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A mussel-derived one component adhesive coacervate ☆

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ABSTRACT

Marine organisms process and deliver many of their underwater coatings and adhesives as complex fluids. In marine mussels one such fluid, secreted during the formation of adhesive plaques, consists of a concentrated colloidal suspension of a mussel foot protein (mfp) known as Mfp-3S. The results of this study suggest that Mfp-3S becomes a complex fluid by a liquid–liquid phase separation from equilibrium solution at a pH and ionic strength reminiscent of the conditions created by the mussel foot during plaque formation. The pH dependence of phase separation and its sensitivity indicate that inter-/intramolecular electrostatic interactions are partially responsible for driving the phase separation. Hydrophobic interactions between the non- polar Mfp-3S proteins provide another important driving force for coacervation. As complex coacervation typically results from charge–charge interactions between polyanions and polycations, Mfp-3S is thus unique in being the only known protein that coacervates with itself. The Mfp-3S coacervate was shown to have an effective interfacial energy of $\leqslant 1$ mJ m^{-2}, which explains its tendency to spread over or engulf most surfaces. Of particular interest to biomedical applications is the extremely high adsorption capacity of coacervated Mfp-3S on hydroxyapatite.

1. Introduction

One of the most fascinating aspects of the underwater adhesion of marine organisms such as mussels and sandcastle worms is the reliance on metastable, water-insoluble fluids that resist being dispersed in the surrounding seawater. In mussels these adhesive fluids consist of highly concentrated, intrinsically unstructured polyelectrolytes known as mussel foot proteins (mfps) that rapidly solidify upon equilibration with seawater. In sandcastle worm cement, given the presence of both polyanions (polyphosphoserine-rich protein) and polycations (lysine-rich proteins), fluid–fluid phase separation is modelled as complex coacervation leading to a polyelectrolyte-depleted equilibrium phase and a denser, protein-rich coacervate phase [1,2]. Complex coacervation results from the coulombic attraction and neutralization of oppositely charged polyelectrolytes coupled with the concomitant release of counterions [3] and confers unusual properties on the coacervate phase, including relatively high diffusion coefficients of the solute and solvent molecules, high concentrations, relatively low viscosity, and a low interfacial energy, all highly conducive to dispensing adhesives underwater [4–8]. Coacervates are used industrially in micro-encapsulation technology [9,10], and are particularly important in food processing, as well as drug and gene delivery [11–15]. Hydrogel formation can also be mediated by coacervation [16].

Polyanions are not known to be involved in mussel adhesion, thus the basis for fluid–fluid phase separation by mfps remains unknown. In this report we show that Mfp-3S (Fig. 1), a zwitterionic protein functioning as both adhesive primer and sealant in mussel adhesion [17], undergoes fluid–fluid phase separation under conditions identical to those imposed by the mussel foot during plaque formation. The outstanding interfacial adhesive and cohesive properties of Mfp-3S over a relatively wide pH range have been previously demonstrated using a surface forces apparatus (SFA) [17], and attributed to its abundant 3,4-dihydroxyphenylalanine (dopa) content and unique hydrophobic sequence. The strategy of achieving efficient phase separation and surface spreading by coacervation is very appealing in its simplicity, in part because it is only rarely observed in single protein solutions: only tropoelastin is known to undergo simple hydrophobically driven coacervation [18,19]. Mfp-3S provides an interesting counterpoint for

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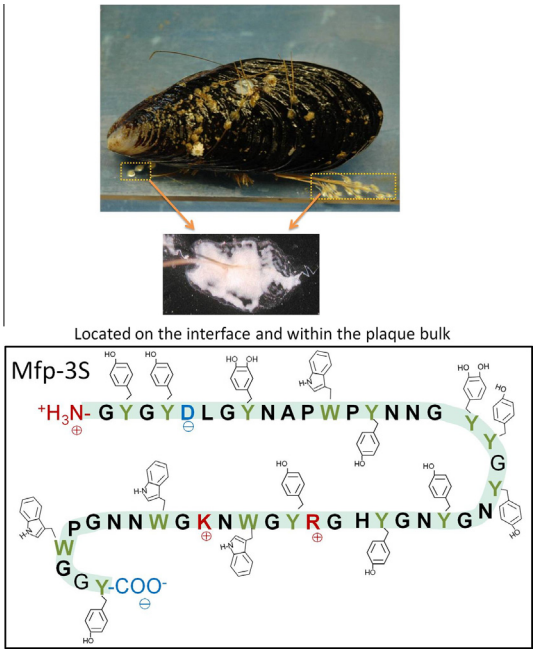

Fig. 1. A representative sequence of Mfp-3S from mussel plaque. Red, blue and green indicate positively charged, negatively charged and aromatic residues, respectively.

understanding the molecular requirements for single component coacervation. Based on how pH, ionic strength, and temperature affect Mfp-3S coacervation, we propose that the electrostatic and hydrophobic driving forces are uniquely balanced in the observed fluid–fluid phase separation of Mfp-3S. These forces will, of course, be subjected to much greater scrutiny as synthetic and recombinant mimics become more readily available. The relevance of coacervates to orthopaedic and dental materials was explored by investigating the adsorption of Mfp-3S coacervates on hydroxyapatite (HAP) ($Ca_{10}(PO_4)_6(OH)_2$,) surfaces.

2. Experimental section

2.1. Mfp-3S purification

Mfp-3S was purified from the plaques of California mussels, *Mytilus californianus*, as described elsewhere [20]. About 1000 accumulated plaques were thawed and homogenized in a small volume (5 ml per 200 plaques) of 5 vol.% acetic acid containing 8 M urea on ice using a small hand-held tissue grinder (Kontes, Vineland, NJ). The homogenate was centrifuged for 30 min at 20,000*g* and 4 °C. The soluble acetic acid/urea plaque extracts were subjected to reverse phase HPLC using a 260 × 7 mm RP-300 Aquapore column (Applied Biosciences Inc., Foster City, CA), eluted with a linear gradient of aqueous acetonitrile. The eluant was monitored continuously at 230 and 280 nm, and 1 ml fractions containing Mfp-3S were pooled and freeze-dried, injected into a Shodex-803 column (5 μm, 8 × 300 mm), which was equilibrated and eluted with 5% acetic acid in 0.1% trifluoroacetic acid. The eluant was monitored at 280 nm. Sample purity was assessed by acid urea PAGE, amino acid analysis, and MALDI time-of-flight mass spectrometry. Fractions with pure Mfp-3S were freeze-dried and redissolved in buffers for further studies. About 3 mg of Mfp-3S can be purified from 1000 freshly (within 24 h) secreted plaques.

2.2. Zeta potential

The zeta potentials of Mfp-3S in solution (~0.1 mg ml^{-1}) were obtained using a Malvern Nano ZS which is calibrated regularly using a Malvern Zeta Potential Transfer standard (P/N DTS1230, batch no. 380901). The zeta potentials of Mfp-3S were +23, +6, and −0.8 mV, respectively, in pH 5.5, 6.5 and 7.5 buffer at 100 mM ionic strength.

2.3. Mfp-3S self-coacervation and turbidity measurement

Stock solutions of 1 mg ml^{-1} Mfp-3S were prepared in 10 mM acetic acid buffer (pH 3). The final protein concentration was fixed at 0.1 mg ml^{-1} by adding stock solution to buffer at a volume ratio of 1:9 (stock:buffer). Coacervation of Mfp-3S at different buffer conditions was measured turbidimetrically at 600 nm by UV-vis spectrophotometry. Mfp-3S absorbance was negligible at 600 nm. The relative turbidity is defined as $\ln(T/T_0)$, where T and T_0 are the light transmittance with and without sample, respectively [21].

2.4. Microscopy

The turbidity associated with coacervate droplet formation was visually inspected by inverted light microscopy. The protein distribution was also investigated using an Olympus model IX81 DSU fluorescence microscope (Olympus, Tokyo, Japan). Images were taken with an ImagEM camera (C9100-13, Hamamatsu, Shizuoka, Japan) under the control of MetaMorph software (Olympus). The desired excitation and emission wavelengths were obtained using a mercury bulb combined with a 89000 Sedat Quad Filter Set (Chroma Tech. Corp.).

2.5. Quantification of adsorbed coacervate by amino acid analysis

A scaled-up version of a microscopic slide with coverslip was made using two rectangular glass slides as follows: Double-sided tape was used to stick two slides together along their margins. A 50–100 μl volume of Mfp-3S coacervate (0.1 mg ml^{-1}) was then injected into the gap and left undisturbed for 1 h to let the coacervate be adsorbed or settle on the glass surfaces. The upper and lower glass slides were then separated and broken into pieces to fit in 1 ml hydrolysis vials. 100 μl of 2 M HCl and 5 μl of phenol were added to the vials containing the glass samples, which were then vacuum sealed, followed by hydrolysis at 158 °C for 1 h. After hydrolysis the solutions were washed twice with water and then twice more with methanol via flash evaporation. The hydrolyzed products were dissolved in 0.02 M HCl and routine amino acid analysis carried out in a Hitachi L-8900 amino acid analyzer.

2.6. Adhesion measurement using a Surface Forces Apparatus (SFA)

The adhesion of Mfp-3S coacervate on mica was measured using a SFA. The details of the SFA technique have been described elsewhere [22]. Coacervate deposition was carried out by placing 40 μl of Mfp-3S coacervate (0.1 mg ml^{-1}) between two mica surfaces. After 1 h settlement and absorption the bottom mica surface were brought in contact with the upper one, and further compressed for another 1 min before separation All experiments were performed at room temperature, thermostatted at 22 °C.

2.7. Quartz crystal microbalance dissipation (QCM-D)

Gold sensors were purchased from BiolinScientific (QSX301) and cleaned according to the protocol suggested before use. QCM-D experiments were carried out in a Q-Sense E4 system using two flow modules in parallel. Samples were introduced into the