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Comparison of adsorption behavior of two *Mytilus edulis* foot proteins on three surfaces

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Abstract

The sea mussel *Mytilus edulis* fabricates a hold-fast (an adhesive plaque) from a proteinaceous mixture that it extrudes into a cavity formed by an organ called the 'foot'. A family of four proteins in the mixture known as *M. edulis* foot proteins (Mefp 1–4) have been purified to homogeneity. Mefp-1 and 2 are the most well-characterized and most easily purified members of the Mefp family. They constitute about 5 and 25% of the content of the plaque, respectively. It has been proposed that Mefp-1 mediates bonding to the intended substratum while Mefp-2 serves more as a structural component. In order to provide data relevant to this hypothesis, the adsorption behavior of Mefp-1 and 2 was compared on three surfaces using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). Surfaces were germanium (Ge), polystyrene (PS) or poly(octadecyl)methacrylate (POMA). Polymer surfaces were prepared by spin casting onto the flat face of Ge trapezoidal internal reflection elements (IRE). Adsorption behavior was characterized by analyzing the kinetics of adsorption using a double exponential fit. The data indicate that the adsorption behavior of Mefp-1 and 2 is similar on the three surfaces both in terms of rate of adsorption and surface coverage attained over a short (<60 min) time period.

Keywords: Mussel adhesive protein; Mefp-1; Mefp-2; Adsorption; Germanium; Polystyrene; Poly(octadecyl)methacrylate

1. Introduction

The sea mussel, *Mytilus edulis*, tethers itself to surfaces by means of a set of collagenous threads each of which is anchored to the surface at the distal end by an adhesive plaque. The plaque is formed within minutes in the cavity of an organ known as the foot. The survival of the mussel depends on the security of byssal attachment which must withstand the considerable hydrodynamic forces exerted in intertidal zones. The sea mussel's ability to form this glue so rapidly in an aqueous environment has inspired both academic and more practical interest.

The plaque is composed primarily of proteinaceous material consisting of at least one collagen [1], at least one catalyst (catechol oxidase [2]) and a family of proteins known as *M. edulis* foot proteins (Mefp). The Mefp family is numbered roughly in the order in which the proteins were

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purified to homogeneity. All proteins in the Mefp family have positive isoelectric points and contain the unusual amino acid, L-3,4-dihydroxyphenylalanine (L-dopa). Of the four proteins in this family Mefp-1, the first to be purified and characterized [3], has received most attention. Mefp-1 consists primarily of tandem repeats of a decapeptide sequence. The motif is well-preserved in the species (relatively few residue substitutions are tolerated) and analogous motifs appear across species [4]. This suggests that Mefp-1 has a specialized function in plaque formation. Mefp-1 has an open [5], non-random [6] conformation that is thought to facilitate interaction of functional groups with the surface. Mefp-1 has a relatively high L-dopa content (11–18 mol.%). A number of energetic interactions are possible between L-dopa and various surface chemistries [7]. A patent has originated from the possible technological applications of the Mefp-1 deca-peptide sequence [8].

Mefp-2, the second protein in the Mefp family to be fully characterized, is about a third the size of Mefp-1 (42–47 kDa compared to 130 kDa) [9]. Whereas Mefp-1 constitutes about 5% of the protein content of the plaque, Mefp-2 makes up about 25%. Mefp-2 has a high density of cystine disulfide cross-links. The L-dopa content is about 2–3 mol.%. Analysis of the cDNA sequence of a closely related species indicates that Mefp-2 incorporates oligo-peptide motifs with sequence homology to epidermal growth factor [10].

It has been proposed that Mefp-1 acts as the component that mediates binding to the intended substratum and that Mefp-2 is a structural component of the adhesive plaque [9]. In order to provide data relevant to this hypothesis Mefp-1 and 2 adsorption behavior was characterized on three surfaces. The surfaces were germanium (Ge), polystyrene (PS) and poly (octadecyl methacrylate) (POMA). The general character of these surfaces is, respectively, oxide, aromatic and aliphatic. The kinetics of adsorption for short times (minutes) are relevant to adhesive plaque formation and thus the focus was on this aspect of the adsorption behavior.

2. Materials and methods

2.1. Materials

Mefp-1 was purchased from Bioscience Laboratory (Floda, Sweden). Mefp-2 was a gift from J. Herbert Waite (Marine Science Institute, University of California, Santa Barbara). Purity of proteins was checked by acetic acid–urea polyacrylamide gel electrophoresis [11]. Stock solutions of protein were made at 1 mg ml^{-1} in filter sterilized, de-aerated dilute HCl (pH 2.5) and stored at 5°C. Solutions for adsorption were in 10 mM sodium phosphate (pH 7.2). Water was ultra pure (Barnstead water purification system, Dubuque, IA). Ethyl alcohol and chloroform were HPLC grade. Isopropyl alcohol was analytical grade.

2.2. Flow chamber

The flow cell was described previously in Ref. [12]. It accommodates a Ge trapezoidal internal reflection element (Harrick Scientific, Ossingen, NY). The flow channel is approximately rectangular ($45 \times 10 \times 0.86$ mm) with entrance and exit ports at each end of the long dimension. The top and bottom walls of the flow channel are comprised of the IRE and a window for microscopic observation made from a coverslip. The thin walls are composed of a sandwich of Teflon™ and Viton™ spacers. Fluid was drawn through the flow chamber at 0.5 ml min^{-1} using a peristaltic pump (Cole-Parmer Instrument, Niles, IL) coupled to the silicone effluent tubing. All influent tubing and fittings were Teflon (0.08 cm ID) which were cleaned by sonicating in base bath. A Teflon valve (Cole-Parmer Instrument) channeled influent feed from among two vessels. All glassware was cleaned in base bath.

2.3. FTIR spectroscopy

The protocol was similar to one described in Ref. [13]. During the course of each experiment infrared spectra were acquired during 4.5 min intervals. A Nicolet 740 Fourier transform infrared spectrophotometer was used to collect the

spectra. Interferograms (100) were averaged per spectrum. Difference spectra were computed using the spectrum acquired immediately preceding introduction of the protein solution as the background.

When necessary water vapor bands were removed by subtraction of a pure water vapor spectrum. In some experiments, fluctuations in absorbance by water resulted in the appearance of the water band at 1640 cm^{-1} in the amide II region. This residual water absorption band was removed by subtracting out a pure buffer spectrum using the ratio of areas of the water absorption band centered at 2120 cm^{-1} as a normalization factor [14]. For experiments with thin films, a change in residual water bands was always accompanied by the appearance of residual bands from the polymer film in the difference spectra. The most typical reason for changes in interfacial water is the formation of an air bubble near the flow cell entrance during a portion of an adsorption experiment (likely resulting from a combination of coalescence of very small bubbles that are dislodged from the tubing, and decrease in the solubility of dissolved oxygen due to the temperature gradient between the ambient air and the spectrophotometer bench). In this case down-going bands from both water and the polymer film were evident. For experiments in which these interfering bands were too large, the data were not analyzed further. In cases in which these interfering bands appeared and were comparable to the size of the spectral features originating from the protein (amide II band) they were removed by subtracting from the difference spectra a pure spectrum of the polymer. This was accomplished by using the ratio of areas of a selected polymer band in the pure polymer spectrum and in the difference spectra to compute a normalization factor. The criterion was to render the area of the selected down-going polymer band zero in the difference spectra. Bands used for the normalization were centered at 1452 and 1730 cm^{-1} for PS and POMA, respectively. Removal of the residual water band as described above preceded removal of residual polymer bands.

2.4. Adsorption protocol

For adsorption of proteins a vial containing 1ml of the appropriate concentration in phosphate buffer was inserted into the flow system and the protein solution immediately pumped through a short (approximately 20 cm) section of leader tubing and through the flow chamber for 100 s. Flow was discontinued for 50 s while a vial containing buffer was inserted. Flow was then resumed for another 30 s. Adsorption of protein was done under stagnant conditions to conserve material.

2.5. Surface preparation

The protocol for cleaning Ge has been described in Ref. [13]. For spin casting polymers the IRE was fixed to a centrifuge rotor by a Teflon holder. It consists essentially of a block of Teflon with an appropriately sized cavity. The Ge surface was covered with toluene and spun while a total of 5 ml toluene was deposited on the surface and allowed to dry. With the rotor stationary the Ge surface was covered with a 10% (v/v) solution of phenyltrichlorosilane (Aldrich Chemical, Millwaukee, WI). (Without this organo-silane coupling layer the polymer films tend to peal off the Ge surface when exposed to water). After 2 min the rotor was spun for 30 s. The surface was then rinsed as above with 5 ml toluene. The surface was then covered with a 2% solution (m/v) of polymer solution (PS or POMA) in toluene and spun immediately until there was no change in coloration (approximately 1 min). The IRE was then placed at 100°C for 2 h.

2.6. Data analysis

Surface coverage of protein was estimated on the basis of a previously derived expression [15] using bovine serum albumin as a standard:

$$c_{B,E} = [(A_{W,T}/A_{W,E})(A_{S,E}/A_{S,T})]c_{B,T} \quad (1)$$

where $A_{W,T}$ is the absorbance of water (band at 1640 cm^{-1}) in transmission mode, $A_{W,E}$ is the absorbance of water probed evanescently (ATR mode), $A_{S,E}$ is the absorbance of the substance of

interest probed evanescently, $A_{S,T}$ is the absorbance of the substance in transmission mode, $c_{B,T}$ is the (known) concentration of the substance in the transmission cell and $c_{B,E}$ is the bulk or volume concentration of the substance probed evanescently.

Kinetic data curves were fit with a double exponential:

$$K + C_1(1 - \exp(k_1 t)) + C_2(1 - \exp(k_2 t)) \quad (2)$$

where t is time. Estimates of K, C_1, C_2, k_1, and k_2 were found by a least squares fit criterion. Kinetic curves were fit using TableCurve 2D (Jandel Scientific). In all cases curves were fit better by the double exponential than the analogous single exponential. This suggests that adsorption of both Mefp-1 and 2 is a two-step first order process on all three surfaces [16]. Use of Eq. (1) is semi-empirical here. The floating parameter, K, releases the constraint that the predicted curve pass through the origin and thus effects of the entrance kinetics that are appreciable during the first 5 min are ignored.

The kinetics are regulated by both diffusion into the interfacial region and the rate of adsorption. A rough estimate of the maximum time required for diffusion of Mefp-1 and 2 into the interfacial region is 12 min for Mefp-1 and 6 min for Mefp-2. This is the time for the solution phase concentration at the surface to reach 90% of the concentration of the bulk solution based on an estimate of the diffusion coefficients [17], an assumption of laminar flow during insertion of the plug of protein ($\mathrm{Re} = 0.88$), the maximum distance of the parabolic-shaped plug from the Ge surface (i.e. at the distal end of the flow cell cavity), and the expression describing time for diffusion through a boundary layer:

$$t_{90} = \gamma^2/D \quad (3)$$

where t_{90} is the time to reach 90% of the bulk concentration, γ is the boundary layer distance, and D is the diffusion coefficient.

Curve fits to spectra were generated by GRAMS/386 software (Galactic Industries) using mixed Guassian–Lorentzian component bands. The goal for fitting was to obtain the best estimate of the ratio of two spectral features (the amide II band and a sharp feature contained within the amide II region). The strategy that was found to be most effective was to subjectively choose centers of component bands on the basis of spectral features. The centers, amplitudes and widths were then adjusted to optimize the apparent randomness of the residual. The best (least squares) fit was then found by the program. The least number of component bands that appeared to give a satisfying fit were used. (4–9 bands were tried).

3. Results

3.1. Amide II region of Mefp-1 and 2 adsorbed onto Ge

Figs. 1 and 2 show the amide II region for adlayers of Mefp-1 and 2, respectively, adsorbed onto Ge. The most pronounced difference in the spectra (mid-infrared range) is the magnitude of the band centered at 1492 cm^{-1}. It was proposed previously that this band originated from the L-dopa and or tyrosine residues of Mefp-1 [13]. Qualitatively the relative size of this band (which appears only as a shoulder for Mefp-2) is consistent with this interpretation. In order to obtain a more precise estimate of the relative areas of this band for Mefp-1 and 2 the spectra were fit with component bands. Using areas of the component bands for the estimation, the ratio of the component band centered at 1492 cm^{-1} to the amide II band (proportional to total protein) is 0.1471 for Mefp-1 and 0.0133 for Mefp-2. Amino acid content of Mefp 1 and 2 (mol.%) yield a ratio of between 3.8 and 6.3 for L-dopa content (Mefp-1/Mefp-2) [9]. The ratio of the sum of L-dopa and tyrosine residues in Mefp-1 compared to Mefp-2 (mol.%) is between 1.7 and 3.0. After normalization to total protein, the ratio of the component bands centered at 1492 cm^{-1} for Mefp-1 to 2 is 11 ($0.1471/0.0133 = 11$). This suggests that the component band centered at 1492 cm^{-1} originates entirely from L-dopa residues. L-Dopa residues are likely to participate in different intramolecular interactions in Mefp-1 and 2. This may account for the lack of complete correspondence

between ratios based on amino acid content and component band areas.

3.2. Repetitions of adsorption experiment for Mefp-1 on Ge

Fig. 3 shows kinetic data curves for three repetitions of adsorption of Mefp-1 onto Ge. Parameters values obtained by the fit of the model (Eq. (2)) are listed in Table 1. Two types of estimations of the error in the measurements appear in Table 1. First, there is the S.E. in the parameter calculated by the fitting program. Secondly, there is the S.E. of the mean of the parameter fits for three repetitions of the experiment. According to both estimates of error, the estimates for k_1, the rate constant for the more rapid adsorption process, and Γ_1, the projected adsorbed amount for the more rapid adsorption process, are relatively reliable (S.E. $< 10\%$ mean). The S.E. for parameters corresponding to the slower adsorption process (C_2 and k_2) are in some cases larger than the mean. This is a consequence of the time period of measurement of adsorption (60 min); The time constant (τ) is 170–420 min for the slower process. For simplicity, subsequent presentation is confined to the more reliably estimated parameters (k_1, Γ_1). The more rapid adsorption process ($\tau = 5$–7 min) is the relevant one for reactions responsible for adhesive plaque formation which occurs in approximately 2 min. The projected surface coverage for both adsorption processes (Γ) is included in the tables below to give an idea of the relative importance of the slower process.

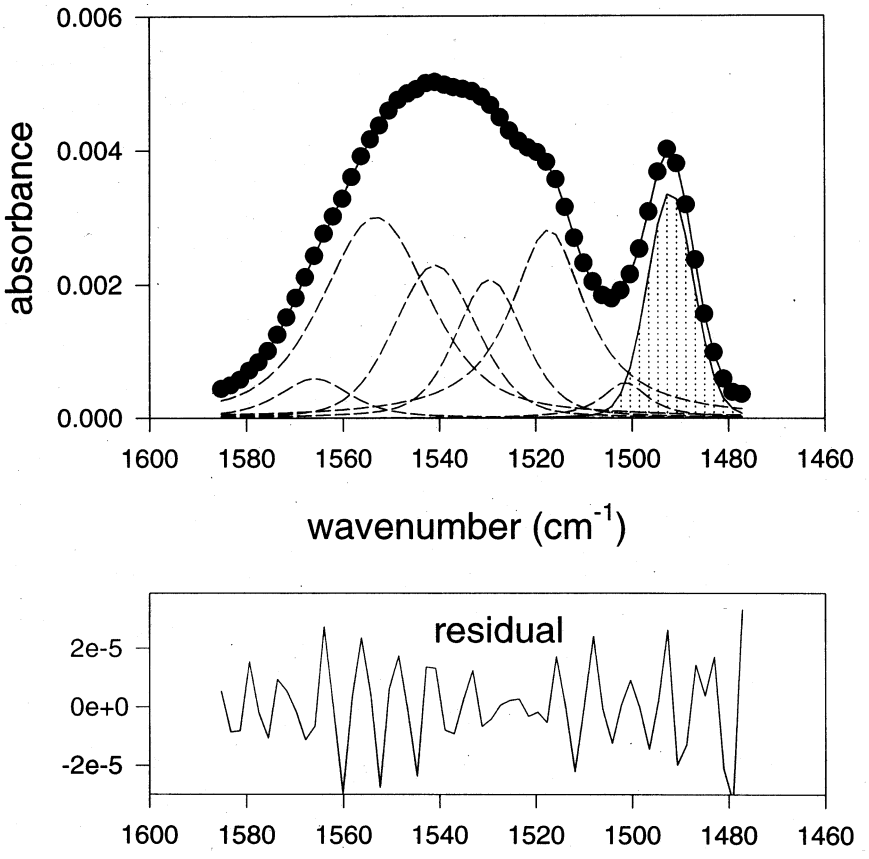

Fig. 1. Amide II region of Mefp-1 and decomposition into component bands. Data (closed circles); fit solid line; component bands (broken lines); cross-hatched area is a putative L-dopa band. The residual is below.

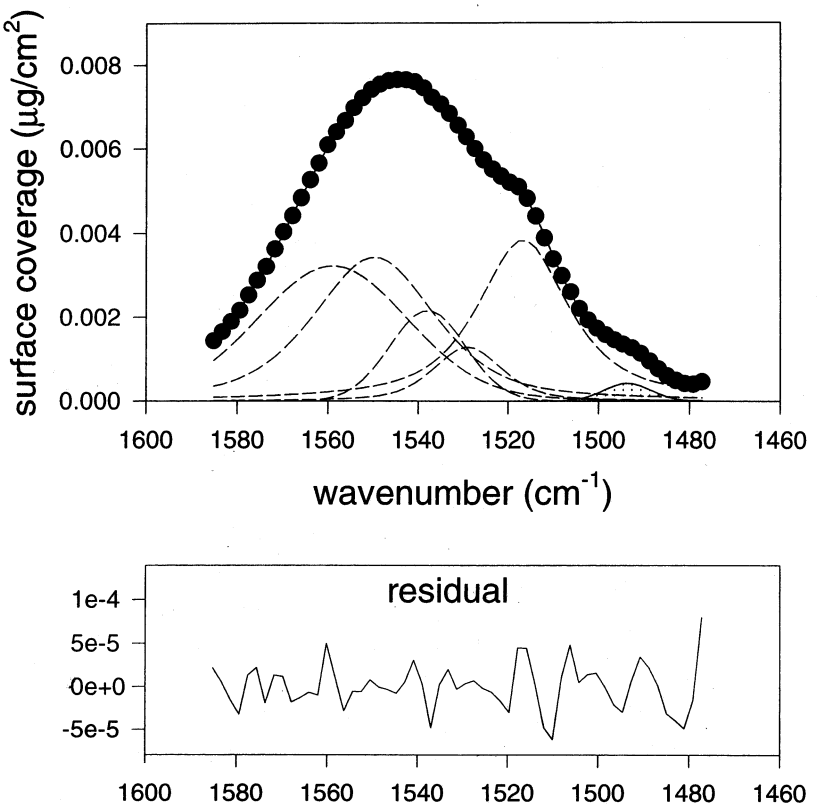

Fig. 2. Amide II region of Mefp-2 and decomposition into component bands. Data (closed circles); fit solid line; component bands (broken lines); cross-hatched area is a putative L-dopa band. The residual is below.

The parameter K relieves the constraint that the model pass through the origin. The parameter t_0 is the time at which the predicted values of the model (Eq. (2)) become positive. Possible complex kinetics associated with the combined effects of flow and diffusion before this time (approximately 5 min) are ignored by treating this time point essentially as time zero.

3.3. Adsorption behavior of Mefp-1 and 2 onto Ge, PS and POMA

Figs. 4 and 5 present kinetic data curves and corresponding fits for Mefp-1 and 2 on Ge, PS and POMA. Tables 2 and 3 list parameter values obtained by the model fit (Eq. (2)). Data for Mefp-1 onto Ge are presented as means and S.E. for the three repetitions. Trends in these data curves are discussed in Section 4.

4. Discussion

The mussel can attach to surfaces having a wide range of surface properties and fabricates its versatile adhesive with impressive efficiency [7]. Characterizing the entire set of interactions responsible for plaque formation is a formidable task. At a molecular level, it is not clear at this point which proteins combine, or whether proteins have specialized roles in the condensation process. Knowledge of the functional group interaction involved in cross-linking is sketchy [18]. Focusing on the interfacial region provides an approach for isolating a portion of the complex process for study. It is essential to the mussel's attachment process, and thus its survival, that components of the plaque matrix bind rapidly and tenaciously to the intended substratum. Adsorption behavior of plaque components

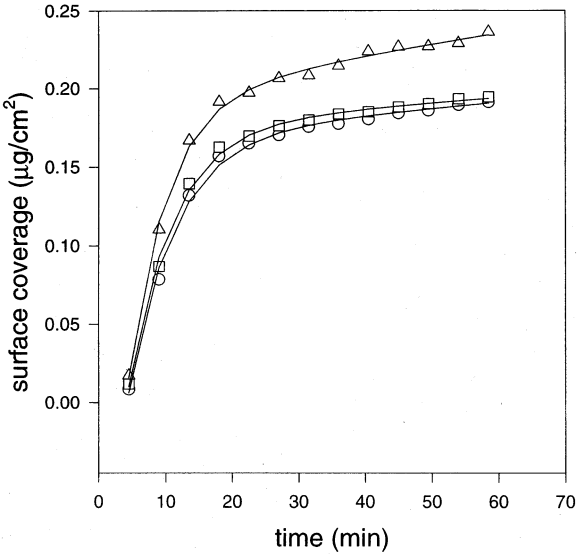

Fig. 3. Kinetic data curves for adsorption of Mefp-1 onto Ge for three repetitions of the experiment (open symbols) and model fit (solid line).

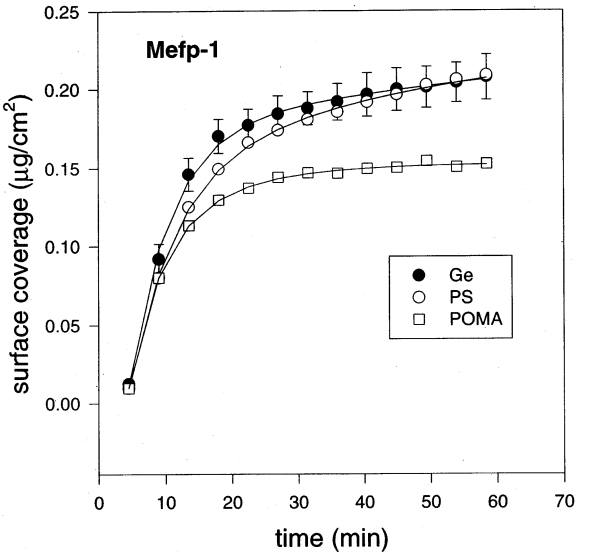

Fig. 4. Kinetic data curves for adsorption of Mefp-1 onto Ge, PS and POMA. Means and S.E. for three experiments are shown for Mefp-1 onto Ge.

yields information that is directly related to this function.

The main conclusion drawn from data presented in Figs. 4 and 5 and Tables 2 and 3 is that there are no pronounced differences between either the rate of adsorption or the surface coverage attained over short (<60 min) time periods for Mefp-1 and 2. According to these criteria Mefp-1 and 2 are ranked about equal in terms of being the putative agent (primer) that mediates bonding to the intended substratum. In fact, despite the speculation that Mefp-1 has special intrinsically adhesive properties, by every measure except long term adsorption (Γ) Mefp-2 adsorbs more effec-

Table 1
Parameter fits for three repetitions: Mefp- 1 on Ge

Exp[a]	C_S[b]	C_1[c]	k_1[c]	C_2[c]	k_2[c]	K[c]	t_0[d]	Γ[e]	Γ_1[f]	r^2
1	0.2128	0.3416	0.1344	0.1935	0.0024	−0.01605	4.32	0.3746	0.1811	0.9963
		(0.0239)[g]	(0.0239)	(4.003)	(0.0580)	(0.0257)		(4.053)	(0.0496)	
2	0.1951	0.3237	0.1271	0.1118	0.0031	−0.1521	4.17	0.2835	0.1717	0.9974
		(0.0219)	(0.0216)	(2.078)	(0.0697)	(0.0232)		(2.123)	(0.0452)	
3	0.2361	0.3849	0.1621	0.1742	0.0057	−0.1980	4.12	0.3611	0.1869	0.9976
		(0.0219)	(0.0260)	(0.9094)	(0.0348)	(0.0318)		(0.9611)	(0.0537)	
Mean	0.2148	0.3501	0.1412	0.1598	0.0035	−0.1702	4.20	0.3397	0.1799	
S.E.[h]	0.0120	0.0182	0.0107	0.0246	0.0008	0.0141	0.06	0.0284	0.0044	

[a] Experiment number.
[b] Surface coverage (μg cm^{-2}) at end of rinse period.
[c] Parameter (see Eq. (2)).
[d] Time zero for fit.
[e] Surface coverage (μg cm^{-2}) at infinite adsorption time predicted by fit to Eq. (2) (C_1+C_2+K).
[f] Surface coverage (μg cm^{-2}) at infinite adsorption time predicted by fit to Eq. (2) for more rapid process only (C_1+K).
[g] S.E. for fit.
[h] S.E. for three repetitions.

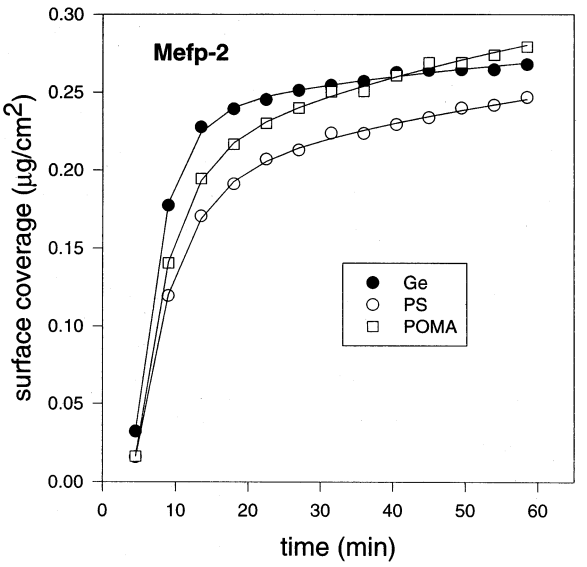

Fig. 5. Kinetic data curves for adsorption of Mefp-2 onto Ge, PS and POMA.

tively (faster and reaching greater surface coverage) than Mefp-1. The greater rate of Mefp-2 adsorption may be due to its smaller size and, thus, greater diffusion coefficient.

The similarity between adsorption behavior of Mefp-1 and 2 onto surfaces having quite different properties (oxide, aromatic, aliphatic) is unexpected. Although the primary structures have in common incorporation of the unusual amino acid, L-dopa, and both exhibit oligo-peptide repeats, the resemblance essentially ends there. The L-dopa content of Mefp-1 is about five times that of Mefp-2. In addition, whereas Mefp-1 is an extended chain, Mefp-2 can be described more as a stiff, highly cross-linked rod. They both have quite positive isoelectric points due to the high lysine contents (approximately 21 and 13 mol.% for Mefp-1 and 2, respectively, [9]). This can explain their affinity for the oxide surface (Ge) and perhaps for the aromatic surface (PS) [19]. Hydrophobic interactions are thought to drive adsorption of globular proteins [20–22]. Part of this driving force (free energy change) is attributed to conformational rearrangements (protein denaturation upon adsorption). The POMA surface is the most hydrophobic of the three surfaces tested [23]. Although hydrophobic interactions may be the driving force behind Mefp1 and 2 adsorption onto POMA, it seems unlikely that either experiences conformational rearrangements upon adsorption comparable to those experienced by globular proteins. The most pronounced difference between the kinetic data curves presented in Figs. 4 and 5 is the lowered surface coverage of Mefp-1 on POMA. One possible explanation is that specific interactions between the lysines and/or L-dopa residues of Mefp-1 and the Ge and PS surfaces (absent for the POMA surface) contribute to the binding site density [24].

Table 2
Parameter fits for Mefp-1

Surface	C_s[a]	k_1[b]	τ[c]	Γ_1[d]	Γ[e]	r^2
Ge[f]	0.2148	0.1412	7.1	0.1799	0.3397	
	(0.0128)	(0.0107)		(0.0042)	(0.0284)	
PS	0.2047	0.1386	7.2	0.1459	0.2484	0.9990
		(0.0201)[g]		(0.0357)	(0.0725)	
POMA	0.1524	0.1995	5.0	0.0876	0.1530	0.9992
		(0.0495)		(0.0765)	(0.1473)	

[a] Surface coverage (μg cm^{-2}) at end of rinse period.
[b] First order rate constant (min^{-1}).
[c] Time constant ($1/k_1$) (min).
[d] Surface coverage (μg cm^{-2}) at infinite adsorption time predicted by fit to Eq. (2) for more rapid process only (C_1+K).
[e] Surface coverage (μg cm^{-2}) at infinite adsorption time predicted by fit to Eq. (2) (C_1+C_2+K).
[f] Mean and S.D. (in parentheses) for three experiments.
[g] S.E. for fit.

Table 3
Parameter fits for Mefp-2

Surface	C_s[a]	k_1[b]	τ[c]	Γ_1[d]	Γ[e]	r^2
Ge[f]	0.2781	0.2726 (0.0155)	3.7	0.2292 (0.0690)	0.2947 (0.0926)	0.9993
PS	0.2646	0.1884 (0.0136)	5.3	0.1806 (0.0278)	0.3110 (0.0719)	0.9994
POMA	0.2864	0.2142 (0.0154)[g]	4.7	0.1961 (0.0430)	0.3725 (0.1165)	0.9993

[a] Surface coverage (μg cm^{-2}) at end of rinse period.
[b] First order rate constant (min^{-1}).
[c] Time constant ($1/k_1$) (min).
[d] Surface coverage (μg cm^{-2}) at infinite adsorption time predicted by fit to Eq. (2) for more rapid process only (C_1+K).
[e] Surface coverage (μg cm^{-2}) at infinite adsorption time predicted by fit to Eq. (2) (C_1+C_2+K).
[f] Mean and S.D. (in parentheses) for three experiments.
[g] S.E. for fit.

The patterns of order exhibited by the primary structures of the Mefp family of proteins suggest that the proteins have specialized roles in a self-assembly process that results in the well-bonded, highly condensed adhesive plaque. If there exists a specialized primer it must not only adsorb rapidly and tenaciously to surfaces having a variety of properties, but must also induce binding of components that are structural members of the plaque. Thus it must be endowed with a bi-functionality, having functional groups that bind to the surface and others that are left free to bind to solution phase components. It has been suggested that another protein in the Mefp family (Mefp-3) has a 'molecular asymmetry' that is consistent with the role of primer [25]

The methodology presented here can be used to further characterize interactions between the Mefp family of proteins on a surface. The influence of a catalyst (e.g. catechol oxidase) can be tested. The ability of a member of the family to induce adsorption of other plaque components (pertinent to primer function) can be probed. It may be possible to use distinctive spectral features of the Mefp family members (e.g. the relative magnitude of the putative L-dopa band (Figs. 1 and 2)) to determine relative densities of Mefp proteins in mixed adlayers, thus making it feasible to do competition studies without labeling the proteins.

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