



Adhesion force measurement of live cypris tentacles by scanning probe microscopy in seawater

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Abstract

The adhesion force of the tentacle of a live cypris on a glass surface covered with polymer brush was directly measured by scanning probe microscopy. Polymer brushes were prepared on the cover glass and silicon wafers by surface-initiated atom transfer radical polymerization of 3-(*N*-2-methacryloyloxyethyl-*N,N*-dimethyl)ammonatopropanesulfonate (MAPS) or 2-hydroxyethyl methacrylate (HEMA). A small amount of glue was placed at the edge of a tipless cantilever supported by the piezo motion control system of the scanning probe microscope and monitored by using an optical microscope. A live cypris swimming in seawater was held down on the slide glass by the cantilever for 30 min until the glue cured. The tentacles of the live cypris immobilized on the cantilever were forced to make contact with the surface-modified cover glass. The adhesion force was determined by the torsion of the cantilever when the tentacle was detached from the cover glass surface. The adhesion force between the cypris and the propylsilane-modified glass surface increased from 1.6 to 40 μN with the increase in age of the cypris larva from 3 to 21 days after metamorphosis from the nauplius larva. Poly(MAPS) and poly(HEMA) brush surfaces exhibited extremely low adhesion to the cypris larva during 21 days in seawater, indicating the effective antifouling property of hydrophilic polymer brushes.

Introduction

Settlement of marine organisms, particularly barnacles, is a serious problem for the marine industry [1]. To develop anti-biofouling materials and coatings, it is important to understand the sessile process and permanent adhesion mechanism of barnacles and their life cycles. For instance, a planktonic nauplius hatched from an adult barnacle grows to a non-feeding cyprid larva within 1–2 weeks. The cypris larva begins exploring suitable locations for settlement by repeated tentative touches using the adhesive discs on its paired tentacles, and eventually settles on a certain position on the substrate surface to metamorphose into the juvenile barnacle [2]. During the surface exploration stage, the

cypris probes the surface morphology and physicochemical properties by using its tentacles, similar to a walking motion, on the substrate in seawater, leaving a “footprint protein” on the surface [3]. The temporary adhesion behavior and footprint adhesive have attracted much attention due to their close relationship with the settlement-inducing protein complex (SIPC) [4], which functions as a settlement cue [5].

Direct measurement of the temporary adhesion strength of live cypris larvae was first reported by Crisp [6] and Yule et al [7]. They attached live cypris larvae of *Balanus balanoides* to a piece of nichrome wire with glue. The wire was perpendicularly hooked on a suspension wire connected to a sensitive electromicrobalance to detect the force required to detach the cypris larvae from a slate substrate placed in seawater by controlling the stage position with a micrometer. They carried out a series of measurements at intervals during a period of 6 weeks from April to May in

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1979 and found that the adhesion strength increased from 1×10^5 to $2 \times 10^5 \text{ N m}^{-2}$ in mid-April to early May, reaching a maximum in the range of $2.4 \times 10^5 \text{ N m}^{-2}$, and then dropped sharply at the end of May. The seasonal variation in the temporary attachment of the cyprids was quantitatively evaluated.

The adhesion force of the footprint protein was first evaluated by Vancso et al. using atomic force microscopy (AFM) [8, 9]. The footprint protein secreted from the paired attachment disks of the ambulatory antennules of larvae contains SIPC [10] and achieves temporary adhesion during surface exploration, acting as a pheromone [11] of cuticular glycoprotein [12] and inducing gregarious settlement of conspecific cyprids [2, 13]. The AFM cantilever was controlled to once contact the footprints deposited by *Amphibalanus amphitrite* (*Balanus amphitrite*) cyprids on a chemically modified glass surface in seawater and was pulled off to obtain a force-distance curve with a characteristic sawtooth profile, corresponding to the visco-adhesive mechanism of footprint proteins.

Vancso and coworkers further investigated the adhesion force of footprint proteins by AFM using a colloidal probe-based cantilever to show the suppression of protein adsorption on poly(sulfobetaine) brushes [14] and the effect of substrate surface hydrophilicity on the interaction forces of the cyprid adhesive protein [15]. They evaluated the adhesion forces of the footprint proteins of the cypris larvae of *A. amphitrite* for temporary attachment during surface exploration by using the surface-modified colloid probe bearing footprint proteins. The measurements revealed a greater adhesion force (21 nN) on the hydrophobic surfaces compared with the more hydrophilic surfaces (7.2 nN). Although they pointed out that the direct scaling of adhesion forces observed by force-curve measurements could not predict the real adhesion of cyprids, this methodology is particularly useful for quantitative evaluation or comparison of the molecular interaction of proteins.

Recently, several research groups observed good antifouling activity against cypris larvae on surfaces covered with poly(2-hydroxyethyl methacrylate) (poly(HEMA)) brushes and zwitterion-containing polymer brushes bearing sulfobetaine [16, 17], carboxybetaine, and phosphorylcholine [18, 19]. Polymer brushes are densely surface-grafted polymers that are covalently bonded to the substrate surface to modify the surface properties, such as wettability, adhesion, and friction, corresponding to the chemical structure of the grafted polymers. It is widely known that hydrophilic polyelectrolyte brushes show antifouling and oil detachment behavior in water due to extremely low adhesion at the interface between the oil and the polymer brush surface in water [20]. In particular, poly(2-

methacryloyloxyethyl phosphorylcholine) (poly(MPC)) has low attractive interactions with proteins and living cells, imparting excellent biocompatibility [21], and antithrombogenicity [22]. However, it was still unclear why the cypris larva disfavors hydrophilic polymer brushes containing not only zwitterions but also simple hydroxyl groups, such as poly(HEMA).

In this study, we used scanning probe microscopy (SPM) to directly measure the adhesion force of live cypris larvae of *Megabalanus rosa* exhibiting temporary attachment to substrate surfaces in seawater. We attached a live cypris larva to a cantilever in such a way that its ambulatory antennules (tentacles) could behave as normally as possible, and we measured the adhesion force between the tentacles of the cypris larvae and the surface of ionic and nonionic polymer brushes in seawater during the surface exploration stage, when the cypris larvae repeated tentative touches and temporary adhesion. The age dependency of the adhesion strength was also investigated for a period of 3 weeks.

Experimental section

Materials

Commercially available copper (II) bromide (Wako Pure Chemicals, 99.9%), 2-hydroxyethyl methacrylate (HEMA, Wako, 95.9%) containing 0.025% hydroquinone methyl ether as a stabilizer, 2,2'-bipyridyl (bpy, Wako, 99.5%), L(+)-ascorbic acid (Wako, 99.6%) and 2,2,2-trifluoroethanol (TFE, Tokyo Chemical Industry, 99.0%) were used without additional purification. The sulfobetaine-type monomer 3-(*N*-2-methacryloyloxyethyl-*N,N*-dimethyl) ammonatopropanesulfonate (MAPS) was synthesized using *N,N*-dimethylaminoethyl methacrylate (Wako, 98%) and 1,3-propanesultone (Aldrich, 99%) [23]. The surface initiator, (2-bromo-2-methyl)propionyloxyhexyltrimethoxysilane (BHM) was synthesized through the hydrosilylation of 5'-hexenyl 2-bromoisobutyrate treated with trimethoxysilane in the presence of a Karstedt catalyst [24]. A square quartz cover glass (size = $18 \times 18 \text{ mm}^2$, thickness = 0.3 mm, Vidtec) was divided into square pieces ($10 \times 10 \times 0.3 \text{ mm}^3$). The silicon (111) wafer (original diameter = $100 \pm 0.5 \text{ mm}^2$, thickness = $500 \pm 25 \mu\text{m}$, Matsuzaki Seisakusho Co., Ltd.) was sliced into rectangular pieces ($10 \times 40 \times 0.5 \text{ mm}^3$). Water was purified with a Direct-Q UV3 system (Merck Millipore, Inc.) and used for substrate cleaning and contact angle measurement. Natural seawater (salinity 35) was filtered by using a Millipore Sterivex unit with a 0.22 μm filter limit. Diluted seawater with salinity 22 was prepared by simple dilution of the filtered natural seawater with deionized water.

Surface modification on glass substrates

The cover glass and silicon wafers were cleaned by washing with piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2 = 7/3$, v/v) at $100\text{ }^\circ\text{C}$ for 1 h, followed by successive rinsing with pure water to give a hydrophilic surface. The resulting hydrophilic cover glass and Si wafers were immersed in a 1.0 wt% BHM toluene solution or 1.0 wt% *n*-propyltrimethoxysilane/toluene solution at $25\text{ }^\circ\text{C}$ for 4 h to form a surface-grafted alkylbromide or propylsilane (PrS) monolayer, as shown in Fig. 1.

Surface-initiated (SI) activators generated by electron transfer (AGET) atom transfer radical polymerization (ATRP) of MAPS and HEMA was carried out as follows. A few sheets of the BHM-immobilized cover glass and silicon wafers, CuBr_2 (0.062 mmol), bpy (0.130 mmol), and 8.0 mL of MAPS/TFE solution (1.50 M) were charged in a well-dried glass tube with a stopcock. Nitrogen gas was passed through the reaction mixture with bubbling for 30 min to remove oxygen, and then 1.50 mL of ascorbic acid aqueous solution (0.200 M) was injected into the reaction mixture. The resulting reaction mixture was stirred in an oil bath at $60\text{ }^\circ\text{C}$ for 24 h under nitrogen. The reaction was stopped by opening the stopcock to air at $0\text{ }^\circ\text{C}$. The substrates were washed with TFE using a Soxhlet apparatus for 6 h to remove the monomers and catalysts adsorbed on the surface. SI AGET-ATRP of HEMA was also carried out with a CuBr_2/bpy catalyst by using a methanol/water (7/3, v/v) solution of HEMA (18 M) at $30\text{ }^\circ\text{C}$ for 24 h. The obtained

poly(HEMA) brush substrates were washed with methanol using a Soxhlet apparatus for 6 h.

Preparation of cyprid larvae

The *Megabalanus rosa* cypris larvae were cultured according to standard procedures [25]. Briefly, adult individuals of *M. rosa* were obtained from the Oga peninsula, Akita, Japan, and deployed by local fishermen. Adult *M. rosa* brood stocks were maintained in a 10-L plastic tank with aeration at a controlled temperature of $20\text{ }^\circ\text{C}$ and were fed a daily diet of naupliar larvae of the brine shrimp *Artemia salina*. Nauplii hatched out in the tank were concentrated using a light source without aeration. Nauplii were collected by pipette, transferred to a 3-L glass beaker at a density of 1 larvae mL^{-1} and fed on the diatom *Chaetoceros gracilis*. The concentration of diatoms in the beaker was kept at 60×10^4 cells mL^{-1} at a temperature of $20\text{ }^\circ\text{C}$ with a photoperiod of 12 h light and 12 h dark. A mixture of streptomycin ($30\text{ }\mu\text{g mL}^{-1}$) and penicillin G ($20\text{ }\mu\text{g mL}^{-1}$) was added to the culture seawater at the beginning of the culture to prevent bacterial growth. After 7–8 days, metamorphosed cypris larvae were collected using $100\text{ }\mu\text{m}$ plankton filter and washed in sterile filtered ($0.22\text{ }\mu\text{m}$) seawater (salinity 33). Cyprids were moved in filtered seawater and kept at $20\text{ }^\circ\text{C}$ in the dark in sterile filtered seawater for 2 days before the adhesion force measurements. Sterile filtered seawater ($20\text{ }^\circ\text{C}$, 20 min, salinity 33) was used in all the culture process. The cyprids were moved and kept in the diluted seawater with salinity 22 [26] in a Falcon conical tube (Corning, Co.) at $20\text{ }^\circ\text{C}$ in the dark until force measurements.

Characterization of polymer brush

The thickness of the polymer brush on the Si wafers was determined with an Alpha-SE KKB spectroscopic ellipsometer (J.A. Woollam Co. Inc.) equipped with a xenon arc lamp ($\lambda = 390\text{--}890\text{ nm}$) at a fixed incident angle of 70° . X-ray photoelectron spectroscopy (XPS) measurements were performed with a Quantum 2000 system (Physical Electronics Inc.) at 5×10^{-8} Torr using a monochromatic $\text{Al K}\alpha$ X-ray source at 1.48 keV. XPS data were collected at a takeoff angle of 45° , and a low-energy (24.7 eV) electron flood gun was used to minimize sample charging. The survey spectra (0–800 eV) and the high-resolution spectra (narrow scan) of the C_{1s} , O_{1s} , N_{1s} and S_{2p} regions were acquired at an energy step of 1.0 and 0.125 eV, respectively. The X-ray beam was focused on an area with a diameter of $\sim 0.1\text{ mm}$. The static contact angles against water ($2\text{ }\mu\text{L}$) were recorded with a Simage Entry system (Excimer, Inc.) equipped with a zoom camera with a USB interface. The average of five measurements was used as the data.

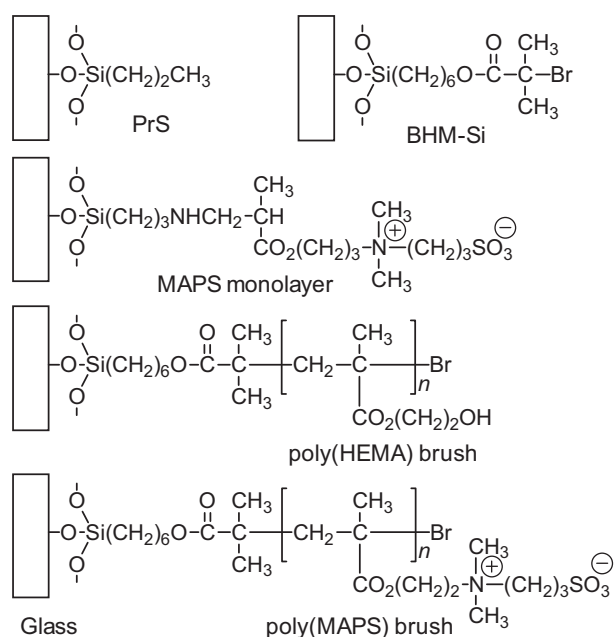
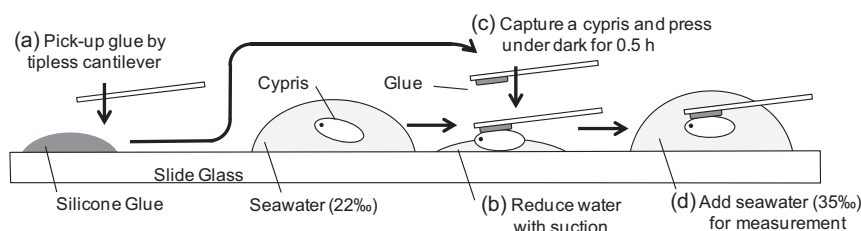


Fig. 1 Chemical structure of polymer brushes and monolayers on glass substrates

Fig. 2 Immobilization of a live cypris on a tipless cantilever with silicone glue under seawater



Adhesion force measurement of live cypris larvae

SPM was performed by using a NanoWizard 3 Ultra system (JPK Instruments) equipped with an inverted optical microscope. An Arrow TL1 tipless cantilever (NanoWorld, $L = 500 \mu\text{m}$, $W = 100 \mu\text{m}$, $t = 1 \mu\text{m}$, normal spring constant $= 0.03 \text{ N m}^{-1}$) was used for the force measurement. The torsion spring constants and sensitivities of the cantilevers were 2.6×10^2 – $2.1 \times 10^3 \text{ N m}^{-1}$ and 9.4×10^2 – $2.1 \times 10^3 \text{ N V}^{-1}$, respectively, as determined by the thermal noise method [27] in the atmosphere before use. A small amount of elastic-type chemically reactive adhesive consisting of modified silicone polymer (60%) and synthetic resin (40%) without solvent (Super X Hyper Wide, Cemedine Co., Ltd.) was placed on the slide glass and was picked up on the edge of the tipless cantilever head (Fig. 2a) by using piezoelectric scanner manipulation. A living cypris larva in a small amount of filtered seawater (23 °C, salinity 22, filter limit $0.22 \mu\text{m}$) was transferred from an incubation vessel to the slide glass. The seawater was carefully reduced through suction by a micropipette to leave the living cypris on the slide glass, as illustrated in Fig. 2b. Then, the adhesive-bearing cantilever was quickly moved down to the center of the larva body to capture the live cypris (Fig. 2c). The direction of the cantilever and craniocaudal axis of the cypris larva was perpendicular, as shown in the photograph in Fig. 3. A small portion of seawater ($100 \mu\text{L}$, salinity 22) was constantly added using a micropipette to avoid drying. After the cypris was held down by the cantilever for 20–30 min, the cantilever was moved up $500 \mu\text{m}$ from the substrate surface to confirm the adhesion of the live cypris on the cantilever. Then, the seawater (salinity 22) was exchanged with filtered seawater (salinity 35) (Fig. 2d). Two pieces of quartz cover glass, one grafted with a polymer brush and one coated with a propylsilane monolayer, were aligned on the flat slide glass and stabilized with glue, as shown in Fig. 3. Using the micrometer of the microscope moving stage, the cover glass was moved near the tentacles of the live cypris. Then, the cypris larva began tentatively touching the cover glass with its tentacles to explore the surface.

After the tentacles had contacted the surface of the cover glass for a few seconds, we moved the cypris away from the sidewall of the cover glass until the tentacles were released. At that time, the cantilever was twisted in accordance with

the adhesion strength between the tentacles and the substrate surface. The torsion of the cantilever was detected by the photodiode as a lateral deflection [V] of the laser, which was recorded at a sampling rate of 5 Hz. The torsion spring constant of the cantilever was determined based on the thermal noise method [27], prior to the immobilization of the live cypris on the cantilever. The lateral deflection was converted to adhesion force [μN] by multiplying the torsion spring constant and the cantilever sensitivity. The time course of both the lateral deflection (Fig. 4) and the adhesion and release behavior of the cypris larva was simultaneously recorded on the computer. We reviewed the recorded digital file and videos to determine the lateral deflection caused by the detachment of the tentacles from the substrate surface and calculated the adhesion force of the cypris larva. The cypris larvae were metamorphosed from nauplii on the same date, and the different individuals were used for the adhesion force measurements at the different ages.

Results and discussion

SI AGET-ATRP of MAPS was carried out from the BHE-immobilized silicon wafer and quartz substrate simultaneously to produce the polymer brush. The static water contact angle on the Si and quartz was 12° and 8° , respectively. XPS spectra of both substrates showed O_{1s} , N_{1s} , C_{1s} , and S_{2p} peaks, for which the atomic ratio agreed well with the poly(MAPS), as shown in Figure S1. The thickness of the brush on the Si wafer was determined by the ellipsometer as 90 nm. These results indicated the formation of poly(MAPS) brushes on the silicon and quartz substrates. Although, the thickness of the brush on the quartz substrate could not be determined by the ellipsometer, the refractive index of quartz and poly(MAPS) are very close in the wavelength range of 400–900 nm, and thus the brush thickness on the quartz substrate is expected to be similar to that on the Si wafer. A poly(HEMA) brush with 100-nm thickness was also prepared on a quartz substrate by surface-initiated AGET-ATRP to measure the static water contact angle of 52° . The MAPS monolayer was formed by the Michael addition of aminopropyl silane, and the water contact angle was 52° . A hydrophobic surface was

Fig. 3 Adhesion force measurement of a live cypris in seawater (salinity 35) on cover glass surface modified with polymer brushes and PrS monolayer

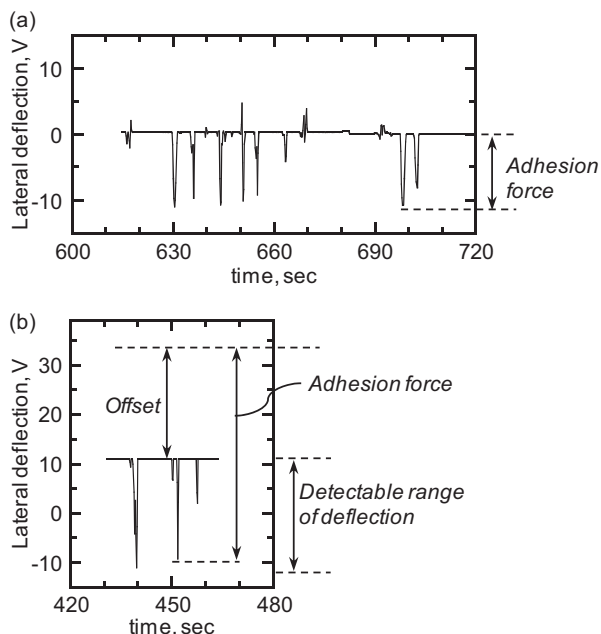
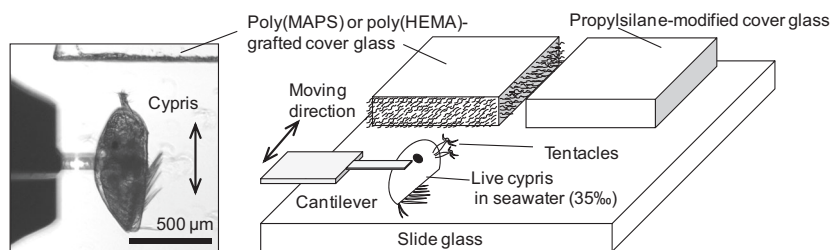


Fig. 4 **a** Typical time course lateral deflection profiles of cantilever detected by photodiode during temporary adhesion and detachment behavior of a live cypris in seawater (salinity 35) at 23 °C. **b** Offset setting example to adjust the peak top of lateral deflection (due to strong adhesion) into the detectable range limit of the photodiode

also prepared by using the PrS monomer on a quartz substrate, and the static water contact angle was 89°.

Until the force measurements, cyprids were kept in the diluted seawater with salinity 22 to prevent the settlement of cyprids on the surface of a Falcon conical tube [26]. Because Nogata et al. previously found that the settlement rate of the cypris larvae of *M. rosa* was remarkably reduced in seawater with low salinity of 22 and was promoted in seawater with salinity 35–46 [26]; we were able to control the settlement behavior of cypris larvae before the force measurement via the salinity of the seawater. A live cypris was picked up by pipet and moved to the slide glass on the microscope. After the cypris was immobilized on the cantilever, the diluted seawater was exchanged for seawater with salinity 35 to activate the settlement of the cypris. A live cypris was successfully immobilized on the cantilever head in seawater using a commercially available adhesive, as described in Experimental section and shown in Fig. 3. An experiment on the temporary adhesion and detachment

of the tentacles of the cypris on the surface-modified cover glass was performed in seawater (salinity 35) at 23 °C by repeating the approach/detachment of the cypris immobilized on the cantilever toward/from the cover glass. For example, when the cypris approached the sidewall of the cover glass, the cypris tried to attach itself to the substrate surface by gripping it with two tentacles. After two tentacles were attached on the surface, the cantilever began to move away from the cover glass. The cypris extended its tentacles to remain adhered to the surface, and one of the tentacles was released from the substrate. Eventually, the cypris retracted the other tentacle from the substrate, resulting in detachment. These processes were recorded as a video file, which is included in Supporting Information.

Figure 4a shows a typical time course lateral deflection profile of the cantilever detected by the photodiode during the adhesion and detachment behavior of the cypris. When the surface-attached tentacles were forced to retract from the sidewall of the cover glass, spike-shaped peaks were detected at the negative voltage region due to the torsion of the cantilever. Both tentacles of the cypris were attached to the substrate surface at the beginning of the experiment, and then one tentacle was detached by moving the cypris away from the substrate. When the other side of the tentacles was finally detached from the substrate, the highest peak top of lateral deflection was observed. Therefore, all forces observed in this experiment were attributed to the adhesion of one tentacle of cypris larvae. Adhesion force [μN] was estimated by multiplying the observed lateral deflection [V], the torsion spring constant [N m^{-1}] of the cantilever, and the sensitivity [N V^{-1}] of the photodiode. Sometimes, the cypris so strongly adhered to the substrate surface that the lateral deflection exceeded the detectable range limit of the photodiode. In that case, the observation position of the photodiode was adjusted to detect the peak of lateral deflection by setting the offset value from the base position, as shown in Fig. 4b. The adhesion force was estimated based on the observed deflection index and the offset value. In advance of the experiment, we observed the deflection voltage at various Z-piezo heights to confirm a proportional relationship between vertical deflection and bending angle of the cantilever in the vertical range from -10 to 10 V for NanoWizard 3 system. In the case of lateral deflection, the

tip displacement per volt of detector signal by torsion is much smaller than the displacement by bending. In other words, the actual detection area for lateral deflection is

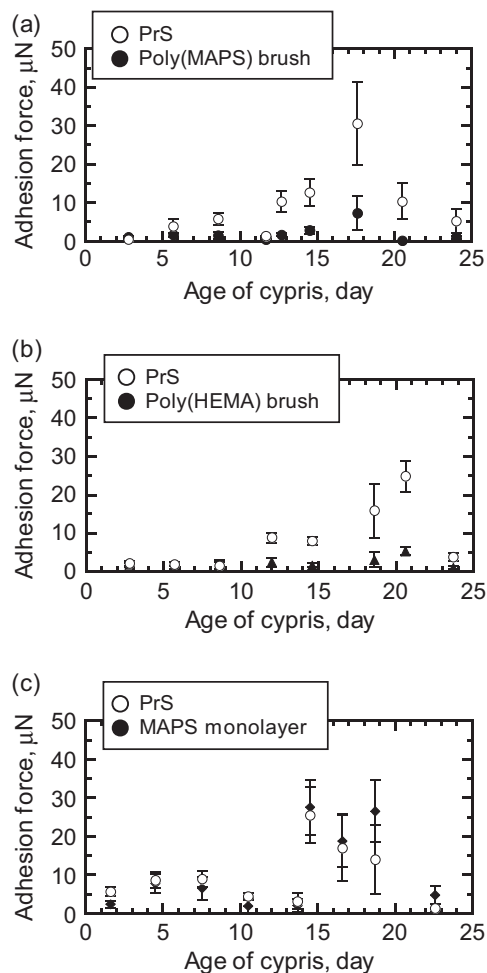


Fig. 5 Age dependency of adhesion force of tentacles of cypris on surface of (a) poly(MAPS) brush, (b) poly(HEMA) brush, or (c) MAPS monolayer in seawater (salinity 35) at 23 °C. As a control experiment, adhesion forces on the surface of hydrophobic propylsilane (PrS) monolayer were measured at each force measurement

Table 1 Adhesion force (F) of tentacles of live cypris on surface-modified glass in seawater^a

Age, days	F on PrS, μN	N	Age, days	F on poly(MAPS) brush, μN	N	Age, days	F on poly(HEMA) brush, μN	N
2.8	0.40 ± 0.20	20	2.8	0.10 ± 0.10	15	1.8	0.20 ± 0.20	58
5.7	2.6 ± 2.1	55	5.7	0.40 ± 0.40	39	4.7	0.40 ± 0.40	67
8.6	3.9 ± 2.8	89	8.6	0.20 ± 0.20	114	7.6	1.1 ± 1.0	52
12.7	9.40 ± 3.6	68	12.7	0.30 ± 0.30	58	11	0.60 ± 0.60	37
14.5	12.7 ± 3.6	39	14.5	1.0 ± 1.0	63	13.6	0.50 ± 0.50	46
17.6	29.6 ± 15.4	56	17.6	4.1 ± 4.0	40	17.6	0.7 ± 0.70	28
20.5	10.3 ± 4.7	12	20.5	0.0 ± 0.0	14	19.6	1.2 ± 1.0	42
22.0	5.2 ± 3.1	5	22.0	0.30 ± 0.25	69	22.7	0.60 ± 0.60	27

N number of samplings (number of times)

^aTypical F measured in seawater (salinity 35) at 23 °C were presented

narrower than that of vertical deflection. Therefore, we supposed that linearity between the lateral deflection and the torsion angle was sufficiently maintained in our measurement range, including offset.

Adhesion experiments with live cypris were carried out every 2 or 3 days after the cyprids metamorphosed from nauplii, using two sets of quartz cover glass: one modified with a hydrophilic polymer brush and one coated with a hydrophobic PrS monolayer, as shown in Fig. 3, to compare the adhesion behavior of the cypris using the two substrates under the same aqueous environment on the same day. Therefore, the adhesion force of the cypris on the PrS-modified glass was measured in every experiment. The histograms of the adhesion force between the surface-modified quartz cover glass and the tentacles of a live cypris in seawater (salinity 35) at 23 °C for 3 weeks are shown in Figure S5–S7 in Supporting Information. Figure 5 and Table 1 summarize the age dependency of the adhesion force.

Young cypris (<10 days old) exhibited adhesion force lower than 4 μN on the hydrophobic PrS surface, but their adhesion force gradually increased with age and reached 30–40 μN after 14–20 days, as shown by the open circle plots in Fig. 5. Much larger adhesion forces of 60–80 μN were exhibited by cypris 17 days old. These results indicated that the adhesive behavior of the cypris was activated with age until 2 weeks after transformation from nauplii. The adhesion force dropped again 22 days later, likely due to deterioration with age. It has already been reported that the settlement activity of cypris is largely influenced not only by their age but also by the season, environment, and water flow rate (stream velocity) near the substrates [28]; therefore, it will be necessary to measure adhesion under various conditions in future work.

On the other hand, the surfaces of the hydrophilic poly(MAPS) and poly(HEMA) brushes exhibited extremely low adhesion to the cypris larva for 21 days in seawater, as shown by filled circle plots and triangle plots in Fig. 5a, b,

respectively. These results indicated the effective antifouling property of superhydrophilic polymer brushes. In contrast, the MAPS monolayer surface showed a similar adhesion trend to that of the PrS-modified surface (filled rhombus plots in Fig. 5c). The adhesion force of the cypris on the MAPS monolayer gradually increased with time, reaching 30 μN after 14–20 days, indicating the poor antifouling property of the MAPS monolayer. The poly(MAPS) brush forms a swollen structure in aqueous solution due to high osmotic pressure [29, 30]. Low adhesion on the poly(MAPS) brush surface may be induced not only by the low chemical interaction between MAPS and the footprint protein but also by the exclusive volume effect of a high-density brush.

Although, the exact attachment area of the tentacles on the substrate surface was not measured in this study, the typical footprint of one tentacle was estimated to be $\sim 20 \mu\text{m}$ in diameter based on optical micrographs and SEM images of the cypris in Figure S8 and S9, respectively. Supposing the temporary adhesion force of the cypris to the PrS surface was 30–60 μN in our experiment, the force per unit area required to detach the cypris from the PrS surface was calculated as 0.95×10^5 – $1.9 \times 10^5 \text{ N m}^{-2}$ (95–190 kPa), lower than the vertical force of $2.16 \times 10^5 \text{ N m}^{-2}$ (216 kPa) of the cypris larvae of *B. Balanoides* for detachment from a slate substrate reported by Crisp et al. [6]. Yule and Walker reported adhesion strength of 69–76 kPa on glass substrate [7]. Vancso and colleagues theoretically predicted that cyprids could attach with a tenacity of 26 kPa based on the pull-off force of the footprint protein measured by AFM [8]. We could not directly compare the adhesion force of different species of cypris larvae on different substrate surfaces; however, it is meaningful that a similar order of magnitude of adhesion force was obtained from a different measurement system.

Regarding other sessile organisms, it is well known that the adhesion strength of mussels is $\sim 750 \text{ kPa}$ [31], owing to the mussel adhesive protein (MAP) containing dihydroxyphenylalanine (DOPA), which coordinates with a metal oxide surface and forms cross-linking organs by oxidation. Yamamoto chemically synthesized an MAP mimetic polypeptide and sandwiched its aqueous solution between two metal pieces, achieving an adhesion strength of 290 kPa [32]. Nishida et al. synthesized a hydrophilic copolymer via radical polymerization of acrylamides bearing DOPA moiety, and hydroxyl and amino groups, and demonstrated adhesion under wet conditions reaching an adhesion strength of 450 kPa [33]. These workers further synthesized a barnacle mimetic polymer containing a self-assembled peptide sequence to achieve the adhesion of poly(methyl methacrylate) substrates with a lap shear adhesion strength of 402 kPa [34]. The adhesion

strength of the attachment/detachment of a gecko's toe pads on a vertical surface is widely known to be ~ 100 – 350 kPa [35, 36]; although, the adhesion is achieved by van der Waals force under air atmosphere. These adhesion behaviors in nature or nature-inspired adhesives showed much larger adhesion strength than that observed in our experiment. It would be difficult to evaluate such large adhesion force by SPM due to the excessive stiffness of the cantilever. However, this method is appropriate for measuring relatively weak adhesion or repeatable temporary adhesion, which varies on the age of living things under various environments.

In general, adhesion strength cannot be determined only by maximum force but also by energy. It is well known that the total energy required to break the adhesion is usually given by the area of the force-distance curve. In the present study, precise distance information between the tentacles and the substrate surface during the push-and-pull process was not obtained very well from the video of the microscope observation. In addition, the exact adhesion area of the attached tentacles was not measured. From these perspectives, further improvement including footprint size measurement by using the dye reagent Coomassie Brilliant Blue (CBB) [13, 37] will be required in the future. However, the system described here makes it relatively easy to measure the adhesion force of live cypris using a commercially available SPM with the established setup. The capture process of live cypris is also a conventional method for the preparation of a spherical colloid probe for force curve measurement by immobilization of a colloidal particle on the tipless cantilever with glue. Therefore, this method would be applicable to not only cypris larvae but also various sessile animals.

Conclusions

Measurement of the adhesion force of live cypris to various surface-modified substrates in seawater by SPM was demonstrated. A live cypris was successfully immobilized on the cantilever of SPM to make contact with the surfaces covered with hydrophilic polymer brushes and hydrophobic PrS. The adhesion force of the tentacles of the live cypris on PrS surface gradually increased with its age, reaching 30–60 μN , which corresponds to 0.95×10^5 – $1.9 \times 10^5 \text{ N m}^{-2}$ after 14–20 days. Age dependency of the adhesion behavior of cypris was clearly observed. On the other hand, the hydrophilic poly(MAPS) and poly(HEMA) brush surfaces exhibited extremely low adhesion over a period of 3 weeks. Further experimentation is in progress using SPM to understand the relationship between the temporary adhesion

strength of the live cypris and the exploration behavior in various solution conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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