Colloids and Surfaces B: Biointerfaces 164 (2018) 98–106

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Formation and elasticity of membranes of the class II hydrophobin *Cerato-ulmin* at oil-water interfaces

Xujun Zhang[a,e], Stephanie M. Kirby[b], Yuwu Chen[c], Shelley L. Anna[b], Lynn M. Walker[b], Francisco R. Hung[c,d], Paul S. Russo[a,e,f,*]

[a] School of Materials Science and Engineering, Georgia Institute of Technology, Atlanta, GA 30332-0245, United States
[b] Department of Chemical Engineering, Carnegie Mellon University, Pittsburgh, PA 15213, United States
[c] Cain Department of Chemical Engineering, Louisiana State University, Baton Rouge, LA 70803-1804, United States
[d] Department of Chemical Engineering, Northeastern University, Boston, MA 02115, United States
[e] Georgia Tech Polymer Network, GTPN, Georgia Institute of Technology, Atlanta, GA 30332, United States
[f] School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, United States

ARTICLE INFO

Article history:
Received 26 September 2017
Received in revised form
29 December 2017
Accepted 15 January 2018

Keywords:
Hydrophobin
Cerato-ulmin
Biomembrane
Protein surfactant
Dilatational modulus

ABSTRACT

Protein surfactants show great potential to stabilize foams, bubbles, and emulsions. An important family of surface active proteins, the hydrophobins, is secreted by filamentous fungi. Two hydrophobin classes have been recognized, with Class II exhibiting slightly better solubility than Class I, although neither is very soluble in water. Hydrophobins are small proteins (8–14 kDa), but they are larger and more rigid than typical surfactants such as sodium dodecyl sulfate. This rigidity seems to be manifested in the strength of adsorbed hydrophobin layers on oil droplets or air bubbles. A particular Class II hydrophobin, *Cerato-ulmin*, was characterized at the oil-water interface (the oil was squalane). The results are compared to measurements at the air-water interface, newly extended to lower *Cerato-ulmin* concentrations. For both oil-water and air-water interfaces, static and dynamic properties were measured during the evolution of the membrane structure. The static measurements reveal that dilute *Cerato-ulmin* solution efficiently decreases the interfacial tension, whether at oil-water or air-water interfaces. The reduction in surface tension requires several hours. Interfacial mechanics were characterized too, and the dilatational modulus was found to reach large values at both types of interfaces: 339 ± 19 mN/m at the squalane-water interface and at least 764 ± 45 mN/m at the air-water interface. Both values well exceed those typical of small-molecule surfactants, but come closer to those expected of particulate-loaded interfaces. Circular dichroism provides some insight to adsorption-induced molecular rearrangements, which seem to be more prevalent at the oil-water interface than at the air-water interface.

1. Introduction

Filamentous fungi secrete small proteins (typically 8–14 kDa) known as hydrophobins [1–5]. About 70 different hydrophobins have been identified [5]. All contain eight cysteine residues [1], which form four intramolecular disulfide bridges in a conserved manner. The second and third cysteine residues follow each other immediately in sequence to create a pair [6]. The sixth and seventh cysteine residues also form a pair. The two pairs of disulfide bridges create four loops connected by three strands. This allows the protein to have a globular secondary structure that does not easily denature. All hydrophobins exhibit strong surface activity [2]. One nominally flat face of the globular protein is coated with aliphatic amino acids occupying 20% of the surface area [7], whereas the other face presents hydrophilic amino acid groups. The reason fungi produce these amphipathic molecules has been the subject of much speculation [3,8–10]. It is thought that hydrophobins play a specific role in the formation of aerial structures that help fungi grow to penetrate the solid substrates on which they feed, and during cell differentiation [2,3,8–10]. These processes expand the surface area of the organism, and could benefit from reduced surface energy.

Hydrophobins are divided into two classes depending on their solubility characteristics. Class I hydrophobins, such as SC3 from the common fungus *Schizophyllum* commune [11] and ABH1 from *Agaricus bisporus* [12], form insoluble aggregates that can only be dissolved in trifluoroacetic acid, formic acid, or other strong acids.

* Corresponding author at: School of Materials Science & Engineering/Georgia Tech/801 Ferst Dr NW/MRDC 3508/Atlanta Georgia, 30332-0245, United States.
E-mail address: paul.russo@mse.gatech.edu (P.S. Russo).

https://doi.org/10.1016/j.colsurfb.2018.01.017
0927-7765/

In contrast, the assemblies of Class II hydrophobins are readily dispersed by agents like ethanol or sodium dodecyl sulfate (SDS), as well as by pressure or cooling [13].

Hydrophobins have potential applications as surface coating agents [14,15], stabilizers for foams and emulsions [16–18], carriers of water-insoluble drugs [19,20], and dispersants [21,22]. They can change hydrophobic surfaces to hydrophilic and vice versa [1]. These large alterations in water contact angle are driven by surface activity. The Class I hydrophobin SC3 lowers the surface tension of water from 72 mN/m to 24 mN/m at a concentration of only 50 μg/mL [9]. Lumsdon et al. measured the surface tension of the Class II hydrophobin HFB II from *Trichoderma reesei* at the air-water interface and found an equilibrium value of 45 mN/m at 200 μg/mL [23]. Another observation related to surface activity is the ability of hydrophobins to stabilize air bubbles [13,24] or foams [18] because of membrane formation. Measurements of the interfacial moduli can be used to quantify the surface elasticity and viscosity of the hydrophobin film. Values up to 500 mN/m have been reported for surface elasticity [25], which is about an order of magnitude higher than the surface elasticity observed for other surface-active proteins such as bovine serum album, BSA [15].

This report concerns a particular hydrophobin, *Cerato-ulmin* or CU. It was discovered and isolated [26] from *Ceratocystis ulmi* in 1973, before the term hydrophobin was coined [27] by Wessel et al., who described the excreted fungal proteins from *Schizophyllum commune* [8]. Due to its reported phytotoxicity, particularly towards elm trees, CU was extensively characterized by Richards and Takai et al. [26,28–34] of the Canadian Forest Service. Microstructures, such as rods, fibrils, and membranes, were seen when the liquid culture was purified by a bubbling technique.[28] Other hydrophobins exhibit unusual structures, too, but those displayed by CU are spectacular; for example, air-filled cylinders with axial ratios (length:width) of up to 35 have been observed [28], as have oil-filled cylinders (length:width ratio of 5) [35]. Despite early studies on the solubility of CU and the stability of its oddly shaped membranes [13,35], little quantitative research had been conducted to understand the adsorption behavior of CU at interfaces.

We recently reported how CU behaves at the air-water interface [36]. Given sufficient time to develop, CU forms solid-like films with very high dilatational moduli of at least 500 mN/m, accompanied by surface pressures of approximately 22 mN/m at 2 μg/mL bulk CU concentration. The CU films were irreversibly adsorbed (stable towards rinsing with water). Sequential addition of a competing surfactant, sodium dodecyl sulfate (SDS), further increased the surface pressure while reducing the dilatational modulus. Only at high SDS concentrations was CU removed from the interface. That analysis, on samples of limited availability, was made possible using a microtensiometer platform [37]. This fully vetted technique is based on capillary tenisometry and has been used to characterize the dynamics and transport of small molecule surfactants [37–40] as well as the irreversible adsorption and interfacial mechanics of polymeric surfactants [41–43], polymer grafted nanoparticles [44], colloidal particles [45,46], and proteins [36].

Here we report the surface activity and dilatational moduli of CU at oil-water interfaces using the same equipment. We are motivated by interest in the interaction of hydrophobins with oil in water. Knowledge of the biomembrane's properties is germane to development of environmentally friendly dispersants for oil spill cleanup, and hydrophobins have been interacting with oils from natural seepage for eons. Additionally, the interaction of hydrophobins with organic molecules offers new opportunities in materials science. For example, CU membranes have been used to stabilize latex-like aqueous dispersions of semiconducting polymer solutions in organic solvents [21]. The oil we chose is squalane (2,6,10,15,19,23-hexamethyltetracosane), which is a branched alkane with a melting point (−38 °C) well below the temperature range of interest for potential CU applications and with no measurable partitioning into water. We characterized the progression and reversibility of membrane formation in the absence of competing surfactants using dynamic and static measurements. Switching to mechanical (oscillatory) measurements, CU membranes were observed to grow quite strong, whether at oil-water or air-water interfaces.

The organization of the remainder of the paper is as follows. After describing the experimental methods, we update our knowledge of CU at the air-water interfaces. Then the main topic, the behavior of CU at oil-water interfaces, is addressed. These observations suggest circular dichroism (CD) measurements to probe CU's secondary structure, especially changes that may be induced by surface adsorption.

2. Materials and methods

Cerato-ulmin was a gift from Dr. Wayne Richards of the Canadian Forest Service. It was produced by an aggressive strain of *C. ulmi* (RDT2) and purified by the methods of Takai and Richards [29] and Stevenson et al. [30]. The purified sample was stored in a sealed vial and placed in a jar filled with Drierite® ($CaSO_4$) at ambient temperature and pressure. The water used to prepare CU aqueous stock solutions was supplied by a Barnstead Nanopure® purification system. A stock solution of CU was prepared at a concentration of 20 μg/mL. As a simple confirmation of potency, exposing glass and poly(tetrafluroroethylene) (Teflon) surfaces to CU, even at this low concentration, altered the water contact angle (See Supporting Information, Fig. S4).

Squalane was purified by gravity filtration through a glass column packed with 1.5 g of basic alumina. The filter medium was held in place with a slug of 400 μm silica beads purchased from OPS Diagnostics, LLC, Lebanon, NJ [41].

The surface tension measurements were carried out using a microtensiometer design based on a capillary tensiometer (Fig. 1). Because the apparatus has been described previously [37], here a brief summary will suffice. Bulk solution is placed into the ~3 mL solution well (A) which is fabricated from cross-linked polydimethylsiloxane (PDMS). The solution chamber is open at the top and sealed by a No. 1 cover slip at the bottom. A glass capillary (E) is inserted from one side of the chamber and connected with a pressure transducer (B) through polyethylene tubing. The capillary is extended to make a 35–38 μm tip using a capillary puller (Micro Data Instrument Inc., South Plainfield, NJ). An air bubble or oil drop is formed at the end of the capillary tip using a constant pressure head (C). The pressure is generated using a column of water attached to a three-way solenoid valve. An inverted light microscope and a camera are used to image the bubble or drop. The images are captured using a LabVIEW code. The radius of the bubble or drop is analyzed in real time by fitting a circle to images and extracting the radius, $R(t)$, of the spherical cap. The surface tension $\gamma(t)$ is calculated from the Laplace equation,

$$\gamma(t) = \frac{R(t)(P(t) - P_H)}{2} \quad (1)$$

where $P(t)$ is the pressure behind the fluid slug inside the capillary and P_H is the hydrostatic pressure of the aqueous solution at the capillary. The difference $P(t) - P_H$ is then the pressure jump across the hemispherical cap trapped at the end of the capillary. Rinsing the bulk solution with deionized water is achieved via the dilution/exchange bath valve (D) and peristaltic pump. The residence time of the reservoir is on the order of tens of seconds.

To determine the dilatational modulus, a reciprocating pump generates periodic pressure fluctuations to expand and contract a single bubble or drop. A low-amplitude pressure (400 Pa) oscilla-