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# Alginate/albumin in incubation solution mediates the adhesion and biofilm formation of typical marine bacteria and algae

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## HIGHLIGHTS

- Porous conditioning layer was characterized by AFM.
- Alginate/albumin in solution enhances adhesion of typical marine microorganisms.
- Alginate/albumin impacts biofilm formation and its surface topography.

## ARTICLE INFO

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## ABSTRACT

Adhesion of microorganisms in the marine environment is one of the initial events responsible for the occurrence of biofouling. A variety of factors play roles in regulating adhesion behaviors and subsequent biofilm formation. Here the study was focused on the influence of the typical marine polysaccharide alginate and the protein albumin on the attachment and colonization of *Bacillus* sp., *Chlorella pyrenoidosa* and *Phaeodactylum tricornutum* to silicon wafers in sterile artificial seawater. The rapid formation of conditioning layers due to the adsorption of the molecules was revealed by atomic force microscopy, and porous layers with the thickness of 3–6 nm further altered the surface roughness and wettability of the substrata. The presence of alginate or albumin in the culture solution tailored the surface properties of *C. pyrenoidosa* and *P. tricornutum*. The thickness, structure heterogeneity, biomass, diffusion distance, and roughness coefficient of the biofilm formed by colonization of the microorganisms were examined and their values showed that alginate/albumin had a significant influence on biofilm formation. The results are relevant to biofouling research on exploring antifouling strategies at the molecular level.

## 1. Introduction

Biofouling caused by unwanted colonization of sea species usually brings about serious impacts on marine industries, such as increased fuel consumption and frictional resistance [1,2], enhanced corrosion [3], the clogging of membranes and heat exchangers [4] and great economic loss [5]. Many efforts have been devoted to clarifying the mechanism of biofouling in order to prevent it. It was established that the attachment and subsequent biofilm formation of microorganisms on man-made surfaces is the key phenomenon involved in biofouling [4,6]. Generally, as the initial step, the formation of conditioning layers on solid surfaces is attributed predominately to the adsorption of ions, organic molecules (proteins, polysaccharides and lipoproteins) and other matter in the marine environment [7]. Conditioning layers can potentially change the physicochemical properties of substrata significantly, for instance surface tension, roughness, electrostatic charges, and wettability, and in turn significantly inhibit or facilitate biofilm formation [8–10].

In recent years, the influence of conditioning layers on microorganisms' adhesion has been widely investigated. Yet, due to the complex composition of biomacromolecules and difficulties in characterizing the layers, knowledge about the impact of physicochemical features of conditioning layers on biofilm remains elusive. As one of the model macromolecules in biofilm research, albumin is a negatively

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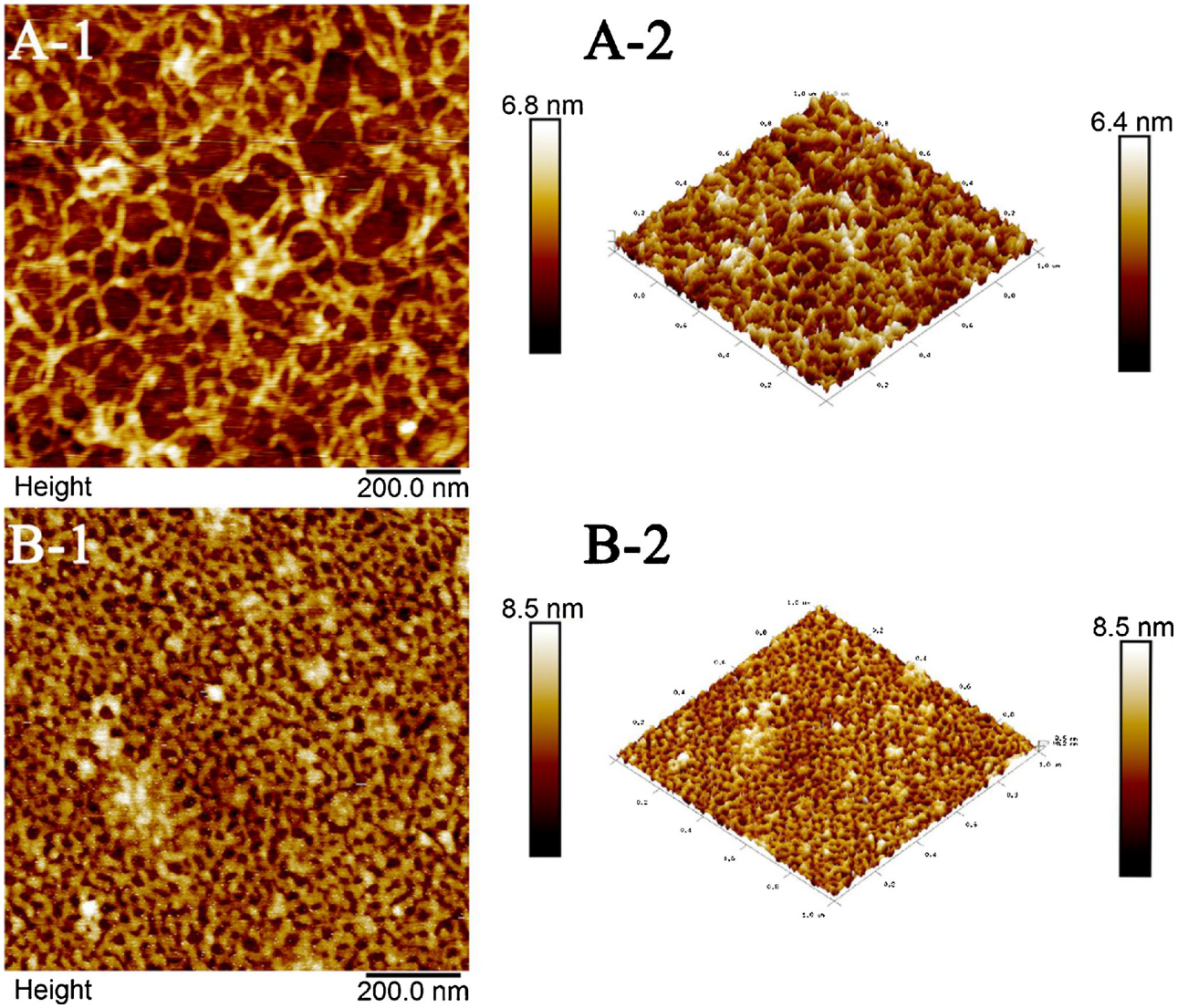


**Fig. 1.** PeakForce QNM AFM height images (-1) and the 3D images (-2) of surface after adsorption of alginate (A) and albumin (B).

charged protein with extraordinary ligand binding capacity [11]. Pre-adsorbed albumin showed a surprising inhibitory effect on bacterial adhesion to polymer, ceramic, and metal surfaces [12,13]. Studies performed by Tang et al. showed that increased concentration of -$NH_2$ group and more hydrophilic albumin inhibited adhesion and colonization of *Staphylococcus Epidermidis* [14]. Meanwhile, albumin suppresses initial bacterial adhesion due to the lack of specific interactions between *Staphylococcus epidermidis* and albumin [15]. It was claimed that, at higher ionic strength, the protein would result in more compressed, giving rise to a denser core with fewer interaction sites for adhesion [16]. However, it was reported that bovine serum albumin conditioning layer significantly promoted the adhesion of *Pseudomonas aeruginosa* PAO1 at a low ionic strength but hindered its adhesion at a higher ionic strength [17]. As an extensively used polysaccharide [18], alginate has been noticed because it is the major component of extracellular polymeric substances (EPS) and possesses similar macroscopic physicochemical properties of conditioning layers [8,19], which triggers severe fouling of membranes and coatings [20]. The presence of alginate conditioning layer enhanced the initial adhesion of *Pseudomonas aeruginosa* PAO1 on microscope slides through polymeric interactions between alginate and bacterial surfaces [17]. Moreover, the adhesion of *Burkholderia cepacia* to alginate-coated slides was enhanced with increasing ionic strength [8]. However, the over-expression of alginate can reduce deposition and the adhesion of *Pseudomonas aeruginosa* due to steric shielding of cell surface features [10]. Hwang et al. extracted EPS from *Burkholderia cepacia* and *Pseudomonas aeruginosa* and then placed bare slides into the EPS solution to form a conditioning layer, and they surprisingly found that the conditioning layer formed by EPS hindered the adhesion of *Burkholderia cepacia* and *Pseudomonas aeruginosa* [21]. It is clear that the influence of biomacromolecule conditioning layers on bacterial adhesion varies depending on the species and conformations of the macromolecules, the surface properties of bacteria, and the ionic strength of culture solutions.

It is well known that the adhesion of microorganisms and subsequent biofilm formation is influenced by the physicochemical features of both the conditioning layers and the microorganism cells [21]. The response of microorganisms to conditioning layers pre-formed by several typical biomacromolecules at the initial stage has been studied [8,11,17,21]. Yet knowledge about the impact of biomacromolecules in suspension on the adherence of microorganisms and subsequent development of biofilm remains elusive. A fundamental understanding of the interactions between macromolecules in suspension and marine microorganisms is essential for understanding biofouling processes. In the present study, alginate and albumin were chosen as the typical biomacromolecules for building simplified models of conditioning layers. The influence of alginate/albumin on adhesion and biofilm

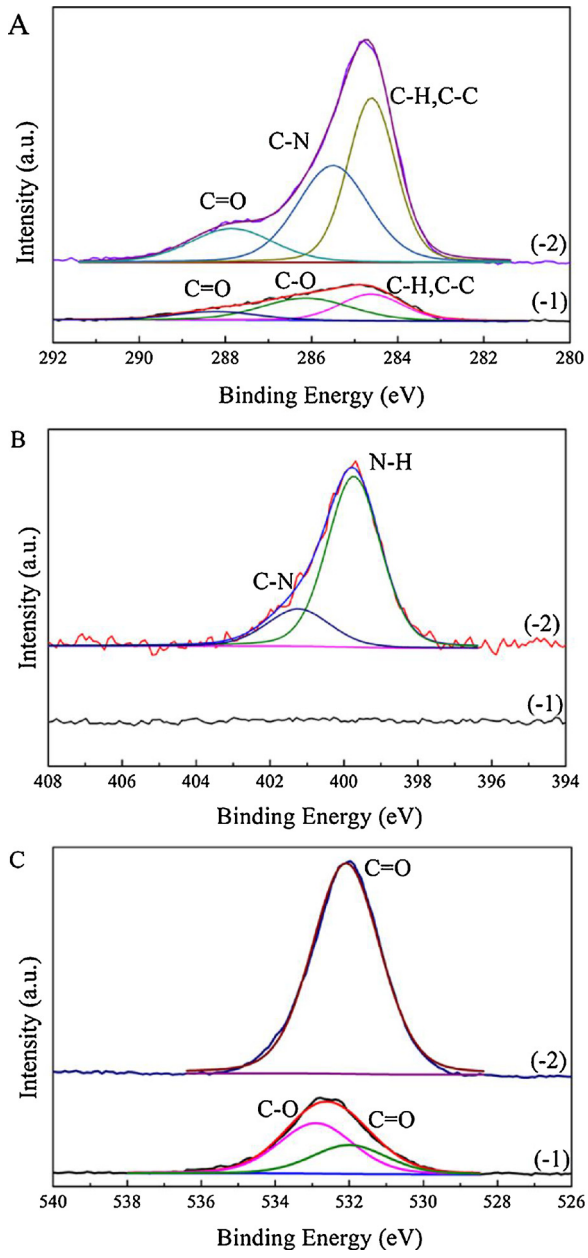


**Fig. 2.** XPS spectra of C 1 s (A), N 1 s (B), and O 1 s (C) electrons detected on the wafer surface after adsorption of alginate (-1) and albumin (-2).

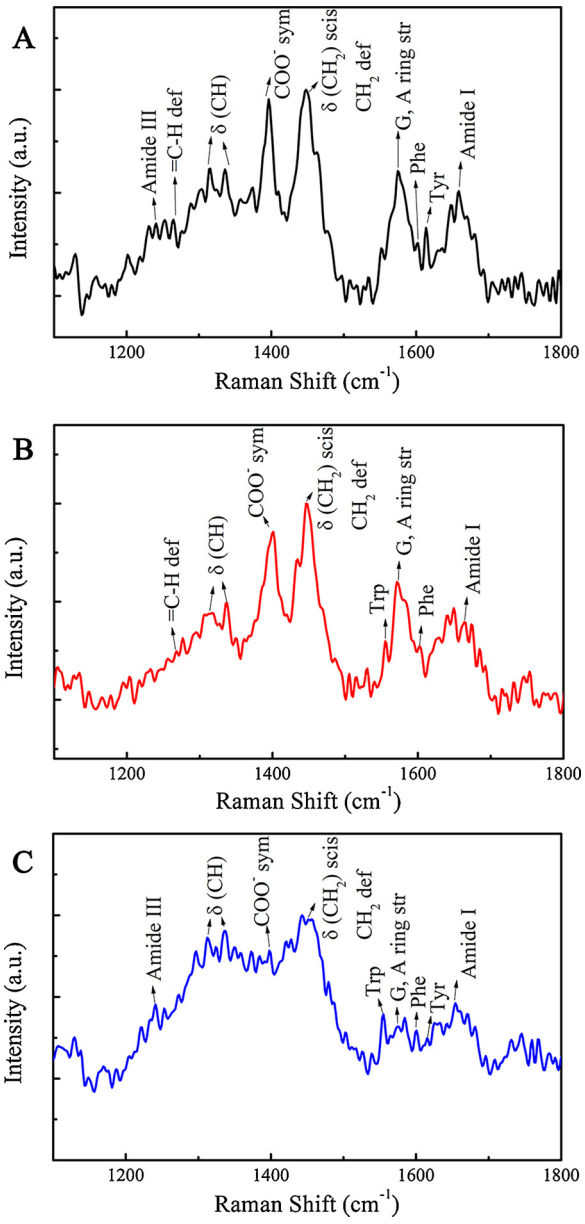


**Fig. 3.** Raman spectra of *Bacillus* sp. after incubation for 4 days in alginate/albumin-free media (A), and alginate-containing media (B), and albumin-containing media (C).

formation of bacterium *Bacillus* sp., green alga *Chlorella pyrenoidosa* and diatom *Phaeodactylum tricornutum* was investigated. *Bacillus* sp. is a typical rod-shaped Gram-positive bacterium, which forms biofilm with its abundantly secreted EPS to cause biofouling [22–24]. The spherically shaped *C. pyrenoidosa* was categorized as one of the colonizers on artificial surfaces in the marine environment [25,26]. *P. tricornutum* is a coastal marine pennate diatom that is usually considered as a model organism for diatom-related research [9,27], and it participates at the early stage in biofouling and often dominates the fouling [28]. CLSM images and COMSTAT analysis were used to investigate the influence of macromolecules on biofilm topographies. The results obtained in this study should shed light on the roles that the biomacromolecules in suspension play in regulating biofouling.

## 2. Materials and methods

### 2.1. Sample preparation

Sodium alginate (Aladdin Industrial Corp., China) and all the other reagents and solvents were used as received without any further purification. Artificial seawater (ASW) was prepared according to ASTM D1141-98. Briefly, ASW was prepared by dissolving 24.53 g NaCl, 5.20 g $MgCl_2$, 4.09 g $Na_2SO_4$, 1.16 g $CaCl_2$, 0.695 g KCl, 0.201 g $NaHCO_3$, 0.101 g KBr, 0.027 g $H_3BO_3$, 0.025 g $SrCl_2$, and 0.003 g NaF in 1 L deionized water and adjusting the pH to 8.2 with 1 mol/L NaOH. For the examination of the conditioning layer formed by alginate, a 0.2 mg/mL sodium alginate solution was prepared by dissolving the sodium alginate in deionized water. The conditioning layer was prepared by soaking glow-discharged silicon wafers (10 mm × 10 mm) for 10 min in a 1 mL aqueous suspension of alginate in a 24-well plate. The samples were subsequently rinsed twice with deionized water for 5 min

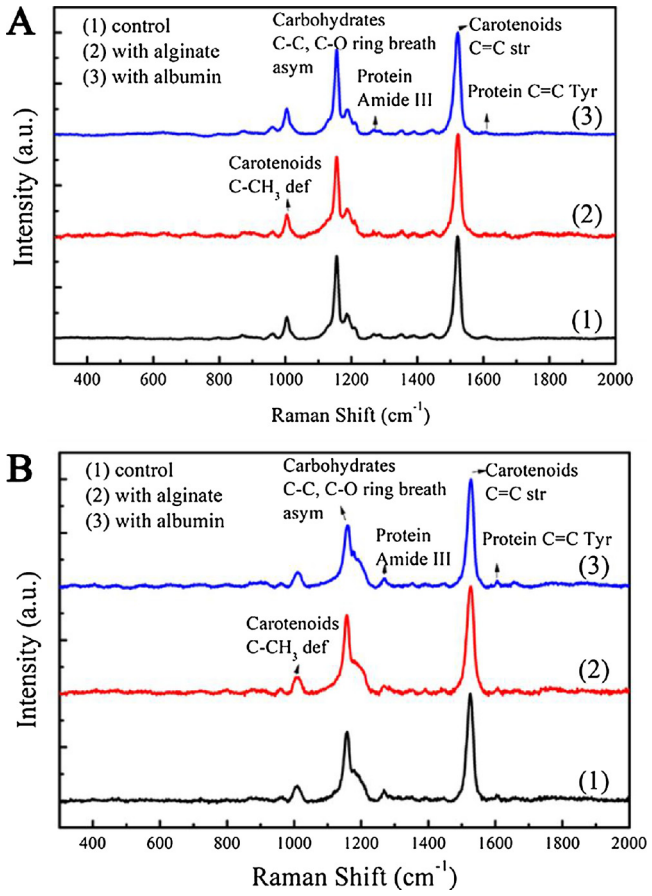


**Fig. 4.** Raman spectra of *C. pyrenoidosa* (A) and *P. tricornutum* (B) after incubation for 7 days in alginate/albumin-free media (1), alginate-containing media (2), and albumin-containing media (3).

**Table 1**
Assignment of the Raman bands of *C. pyrenoidosa* after incubation for 7 days.

| Assignments | Intensity | | |
|---|---|---|---|
| | control | In alginate-containing media | In albumin-containing media |
| Protein C = C Tyr | 0.01819 | 0.02987 | 0.02352 |
| Carotenoids C = C str | 1 | 1 | 1 |
| Protein Amide III | 0.04104 | 0.03594 | 0.04465 |
| Carbohydrates C-C, C-O ring breath asym | 0.80657 | 0.78258 | 0.83271 |
| Carotenoids C-$CH_3$ def | 0.20878 | 0.21385 | 0.25311 |

**Table 2**
Assignment of the Raman bands of *P. tricornutum* after incubation for 7 days.

| Assignments | Intensity | | |
|---|---|---|---|
| | control | In alginate-containing media | In albumin-containing media |
| Protein C = C Tyr | 0.05532 | 0.05545 | 0.05311 |
| Carotenoids C = C str | 1 | 1 | 1 |
| Protein Amide III | 0.10234 | 0.07298 | 0.07405 |
| Carbohydrates C-C, C-O ring breath asym | 0.64091 | 0.73146 | 0.56884 |
| Carotenoids C-$CH_3$ def | 0.1392 | 0.15378 | 0.13362 |

to remove unabsorbed alginate and then dried by flowing air at 37 °C. To form the conditioning layer with albumin, a 0.1 mg/mL albumin solution (BSA, 98% purity, Sigma-Aldrich) was prepared. The subsequent procedures were the same as the ones used to prepare the conditioning layer formed by alginate.

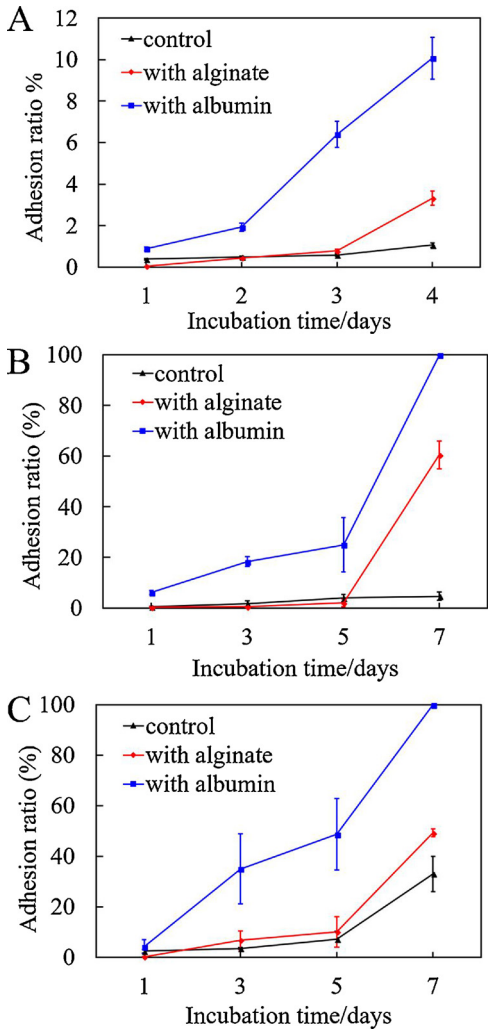


**Fig. 5.** Statistical analyses of the adhesion of *Bacillus* sp. (A), *C. pyrenoidosa* (B) and *P. tricornutum* (C). Error bars are shown as ± SD.

### 2.2. Characterization of the samples

PeakForce quantitative nanomechanical mapping (PeakForce QNM) was performed to examine the morphology and roughness of the conditioning layer formed by alginate/albumin by using Bruker Dimension FastScan™ atomic force microscopy (AFM). ScanAsyst-Air cantilevers (Bruker) with a resonance frequency of 70 kHz were used and the spring constant was 0.4 N/m. Topographic height images were recorded at 1 kHz at a resolution of 1024 × 1024 pixels. All images were flattened and plane-fitted using the NanoScope analysis software (Bruker). The absorbed alginate or albumin was also characterized by X-ray photoelectron spectroscopy (XPS, AXIS Ultra DLD, Japan) using Al-kα as a radiation resource. For XPS detection, the samples were soaked for 12 h in a 1 mg/mL alginate/albumin-containing ASW suspension, rinsed twice in deionized water for 5 min, and then dried by flowing air at 37 °C. The wettability of the samples after soaking in different solutions was determined by measuring the contact angle of deionized water droplets on their surfaces, using a contact angle measurement instrument (Dataphysics OCA35, Germany).

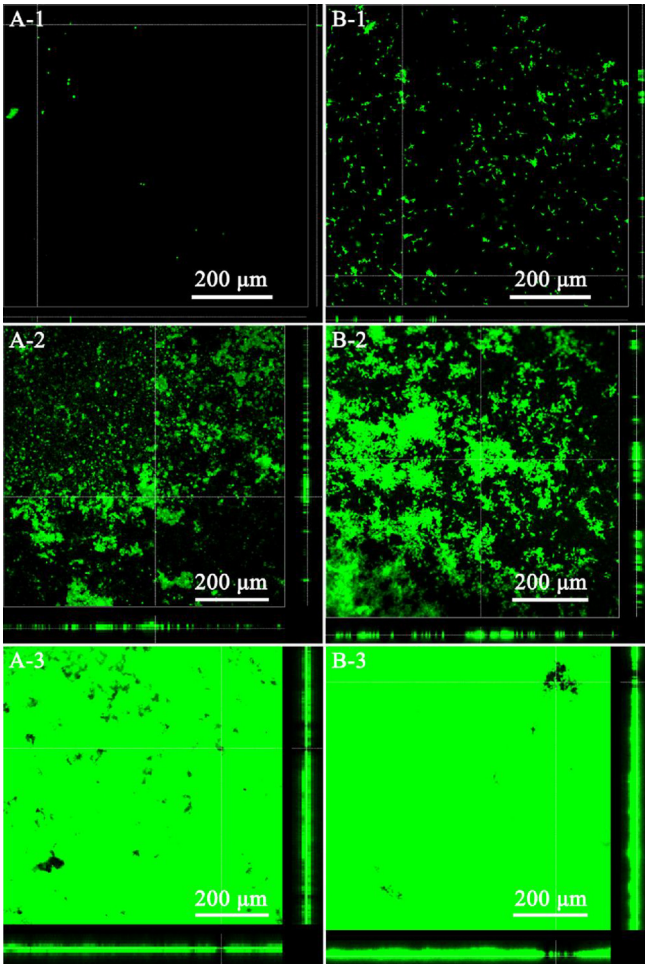


**Fig. 6.** CLSM images of the biofilm formed by *C. pyrenoidosa* (A) and *P. tricornutum* (B) after incubation for 7 days in alginate/albumin-free media (-1), alginate-containing media (-2) and albumin-containing media (-3).

### 2.3. Preparation of bacterial and algal strains

The gram-positive *Bacillus* sp. (MCCC1A00791) from the Marine Culture Collection of China was selected for adhesion testing and cultured in CM 0471-2216E media (1 *g* yeast extract, 1 *g* beef extract, 0.01 *g* $FePO_4$ and 5 *g* peptone in 1000 mL sterile ASW). The sterile media containing the bacterial strains were shaken at 120 rpm for 24 h at 25 °C. *Bacillus* sp. was washed with sterile ASW 3 times by centrifugation at a rotational speed of 2000 *g* for 5 min and then re-suspended in sterile ASW. Marine strains *C. pyrenoidosa* (NMBluh015-1) and *P. tricornutum* (NMBguh001) (Ningbo University, China) were used for adhesion testing. *C. pyrenoidosa* was cultured in enriched sterilized seawater with Guillard's F/2 growth medium, while *P. tricornutum* was cultured in sterilized seawater with silicate-enriched Guillard's F/2 growth medium. The algae were cultured in an incubator with a 12 h: 12 h light/dark cycle at 22 °C. The algae in the exponential growth phase were used in the experiments.

### 2.4. Analyses of the microorganisms

The chemistry of *Bacillus* sp. and algae was examined by Raman spectroscopy (Renishaw inVia Reflex, Germany). The air-drying cells can create a static environment that allows scattered photons to be easily collected from single cells, thus producing spectra with higher signal-to-noise ratios and more easily distinguishable peaks [29]. *Bacillus* sp. suspensions with/without 1 mg/mL alginate or albumin were put in a shaker at 25 °C for 4 days. The bacteria were washed sequentially with sterile ASW, sterile 50% ASW, and sterile deionized water by centrifugation at 2000 *g* for 5 min. They were then re-suspended in sterile deionized water, and the suspensions were diluted to $10^9$ cells/mL. By reducing the cell concentration to approximately $10^9$ cells/mL, it was possible to focus on individual cells to produce clear Raman signals without fluorescence saturating the detector [29]. For Raman analysis, 20 μL samples of *Bacillus* sp. in suspension were air dried on silicon wafers. Samples were illuminated with a frequency-doubled 532 nm laser emitting approximately 10 mW laser power at the sample. The procedures for obtaining algal Raman spectra were the similar to those used for characterizing the bacteria. The media without alginate and albumin were used as controls. Each test was performed in triplicate.

### 2.5. Bacterial/algal adhesion testing

The *Bacillus* sp. suspension with a concentration of $10^7$ cells/mL was prepared in sterile ASW. Silicon wafers with three specimens for each were put into 24-well plates after being ultrasonically washed with deionized water and then dried by an air flow at 37 °C. The *Bacillus* sp. suspension was added to each well for soaking at 25 °C in a shaker operated at 120 rpm for 1 day, 2 days, 3 days, or 4 days. After the incubation, the samples were washed three times with ASW to remove the bacteria that did not adhere to the samples, and then fixed by 2.5% glutaraldehyde in ASW. Morphological features of the adhered microorganisms were characterized by field emission scanning electron microscopy (FESEM, FEI Quanta FEG 250, the Netherlands). For FESEM observation, dehydration of the samples was performed through the critical point drying using 25%, 50%, 75%, 90%, and 100% ethanol solution sequentially. To investigate the influence of alginate and albumin on *Bacillus* sp. adhesion, 1 mg/mL of alginate- and albumin-containing *Bacillus* sp. suspensions were prepared. Media without alginate and albumin were used as controls.

Algae-containing suspensions at a concentration of $10^6$ cells/mL, with/without 1 mg/mL alginate and albumin, were prepared for adhesion testing. Silicon wafers with three specimens for each in 24-well plate were soaked in algal suspensions with a 12 h:12 h light/dark cycle at 22 °C for 1 day, 3 days, 5 days or 7 days. After incubation, the wafers were washed with sterile seawater to remove the algae that did not adhere to the wafers and then fixed with 2.5% glutaraldehyde in ASW for 2 h. The samples were observed by confocal laser scanning microscopy (CLSM, Leica TCS SP8, Germany). Biofilm formation after 7 days was also assessed by CLSM. CLSM pictures were captured layer by layer and then were 3D projected by LAS AF Lite software to reconstruct the spatial structure of the biofilm. Biofilm parameters, namely, average thickness, maximum thickness, total biomass, surface to biovolume ratio, and roughness coefficient, were quantified using the program COMSTAT [30]. Media without alginate and albumin were used as controls.

### 2.6. Statistical analyses

All of the data were expressed as the mean ± standard deviation of the triplicate experimental data. Statistical analysis of the adhesion of *Bacillus* sp. was made from SEM images using ImageJ software. Statistical analyses of the adhesion of algae to silicon wafers were made by *ImageJ* software of CLSM images. The adhesion ratio is defined here as the ratio of the adhesion area of the bacteria/algae to the total surface area of the substratum. A standard *t*-test was used to determine the difference between control and experimental groups. For statistical analysis data, *p*-values less than 0.05 were considered to be statistically significant while *p*-values less than 0.01 were considered to be statistically very significant.

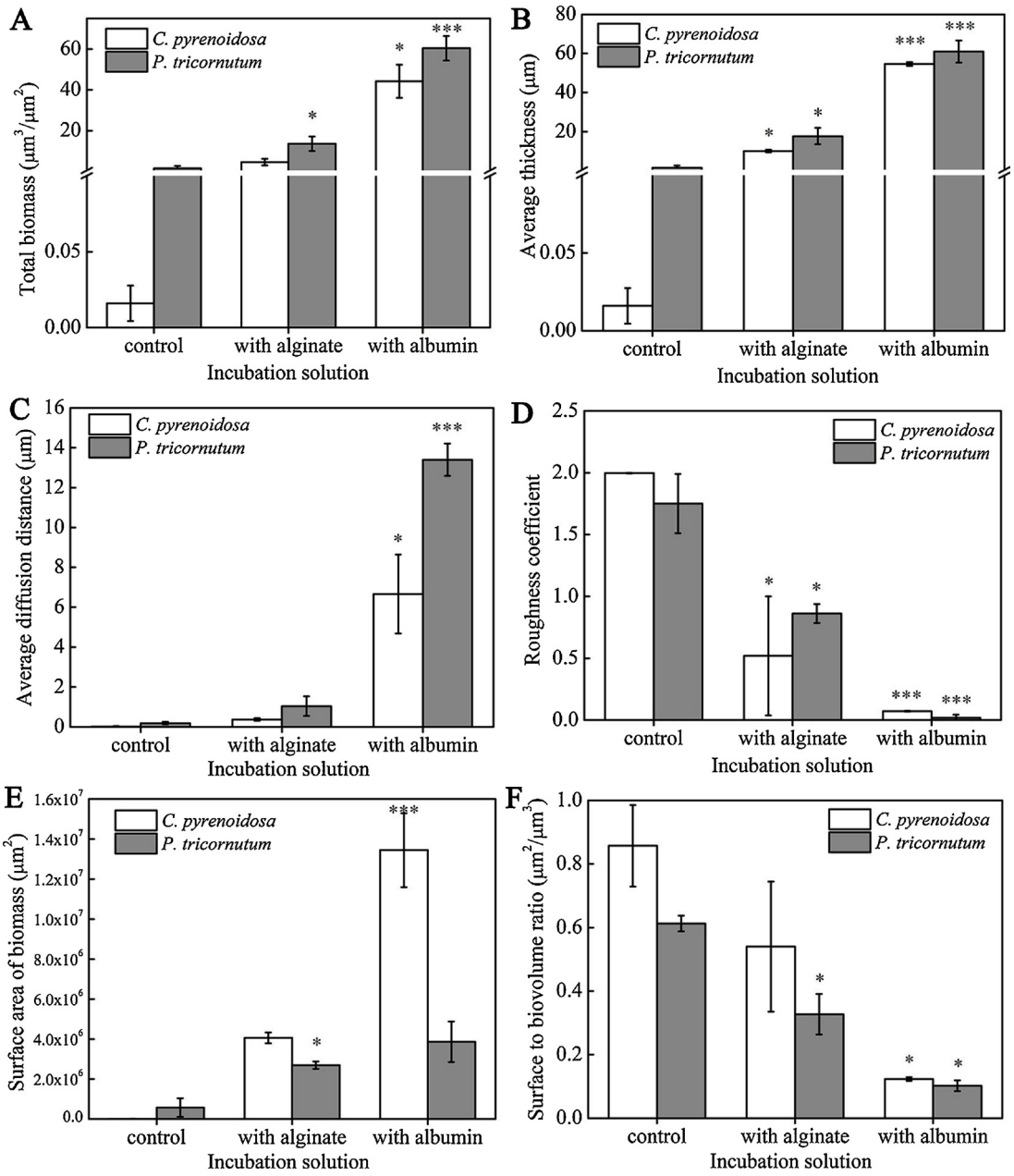


**Fig. 7.** Examination of the key parameters of the biofilm formed by *C. pyrenoidosa* and *P. tricornutum*, A: total biomass, B: average thickness, C: average diffusion distance, D: roughness coefficient, E: surface area of biomass, and F: surface to biovolume ratio. * $p < 0.05$ and *** $p < 0.01$, as compared with control biofilm grown in alginate/albumin-free media.

## 3. Results and discussion

After the adsorption of alginate/albumin to silicon wafers, the formation of conditioning layers was clearly seen (Fig. 1). Measurement by the NanoScope equipped with AFM provided an estimate of the size of alginate strands, which was ~20 nm in width (Fig. 1A). This is much larger than the actual size of the alginate molecule, which has been well characterized [31,32]. Apart from the probe-broadening effect caused during the AFM measurement, the altered size might also indicate side-by-side association of the molecules by hydrogen bonding [32]. The measured thickness of alginate strands ranged from 0.5 nm to 3 nm, suggesting that individual strands were aggregates of several molecules. In addition, the tangled alginate molecules formed a conditioning layer that covered the substrate evenly. It is speculated that the empty spaces are localized for bound and unbound water to form a highly-hydrated matrix in the liquid environment [33]. In addition, the layer exhibited a slightly rougher surface than the silicon wafer substratum (Ra value of 0.942 nm versus 0.347 nm). Surprisingly, AFM images of the adsorbed albumin on silicon wafers also suggested the presence of layers with a network structure (Fig. 1B). In this case, it was difficult to observe individual albumin molecules since albumin readily formed a three-dimensional network of aggregates exceeding certain critical levels [34]. It is likely that albumin spreads over the wafer surface to form a highly hydrated matrix in liquid environment. The thickness of the albumin conditioning layers was ~3–6 nm (Fig. 1B). The layer also exhibited a much rougher surface than the silicon wafer substratum did (Ra value of 0.942 nm versus 0.347 nm). The conditioning layers formed by alginate/albumin had similar network-like structures at the nanoscale as biofilm [19], which could significantly influence the deposition and initial adhesion of microorganisms.

High-resolution XPS spectra further demonstrated the adsorption of alginate and albumin on the surfaces of the samples (Fig. 2). For the

XPS analyses, carbon peak was used as the internal calibration standard. Polysaccharide usually exhibits −COOH peaks. The C 1 s spectrum that was acquired from the silicon wafer after incubation in alginate-containing solution was resolved into three components, namely C═O (288.2 eV), C–O (286.1 eV), and C−C, C–H (284.6 eV) [35]. The O 1 s spectrum suggested the existence of C–OH (532.9 eV) and C═O (532.0 eV) peaks. The XPS spectra of alginate detected on the surfaces of the samples suggested rapid adsorption of alginate after immersion of the samples in the alginate-containing ASW (Fig. 2). It is known that proteins usually have functional groups -$NH_2$ and −COOH. The C1 s spectrum detected on the silicon wafer after the incubation in alginate solution could be fitted by three components, namely C═O (287.8 eV), C–N (285.5 eV), and C−C, C–H (284.6 eV) [36]. The N 1 s spectrum was fitted by two components, C–N (401.2 eV) and N−H (399.7 eV) [37]. The O 1 s spectrum revealed the presence of C═O (532.0 eV) [35]. The results clearly evidenced the presence of -$NH_2$ and −COOH groups, which further confirmed the adsorption of albumin on the wafers. Further wettability testing suggested that the adsorption of alginate/albumin already weakened the hydrophobicity of silicon wafer, showing reduced contact angle from 59.2 ± 2.8° to 36.2 ± 0.3° and 40.2 ± 0.4°, respectively. This result is in agreement with a previous study that the surface becomes more hydrophilic after adsorption of alginate/albumin [15].

The characteristics of the microorganisms were examined by Raman spectroscopy (Fig. 3). The band for = C–H in-plane deformation at 1265 $cm^{-1}$ was detected from individual *Bacillus* sp. cell in the alginate-containing solution. It was weaker than that detected from control samples. The bands for, amide III and C═C of tyrosine (Tyr) at 1241 $cm^{-1}$, and 1614 $cm^{-1}$, respectively, were disappeared. It is speculated that the incubation in alginate-containing solution changed the composition of biomacromolecules existing on the surfaces of *Bacillus* sp.. In addition, it was noted that the amide I peak for individual *Bacillus* sp. cell in the albumin-containing solution was not as evident as that of the control samples. The bands for amide III at 1241 $cm^{-1}$, ring vibrations for aromatic residues of tryptophan (Trp) at 1550 $cm^{-1}$, and phenylalanine (Phe) at 1607 $cm^{-1}$, for individual *Bacillus* sp. cell in the albumin-containing solution were stronger than those of control samples. Moreover, the vibration of G, A ring stretching from RNA/DNA at 1575 $cm^{-1}$, symmetric stretch bands of the carboxyl ion ($COO^-$) from carbohydrates at 1397 $cm^{-1}$, and =C–H in-plane deformation from lipids at 1265 $cm^{-1}$ for individual *Bacillus* sp. cell in the albumin-containing solution were weaker than those of the control samples, further indicating that the composition of biomacromolecules on the surface of the bacteria was altered after incubation in the albumin-containing solution.

However, it was noted that neither alginate nor albumin seemed to have an effect on the surface properties of *C. pyrenoidosa* and *P. tricornutum* (Fig. 4). Raman spectra of *C. pyrenoidosa* and *P. tricornutum* revealed bands in three major regions: carbohydrates, proteins, and carotenoids. The characteristic peaks of carbohydrates showed the peaks at 1157 $cm^{-1}$, which were attributed to C−C, C–O ring asymmetric stretching vibration, and carotenoids show the C═C stretching vibration bands at 1527 $cm^{-1}$ [38]. The vibrations of proteins were not obvious due likely to the high intensity of the vibrations from carbohydrates and carotenoids. The Raman spectra only showed very weak peaks for amide III at 1267 $cm^{-1}$ and ring vibrations for aromatic residues of tyrosine (Tyr) at 1607 $cm^{-1}$. The algae incubated in different environments showed minor differences in Raman spectra. Nevertheless, the presence of alginate or albumin in the culture solutions altered the intensity of the Raman peaks.

For further clarification, all the spectra were normalized in relation to the carotenoids C═C stretching peak. The vibration of carbohydrates C−C, C–O ring asymmetric breathing became stronger when *C. pyrenoidosa* was incubated in albumin-containing media (Table 1). Meanwhile, the vibration of carbohydrates C−C and C–O ring asymmetric breathing peaks were enhanced when *P. tricornutum* was incubated in alginate-containing media (Table 2). It was clear that alginate and albumin influence the surface properties of *C. pyrenoidosa* and *P. tricornutum* and likely further influence their subsequent adhesion behaviors on silicon wafers.

The data analysis provided intuitive information about the influence of alginate/albumin on the adhesion of *Bacillus* sp. to silicon wafers (Fig. 5A). Both alginate and albumin remarkably affected the adhesion of *Bacillus* sp.. After 1 day incubation, the samples exhibited few differences in the adhesion ratios of the bacteria. Interestingly, the adhesion ratio reached 0.38% in the albumin/alginate-free media, while showed the value of 0.04% in alginate-containing media and 0.89% in albumin-containing media. When the incubation time was lengthened to 2 days, a similar trend in differences in the adhesion ratios was observed. The ratio was 0.49% in the alginate/albumin-free media, 0.44% in the alginate-containing media, and 1.93% in the albumin-containing media. Similar results were also found for the incubation durations of 3 days and 4 days (Fig. 5A). Albumin triggered more pronounced bacterial colonization. Alginate seemed to have no impact on the adhesion of *Bacillus* sp. in early stage (less than 3 days). The same phenomena were also realized for the adhesion of *C. pyrenoidosa* and *P. tricornutum*, in that both alginate and albumin promote their adhesion (Fig. 5B, C). The adhesion of the microorganisms that was slightly inhibited by alginate in the early stages of culture might be attributable to the hydrophilic nature of alginate. This effect becomed weakened over time, since the hydrophilicity simply faded away. The tailored adhesion of the microorganisms by albumin and alginate could potentially affect further development of the biofilm.

The effects of alginate and albumin on the physical characteristics of algal biofilm was also assessed by CLSM characterization (Fig. 6). It showed that both alginate and albumin influenced the architecture and topography of the algal biofilm after 7 days of incubation. The formation of *C. pyrenoidosa* biofilm in the presence of 1 mg/mL alginate was associated with the uniform distribution of cell aggregates. However, inhomogeneous distribution of cell aggregates with distinct voids was seen in the biofilm grown in the presence of 1 mg/mL albumin. Meanwhile, the presence of 1 mg/mL alginate in the culture solution gave rise to the formation of the *P. tricornutum* biofilm comprising clusters of glued cells, indicating that cohesion was enhanced in the presence of alginate. However, the presence of albumin in the solution brought about comparatively more compact biofilm structures than alginate. CLSM images clearly showed the topographical changes of the algal biofilm in the presence of alginate and albumin.

To elucidate the regulating regimes of the macromolecules on the biofilm, several key biofilm parameters were analyzed by COMSTAT program from the CLSM images. Result showed that, compared to the biofilm formed in the absence of alginate/albumin, total biomass of the biofilm increased significantly in the presence of alginate or albumin (Fig. 7A). A similar trend was also noted for the average thickness of the biofilm, which provided a measure of the spatial size of the biofilm. The average thickness was dramatically higher for the biofilm grown in albumin-containing media as compared to the biofilm grown in albumin-free media (Fig. 7B). In addition, the average diffusion distance, which reflects the length a substrate needs to travel to get to the middle of a micro-colony [39], was also examined in this study (Fig. 7C). The presence of alginate or albumin increased the average diffusion distance. Furthermore, the roughness coefficient, an indicator of heterogeneity degree of a biofilm, was found to be sharply reduced by alginate or albumin (Fig. 7D), which was in agreement with the CLSM observations (Fig. 6). These values suggested that the addition of albumin or alginate in the culture solution could effectively facilitate the buildup of a thicker and smoother biofilm. Further, the markedly increased ratio of surface area to biomass of the biofilms in the presence of alginate/albumin was also revealed (Fig. 7E). The decreased surface to biovolume ratio further indicated that alginate and albumin promote the development of algal biofilms (Fig. 7F).

The adhesion of microorganisms and following biofilm development

is believed to be influenced by conditioning layers formed by biomacromolecules [40]. The influence of conditioning layers pre-formed by several typical biomacromolecules on the adhesion of microorganisms to target surfaces at the initial stage has been well studied [8,11,17,21]. While these studies were focused on the responsible of the physicochemical properties of the target surfaces to the adsorption of biomacromolecules. An important factor for the adhesion of microorganisms, the characteristics of the microorgansims, was neglected in their studies. Our study further revealed the impact of typical protein/polysaccharide on the adhesion of microorganisms by varying the properties of the target surfaces and the microorganisms' surfaces. The protein/polysaccharide continued regulating their subsequent biofilm formation.

## 4. Conclusions

The adhesion and subsequent biofilm formation of *Bacillus* sp., *C. pyrenoidosa* and *P. tricornutum* on silicon wafers in response to the presence of alginate and albumin were investigated and elucidated. Conditioning layers constituted by alginate or albumin were manifested by AFM and XPS. The similar network-like structures in the conditioning layers were revealed at the nanoscale. The conditioning layers altered the surface roughness and wettability of substrate surfaces. The presence of alginate and albumin in incubation solutions changed the surface properties of the microorganism cells. The initial adhesion and biofilm development of the microorganisms to silicon wafers were significantly influenced by the adsorption of alginate/albumin to both the individual cells and the wafer surfaces. The protein/polysaccharide continued regulating subsequent biofilm formation and tailoring the surface topographies of biofilms. These results provide insight into understanding the roles that the biomacromolecules play in regulating biofouling processes.

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