

Mucin from Loach Skin Mucus and Its Interfacial Behavior on Gold Surface^{*}

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Abstract Loach skin mucin was isolated from loach skin mucus and found to be similar to mammalian mucins in many aspects, *i.e.*, low amino acid residue content, high molecular weight, presence of hydrophobic blocks and gel-forming characteristics in water. However, loach skin mucin can form a weak gel in water at a much lower concentration (3 mg/mL) than mammalian mucins, indicating its good hydrophilicity. Loach skin mucin can also form a stable adsorption layer on gold surface in aqueous environment, owing to the existence of hydrophobic blocks within mucin. The nature of high hydrophilicity and interfacial behavior give loach skin mucin potential as excellent material for use in solid-water interfaces for antifouling and lubrication, and should be crucial to the versatile functions of loach skin mucus.

Keywords: Fish mucus; Mucin; Hydrophilicity; Adsorption; Interface.

INTRODUCTION

Loaches are slender freshwater cyprinoid fish of the family *Cobitidae* from Eurasia and Africa. They have long narrow bodies (length of 15 cm and diameter of 2 cm or so), live at the bottom sludge of a pond, and can move very fast in sludge. Despite such dirty and complex environmental conditions, they can keep their bodies very clean and avoid being hurt by the sharp and hard solids in sludge. These characteristics may be attributed to the slippery mucus wrapped around loach skin. The mucus serves as a lubricant and a protective barrier that shields epithelial cells from damaging substances. Aside from extensive biological roles of fish mucus, such as respiration, ionic and osmotic regulation, reproduction, excretion, disease resistance, communication, feeding and nest building^[1], the excellent antifouling and lubricating functions of loach skin mucus arouse our attention. Researches on loach skin mucus may help us to design and construct high-performance biointerfaces.

According to our previous rheological study, loach skin mucus behaves as a weak gel^[2]. The mucus can flow, but shows solid-like rheological behavior under oscillatory sweep: the storage modulus (G') surpasses the loss modulus (G'') over the frequency (ω) range from 0.01 rad/s to 10 rad/s. This characteristic is also possessed by mammalian epithelial mucus, *e.g.*, gastric mucus and saliva, in which the mucin (mucous glycoproteins) forms gel matrix and plays a key role in its functions^[3–5]. The molecular structure and physicochemical properties of mammalian mucins have been widely investigated^[6–9], whereas what constructs the gel structure of fish mucus still lacks strong evidence. Although histochemical study has confirmed that glycoproteins exist in fish mucous cells^[10], the glycoproteins are rarely isolated and their properties receive not much attention^[11, 12].

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Considering that various substances (*i.e.*, proteins, saccharides, lipids and glycoproteins) exist in loach skin mucus^[1], figuring out what forms the gel structure should be a key step to understanding its functions.

Another fascinating characteristic of loach skin mucus is its ultrahigh water content, *i.e.*, water accounts for more than 99% of its total weight^[2]. The non-water matter, which includes proteins, polysaccharides, lipids and glycoproteins, only accounts for about 0.5%. In most mammalian epithelial mucus, the weight percentage of non-water matter is almost 10 times higher^[13, 14]. Therefore, the non-water matter of loach skin mucus can absorb water more than 100 times of its own weight after release and then maintain itself as a weak gel surrounding the loach skin in water. Thus, we deduce that some gel-forming materials with high hydrophilicity exist in loach skin mucus. In this article, we isolate these materials, and prove that they are mucin-type glycoproteins. We also explore the interfacial behavior of as-obtained loach skin mucin on a hydrophobic gold surface by quartz crystal microbalance and scanning probe microscopy, as it has been reported that mucins have excellent functions such as antifouling and lubrication when adsorbed by a hydrophobic surface^[6].

MATERIALS AND METHODS

Isolation of Mucin from Loach Skin Mucus

Loaches were purchased from commercial sources and kept in tap water. When collecting mucus, we put loaches into a clean polyethylene bag and softly squeezed the loaches to stimulate them to secrete mucus. The loaches were then carefully released, leaving foam-like mucus in the bag. The foam-like mucus was centrifuged (2000 r/min) at 4 °C for 5 min, and about 100 g fluid-like mucus was obtained. The obtained mucus was diluted to 500 mL with ultrapure water (resistivity 18.5 MΩ·cm), and then thoroughly mixed with 125 mL Sevag reagent (4CHCl₃:1*n*-BuOH, *V/V*) to deproteinize. The mixture was centrifuged at 15000 r/min for 1 h to remove denatured proteins and the Sevag reagent; the aqueous phase was reserved. The aqueous phase was poured into absolute alcohol four times its volume and kept at 4 °C for a week to obtain precipitate. The obtained precipitate was dissolved in 0.05 mol/L Tris-HCl buffer (pH = 8.0) with a minor amount of nuclease, and dialyzed (molecular weight cutoff: 2×10^5) against pure water at 4 °C for 72 h to remove salts, nuclease, degraded nucleic acid and saccharides. The aqueous solution was then lyophilized to obtain loach skin mucin.

Characterization of Loach Skin Mucin

Amino acid residue and sialic acid content determination

The contents of amino acid residue and sialic acid in loach skin mucin were measured using a spectrophotometer (Cary, U.S.). The amino acid residue content was determined using BCA Protein Assay Kit (Sangon Biotech, China). The sialic acid content was determined according to the periodate thiobarbituric acid method using a commercial kit (Genmed, U.S.)^[15]. For these two measurements, loach skin mucin was dissolved in ultrapure water to a concentration of 0.5 mg/mL.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS page)

SDS PAGE was conducted using a vertical gel system (Liuyi, China). The concentration of the stacking gel was 5 wt%, and that of the separating gel was 7.5 wt%. For electrophoresis, 1 mg/mL loach skin mucin aqueous solution was mixed with same volume of non-reducing buffer and heated in boiled water for 5 min. The non-reducing buffer (10 mL) contained 1.0 mL ultrapure water, 2.5 mL 0.5 mol/L Tris-HCl buffer, 2.0 mL glycerol, 4.0 mL 10 wt% SDS solution, and 0.5 mL 0.1 wt% bromophenol blue solution. After heating, 20 μL mixed solution was applied to each application point, and electrophoresed at a constant current of 20 mA at 4 °C for 5 h. After electrophoresis, some gels were washed with ultrapure water for 15 min, incubated in Blue-Bandit Protein Stain (Amresco, U.S.) for 30 min, and destained in ultrapure water overnight. Other gels were subjected to Periodic Acid Schiff's (PAS) staining. These gels were fixed overnight in 7% (*V/V*) acetic acid after electrophoresis, and incubated in 0.2 wt% periodic acid solution (dissolved in 7% (*V/V*) acetic acid) for 1 h. They were then washed with ultrapure water thoroughly, and incubated in Schiff's fuchsin-sulfite reagent (Sigma, U.S.) at 37 °C for 30 min. The validity of PAS staining for carbohydrate of mucin after electrophoresis has been proved^[16]. After staining, all gels were scanned with a Gel Doc XR+ imaging system (Bio-Rad, U.S.).

Dynamic light scattering (DLS)

0.05 mg/mL loach skin mucin was dissolved in 6 mol/L guanidine hydrochloride (Sangon Biotech, China) and then subjected to DLS measurements using BI200SM (Brookhaven, U.S.) with a correlator (BIO9000) and He-Ne laser ($\lambda = 636$ nm), with the scattering angle being set at 20 degrees. To obtain the molecular size of the mucin in the solution, CONTIN program was used to analysis the correlation functions. Before measurements, the solution was filtered (Millipore, U.S.) to remove dust.

Rheological tests

The mucin isolated from loach skin mucus was dissolved in ultrapure water (containing 0.02 wt% NaN_3) with very gentle stirring at ice-water bath for 40 h, reaching a final concentration of 3 mg/mL. Oscillatory frequency sweep tests were performed on 3 mg/mL loach skin mucin using an AR-G2 rheometer (TA Instruments, U.S.). The strain was set to 2% for the tests, in the linear viscoelastic region of 3 mg/mL loach skin mucin.

Steady-state pyrene fluorescence study

Loach skin mucin dissolved in 0.1 mol/L NaCl/0.02% NaN_3 with concentrations in the range being 0.015–1.5 mg/mL were prepared, and their properties were assessed by steady-state pyrene fluorescence study using a LS-55 FL fluorescence spectrophotometer (Perkin-Elmer, U.S.) and a 10 mm path length quartz cell under room temperature. A stock solution of 1 mmol/L pyrene in absolute methanol was prepared, from which 1 μL was added to 1 mL aqueous sample. The sample was then allowed to equilibrated overnight with shaking, and emission spectra ($\lambda_{\text{ex}} = 335$ nm) were recorded. Each spectrum was obtained by averaging three scans, corrected for scatter using equivalent blank loach skin mucin solution.

Quartz Crystal Microbalance (QCM) Measurements

The adsorption of loach skin mucin onto a gold-coated quartz crystal was monitored by a D-300 quartz crystal microbalance (Q-Sense, Sweden). The gold surface was hydrophobic, with a static water contact angle of $(101 \pm 3)^\circ$. Prior mucin adsorption, a stable base line was obtained in the pure solvent 50 mmol/L NaCl. After this, 200 $\mu\text{g/mL}$ loach skin mucin in 50 mmol/L NaCl was injected, and adsorption procedure was followed for *ca.* 70 min. Then mucin-free 50 mmol/L NaCl was injected with a rinsing time of 10 min. The sensed mass was calculated using the Sauerbrey^[17] equation $\Delta m = -C\Delta f/\nu$, where Δm is the sensed mass, C is the mass sensitivity constant ($17.7 \text{ ng/cm}^2 \cdot \text{Hz}$ at 5 MHz), Δf is the frequency shift and ν is the overtone number.

Scan Probe Microscope (SPM) Imaging

The hydrophobic gold surface before and after adsorbing loach skin mucin from 200 $\mu\text{g/mL}$ aqueous solution was imaged using a SPI3800N scanning probe microscope (Seiko, Japan). In order not to disturb the adsorbed layer of loach skin mucin, soft contact mode was chosen to do the imaging using silicon nitride tips with a nominal force constant of 0.06 N/m.

RESULTS AND DISCUSSION

Properties of Loach Skin Mucin

According to the BCA method and periodate thiobarbituric acid method^[15], the amino acid residue content and sialic acid content of loach skin mucin are 10 wt% and 7.5 wt% respectively. Figure 1 shows the SDS PAGE results of loach skin mucin. Both the proteins staining band (Fig. 1a) and PAS staining band (Fig. 1b) appeared near the outset position of SDS PAGE gels after electrophoresis. This is typical of mucin-type glycoproteins^[18, 19], and suggests that loach skin mucin has high molecular weights and size qualitatively. The accurate molecular weights of loach skin mucin cannot be obtained in this study. We have tried to measuring them using gel permeation chromatography (GPC) and static light scattering, but failed due to the strong adsorption on GPC column and the huge size of loach skin mucin macromolecule respectively.

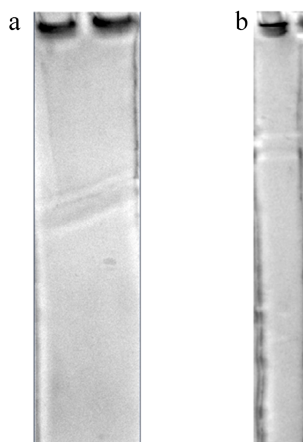


Fig. 1 Staining of loach skin mucin after SDS PAGE: (a) protein staining result and (b) PAS staining result

The accurate molecular size of loach skin mucin was obtained by DLS measurements, as shown in Fig. 2. It shows that loach skin mucin has a size spanning from 50–800 nm, and most of the mucin molecules have a size between 200–400 nm. That is, both the DLS and SDS PAGE results indicate that loach skin mucin has high polydispersity, high molecular weights, and a much larger size than ordinary macromolecules.

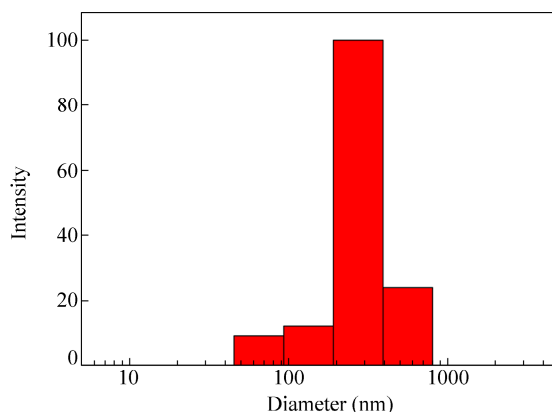


Fig. 2 DLS result of 0.05 mg/mL loach skin mucin in 6 mol/L guanidine hydrochloride

Figure 3 gives G' and G'' of loach skin mucin aqueous solution with a concentration of 3 mg/mL during oscillatory frequency sweep. G' is higher than G'' over the entire test frequency (ω) range, indicating that loach skin mucin forms a weak gel in water at the concentration of 3 mg/mL. Considering that the non-water matter in native loach skin mucus is around 5 mg/mL, loach skin mucin should be responsible for the weak gel structure of native loach skin mucus^[2], despite that proteins, polysaccharides and lipids are also contained in the native mucus^[1]. In addition, it is noted that G' and G'' of 3 mg/mL loach skin mucin aqueous solution are almost parallel, indicating that 3 mg/mL loach skin mucin is near the critical state^[20].

Figure 4 presents the fluorescence intensity ratio between the first (373 nm) and third (384 nm) vibronic peaks (I_1/I_3) of excited-state monomeric pyrene in loach skin mucin aqueous solutions under room temperature. The I_1/I_3 ratio is known to be sensitive to the micropolarity of the local environment, decreasing as environmental polarity decreases. It has been widely used as an indicator of hydrophobic associations in polymeric systems for decades^[21–23]. In Fig. 4, the I_1/I_3 ratio is around 1.7 at concentration $c < 0.06$ mg/mL, reflecting the polarity of the aqueous solvent. The sharp decrease at $c > 0.06$ mg/mL indicates the onset of hydrophobic association. Then, the ratio reaches a constant value of 1.15 at $c > 1$ mg/mL. This implies that loach

skin mucin contains hydrophobic blocks and hydrophobic association dominates at $c > 0.06$ mg/mL. There is strong hydrophobic association between mucin macromolecules at the concentration of 3 mg/mL. Therefore, it is reasonable to deduce that intermolecular hydrophobic association should be responsible for the formation of weak gel structure of loach skin mucin in water at the concentration of 3 mg/mL. It is impossible to form a weak gel in such extremely low concentration for a water-soluble macromolecules without hydrophobic blocks, such as hyaluronic acid, polyethylene oxide and so on^[24].

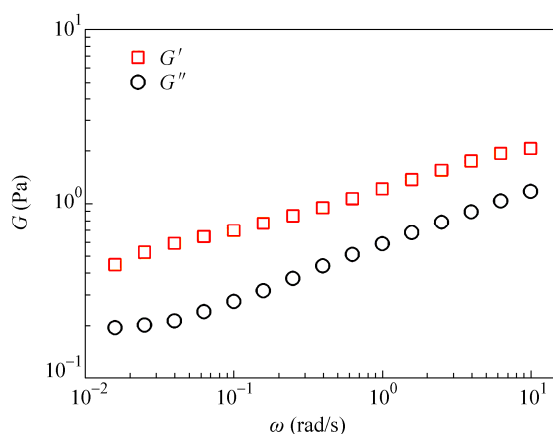


Fig. 3 G' and G'' of 3 mg/mL loach skin mucin during oscillatory frequency sweep at a strain of 2% (in the linear viscoelastic region)

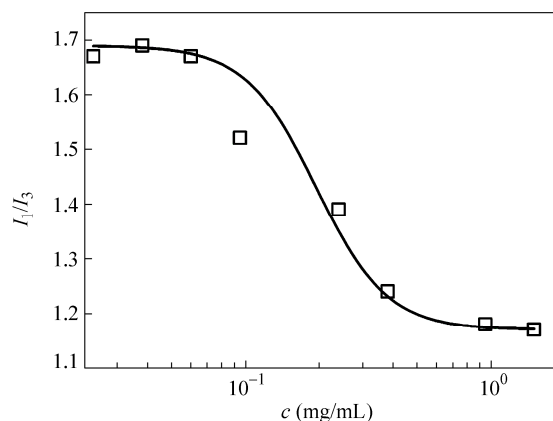


Fig. 4 Intensity ratio (I_1/I_3) for pyrene in loach skin mucin dissolved in 0.1 mol/L NaCl/0.02% NaN₃ as a function of loach skin mucin concentration

Hydrophilicity of Loach Skin Mucin

Despite similar properties to mammalian mucins (low amino acid residue content, high molecular weight, gel-forming characteristics in water and hydrophobic blocks)^[6, 8], loach skin mucin has its own unique characteristics. It can form gels in water under a much lower concentration (3 mg/mL), whereas most mammalian mucins cannot form gels below 20 mg/mL and none of them can form gels below 10 mg/mL^[6, 25]. Loach skin mucin can absorb water more than 300 times of its own weight in the form of hydrogel, exhibiting good hydrophilicity. To characterize their strong affinity for water, we compared loach skin mucin with two typical mammalian mucins (*i.e.*, human tracheobronchial mucin and porcine submaxillary mucin) on the basis of gel elasticity and swelling theories.

McCullagh *et al.* reported that the required concentration for human tracheobronchial mucin to form critical gel in water is 12 mg/mL, and that G' of this critical gel at the angular frequency of 0.63 rad/s is about 0.04 Pa^[26]. Similarly, Marquart *et al.* reported that the critical gel concentration of porcine submaxillary mucin in water is about 40 mg/mL, and that G' of this critical gel at the angular frequency of 0.1 rad/s is about 2.5 Pa^[27]. For 3 mg/mL loach skin mucin, G' at 0.63 rad/s and 0.1 rad/s are 1.1 Pa and 0.7 Pa respectively (Fig. 3). The classical equation used for gel elasticity is^[28]

$$G = \nu kT \quad (1)$$

where G is the storage modulus, ν is the density number of network strands, k is the Boltzmann constant, and T is the thermodynamic temperature. In the case of constant temperature, $\nu \propto G$. The ratios of the density number of network strands in 3 mg/mL loach skin mucin to that in 12 mg/mL human tracheobronchial mucin and that in 40 mg/mL porcine submaxillary mucin were

$$\frac{\nu_0}{\nu_1} = \frac{G_0}{G_1} = \frac{1.1}{0.04} = 27.5, \quad \frac{\nu_0}{\nu_2} = \frac{G_0}{G_2} = \frac{0.7}{2.5} = 0.28, \quad (2)$$

where subscripts 0, 1, and 2 represent loach skin mucin, human tracheobronchial mucin and porcine submaxillary mucin, respectively. Note that G in Eq. (1) should be the plateau modulus of a polymeric network at low frequency. However, for critical and weak gels, the plateau modulus cannot be attained at the test frequency range. Thus, we chose the storage moduli at 0.63 rad/s and 0.1 rad/s for calculation.

Flory proposed that the ability of an ionic gel to adsorb water could be calculated using the following equation^[29]:

$$q_m^{5/3} \cong \left[\left(i/2v_u S^{*1/2} \right)^2 + (1/2 - \chi_1)/v_1 \right] / (v_e/V) \quad (3)$$

where q_m is the volumetric swelling ratio at equilibrium, i/v_u is the concentration of the fixed charge referred to the unswollen network, S^* is the ionic strength, v_u and v_1 are the molar volumes of dry uncrosslinked macromolecules and water respectively, χ_1 is the Flory interaction parameter, v_e is the number of network strands in the network and V is the volume of the gel before adsorbing water. In this study, we define,

$$Q \equiv \left(i/2v_u S^{*1/2} \right)^2 + (1/2 - \chi_1)/v_1 \quad (4)$$

where Q is a quantity-independent parameter determined by i/v_u , χ_1 , v_1 , and S^* . For hydrogels absorbing pure water to equilibrium, v_1 and S^* are constant, and Q is determined by i/v_u and χ_1 , both indicating the affinity of the gel-forming macromolecules for water. A higher value of Q represents higher hydrophilicity of macromolecules.

We compared the Q value of loach skin mucin with those of human tracheobronchial mucin and porcine submaxillary mucin. It is worth noting that Eq. (3) is valid only when the hydrogel has absorbed water to its maximum extent. Human tracheobronchial mucin gel of 12 mg/mL and porcine submaxillary mucin gel of 40 mg/mL in pure water are under critical state and certainly cannot absorb water anymore^[26, 27]. Loach skin mucin of 3 mg/mL is near the critical state, as demonstrated by that G' and G'' of 3 mg/mL loach skin mucin are almost parallel (Fig. 3). Combining Eq. (3) with Eq. (4), we obtain

$$\begin{aligned} Q_0/Q_1 &\cong (q_{m0}/q_{m1})^{5/3} \cdot \frac{(v_e/V)_0}{(v_e/V)_1} \\ Q_0/Q_2 &\cong (q_{m0}/q_{m2})^{5/3} \cdot \frac{(v_e/V)_0}{(v_e/V)_2} \end{aligned} \quad (5)$$

Assuming that the densities of loach skin mucin, human tracheobronchial mucin and porcine submaxillary mucin are the same in the dry state, which are represented by ρ in the following equation, we obtain

$$\begin{aligned} (q_{m0}/q_{m1})^{5/3} &= [(1000\rho/3)/(1000\rho/12)]^{5/3} = 4^{5/3} \\ (q_{m0}/q_{m2})^{5/3} &= [(1000\rho/3)/(1000\rho/40)]^{5/3} = (40/3)^{5/3}, \end{aligned} \quad (6)$$

$$\begin{aligned} \frac{(v_e/V)_0}{(v_e/V)_1} &= \frac{v_0}{v_1} \cdot \frac{V_1}{V_0} = \frac{v_0}{v_1} \cdot \frac{12/\rho}{3/\rho} = \frac{4v_0}{v_1} \\ \frac{(v_e/V)_0}{(v_e/V)_2} &= \frac{v_0}{v_2} \cdot \frac{V_2}{V_0} = \frac{v_0}{v_2} \cdot \frac{40/\rho}{3/\rho} = \frac{40v_0}{3v_2} \end{aligned} \quad (7)$$

Combining Eqs. (2), (5), (6), and (7), we obtain

$$Q_0/Q_1 \cong 4 \times 27.5 \times 4^{5/3} \cong 1100, \quad Q_0/Q_2 \cong (40/3)^{8/3} \times 0.28 \cong 280.$$

The Q value of loach skin mucin is hundreds of times higher than those of human tracheobronchial mucin and porcine submaxillary mucin, indicating that loach skin mucin is much more hydrophilic than mammalian

mucins, which have been widely considered as very hydrophilic macromolecules^[30]. Therefore, it is believed that loach skin mucin is a kind of very hydrophilic macromolecules.

Interfacial Behavior

It is reported that hydrophilic macromolecules on solid-water interfaces can bring some excellent functions, *e.g.*, antifouling and lubrication^[31–33]. So we explore the interfacial behavior of loach skin mucin on a hydrophobic gold surface in aqueous environment using QCM and SPM.

Figure 5 presents the QCM raw data for the adsorption of loach skin mucin on a gold surface, as monitored by the change in the resonance frequency for the third overtone ($\Delta f_3/3$). The sharp decrease in $\Delta f_3/3$ at the first several minutes indicates the rapid adsorption of loach skin mucin on the gold surface. $\Delta f_3/3$ was then slightly decreased and reached a constant value at about 60 min, demonstrating to achieve adsorption equilibrium. At 70 min, the adsorption procedure was interrupted by rinsing the gold surface with mucin-free 50 mmol/L NaCl for 10 min. Surprisingly, after a slight increase due to the flow effect of 50 mmol/L NaCl at the beginning of rinsing, $\Delta f_3/3$ became the same as before rinsing. This indicates that loach skin mucin could adsorb very tightly on the gold surface. According to Sauerbrey equation^[17], the sensed mass of loach skin mucin adsorbed on the gold surface was *ca.* 4.4 mg/m².

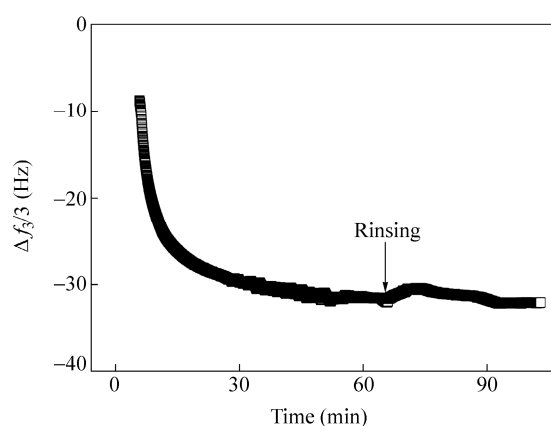


Fig. 5 QCM data for loach skin mucin adsorption on a hydrophobic gold surface from 50 mmol/L NaCl, followed by rinsing with 50 mmol/L NaCl

Figure 6 shows the topographies of the clean gold surface and the gold surface adsorbed with loach skin mucin in 50 mmol/L NaCl. It is obvious that the adsorption of loach skin mucin can reduce the root mean square surface roughness of the gold surface from 10.7 nm to 4.3 nm. When loach skin mucin adsorbs on the hydrophobic gold surface, its hydrophobic blocks tend to adhere to the gold surface, while hydrophilic blocks extend to the aqueous phase as brushes. It is believed that these extended brushes flatten the valleys on the gold surface. The brush conformation has been reported on mammalian mucins adsorbed on hydrophobic gold surface^[34], so it is rather reasonable to deduce that the adsorbed loach skin mucin also adopts brush conformation here.

It has been found that surfaces bearing hydrophilic macromolecules have extremely low friction coefficients and antifouling properties in aqueous environment^[35–37]. The highly hydrophilic nature of loach skin mucin should be vital in everyday life of loach. The mucin wrapping around loach skin in water could largely reduce the friction during the motion of loach in sludge, and keep dirty substances from adhering to loach skin. Moreover, the nature of high hydrophilicity and tight adsorption on gold surface in aqueous environment afford loach skin mucin potential as excellent boundary lubricants and antifouling materials for use in solid-water interfaces.

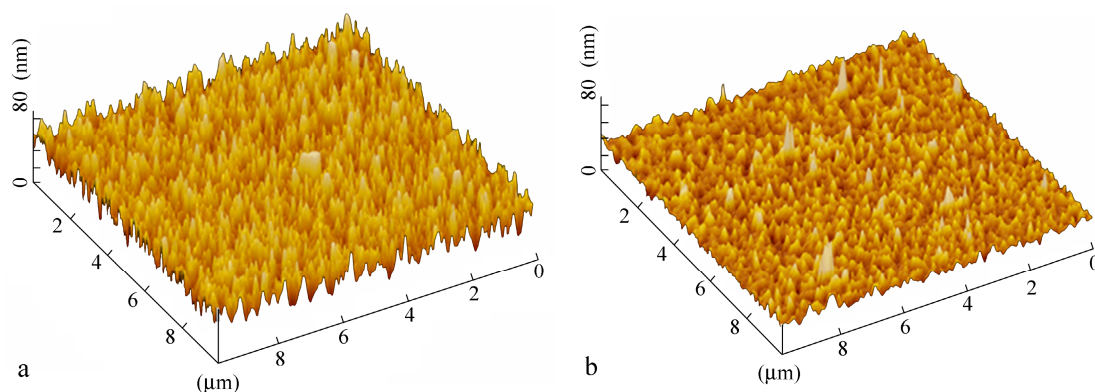


Fig. 6 Topographies of the gold surface before (a) and after (b) adsorbing loach skin mucin in 50 mmol/L NaCl aqueous solution

CONCLUSIONS

Loach skin mucin isolated from loach skin mucus is similar to mammalian mucins in many aspects, *e.g.*, high molecular weight, low amino acid residue content ($\sim 10\%$), hydrogel-forming characteristics, and presence of hydrophobic blocks. However, it can form a weak hydrogel in water at a much lower concentration (3 mg/mL) than mammalian mucins, indicating its high hydrophilicity. In addition, loach skin mucin can adsorb stably on gold surface, and reduce the surface roughness in aqueous environment to some extent. The nature of high hydrophilicity and interfacial behavior on the solid-water interface not only give loach skin mucin potential as a material for constructing low-fouling and low-friction biointerfaces, but also shed light on the versatile functions of loach skin mucus.

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