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Under-water superoleophobicity of fish scales

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Recent surge in the development of superhydrophobic/superoleophobic surfaces has been motivated by surfaces like fish scales that have hierarchical structures, which are believed to promote water or oil repellency. In this work, we show that the under-water oil repellency of fish scales is entirely due to the mucus layer formation as part of its defense mechanism, which produces unprecedented contact angle close to 180°. We have identified the distinct chemical signatures that are responsible for such large contact angle, thereby making fish scale behave highly superoleophobic inside the water medium. In absence of the mucus layer, it is found that the contact angle decreases quite dramatically to around 150°, making it less oleophobic, the degree of such oleophobicity can then be contributed to its inherent hierarchical structures. Hence, through this systematic study, for the first time we have conclusively shown the role of the fish's mucus layer to generate superoleophobicity and negate the common notion that hierarchical structure is the only reason for such intrinsic behavior of the fish scales.

Fish scales are excellent examples of under-water superoleophobic or oil-repellent surfaces. In fact such repelling action of the fish scales extends to different oils, and is considered to be one of the primary reasons responsible for the survival of the fishes in polluted water¹. Beyond this remarkable biophysical consequence, fish scales, like other natural liquid-repellent surfaces (such as lotus or rice leaves or butterfly wings)^{2–5}, have inspired development of artificial surfaces that mimic the fish scale wetting behavior by reproducing the corresponding micro-nanoscale structural features present on the fish scales. Successful realization of such attempts is naturally based on the exact understanding of the factors responsible for the typical wetting behavior of the fish scales. Like most other extreme liquid-repellent surfaces^{6,7}, the wetting behavior of the fish scales, too, results from a combination of its intrinsic material and hierarchical micro/nanostructures on its surface. In addition, and this is where the fish scales are different from other natural examples, it is believed that the slippery mucus layer present on the fish scales^{1,2,8,9} substantially aids in ensuring the under-water superoleophobic behavior of the fish scales. Remarkably, this entire hypothesis on the combined consequence of the mucus and the fish scale architecture in dictating the fish scale wettability has never been truly verified by testing the wettability of the fish scales with and without the mucus layer. Since fish always secretes mucus as a defense mechanism¹ when it comes in contact with any foreign agent, which can be the polluting oil – a conclusive understanding about the role of mucus, in dictating fish scale wettability, is central to complete the picture of mucus as a key defense mechanism of the fishes.

Liu et al.⁵ measured static contact angle of fish scales both in air (showing complete wetting in air) and in water ($156.4 \pm 3.0^\circ$: degree of oleophobicity) and found that the wetting properties of these scales changed from completely oleophilic in air to superoleophobic under-water due to presence of hierarchical structures. However, their study does not pin-point the role of mucus layer towards this superoleophobic behavior. Bixler and Bhushan^{3,10} studied the wettability of two different species – fish scales (*Oncorhynchus mykiss*) and shark skins (*Insurus oxyrinchus*) with respect to water drop in air medium in presence and absence of mucus layer for each case. They found that for their fish scales, the static contact angle for water in air is less than 10°, which changes to 58° on removal of the mucus layer – thereby still having the fish scales hydrophilic for water in air medium. The limited studies, reported so far, have a huge challenge in determining accurate static contact angle, particularly in situations under-water, where the pendant drop technique is often used with the needle being attached to the drop when brought in close contact to the target substrate for contact angle measurements. Coupled with this challenge is the realization of a completely flat substrate requirement for contact angle measurement, which is exceedingly difficult to achieve for bio-organisms with complex surface structures like those seen on the fish scales^{3,4,10,11}.



Fish, in general, brings added complexity towards understanding the hydrophilic/oleophilic characteristics, compared to other substrates commonly found in nature like lotus leaves, butterfly wings^{3–6}, etc. because of its inherent ability to secrete slippery mucus layer as form of a defense mechanism towards any externally induced stresses, like touch, environmental pollutants, toxins in water, etc. The mucus is secreted from the cells located at the outer skin (epidermis) covering the fish scales. It is also known and well-studied that the mucus helps in reducing the drag on the fish and thereby helping fish to swim in water with less friction^{3,9,12}.

However, as mentioned earlier, little attention has been focused on understanding the role of such mucus layer towards the wetting characteristics of the fish scales. In this work, through the under-water wettability measurement of the fish scales of *Oreochromis niloticus* (commonly known as Tilapia fish), with and without the mucus, we analyze the relative importance of the mucus and the fish scale architecture in dictating the under-water superoleophobicity. Here, for the first time, we are able to accurately determine the static contact angle of oil droplets on fish scale under-water using a needle-free drop deposition technique. Our results establish that presence of mucus enhances the under-water superoleophobicity. Through careful experiments conducted by removing the mucus layer from the fish scale, it emphasized that instead of the hierarchical micro/nano-structures present in the fish scales, the mucus layer plays a dominant role in changing the wetting characteristic of fish scales towards oil.

Results and Discussion

Figure 1 provides the contact angle measurements for the fish scales with and without mucus layer. It is found that in air medium, the contact angle with oil drop changes from $30.6 \pm 7.7^\circ$ in case of scales with mucus to $24.1 \pm 0.7^\circ$ for scales without mucus. However, in air medium with water drop, the contact angle is found to be $34.1 \pm 2.7^\circ$ with mucus, which changes to $85.7 \pm 4.7^\circ$ for scales without mucus (please refer to Table S1 in Supplementary Information for the magnitude of contact angles for different cases). Such increase in contact angle for water in air medium has also been reported for *Oncorhynchus mykiss* fish^{3,10}. In case of water medium, with oil drop, it is found that the fish scale with mucus layer behaves as a superoleophobic surface with unprecedented contact angle $\approx 180^\circ$ (measured value is $178.4^\circ \pm 1.3$). Such superoleophobicity has not been determined accurately in earlier studies^{4,5,11} for fish scales. Through our careful mucus removal process, it is found that the same fish scales without mucus shows a reduced superoleophobic behavior, with a measured contact angle of $148.9 \pm 5.5^\circ$. The super-

oleophobicity of fish scales without mucus layer can be considered as the contribution purely due to the geometrical asperities. However, our study clearly demonstrates that such hierarchical structures cannot alone contribute to unprecedented superoleophobic behavior of fish scales under-water. The inherent mucus layer, which is produced due to external stress like a pollutant oil drop in marine ecosystem, is responsible for this added superoleophobicity of fish scales under-water. The role of surface roughness on the wetting nature of fish scales can theoretically be presented using the Cassie-Baxter and Wenzel theories where the prior information regarding roughness parameters (height, pitch, width, etc. of the individual micro feature) and equilibrium contact angle on a flat smooth surface of the same material are needed. Hence, in the case of fish scales the knowledge of the geometrical micro features of fish scale and equilibrium contact angle on the smooth fish scale is an utmost requirement. The microscopic images (SEM as presented in this study) can provide geometrical features but on the other hand obtaining perfectly flat and smooth fish scale for equilibrium contact angle measurement is impractical. Hence, in most of the cases the fish scales are mimicked on the glass, silicon or the PDMS, using cumbersome micro-nano fabrication techniques, and the role of surface roughness is studied, using Cassie-Baxter and Wenzel theories^{3,6,11}.

Further the role of surrounding medium and wettability of substrate can be understood using mathematical argument as presents here. Let us consider a substrate in two different media i.e., in air and in water. Let us denote the interfacial surface tensions and contact angles for different combinations as γ_{ij} , γ_{ik} and θ_{ij} , respectively, where i and j represent different phases i.e., air (a), water (w) or oil (o), and k represents the solid substrate.

Based on Young's equation, one can write¹³:

$$\gamma_{ij} \cos \theta_{ij}^k = \gamma_{ik} - \gamma_{jk} \quad (1)$$

Therefore, one can write,

$$\gamma_{ow} \cos \theta_{ow} = \gamma_{oa} \cos \theta_{oa} - \gamma_{wa} \cos \theta_{wa} \quad (2)$$

For two different substrates i.e., with and without mucus, one can obtain two different equations using Eq. (2). Further, the difference in the wettability of these two substrates can be presented as

$$\begin{aligned} \cos \theta_{ow}^m - \cos \theta_{ow}^{nm} &= \frac{\gamma_{oa}}{\gamma_{ow}} (\cos \theta_{oa}^m - \cos \theta_{oa}^{nm}) \\ &- \frac{\gamma_{wa}}{\gamma_{ow}} (\cos \theta_{wa}^m - \cos \theta_{wa}^{nm}) \end{aligned} \quad (3)$$

Here, superscript 'n' and 'nm' denote two substrates with mucus and without mucus, respectively. It is to be noted that change in the wettability due to presence and absence of mucus i.e., $(\cos \theta_{ow}^m - \cos \theta_{ow}^{nm})$ is a not only a function of contact angles in air and water media ($\theta_{wa}^m, \theta_{wa}^{nm}, \theta_{oa}^m$ and θ_{oa}^{nm}) but it also depends on magnitudes of different interfacial tension (γ_{wa} , γ_{ow} and γ_{oa}). Therefore, it can be inferred that apart from the equilibrium contact angles on such surfaces, the interfacial tension also dictates the decrease in the oleophobicity.

To further delineate the effect of mucus layer on the wetting characteristics of the fish scales, we have scraped the mucus layer from the whole fish using the backside of the sterile surgical knife and collected the mucus sample on a glass slide. The mucus sample was sandwiched between the two glass slides for uniform spreading of the sample and later taken apart and dipped into the glass couvette filled with DI water to measure the contact angle of oil drop on mucus layer alone. It is found that mucus layer on glass surface produces a similar unprecedented contact angle $\approx 180^\circ$, which further collaborates our earlier findings that mucus layer is solely responsible for such unprecedented superoleophobic behavior of fish scales (refer Supplementary Video S1). However, the mucus layer on a glass substrate looks wavy and rough as these are soft gel like material which

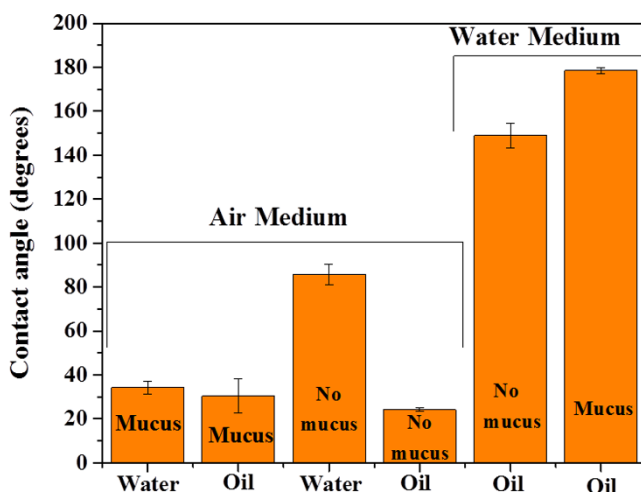


Figure 1 | Contact angle measurement on fish scales. Static contact angle of oil and water drops on fish scales with and without mucus layer for both in air and water media.

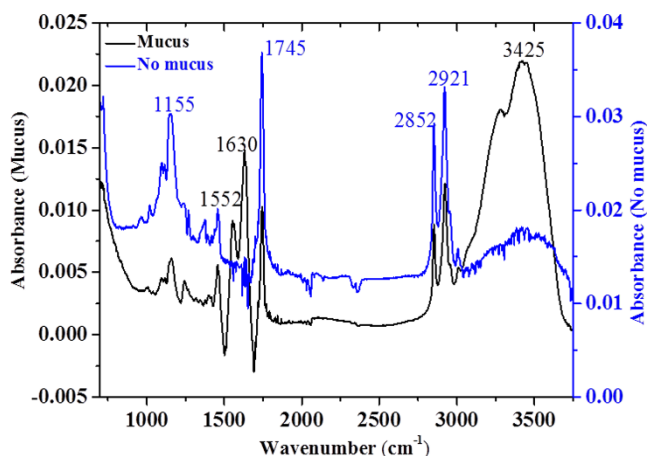


Figure 2 | FTIR data for fish scales with and without mucus along with key chemical signatures.

has confirmed to its own minimum energy state, when deposited on a flat glass substrate. The key here is to emphasise that such conformity of the mucus layer on a glass surface has no bearing on the underlying roughness of the glass substrate, which in this case is almost flat. Therefore, through such exercise (as demonstrated in Video S1), we are able to demonstrate the role of chemical signature, which dictates such oil repellency behavior.

An Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscope was used to obtain the chemical signature of the fish scales, with and without mucus.

The chemical signature of mucus layer obtained from FTIR data, which is presented in Fig. 2, shows the presence of functional groups in the range from 1600 cm^{-1} to 3400 cm^{-1} , which are hydroxyl ($-\text{OH}$), amine ($-\text{NH}_2$) and carbonyl (CO)¹⁴. Also, the mucus layer exhibits three distinct peaks that correspond to molecules, representing alcohols and phenols, which disappears for the mucus free sample. These functional groups along with several other components of fish mucus may lead to the formation of an oil repelling layer between the fish scale and the polluting oil through its interaction with the surrounding water medium. Due to this, the foreign oil drop virtually does not feel the presence of the underlying micro/nano-structured surface and probably creates an unfavorable spreading parameter¹³, which thereby produces such unprecedented superoleophobicity.

Materials

Experiments are conducted on fish scales extracted from a Tilapia fish obtained from local grocery store in Edmonton, Canada. Small portions of the fish, as shown in Fig. 3, are sectioned out using sterile surgical knife and the underlying fish body below the dermis has been carefully removed to obtain almost flat substrates for contact angle measurement. These sections were further transferred on microscope glass slides to provide rigid support to the fish scales. In case, where mucus layer from the fish scales need to be removed, they were done prior to transferring the sections on the glass slide. Chemical reagents like Ethylenediaminetetraacetic acid (EDTA), Dithiothreitol (DTT), phosphate buffer solution (PBS), Dpco's modified eagle medium (DMEM), Penicillin streptomycin, Gibco, Glucose are obtained from Fisher Scientific (Canada) Silicon oil (Cargille Inc., USA) and de-ionized (DI) water (obtained from PURELAB Ultra, ELGA) are used as measurement fluids for contact angle measurement using Drop Shape Analyzer DSA 100 (Krüss, Germany).

Methods

All experimental protocols were approved by Risk Management Services, University of Alberta and methods were carried out in accordance with Biosafety Officer, Environment, Health and Safety Risk Management Services, University of Alberta.

Accurate Determination of Under-water Contact Angle. One of the key challenges in measuring the static contact angle for substrates like fish scale under-water is to have a mechanism in place where the needle producing the desired oil droplet (for pendant drop technique¹⁵) does not remain attached to the drop once it is brought in close contact to the characterizing substrate, i.e., fish scales. When the needle is attached to the drop, it creates additional force on the drop-substrate combination, which further spreads the drop on the substrate, thereby providing erroneous contact angle values^{3,5}. Our recent under-water needle-free drop deposition technique¹⁵ overcomes this limitation by bringing in an additional oil-water interface (as shown in Fig. 4(b) with canola oil used at the interface between water and air) and hence this has been used for accurate estimation of the static contact angle of oil drop on fish scales kept under water, for both with and without mucus, as shown in Fig. 4 (a). This accurate measurement of contact angle is more critical in this case as fish scale with or without mucus is essentially a soft deformable substrate (soft matter) and hence any force exerted by the traditional needle-drop assembly on the characterizing substrate during the contact angle measurement (as done in conventional pendant drop method) will generate additional stresses on the substrate, which will result in erroneous contact angle value, which has often been the case so far^{4,5,11}.

The second challenge for accurate quantification of the contact angle is to distinctly identify the demarcation contour between the oil drop base contact area and the fish scales, particularly more so in case of scales with mucus layer. As observed in case of existing literature^{3,5}, the absence of clear demarcation results in possible inaccurate quantification of the contact angle, which is also shown in Fig. 4(c). Hence, proper illumination is required to accurately determine the drop base contact area. This requires modification of the existing DSA100 system, by bringing in an additional light source (Dolan-Jenner, USA.) with a diffuser (Edmund Optics, USA), behind the glass couvette (Krüss, Germany), as shown in Fig. 4(b), so that accurate quantification of the contact angle can be obtained (refer Supplementary Video S2). Erroneous representation of contact angle due to improper illumination is presented in Fig. S1 (see Supplementary Information) and the repeatability in the measurement is depicted in Fig. S2 (see Supplementary Information).

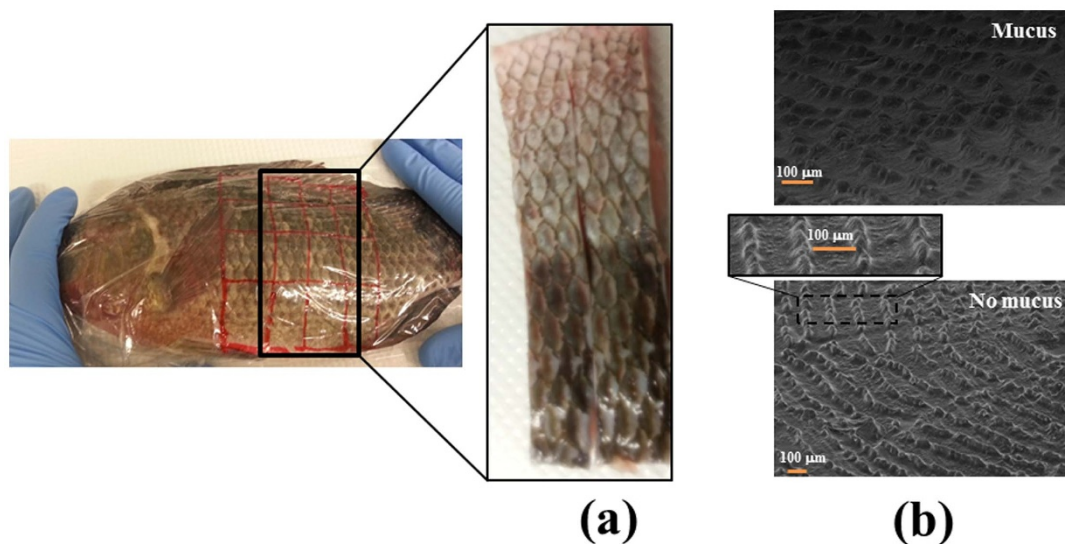


Figure 3 | Tilapia fish used for quantification of wetting characteristics of its scales. The red marked parts of the skin was sectioned out for further analysis. (a) Sectioned out skin from the fish. (b) Scanning Electron Microscopy (SEM) image of fish scales with mucus and without mucus.

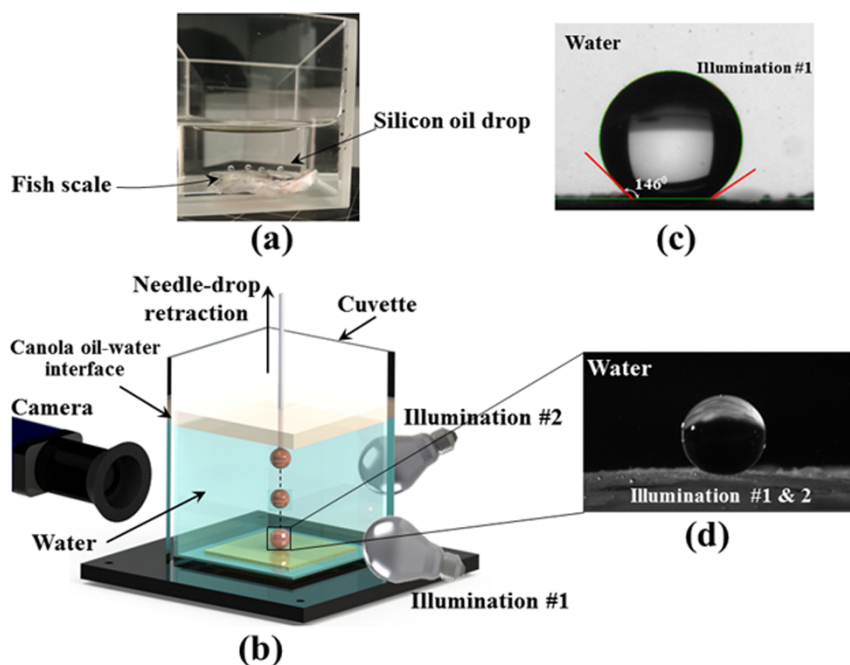


Figure 4 | Measurement of contact angle on fish scales. (a) Silicon oil drops, used as model fluid, resting on fish scales kept under water in a glass cuvette. (b) Technique used to achieve under-water needle-free drop deposition of silicon oil on soft deformable fish scale surface. (c) Commonly used procedure of contact angle measurement which inaccurately quantifies the static contact angle by using normal DSA 100 set-up due to unresolved drop base contact area. (d) Accurate quantification of contact angle using an additional illumination arrangement and modified experimental set-up. This figure was drawn by PRW.

Protocol for removal of mucus layer from fish scales. We have followed an established protocol^{16,17} for removing the mucus layer from the fish scales and we have further performed Fourier transform infrared spectroscopy (FTIR) to distinctly demarcate the presence and absence of mucus layer from the fish scales. All experimental protocols were approved by Risk Management Services, University of Alberta and methods were carried out in accordance with Biosafety Officer, Environment, Health and Safety Risk Management Services, University of Alberta. Dithiothreitol (DDT) solution was prepared by dissolving 111 mg of Ehtylenediamine tetracetic acid (EDTA) and 42 mg of DTT in 300 ml PBS. Sectioned out fish scales (see Fig. 3(a)) were kept for 30 mins. in DTT solution, after which the fish scales were rinsed with DI water to remove the residual DTT solution. Further, sufficient amount (~100 ml for 10 cm × 10 cm sample) of dipco's modifies eagle medium (DMEM) was used to soak the fish scales for 30 mins. After removing the scales from DMEM solution, they were rinsed with DI water and kept completely soaked in the washing media (90 ml PBS+5 ml serum-research grade serum from Fisherbrand + 5 ml P/S- Penicillin streptomycin, Gibco) for 30 mins. Finally, the fish scales were rinsed with another cycle of DI water and used for contact angle measurements.

ATR-FTIR spectroscopy. An Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscope (Thermo Nicolet NEXUS 670 FTIR, USA) was used to obtain the FTIR data. Fish scale samples were kept on the ATR crystals in such a way that there is no air-gap between the fish scale and ATR crystal. At room temperature (22°C), 128 scans of 2 cm⁻¹ were performed for samples with and without mucus. The data were collected and processed using Omnic software.

Conclusion

Here we have performed systematic static contact angle characterization of fish scales with and without mucus layer. The model fluid used is silicon oil and we have measured the contact angle of silicon oil on fish scales submerged in water using needle-free drop deposition technique coupled with proper illumination to decipher the drop contact area on the fish scale. This measurement technique allowed us to correctly quantify the contact angle for fish scales with mucus layer and we have observed an unprecedented contact angle of around 180° for under-water fish scales. Also, through careful characterization of the mucus layer, we found that certain functional groups in mucus layer (hydroxyl (-OH), amine (-NH₂) and carbonyl (CO)) are responsible for such extreme superoleophobicity of the fish scales. By removing the mucus layer, we found that the degree of

superoleophobicity of the fish scales reduces significantly to a static contact angle of around 150°, which can then be attributed to the hierarchical micro/nano-structures on the fish scales. Hence, the present study conclusively proves that under-water superoleophobicity of fish scales is due to its inherent mucus layer formation, generated through its own defense mechanism (for e.g., foreign oil droplet coming in contact with its scales), rather than the common notion of the presence of hierarchical structures of the fish scales. This study further opens up new paradigm towards proper understanding of under-liquid wettability of such marine life, which is often been biomimicked through extensive micro/nanofabrication process to generate superoleophobic/superhydrophobic/omniphobic surfaces.

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Author contributions

S.K.M. conceived the problem and supervised the project. P.R.W. designed the entire experimental set up and performed all the experiments related to contact angle measurements and the FTIR analysis. NSKG provided the SEM images and cleaning protocol of fish samples. S.K.M. and P.R.W. wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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