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Probing biofouling resistant polymer brush surfaces by atomic force microscopy based force spectroscopy

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ABSTRACT

The protein repellency and biofouling resistance of zwitterionic poly(sulfobetaine methacrylate)(pSBMA) brushes grafted via surface initiated polymerization (SIP) from silicon and glass substrata was assessed using atomic force microscopy (AFM) adherence experiments. Laboratory settlement assays were conducted with cypris larvae of the barnacle *Balanus amphitrite*. AFM adherence includes the determination of contact rupture forces when AFM probe tips are withdrawn from the substratum. When the surface of the AFM tip is modified, adherence can be assessed with chemical specifity using a method known as chemical force microscopy (CFM). In this study, AFM tips were chemically functionalized with (a) fibronectin- here used as model for a nonspecifically adhering protein – and (b) arginine-glycine-aspartic acid (RGD) peptide motifs covalently attached to poly(methacrylic acid) (PMAA) brushes as biomimics of cellular adhesion receptors. Fibronectin functionalized tips showed significantly reduced nonspecific adhesion to pSBMA-modified substrata compared to bare gold (2.3 ± 0.75 nN) and octadecanethiol (ODT) self-assembled monolayers (1.3 ± 0.75 nN). PMAA and PMAA-RGD modified probes showed no significant adhesion to pSBMA modified silicon substrata. The results gathered through AFM protein adherence studies were complemented by laboratory fouling studies, which showed no adhesion of cypris larvae of *Balanus amphitrite* on pSBMA. With regard to its unusually high non-specific adsorption to a wide variety of materials the behavior of fibronectin is analogous to the barnacle cyprid temporary adhesive that also binds well to surfaces differing in polarity, charge and free energy. The antifouling efficacy of pSBMA may, therefore, be directly related to the ability of this surface to resist nonspecific protein adsorption.

1. Introduction

Marine biofouling has considerable economic and environmental impact [1–5]. It poses a major problem for harbor installations, oil rigs, vessels, underwater sensors, aquaculture, and for a range of other maritime industries. Prevention of biofouling is usually achieved through use of toxic heavy metals such as copper and/or organic biocides in paints which are slowly released into the environment [6]. Currently, there is legislative and societal pressure to reduce the use of such biocides and provide alternative, environmentally compatible, approaches to biofouling prevention [7]. In this regard tributyltin (TBT) was of great importance but it has officially been banned by the International Maritime Organization as of 2008. Thus the need to find new solutions is pressing.

Some experimental antifouling strategies make use of protein resistant polymers and surface modifications [7–9]. Such fouling-resistant surfaces may directly prevent attachment by fouling organisms and, thus, development of a fouling community. They rely upon weakening intermolecular forces between extracellular biomolecules in the organism's adhesive system and the surface, so that the organism is either unable to attach, or is easily released under low hydrodynamic stress during e.g. movement of a ship.

Various strategies for chemical modification of surfaces have been developed to inhibit protein adsorption, including approaches stemming from the optimization of biosensors and biomedical implants [10]. In this regard surface modification using polymer brushes represents a promising approach for the molecular design of novel experimental antifouling surfaces [11–13]. Zwitterionic brushes comprising functional groups such as phosphorylcholine (PC) [14], sulfobetaine (SB) [15], and carboxybetaine (CB) [16] have been introduced as effective strategies for rendering surfaces resistant to biofouling [17]. The non-fouling properties of zwitterionic bushes are thought to be determined by a combination of functional groups, surface packing and brush length. The fundamental principle of their non-fouling behavior lies in their capacity to form a strongly bound hydration layer at the interface between

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the zwitterionic polymer layer and the bulk water via electrostatic interactions, rather than hydrogen bonding as is the case in polymer layers containing poly(ethylene glycol) (PEG). Importantly zwitterionic polymer brushes show better stability than most commonly used PEG-containing modifications, which undergo rapid oxidation in the presence of transition metals and oxygen, leading to aldehyde formation accompanied by undesired covalent attachment of proteins [15].

Recent studies have demonstrated that zwitterionic poly(sulfobetaine methacrylate) (pSBMA) brushes grafted from surfaces are highly resistant to protein adsorption and reveal excellent antifouling properties [15,17–19]. Being environmentally benign, they are particularly promising as ultralow fouling marine coatings.

Barnacles are a commercially significant group of biofouling organisms and cyprids, the settlement stage larvae, are of particular importance to biofouling research [20]. During exploration of potential settlement surfaces, cyprids make use of a proteinaceous temporary adhesive to facilitate reversible attachment [20–22]. A deposit of this material, referred to as a 'footprint' (FP), is left on the explored surface [23]. The exact physiochemical nature of the FP adhesive material remains largely unknown. However, a settlement-inducing protein complex (SIPC), which functions as a settlement pheromone for cyprids, has been found in FP adhesives [24] and shows strong non-specific adsorption to a range of surface chemistries (unpublished data), allowing cyprids to attach to most immersed surfaces. It is postulated that this large (~200 kDa) glycoprotein complex may serve a dual role as a pheromone and nonspecific adhesive molecule. Importantly, there is no evidence that the adult barnacle 'cement' proteins identified by Kamino [25] play any role in the adhesion of cyprids to surfaces.

Fibronectin is a multifunctional, high-molecular weight glycoprotein (~440 kDa) present in blood, connective tissue and at cell surfaces where it mediates cellular adhesion, binding to a variety of cellular receptors. In addition to its central role in cellular binding via RGD motifs, fibronectin shows unusually high non-specific adsorption to a wide variety of materials, including 'non-biological surfaces' such as metals, plastics, polymers, polysaccharides, ceramics and glass [26–32]. Fibronectin, therefore, serves as a reasonable model for the nonspecific surface adsorption of cyprid temporary adhesive/SIPC in the absence of purified SIPC, and facilitates discussion of the possible mode of action of SBMA against exploring cyprids. It must be stressed that since the exact composition of the cyprid temporary adhesive is unknown, these materials should be considered analogous in terms of their behavior, rather than their functionality. For this purpose we use fibronectin functionalized AFM tips to probe protein repellency at modified surfaces.

Atomic force microscopy (AFM) has become a pivotal tool for the visualization and force probing of materials and biological samples across the length scales, providing nanometer spatial resolution [33–36]. Importantly, AFM measurements can be carried out under ambient conditions, or in a liquid environment relevant to the systems under observation. The approach used here is chemical modification of the AFM tip [37,38] which now often includes the covalent attachment of biomolecules [39,40]. These techniques are an invaluable tool for chemistry and biology-specific probing of surfaces, including polymeric [41,42] and biological systems [43].

Based on the qualitative similarity of fibronectin and SIPC adsorption to surfaces, we propose to use AFM-based fibronectin adherence measurements as an assay to predict cyprid settlement to surfaces. From a methods perspective, fibronectin also offers the important advantage that it is linkable to an AFM tip without losing its adhesive properties; including biochemical binding via RGD motifs. Finally, we propose novel AFM colloidal probe modifications with RGD-peptide motifs covalently attached to poly(methacrylic acid) (PMAA) brushes as biomimics of cellular adhesion receptors.

2. Experimental

Chemicals were purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands) and used as received unless stated otherwise. Organic solvents were purchased from Biosolve (Valkenswaard, the Netherlands), hydrogen peroxide and ethanol were purchased from Merck (NW Schiphol-Rijk, the Netherlands). CuBr was cleaned with 98% acetic acid solution, stirred overnight, filtered, washed with methanol and dried under vacuum. Deionized water was taken from a MilliQ Advantage A10 purification system (Millipore, Billerica, Ma, USA).

2.1. Preparation of pSBMA brushes

Zwitterionic pSBMA brushes were grafted from silicon substrates according to the literature to obtain non-fouling substrates [44]. For the preparation of pSBMA brushes on silicon, initiator molecules were first immobilized on freshly cleaned silicon substrates, under vacuum, by overnight vapor-phase deposition. The same deposition time and silane concentration was used in each case. In this way the initiator grafting density was controlled. Prior to the deposition, substrates were cleaned by sonication in ethanol (15 min) and then in purified water (15 min). Subsequently, the substrates were cleaned in piranha solution (7:3 v/v mixture of H_2SO_4 (95–98%) and H_2O_2 (30%)) for a few minutes, rinsed extensively with water, ethanol, and then dried (Caution: Piranha solution reacts violently with many organic materials and should be handled with great care!). After initiator deposition, substrates were sonicated in ethanol for 2 min, rinsed with ethanol and dried under nitrogen gas.

Initiator-immobilized substrates were immediately used for surface-initiated ATRP. For polymerization, the initiator-covered substrates were placed in dry vials and purged with argon for 2 h. The monomer, [2-(methacryloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide (SBMA) (5.58 g, 20 mmol), was dissolved in the ATRP medium (40 ml water/methanol mixture with ratio 1:4) and the solution was degassed for 2 h. CuBr (286 mg, 2 mmol), $CuBr_2$ (53 mg, 0.24 mmol), and 2,2′-bipyridine (626 mg, 4 mmol) were added to a flask equipped with a magnetic stir bar, and deoxygenated by 3 vacuum-argon backfilling cycles. Next, the degassed monomer solution was added and the resulting mixture was stirred under argon for further 15 min until a clear brown solution was observed. Afterwards, the polymerization mixture was injected into each reaction vial, adding enough solution to submerge each sample completely. To obtain polymer brushes with different thicknesses, the polymerization time was varied and carried out according to previously determined kinetics in our lab (data not yet published). After polymerization the samples were removed from the vials and washed with Milli-Q water. After brief sonication in EDTA solution (0.1 M, pH 7) to extract residual copper ions, substrates were immersed in water overnight to remove any physisorbed polymer. Finally, the substrates were rinsed with ethanol and dried under nitrogen gas. Brush (dry) thicknesses were determined with ellipsometry according to a recent publication from our group including further information on the pSBMA brush synthesis and reaction schemes [44].

2.2. Preparation of self-assembled monolayers

Self-assembled monolayers of alkanethiols were chosen as model systems for highly protein-adhering and protein-repelling surfaces. Before modification, gold substrates (100 nm gold evaporated on Si wafer over a 10 nm Cr adhesion layer) were

thoroughly cleaned. Surfaces were rinsed first with chloroform and piranha solution, then with Milli-Q water, ethanol and chloroform, and finally immersed in the monolayer solution overnight. Monolayer solutions consisted of degassed ethanolic solutions of either 1-octadecanethiol ($CH_3(CH_2)_{17}SH$) (10 mM), triethylene glycol mono-11-mercaptoundecyl ether ($C_{17}H_{36}O_4S$) (1 mM) or cysteamine (∼nM) for 24 h. Substrates were then rinsed with ethanol and dried under argon.

2.3. Modification of AFM tips with fibronectin

AFM tips were modified with fibronectin and used to locally probe nonspecific protein adherence to pSBMA brush-modified substrates and reference samples. Prior to modification, gold-coated AFM probes (Bruker probes, Santa Barbara, CA, USA) were first rinsed with chloroform followed by immersion in freshly prepared piranha solution for a few seconds. Freshly cleaned AFM cantilevers were immersed in a freshly prepared solution of 10 μg/ml fibronectin in phosphate buffered saline (PBS, pH 7.4, 0.1 M NaCl) and kept overnight at 4 °C. Subsequently the cantilevers were carefully rinsed with PBS and used immediately or stored in protein free PBS solution at 4 °C. The adsorbed fibronectin forms a stable linkage to the gold-coated AFM tips without loss of biological receptor binding function [43,45].

2.4. Modification of AFM probes with PMAA-RGD brushes

Conventional gold-coated AFM tips and gold colloidal probes were modified with PMAA-RGD brushes according to the literature [46]. These probes were used to locally probe protein adherence at pSBMA brush modified substrates and reference samples. First the initiator was deposited on gold surfaces followed by SI-ATRP of PMAA and covalent attachment of the RGD peptide.

2.4.1. Initiator deposition on gold surfaces and AFM probes for polymer grafting

A monolayer solution was prepared with 2-bromo-2-methyl-propionic acid 11-[11-(2-bromo-2-methyl-propionyl-oxy)-undecyldisulfanyl]-undecyl ester $[(BrC(CH_3)_2COO(CH_2)_{11}S)_2$ in chloroform (20 ml with the concentration of 0.2 mM) [47]. Either planar gold surfaces, conventional AFM probes or colloid probes (SQube, CP-FM-Au, Nano and more, Wetzlar, Germany) were immersed in the solution. Planar gold surfaces were used as a references for contact angle and FTIR characterization.

2.4.2. SI-ATRP of PMAA from gold coated AFM probes

The initiator-covered substrates were placed in dry vials and purged with Ar for half an hour. The monomer sodium methacrylate (3 g, 0.027 mol) was dissolved in the ATRP medium (10 ml water/methanol mixture with ratio 1:1) and deoxygenated by bubbling with argon for half an hour. Concurrently, 2,2′-bipyridine (31 mg, 0.2 mmol) and CuBr (28 mg, 0.2 mmol) were added to a flask equipped with a magnetic stir bar and deoxygenated by 3 vacuum-argon backfilling cycles. The degassed monomer solution was transferred in and was stirred under argon for a further 15 min until a clear brown solution was observed. The polymerization mixture was injected into each reaction vial in such a way that each sample was immersed completely in the solution. The polymerization was conducted for 3 h at room temperature and terminated by exposing the solutions to air. The samples were removed from the vials and washed with MilliQ water. PMAA brush-modified surfaces were kept in 1 M EDTA (pH 7.4) solution overnight to remove all residual copper.

2.4.3. RGD functionalization of the PMAA grafted AFM probes

To activate the carboxylic acid groups of surface-immobilized PMAA, active ester chemistry was applied using an EDC/NHS solution: 38 mg/ml *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and 6 mg/ml *N*-hydroxysuccinimide (NHS) in phosphate buffer (pH 7.4, 0.1 M NaCl). AFM probes were immersed in the activation solution for 1 h. After removal they were rinsed with buffer solution and subsequently immersed in the RGD tripeptide solution (2 mM in phosphate buffer) overnight. Finally, the PMAA- modified and RGD functionalized AFM probes were rinsed gently with phosphate buffer solution and stored in buffer at 4 °C. Flat substrata were kept under N_2 atmosphere.

2.5. Atomic force microscopy-based adherence probing

AFM-CFM measurements were performed with a PicoForce AFM using a NanoScope IVa controller, a PicoForce vertical engage scanner and a liquid cell. Gold-coated, V-shaped Si_3N_4 cantilevers or gold colloid probes were functionalized as described above. The modified AFM probes were taken out of the storage solutions and immediately mounted to the AFM liquid cell. All experiments were done at pH 7.4 in phosphate buffered saline (PBS) 150 mM NaCl (B. Braun Melsungen AG, Melsungen, Germany). 500 mM and 1 M NaCl buffer solutions were prepared by supplying additional NaCl to the starting buffer solution. Surfaces were subsequently measured with the same colloidal probe: first the polymer brush, next the Si reference surface, followed by the amine-terminated monolayer surface. Here the salt concentration was increased and finally the polymer surface was measured at 1 M NaCl concentration to avoid errors due to ions remaining in the polymer brush grafted onto the probe surface. Cantilever spring constants were determined after each experiment using the thermal tune method [48] and showed values in the range of 0.03–0.12 N/m. Data analysis was performed with the NanoScope software version 8.10 and PUNIAS version 3D. For each AFM experiment (tip-substrate combination) at least 500 force curves were captured. For CFM adhesion probing, different locations with minimal 1 μm lateral separation distance were selected on the modified substrates.

2.6. Lab settlement assay

To probe the biofouling resistance of pSBMA modified glass slides against *Balanus amphitrite* cypris, a laboratory settlement assay was done. Glass microscope slides, either cleaned by acetone and dried under a nitrogen atmosphere or with pSBMA polymer coatings, were placed inside polystyrene Quadriperm™ dishes – four slides per dish. 1 ml of 0.2 μm-filtered natural seawater was added in a drop on each slide and 20 3-day old cyprids [49] were introduced to each drop. The dishes were then sealed and wrapped in moist laboratory tissue for the duration of the assay to minimize evaporation. Although the water droplet spread significantly on the microscope slides, the hydrophilic nature of the surfaces prevented it from spilling off and the meniscus produced was suffciently high to allow cyprids free movement. After addition of the cyprids, the assay was incubated at 28 °C in the dark for 72 h and settlement was enumerated every 24 h. The mean percentage settlement was calculated per surface type and used for comparison.

3. Results and discussion

The objective of this study was to assess the protein repellence and biofouling resistance of zwitterionic pSBMA brushes by AFM adherence experiments and to provide complementary laboratory fouling data with cypris larvae of the barnacle *B. amphitrite.* pSBMA brushes were grafted from silicon and glass substrates [44]. The use of chemical functionalization of AFM tips encompassed adsorbed

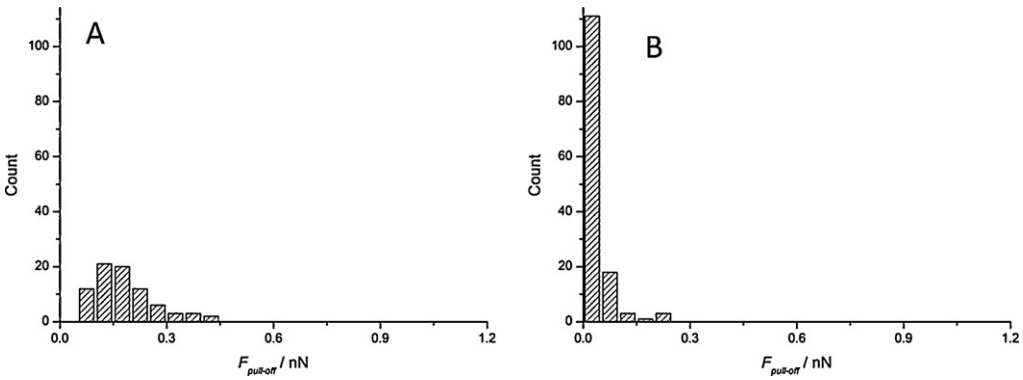

Fig. 1. Distribution of pull-off forces recorded with a fibronectin-functionalized AFM tip on pSBMA with (A) 14 ± 3 nm and (B) 48 ± 5 nm thick brushes on silicon.

fibronectin as a model protein showing unusually high non-specific adsorption to a wide variety of materials. Further, we introduce a novel type of AFM colloidal probe modification composed of RGD-PMAA brushes. The results of the AFM adherence measurements obtained with fibronectin-modified AFM tips on pSBMA brushes and reference samples are first discussed.

3.1. AFM adherence probing using fibronectin modified AFM tips

The surface grafted pSBMA brushes used in these experiments exhibited two different (dry) thicknesses; a thin (14 ± 3 nm) and a thicker (48 ± 5 nm) structure to test the impact of brush thickness on protein repellent properties. In Fig. 1 the distributions of the recorded pull-off forces on pSBMA brushes of different thicknesses are shown obtained with fibronectin modified AFM tips. The measured mean pull-off forces were 0.25 ± 0.18 nN and 0.03 ± 0.003 nN for the thin and the thick brushes respectively. Hence, only weak adhesion of the fibronectin-modified AFM tip on the thinner brush, to almost zero level on the thick pSBMA brush was measured.

It is suggested that for the thin polymer brushes, the increased adhesion results from an incomplete surface modification layer, exposing the underlying silicon substrate. Therefore, for maximal protein repellence of the modified substrate, an optimal brush thickness is crucial. The thicker brush layer provides a more complete modification layer and stronger shielding of the underlying silicon. This is in agreement with observations made recently by Jiang and co-workers [50]. Using surface plasmon resonance (SPR) Jiang and co-workers observed that fibrinogen adsorbs weakly from solution onto thin pSBMA layers of 15 nm and was completely repelled by brushes of 50–60 nm thickness. Interestingly, increasing the pSBMA brush layer thickness to 90 nm resulted in substantial fibrinogen adsorption, which the authors attributed to self-condensation of the brush molecules and thus weakening of the surface hydration layer. Hence there exists an optimal thickness regime of grafted pSBMA brushes to maintain maximal protein repellence.

pSBMA brush-modified substrates were compared with protein-adhering ODT monolayers on gold and bare gold substrates. In Fig. 2, the distributions of the recorded pull-off forces obtained on an ODT SAM and bare gold are shown, both in comparison to the results obtained on the thicker (48 ± 5 nm) pSBMA brush (Fig. 1B).

For ODT monolayers the mean pull-off force was measured to be 1.3 ± 0.75 nN while on bare gold substrates the strongest fibronectin adhesion was observed at 2.3 ± 0.75 nN. These pull-off forces are significantly larger than those observed on the pSBMA brushes indicating strong adhesion of fibronectin to ODT SAMs and bare gold, respectively.

It was found that proteins adsorb spontaneously onto hydrophobic surfaces such as ODT, thereby minimizing the interfacial tension between the surface and water [51–54]. This is in good agreement with our observations as reflected in the large pull-off forces we recorded with the fibronectin modified AFM tips on ODT SAMs (Fig. 2B). Nonspecific protein adsorption is of technical relevance in the fields of biosensors, medical implants and biofouling [10]. Numerous studies on nonspecific protein adsorption can be found in the literature with special attention given to the development of strategies to inhibit protein adsorption at surfaces, because it leads to malfunctioning biosensors, non-compatibility of medical implants and biofouling. Self-assembled monolayers of alkanethiols were introduced as one of the first model systems used to study nonspecific protein adsorption systematically, as a function of the chemical nature of the terminal headgroup and its polarity [55,56].

Strongest adherence of fibronectin was observed on bare gold surfaces, which is in accordance with numerous reports documenting nonspecific protein adsorption and chemisorption at gold

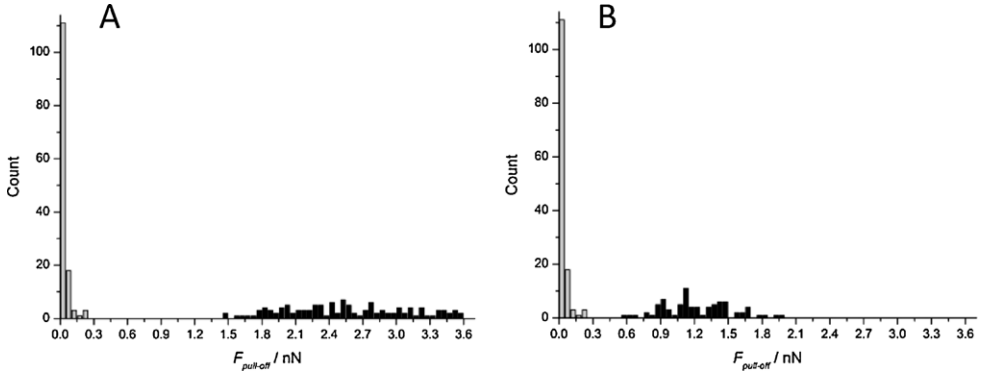

Fig. 2. Distribution of pull-off forces recorded with fibronectin modified AFM tips. pSBMA brushes (48 ± 5 nm) on silicon. (A) bare gold surface, (B) ODT modified gold surface.

Table 1
Results of a *Balanus amphitrite* cyprid settlement assay after 48 h on either glass or pSBMA modified glass substrate, including mean value and standard deviation.

Replicate	Glass	pSBMA
1	5.9	0
2	22.7	0
3	43.8	0
4	15	0
5	4.8	0
6	4.5	0
7	21	0
8	–	0
mean	16.8 ± 14.1	0

surfaces [57]. In many cases adsorption is irreversible and also leads to denaturation of the protein [51–54]. Covalent gold–sulfur linkages might occur from free cysteine or disulfide bridges within or between adsorbing proteins. Since fibronectin is rich in sulfur atoms it may be that temporary gold–sulfur bonds were formed between the fibronectin and the bare gold surface during a force curve cycle. This explains the largest pull-off forces recorded during our measurements which were within the expected regime for gold–sulfur bond strength (1.4 ± 0.3 nN) [58].

As reference samples for a protein-repelling substrate a well-established self-assembled monolayer of triethylene glycol terminated undecanethiol on gold was used [55,56,59]. The observed mean pull-off force of 0.025 ± 0.003 nN (histogram data not shown) was in good agreement with those measured on thick pSBMA brushes (Fig. 1), suggesting a similar degree of protein resistance for the two materials. The primary drawback of PEG or OEG-based modifications is that ethylene oxide chains are, over time, auto-oxidized in aqueous solutions. This results in the cleavage of ethylene oxide units and formation of aldehyde-terminated chains which can covalently bind proteins and thus mitigate any innate protein-resistance. In this critical regard, zwitterionic pSBMA brushes provide a superior alternative to traditional PEG or OEG modifications.

3.2. Laboratory fouling studies

In complementary laboratory fouling studies, larvae of *B. amphitrite* were exposed to pSBMA-modified glass in seawater to determine the potential resistance of pSBMA to a economically important macro-fouling organism. While on the pSBMA surfaces no settled larvae were observed, the clean glass standard surfaces received significant settlement after 48 h (Table 1). These results confirm the excellent anti-biofouling properties of pSBMA brushes previously reported [15] under laboratory conditions, where cyprids are considered to be either unwilling or unable to settle.

In conclusion, the results confirm that modification of surfaces with pSBMA brushes provides an effective strategy to repel non-specific protein adsorption. The surfaces also prevent settlement of cyprids. It seems plausible to suggest, therefore, that the property of the SBMA surface that prevents non-specific adsorption of fibronectin, may also prevent non-specific adsorption of the cyprid temporary adhesive, thus reducing settlement of larvae to the surfaces. For these surfaces it appears that AFM-based force measurements using fibronectin modified AFM tips may provide a good indication of anti-fouling efficacy.

3.3. PMAA-RGD brushes for AFM adherence probing

We introduce a novel type of AFM colloidal probe modification composed of PMAA-RGD brushes as biomimics of cellular adhesion receptors. In future studies these probes shall be used to explore the adhesive proteins of macrofoulers for RGD-like interactions, for example by force probing of barnacle footprints. Here we focus exclusively on the preparation and characterization of these novel PMAA-RGD probes.

The RGD tripeptide sequence is the main binding site of fibronectin to integrin receptors on living cells and also plays an important role in adherence to different material surfaces [29–32]. Here PMAA brushes grafted from AFM tips and colloidal probes were used for the covalent immobilization of the RGD binding site prepared according to the literature [31,46]. As chemically selective probes offering multiple RGD binding motifs those were applied to measure adherence to zwitterionic pSBMA brushes on silicon and other modified surfaces as illustrated in Fig. 3.

The distributions of pull-off forces obtained using PMAA and PMAA-RGD modified colloidal probes on pSBMA brushes are shown in Fig. 4. Neither PMAA nor PMAA-RGD modified colloidal probes showed significant adhesion to the pSBMA modified silicon substrate. Adhesion forces observed between the different surfaces were between 0 to 1.5 nN. In the case of a colloidal probe, the contact area is larger compared to a conventional AFM tip and therefore more sensitive to attractive forces when compared to conventional sharp AFM tip; hence the total measured forces are shifted to larger values compared to those in previous figures. In most cases no adhesion was observed, as shown in the frequency histograms (Fig. 4).

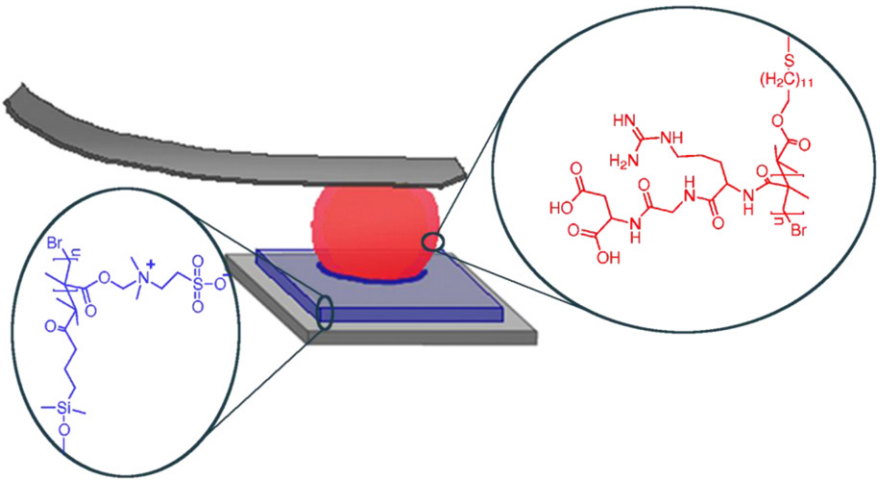

Fig. 3. Schematics of a PMAA-RGD modified AFM colloid probing a pSBMA modified substrate.

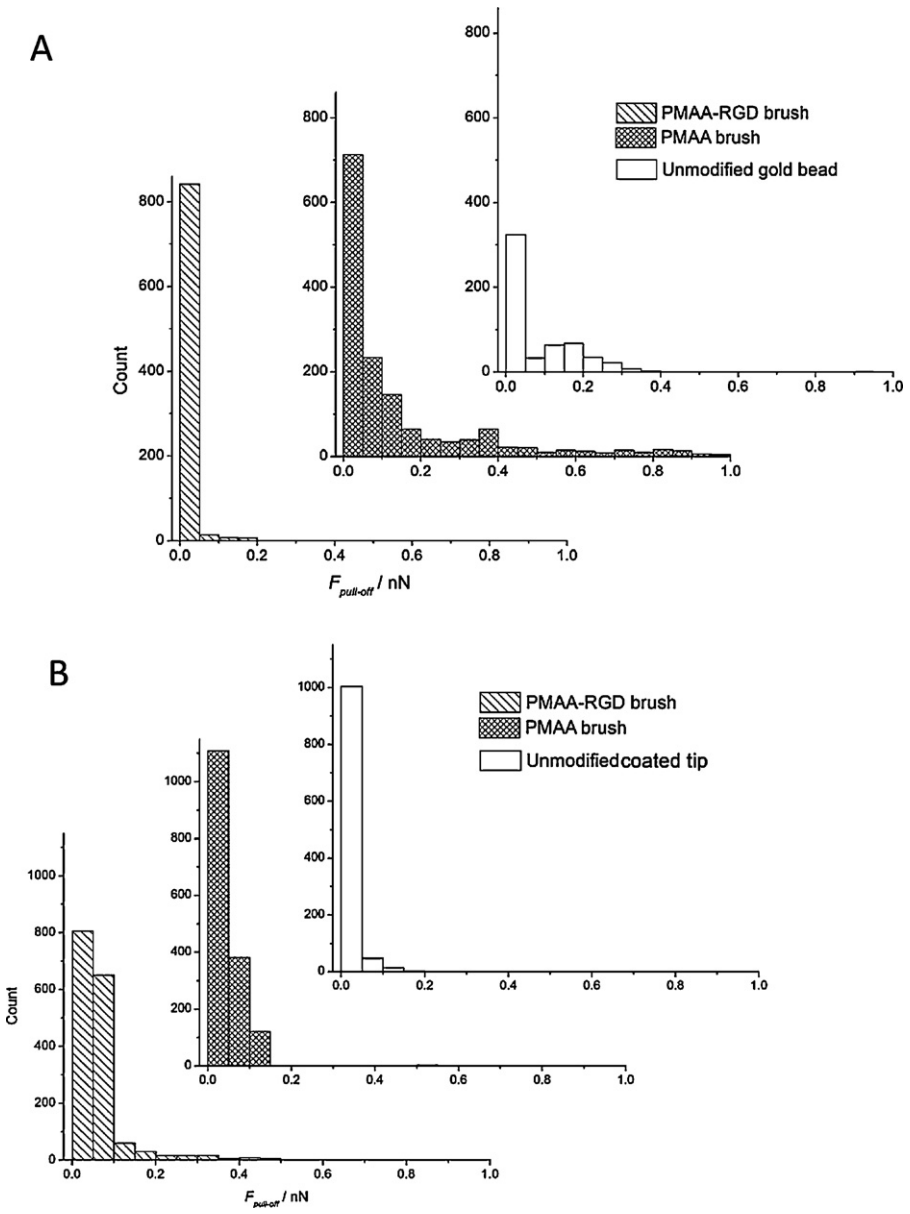

Fig. 4. Distribution of adhesion forces on pSBMA brush-modified silicon substrates, measured using modified colloidal gold probes (A) and modified conventional gold coated AFM tips (B).

An unmodified gold probe has no innate adhesion to the test surfaces since it is relatively rough (see supplementary material). In this case, different loads were applied from 1 to 10 nN, but no tendency to increase the adhesion force was observed.

After adhesion testing on the pSBMA brush, experimental data were collected to confirm successful modification of the colloidal AFM probe and also formation of the PMAA-RGD conjugate. In fact, the force measurement itself, conducted using a representative probe and a well-characterized substrate, can give important indications of a successful AFM probe modification. Initially we studied the mechanical response of PMAA and PMAA-RGD modified colloidal brushes in contact with pSBMA brush coated surfaces. Following established theory, the indentation element of the force curve indicated the stiffness and elastic modulus of the polymeric modification [46]. To obtain the same cantilever deflection as a bare colloidal gold probe, a significantly higher force was needed when using probes terminated with PMAA brushes. In addition, the apparent points of contact shifted by ca. 40 nm for the PMAA brushes and ca. 90 nm for the PMAA-RGD brushes with respect to the bare gold probe; indicating the presence of the brush modification [46]. To gather further experimental evidence of successful colloidal probe modification, adhesion measurements were done on cysteamine modified gold substrates under variation of the ionic strength (NaCl concentration) of the buffer solution. Cysteamine-modified gold substrates were prepared by adsorption of cysteamine from ethanolic solution. Both, PMAA and PMAA-RGD interact mainly electrostatically with the cysteamine modified substrate. This clearly reflects the electrostatic screening, pull-off forces significantly decrease with increasing electrolyte concentration. At pH 7.4 the cysteamine is protonated and positively charged while PMAA and PMAA-RGD will significantly differ in their electrostatic nature. This allows to discriminate the colloidal probe modification which is more pronounced at low ionic strength. In the following the respective results are discussed in detail.

In Fig. 5, the distributions of pull-off forces obtained with PMAA and PMAA-RGD modified colloidal probes on cysteamine-modified gold substrates are shown. At high ionic strength (1 M NaCl), PMAA and PMAA-RGD both showed low adhesion to

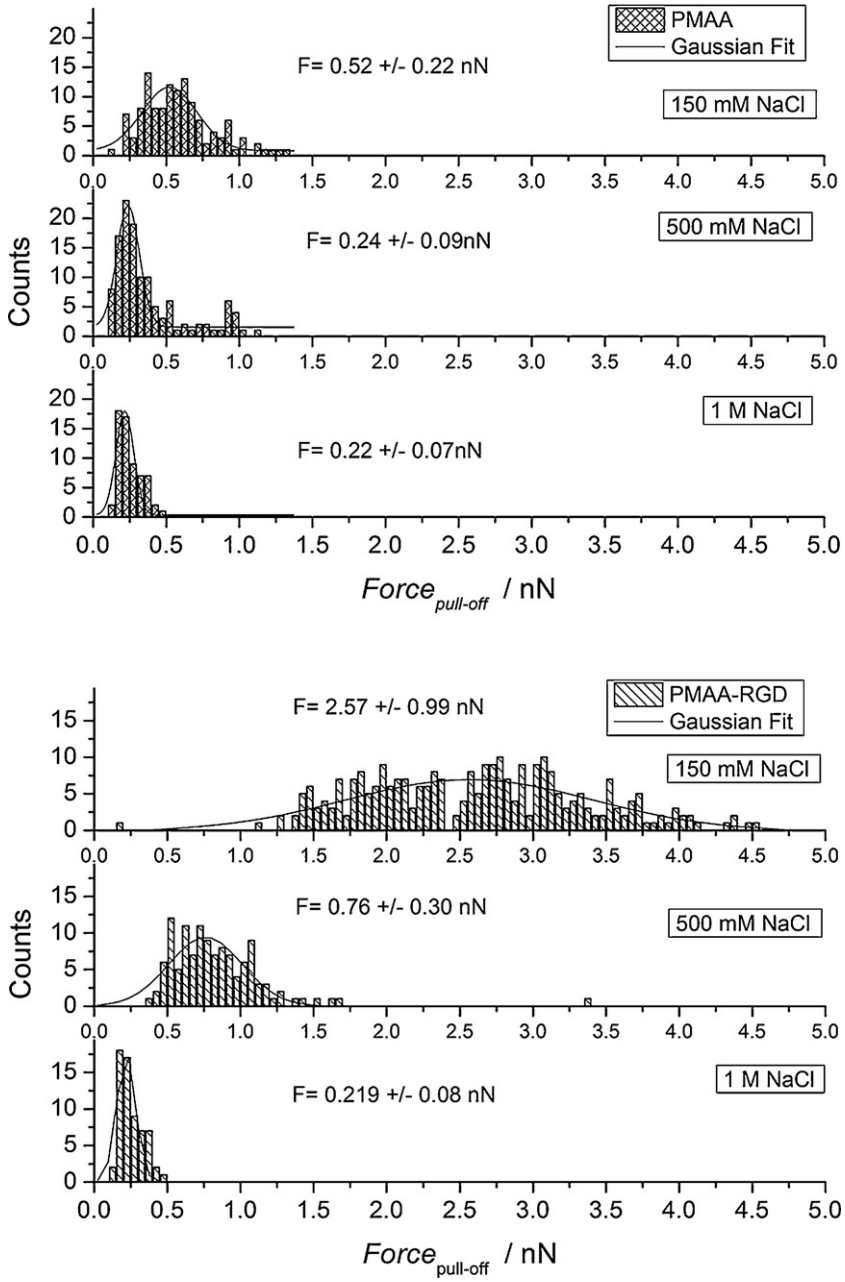

Fig. 5. Adhesion forces measured with PMAA and PMAA-RGD modified colloidal probes on cysteamine modified gold in dependence of the ionic strength (NaCl) of the buffer.

cysteamine-modified gold substrates, providing similar pull-off force values of 0.22 ± 0.07 nN and 0.219 ± 0.08 nN for PMAA and PMAA-RGD, respectively. A different situation was observed for 500 mM NaCl, where forces of 0.24 ± 0.09 nN were recorded for PMAA while PMAA-RGD forces were threefold higher at 0.76 ± 0.30 nN. Even more significant was the difference in observed pull-off forces at lowest ionic strength of 150 mM NaCl. Here, the PMAA-RGD colloidal probe showed a fivefold higher pull-off force of 2.57 ± 0.99 nN compared to the PMAA-modified probe (0.52 ± 0.22 nN). These results can be explained by electrostatic screening of surface confined charges at the polymer interface, increasing with ionic strength. This process leads to adhesion forces that are minimized at high ionic strength. The experimental approach employed here thus facilitated discrimination between PMAA and PMAA-RGD modifications in this context. Corresponding pull-off energies were derived from integration of pull-off force curves and are shown in the supplementary materials.

4. Conclusion

Our results demonstrate the efficient suppression of protein adsorption on zwitterionic pSBMA brushes; pull-off forces were hardly detectable in AFM adhesion measurements using fibronectin-modified AFM tips that were used here as model for a strongly nonspecifically adhering protein. In contrast, on surfaces reported to be strongly protein adhering, significant pull-off forces were detected. These results were in agreement with laboratory fouling studies using a commercially relevant target organism–the cypris larva of barnacles – and suggest that inhibition of settlement may be effected by interference with adhesion during surface exploration by cyprids. For these surfaces, it appears that AFM-based force measurements using fibronectin be a good indicator of anti-fouling efficacy, although this hypothesis must be validated with a wider range of surfaces. Use of the fibronectin AFM adherence measurements as an assay to predict cyprid settlement

to surfaces will open novel possibilities to locally probe biofouling potential/tendency on a variety of material surfaces. Finally, we introduced a novel type of AFM colloidal probe, comprising PMAA-RGD brushes that will be used in future studies to explore adhesive proteins of macrofouling organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2012.09.021.

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