microscopy.

For Raman microscopy of purified mfps, a purified solution of mfp-2 (1 mg/ml) in 0.1M sodium acetate, 0.25M KNO_3, 1mM bis-tris (pH 5.5) was mixed with a solution of $FeCl_3$ in 10mM Bis-Tris (pH 5.5) at ratios of dopa:Fe^{3+} of 2:1 and 20:1 and allowed to equilibrate for 10 minutes. The protein precipitated as the pH was raised to ~8.0 with 0.1M NaOH. The droplet was allowed to evaporate on a glass slide, and Raman spectra (30 accumulations with 1 s integration time each) were taken from the precipitated protein residue at the edges. Raman spectra were also measured from the protein solution prior to iron complexation and from solutions of mfp-1 and mfp-2 in which Fe^{3+} was not limiting for comparison.

Force vs distance profiles measurement by the Surface Forces Apparatus (SFA)- The SFA technique has been used for many years to measure both normal and lateral forces between surfaces in vapors and liquids, e.g., van der Waals forces, electrostatic forces, adhesion forces, friction and lubrication forces, hydrophobic interactions, specific and non-specific biological interactions (22-25). A typical SFA experiment setup for measuring normal forces between two surfaces is shown in Scheme S3. SFA can accurately measure normal (attractive adhesion or repulsive) forces F as low



Scheme S3. Surface forces diagram. *Experiment setup using a Surface Forces Apparatus (SFA) to measure the normal interactions between two surfaces, (a) schematic of SFA experiment setup for normal force measuremment, (b) illustration of typical force-distance profile (positive force values present repulsion, and negative values show attraction), (c) illustration of the experimental geometry for the study of interfactions between two protein layers.*

as 10 nN as a function of surface separation distance D with a resolution of less than 1 Å monitored in situ using the fringes of equal chromatic order (FECO) in multiple beam

interferometry. The force between the two surfaces is measured according to Hooke's law, $F = k\Delta x$, Where k is the spring constant supporting the lower surface, and $\Delta x = D_{actual} - D_{applied}$ is the difference between applied and actual surface separations.

The normal force-distance profiles and adhesion forces (F_{ad}) of mfp-2 were determined using an SFA in a configuration reported previously (9). Briefly, a thin mica sheet of 1-5μm was glued onto a cylindrical silica disk (radius R=2 cm). 100 μL of a stock mfp-2 solution (20 μg/mL) diluted in 0.1 M sodium acetate with 0.25 M potassium nitrate at pH 5.5, was injected onto one mica surface. [*n.b.* In the SFA, potassium nitrate is used in place of NaCl to reduce chloride ion induced corrosion of the semi-reflecting silver layers under the mica substrates.].

For experiments with Fe, 1mM bis-tris was added to stabilize the solubility of Fe^{3+} (26). Although the test pH is significantly lower than seawater pH 8.2, it is consistent with the pH the adhesive secretion by the foot (unpublished observations) and was necessitated by the poor solubility of Fe and the mussel proteins at higher pH in the SFA. The two curved and coated mica surfaces were then mounted in the SFA chamber in a crossed-cylinder geometry, which roughly corresponds to a sphere of radius R on a flat surface based on the Derjaguin approximation: $F(D) = 2\pi RW(D)$, where $F(D)$ is the force between the two curved surfaces and $W(D)$ the interaction energy per unit area between two flat surfaces. The measured adhesion or "pull-off" force F_{ad} is related to the adhesion energy per unit area W_{ad} by $F_{ad}=2\pi RW_{ad}$ for rigid (undeformable) surfaces with weakly adhesive interactions, and by $F_{ad}=1.5\pi RW_{ad}$ (used in this study) for soft deformable surfaces with strong adhesive contact (27, 28). All experiments were performed at room temperature (23 °C).

We applied proteins to mica surfaces according to the whether asymmetric and symmetric testing mode was to be used. In the asymmetric mode, protein was applied to one mica surface only. In contrast, protein was applied to both mica surfaces in symmetric mode.

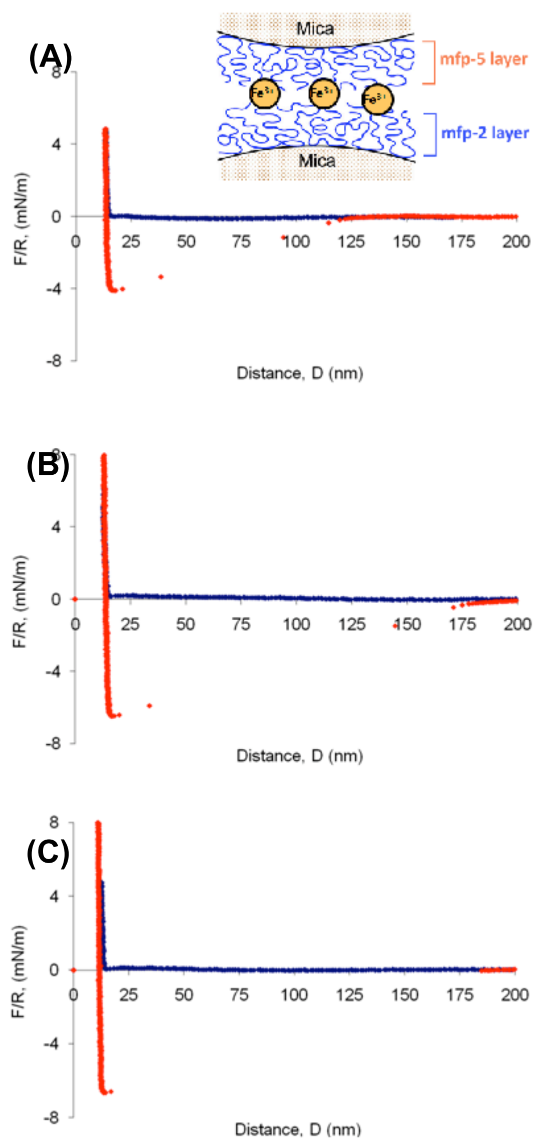


Figure S1. Interaction of mfp-5 and mfp-2 films in the presence of 5μM Fe^{3+}. Contact times in min (0, A), 10 (B), 60 (C)

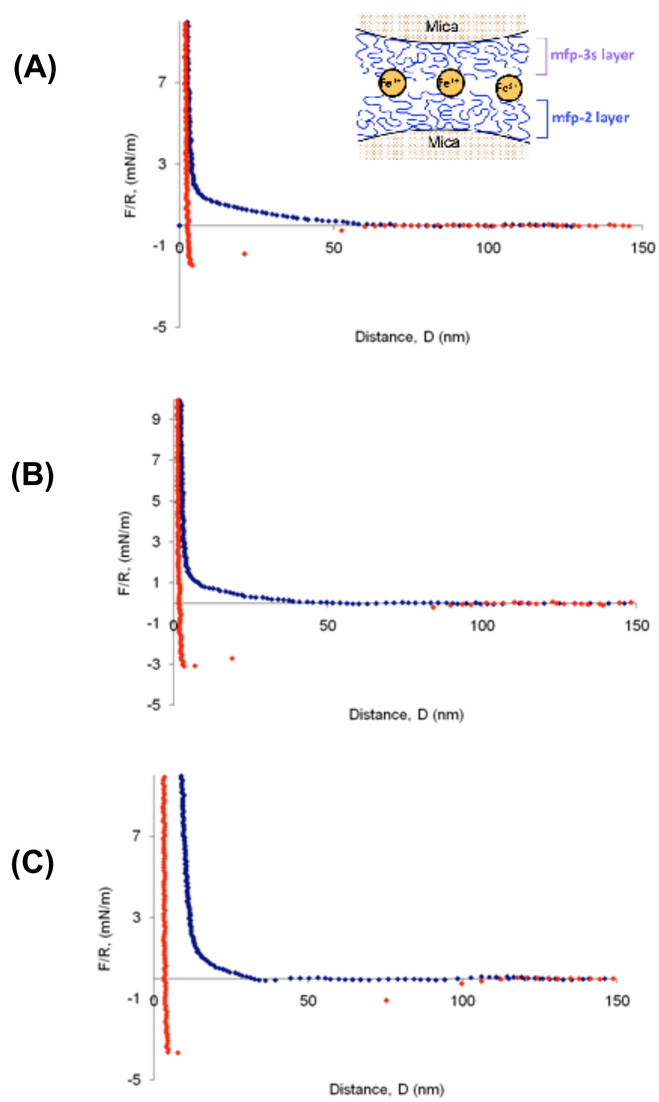
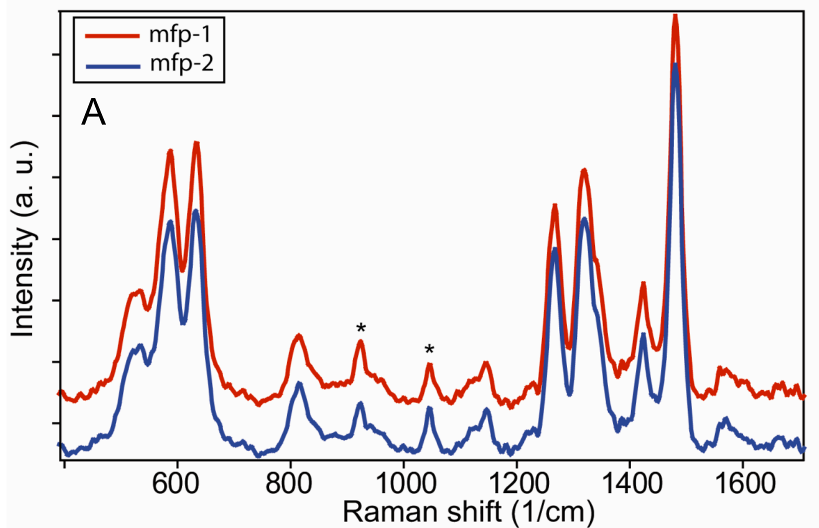


Figure S2. Interaction of mfp-3 and mfp-2 films in the presence of 5μM Fe^{3+}. Contact times in min (0, A), 10 (B), 60 (C). Blue –approach, red - separation. Contact position was the same.



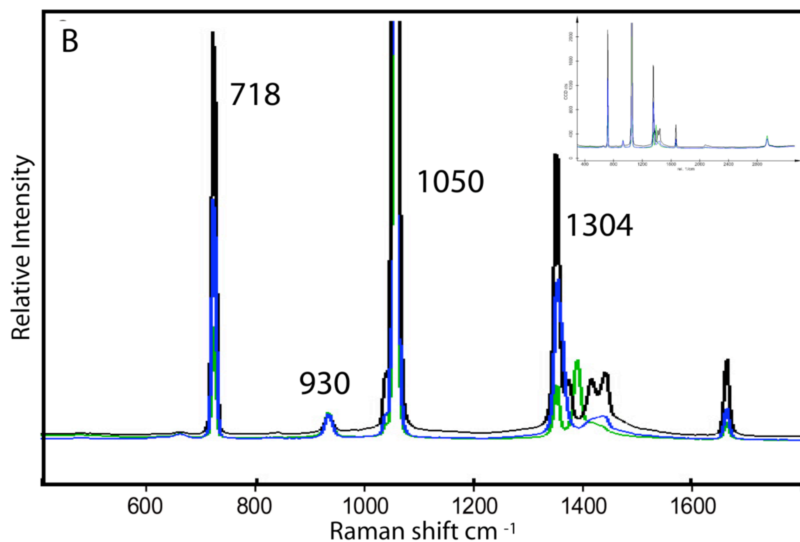


Figure S3. A. Raman spectra of purified mfp-1 and mfp-2 precipitated by raising pH to from 5 to 8.0 in the presence of Fe^{3+} at an iron-Dopa ratio of 1:2. The two spectra are essentially identical. Peaks denoted by * at 930 and 1050 cm^{-1} are buffer artifacts. B. Three Raman spectra of crystals formed upon drying the buffer 0.1M sodium acetate, 0.25M KNO_3, 1mM bis-tris (pH 5.5) on a glass slide. Inset is full scale.