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Hydrophobins as aqueous lubricant additive for a soft sliding contact

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Abstract

Two type II fungal hydrophobins, HFBI and FpHYD5, have been studied as aqueous lubricant additive at a nonpolar, compliant sliding contact (self-mated poly(dimethylsiloxane) (PDMS) contact) at two different concentrations, 0.1 mg/mL and 1.0 mg/mL. The two hydrophobins are featured as non-glycosylated (HFBI, m.w. ca. 7 kDa) vs glycosylated (FpHYD5, m.w. ca. 10 kDa) proteins. Far UV CD spectra of the two hydrophobins were very similar, suggesting overall structural similarity, but showed a noticeable difference according to the concentration. This is proposed to be related to the formation of multimers at 1.0 mg/mL. Despite ten-fold difference in the bulk concentration, the adsorbed masses of the hydrophobins onto PDMS surface obtained from the two solutions (0.1 and 1.0 mg/mL) were nearly identical, suggesting that a monolayer of the hydrophobins are formed from 0.1 mg/mL solution. PDMS-PDMS sliding interface was effectively lubricated by the hydrophobin solutions, and showed a reduction in the coefficient of friction by as much as ca. two orders of magnitude. Higher concentration solution (1.0 mg/mL) provided a superior lubrication, particularly in low-speed regime, where boundary lubrication characteristic is dominant via ‘self-healing’ mechanism. FpHYD5 revealed a better lubrication than HFBI presumably due to the presence of glycans and improved hydration of the sliding interface. Two type II hydrophobins function more favorably compared to a synthetic amphiphilic copolymer, PEO-PPO-PEO, with a similar molecular weight. This is ascribed to higher amount of adsorption of the hydrophobins to hydrophobic surfaces from aqueous solution.
1. Introduction

Hydrophobins are small (m.w. 7-10 kDa) amphiphilic proteins originating from filamentous fungi displaying a variety of biological functions in their growth and morphogenesis [1-7]. Based on the comparison of amino acid residue sequence, hydrophobins are classified into two groups, type I and type II [1-7]. Surface-active properties of hydrophobins have drawn particular interests in self-assembled adsorption behavior of hydrophobins at air/water [8-10], water/oil [11-14], and water/solid interfaces [15-22]. This, in turn, sparked intensive researches to utilize hydrophobins as coating materials for biomedical, technical, and personal care products [23-28].

Hydrophobins’ surface-active properties have recently started to draw attention for tribological applications as well [29-31]. Nanotribological studies of a layer of Sc3 from *schizophyllum commune* on polymeric surfaces with atomic force microscopy (AFM) showed a reduction of friction forces in ambient condition [29]. More recent studies of type II hydrophobins, namely HFBI and FpHYD5, have shown a potential as boundary lubricant additive to lubricate stainless steel in aqueous environment [30,31]. Despite different environment and substrates, the efficacy of hydrophobins as lubricant additive in these studies is commonly based upon a strong adsorption onto material surfaces.

In this study, we have investigated two type II hydrophobins, namely HFBI and FpHYD5, as boundary lubricant additive for a sliding contact of elastomeric, hydrophobic interface by employing a self-mated poly(dimethylsiloxane) (PDMS) pair in aqueous environment. Based on distinct amphiphilicity of hydrophobins, it is hypothesized that they are ideally suited to hydrate and lubricate hydrophobic interfaces in aqueous environment. While various synthetic [32-34] and biopolymeric [35-37] amphiphiles have shown facile lubricating effects for hydrophobic interfaces, comparative studies of the two hydrophobins in this study are particularly interesting in the following viewpoints. Firstly, the molecular weight of the hydrophobins in this study, ca. 7 – 10
kDa, is much smaller than those of other amphiphilic biomacromolecules that are related to biological lubrication, such as lubricin (in the range of ca. 250 kDa [38,39]) or mucins (0.5 to 20 MDa [40]), and is rather comparable to those of synthetic amphiphilic polymers that have been used as aqueous lubricant additive [32]. Thus, it is of interest to study whether small biomolecules as hydrophobins can also display as effective lubricating capabilities as much larger ones. Secondly, in the same context, it is also of interest to compare the lubricating properties of hydrophobins with synthetic copolymers, especially those showing comparable molecular weights. Thirdly, despite the differences in the fungi of origin, structural and sequence homology of hydrophobins are very high [1-7]; both hydrophobins contain eight cystein residues, i.e. four disulfide bonds, one α-helix and two β strands, and a number of aliphatic hydrophobic residues. A major structural difference between the two hydrophobins is N-glycosylation (ca. 1.7 kDa) of FpHYD5 [31,41], and thus its influence on the lubricating properties can be studied. Lastly, it is well known that hydrophobins form multimers as a result of self-assembly in bulk aqueous solution [42-44], which may have an influence on the surface adsorption and boundary lubricating properties too; for this reason, all the experiments were performed at two concentrations, namely at 0.1 and 1 mg/mL.

2. Materials and Methods

2.1 Hydrophobins and hydrophobin solutions

Two type II hydrophobins, namely HFBI from *T. reesei* [5-9] and FpHYD5 from *Fusarium poae* [41], were employed. Details on culture, extraction, and purification processes of the two hydrophobins are found in literature [8,9,41]. Molecular weights of HFBI and FpHYD5 are 7.54 kDa [31] and 9.21 kDa [31,41], respectively. For FpHYD5, a molecular weight of ca. 1.7 kDa is indebted from N-glycosylation at N-37 site (2 N-acetyl glycosamines and 7 hexoses [41]). For comparison, a triblock copolymer, poly(ethylene oxide)-block-poly(propylene oxide)-block-
poly(ethylene oxide) (PEO-PPO-PEO) with a similar molecular weight with HFBI, namely Synperonic® PE P105 (m.w. ca. 6.5kDa, abbreviated as “P105” hereafter, Sigma-Aldrich Denmark ApS, Broendby, Denmark), was employed.

Hydrophobin and P105 solutions were prepared by dissolving in sodium acetate buffer (pH 5) at 0.1 mg/mL and 1.0 mg/mL, respectively. A slightly acidic buffer solution was selected based on the previous studies, where an optimum adsorption [45] and lubrication [31] were observed in this buffer, especially for HFBI.

2.2 Circular Dichroism Spectroscopy

Far UV circular dichroism (CD) spectra of the hydrophobin solutions were acquired with a Chirascan spectrophotometer (Applied Photophysics Ltd., Surrey, UK) at room temperature (ca. 22 °C). A cylindrical quartz cuvette with 10 mm path length (Hellma GmbH & Co. KG, Müllheim, Germany) was used. The wavelength range was selected from 280 to 190 nm with step size of 2 nm and bandwidth of 1 nm. The far-UV CD signals of the buffer background were subtracted from the data. The presented data are average of three independent measurements, each averaged of three scans.

2.3 Optical Waveguide Lightmode Spectroscopy (OWLS)

OWLS (Microvacuum, OWLS model 210, BioSense software version 2.6.10, Hungary) is an optical, non-labeling technique to monitor the adsorption characteristics of macromolecules from liquid to interfacing solid surfaces. OWLS is based on the in-coupling of incident linear polarized laser light (He-Ne, 633 nm) with diffraction grating waveguides. Upon adsorption of macromolecules onto or at the vicinity of the waveguide surface, specific incidence angle, where total internal reflectance occurs, is changing due to the changes in refractive index at the interface.
Adsorbed mass on the waveguide surface can be deduced using de Feijter equation [46]. Refractive index increment values, $dn/dc$, for the two hydrophobins and P105 were assumed to be 0.182 cm$^3$/g and 0.150 cm$^3$/g [47], respectively. OWLS experiments started from exposing waveguides to the buffer solution until a stable baseline was obtained. Then, hydrophobin or P105 copolymer solution was injected into the flow cell by means of a programmable syringe pump. Upon initiation of adsorption, the pump was stopped and the adsorption was allowed to proceed under static condition for 1 h. Since the signal at this stage includes the contribution from not only adsorbed polymers but also from the change in refractive index at the vicinity of the surface, the adsorbed mass was assessed after rinsing the flow cell with buffer solution, presumably leaving only strongly bound polymers on the surface.

In order to emulate the tribopair surface (see the section 2.4), the waveguides for OWLS adsorption experiments were coated with a thin layer of PDMS. To this end, waveguides were ultrasonicated in EtOH for 10 minutes and spin-coated with a Sylgard® 184 PDMS kit mixture (base component and crosslinker 3:1 wt. ratio dissolved in heptane to give a spin coating solution of 0.5 wt. %) at 2 000 rpm for 60 s. After spin coating, the waveguides were cured overnight at 70 °C. The reference thickness of the spin-coated PDMS layer as measured on silicon wafers by ellipsometry was $16.4 \pm 0.17$ nm [34].

2.4 Pin-on-disk tribometry

The lubricating properties of hydrophobin or P105 solutions have been assessed by acquiring the coefficient of friction vs. speed plots with a pin-on-disk tribometer (CSM Instruments, software version 4.4 M, Switzerland). In this approach, a loaded pin is placed on disk surface, and the disk was allowed to rotate over a defined sliding track using a motor underneath the disk. Dead weights were employed to apply external load. The friction forces were detected by strain gauge on the arm
holding the pin. Coefficient of friction, \( \mu \), is defined as \( F_f/L \), where \( F_f \) is friction force and \( L \) is load, under a fixed load (5 N). This corresponds to the Hertzian contact pressure of 0.36 MPa. Variation of speed, from 0.25 mm/s to 100 mm/s, gives \( \mu \) vs. speed plots.

PDMS discs and pins were prepared with the PDMS kit mentioned above. Base and crosslinker were mixed at 10:1 wt. ratio. Dispersed foams generated during mixing were removed by vacuum. The mixture was then poured into molds and cured overnight at 70 °C. Home-machined aluminum was used for disc mold (diameter; 30 mm, thickness; 5 mm), and Nunc™ U96 MicroWell™ plates (Thermo Scientific, Denmark) were used for pin (radius; 3.0 mm) mold. The roughness of the PDMS discs and pins was measured by AFM tapping mode. The root-mean-square roughness (\( R_q \)) was measured to be 1.34 nm and 4.62 nm for discs and pins, respectively, over a 2 \( \mu \)m \( \times \) 2 \( \mu \)m area. Water contact angle on PDMS surfaces were 105.6 ± 2.2° (tested with Millipore water, standard deviation from 5 measurements).

3. Results & Discussion

3.1 Secondary structures of hydrophobins

The far UV CD spectra obtained from HFBI and FpHYD5 at 0.1 mg/mL and 1.0 mg/mL are presented in Figure 1.
Figure 1. Far UV CD spectra of HFBI (blue lines) and FpHDY5 (red lines) at 0.1 mg/mL (dotted lines) and 1.0 mg/mL (solid lines) in Na-acetate buffer (50 mM), pH 5.

The overall features of the far UV CD spectra are very similar at both concentrations, supporting the structural similarities of the two hydrophobins. The far UV CD spectrum of HFBI at 0.1 mg/mL in Figure 1 is similar to that reported in a previous study in the same buffer [12]. This also suggests that a minor difference in amino acid residue composition and sequence as well as the presence of glycans for FpHYD5 do not induce significant differences in the structure of the two hydrophobins.

Nevertheless, un-ignorable differences between the two hydrophobins are also noticeable according to the type and concentration. For instance, while the far UV CD spectra of the two hydrophobins at both concentrations are generally similar to each other, the major negative peaks are clearly narrower for the spectra obtained from 1.0 mg/mL than 0.1 mg/mL, in particular in the region from 190 to 205 nm (Figure 1). It is also noted that the minima of the major negative peaks of the two hydrophobins shift to higher wavenumbers by ca. 2 nm. In the comparison of the far UV CD spectra of the two hydrophobins, somewhat weaker, yet similar difference is observed too; the far CD UV spectra of HFBI is slightly narrower compared to those of FpHYD5 in the region from 190 to 205 nm, and this contrast slightly stronger at higher concentration (1 mg/mL) than at lower concentration (0.1 mg/mL). In contrast, the far UV CD spectra in the wavelength region higher than
ca. 203 nm are much closer to each other despite the variation of the type or concentration of hydrophobins.

We propose that the concentration-dependent changes of far UV CD spectra of the hydrophobins are related to the self-assembly to form multimers in high concentration [42-44]. Previous studies have shown that HFBI molecules tend to assemble and form dimers/tetramers at high concentration for its amphiphilicity in aqueous environment [43, 48]; a threshold to display the presence of multimer was reported to be ca. 0.15 mg/mL [43]. Thus, in this study too, monomers are likely to be the dominant form of the hydrophobins at 0.1 mg/mL solutions, whereas multimers are more dominant species at 1.0 mg/mL solutions. As the formation of dimer/tetramer is essentially driven by the interaction between surface hydrophobic patches, major secondary structures, such as $\alpha$-helix or $\beta$-strand, are expected to be preserved in this process. Thus, the fact that only minor changes occur in the far UV CD upon increasing the concentration by 10 times (Figure 1) is consistent with the scenario of multimerization without disturbing the major protein structural features of the hydrophobins. In the same context, a slight difference between the two hydrophobins shown at 1.0 mg/mL can also be related to bulkier structure of FpHYD5 than HFBI due to the presence of glycans, and consequent alteration in the conformation of the assembled multimers. Nevertheless, it is more important to emphasize that all these changes are minor in magnitude, and the far UV CD spectra shown in Figure 1 mainly support the structural resemblance of the two hydrophobins in this study.

3.2 Adsorption of hydrophobins onto surface

Upon exposure of the PDMS-coated waveguides to the solutions of hydrophobin or P105, a rapid surface adsorption, followed by saturation was observed; more than 90% of saturated adsorption signals were achieved within the first 5 min (representative adsorption profiles shown in
Fig S1 in Supplementary Information). No meaningful difference was observed in the adsorption profiles according to the type or concentration of the hydrophobins. Adsorbed masses, as defined after rinsing the flow-cell with buffer solution, from three measurements of the hydrophobins and two concentrations, 0.1 mg/mL and 1.0 mg/mL, are presented in Figure 2.

Figure 2. Adsorbed masses of HFBI, FpHYD5, and P105 onto PDMS-coated OWLS waveguides from 0.1 mg/mL and 1.0 mg/mL solutions in Na-acetate buffer (50 mM), pH 5.

Table 1. Adsorbed masses, surface density of the hydrophobin molecules, and area per molecule on PDMS surface, as characterized by OWLS.

<table>
<thead>
<tr>
<th></th>
<th>HFBI</th>
<th>FpHYD5</th>
</tr>
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<tbody>
<tr>
<td>Adsorbed mass (ng/cm²)</td>
<td>165.7 ± 5.7</td>
<td>137.7 ± 22.7</td>
</tr>
<tr>
<td>Surface density of the hydrophobin molecules (/nm²)</td>
<td>0.132 ± 0.005</td>
<td>0.110 ± 0.018</td>
</tr>
<tr>
<td>Area per molecule (nm²)</td>
<td>7.58 ± 0.29</td>
<td>8.62 ± 1.53</td>
</tr>
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The average adsorbed masses of hydrophobins were 165.7 ± 5.7 ng/cm² for HFBI and 177.7 ± 16.0 ng/cm² for FpHYD5, respectively, from 0.1 mg/mL solutions, and 137.7 ± 22.7 ng/cm² for HFBI and 162.3 ± 33.2 ng/cm² for FpHYD5, respectively, from 1.0 mg/mL solution (Table 1). Despite the
10-fold difference in the concentration of bulk solution, the adsorbed masses of the hydrophobins from 0.1 mg/mL and 1.0 mg/mL solutions were statistically indistinguishable. This means that the hydrophobin solutions at 0.1 mg/mL provide a saturated monolayer on PDMS surface, and an increase of the concentration of bulk solution to 1.0 mg/mL does not contribute to further surface adsorption, via e.g. multilayer formation. On the other hand, at even further lower concentration, e.g. at 0.01 mg/mL, the adsorbed masses of both HFBI and FpHYD5 were much smaller than those at 0.1 or 1 mg/mL (< 70 ng/cm$^2$, data not shown). Lastly, the adsorbed masses of P105 at 0.1 and 1 mg/mL concentration were 64.0 ± 35.3 ng/cm$^2$ and 100.0 ± 35.3 ng/cm$^2$, respectively. Based on the molecular weights of the hydrophobins and the adsorbed masses, the number of hydrophobin molecules per unit area (1 nm$^2$), and in turn, the area occupied per hydrophobin molecule can be estimated under the assumption of random close packing. The results are shown in Figure 3 and Table 1; 7.58 ± 0.29 nm$^2$ (0.1 mg/mL) and 8.62 ± 1.53 (1.0 mg/mL) for HFBI, and 9.09 ± 1.37 nm$^2$ (0.1 mg/mL) and 9.43 ± 1.65 nm$^2$ for FpHYD5 (1.0 mg/mL), respectively. Again, the area per molecule was statistically indistinguishable for the two hydrophobins at both concentrations.

![Figure 3](image-url)

Figure 3. Estimated areas per hydrophobin molecule from the adsorbed masses (Figure 2) and the molecular weights of HFBI and FpHYD5 from 0.1 mg/mL and 1 mg/mL solutions.
Many previous experimental studies have shown facile adsorption of HFBI or HFBII onto a variety of hydrophobic substrates, including graphite [8], alkylated gold [23], polystyrene (PS) [16], and PDMS [18,20] from aqueous solution for its distinct amphiphilicity. An MD simulation study also confirmed that the binding of HFBI onto PDMS surface is energetically most favorable when the adsorption occurs exclusively through the interaction of hydrophobic patches with PDMS substrate [15]. As FpHYD5 is relatively a new molecule, its surface adsorption has been studied to a much less extent to date. Based on structural homology though, a similar adsorption mechanism and conformation with HFBI, i.e. aliphatic hydrophobic patches on the protein surface acting as anchoring units, is expected in the adsorption onto PDMS surfaces. Additionally, one N-glycosylation (1695 Da) residing on the opposite side of hydrophobic patches [41] further improves the amphiphilicity of FpHYD5.

3.3 Aqueous lubricating properties

Figure 4 presents the speed-dependent lubricating properties, namely $\mu$-vs-speed plots, of the two hydrophobins and P105 solutions, at the concentration of 0.1 mg/mL (Figure 4(a)) and 1.0 mg/mL (Figure 4(b)), respectively.
Figure 4. $\mu$ vs speed plots for HFBI, FpHYD5, and P105 from (a) 0.1 mg/mL and (b) 1.0 mg/mL, respectively.

The $\mu$-vs-speed plots of additive-free acetate buffer solution are also presented as reference. The $\mu$ values of PDMS-PDMS sliding contacts lubricated either by the hydrophobins or P105 solutions were lower than those of buffer solutions, yet to different extents depending on the type of additive, concentration, and speed range.

Between the two hydrophobins, FpHYD5 showed clearly superior lubricating capabilities, especially at low-speed (< 10 mm/s) and low-concentration (0.1 mg/mL) regime, where boundary lubrication mechanism is dominant. This can be firstly linked to the slightly higher adsorbed mass of FpHYD5 than HFBI (Figure 2). But, as this difference is very small, and the standard deviations are larger than the differences at both concentrations, it cannot account for the large difference in the $\mu$ values between them. Instead, the superior lubricating properties of FpHYD5 in this regime can be related to the presence of glycosylated region in it and consequently more effective hydration, as confirmed by QCM-D study in a previous study [41]. Glycosylation is a common strategy for biomacromolecules, such as mucins [35,36] and lubricins [38,39], to enhance the hydrophilicity and entrainment of base lubricant, i.e. water, at the gliding interface. The present study with hydrophobins further confirms the significance of glycosylation for aqueous lubricating properties of biopolymeric additives. Judging from the $\mu$ vs speed plots obtained in this study, overall aqueous lubricating properties of FpHYD5 can be assessed fairly comparable to those by much larger biomacromolecules, such as mucins at the same tribopair [49,50]. Both hydrophobins showed superior lubricating behavior compared to P105 as aqueous lubricant additive for this tribopair. This is expected from that the adsorbed masses of the two hydrophobins are higher than that of P105 at the same concentrations (Figure 2). With increasing speed, however, the difference in $\mu$ values for the two hydrophobins, as well as with P105, started to disappear. This difference is
much smaller in high-concentration regime (1.0 mg/mL, Figure 4(b)). This may be an indication that fluid-films started to form at the interface in this speed regime [32,33].

Direct comparison of each additive at different concentrations is not shown, as all of them showed clearly superior lubricating effects (i.e. lower $\mu$ values) at high concentration (1.0 mg/mL) than at low concentration (0.1 mg/mL), with the difference being larger in low-speed regime. A superior lubricating effect of hydrophobins at 1 mg/mL concentration than at 0.1 mg/mL is not directly related to the surface adsorption properties (Figure 2), according to which the adsorbed masses from 1.0 mg/mL solutions are comparable to those from 0.1 mg/mL solutions. In other words, higher concentration of the hydrophobin molecules in 1.0 mg/mL solution contributes to lubrication, even though they do not contribute to higher surface adsorption. This is, however, not due to an increase in viscosity; proteins or glycoproteins at 1.0 mg/mL concentration are virtually identical with water in viscosity [36]. Furthermore, the improvement of lubricating properties at high concentration is evident in slow-speed regime, where boundary lubrication is most active, rather than in high-speed regime, where viscosity plays a significant role due to a higher likelihood of forming fluid-films. The improved lubrication at high concentration of hydrophobins can be related to the more effective *recovery* of the lubricating film under cyclic tribological stress in pin-on-disk tribometry [51]; hydrophobins on PDMS surface are easily rubbed away from the tribostress whereas excess proteins in bulk solution can readily reform the lubricating film as well due to non-covalent bonding characteristic, and this process is continuously repeating. In this context, higher concentration of the additives is advantageous for faster regeneration of the lubricating layer due to the steeper concentration gradient near the contact area. A control experiment involving a monolayer coating only, as prepared by replacing hydrophobin solution with buffer solution after the formation a monolayer showed that the degradation in lubricating properties started to occur immediately after the initial contacts (Figure S2, in Supplementary
Information). Thus, excellent lubricating performance of the hydrophobins for the sliding contacts of PDMS-PDMS tribopair is mainly indebted from fast (re)adsorption kinetics on to the surface when excess hydrophobins are present in bulk solution, which is, in turn, indebted from the presence of distinct hydrophobic patches on the protein surface and enhanced amphiphilicity.

5. Conclusions

In this study, tribological properties of type II hydrophobins, HFBI and FpHYD5, as aqueous lubricant additive for a soft hydrophobic sliding interface, PDMS-PDMS, were studied at the concentration of 0.1 mg/mL and 1.0 mg/mL. Far UV CD spectra of the two hydrophobins were very similar, suggesting overall structural similarity of the two hydrophobins, yet showed somewhat different secondary structural features according to the concentration change from 0.1 to 1.0 mg/mL. This is suggested to be related to the dominance of monomers at 0.1 mg/mL and multimers at 1.0 mg/mL, respectively. However, its influence on adsorption was insignificant due to the formation of monolayer already at lower concentration (0.1 mg/mL). The adsorption strength of the hydrophobins was not sufficient to withstand the tribostress under 5 N with a monolayer coating on the surface. But, the hydrophobins displayed very efficient aqueous lubricating capabilities as a solution based on “self-healing” mechanism. Despite its much smaller molecular weight (within the range of 7 to 10 kDa) compared to other biomolecular amphiphiles, such as mucins or lubricins, the two hydrophobins in this study, especially FpHYD5, showed comparable aqueous lubrication capabilities for PDMS-PDMS tribopair. This is mainly due to the presence of distinct hydrophobic patches on protein surface, rather than being entirely buried inside, and consequently enhanced amphiphilicity of the molecules. In low-speed regime, where boundary lubrication character is dominant, FpHYD5 showed relatively superior lubricity to HFBI, presumably related to the presence of glycans and consequently more efficient hydration. With increasing speed though, the
difference in the lubricating properties of the two hydrophobins as well as with P105 started to disappear, due to more feasible entrainment of base lubricant, water, into the sliding interface.

6. Acknowledgements

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References


Highlights

- FpHYD5 and HFBI are effective as aqueous lubricant additives for soft contacts
- Hydrophobins showed superior lubricity than synthetic copolymer P105
- FpHYD5 showed an enhanced lubricity, presumably due to glycosylation