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Protocols

Participation of copper ions in formation of alginate conditioning layer: Evolved structure and regulated microbial adhesion

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ABSTRACT

Antifouling function of copper-based layers is usually gained through the release of cuprous or copper ions to damage most fouling species. In this research the intervening mechanisms of copper ions in formation of simplified conditioning layer comprising marine polysaccharide alginate and subsequent adhesion of typical marine bacteria and algae were studied. Fast interaction of Cu^{2+} with alginate with the formation of copper alginate multimers was observed for the first time by negative-staining electron microscopy. Interconnecting chains of alginate and copper alginate upon adsorption on silicon wafer and tangled structure of the conditioning layer were further characterized by atomic force microscopy. Adhesion testing showed that consumption of copper ions by their linking with alginate in incubation solutions resulted in mitigated toxicity of the ions to the microorganisms *Bacillus* sp., *Chlorella pyrenoidosa* and *Phaeodactylum tricornutum*. The results would give insight into understanding and regulating the formation of conditioning layer for desired antifouling performances.

1. Introduction

Biofouling is ubiquitous in the marine environment, causing severe problems such as increased operating costs, fuel consumption and CO_2 emission [1,2]. Varieties of techniques have been developed to alleviate or prevent occurring of biofouling, among which construction of superhydrophilic or superhydrophobic coatings [3], photocatalytic coatings [4], and enzyme-based antifouling coatings [5] was already reported. Yet, due to complexity of the marine environment and diversity of the fouling organisms, challenges persist in developing antifouling techniques with favorable universality and sustainability, for biofouling is a complex process involving many factors [6,7]. To date, painting with the use of biocides remains as the most effective approach to attain antifouling performances [8]. In response to the banned use of TBT-containing coatings [9], Cu-based antifouling coatings recaptured extensive attention [10]. In fact, copper plates used on vessel hulls were documented as the first antifouling devices combating formation of biofilm [8,10]. Copper and copper-based coatings are the most commonly used broad-spectrum antifouling layers due to the toxicity of copper to marine organisms [10–12]. Understanding at molecular level the antifouling mechanisms of copper is therefore essential for developing copper-related antifouling technology for widespread applications.

It is established that antifouling performances of copper-based coatings are achieved primarily through the release of cuprous and copper ions by reaction of copper with Cl^- in seawater [13]. Copper is usually harmful to microorganisms at elevated concentrations in water [10,14]. It has been reported that the toxicity usually prevails through several regimes. Copper results in different algal cell dysfunctions such as inhibition of photosynthesis, suppression of cell division, and increase of membrane permeability for algae [15]. In addition, negative effect of copper on acid-base equilibrium and ammonia excretion for marine fish and invertebrates was also reported [16]. It is noted however that the copper-associated toxicity varies depending on microorganism species and might be affected by many other factors. Knowledge about influence of copper ions on early stages of biofouling, for instance formation of conditioning layer and subsequent biofilm, is yet lacking. Moreover, there has been no report available on direct visualization of the interaction of marine biomolecules with Cu^{2+} and the interaction keeps elusive. Synergistic effect brought about by marine biomolecules and copper ions on formation of biofilm is also to be clarified.

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As typical marine polysaccharide, alginate possesses macroscopic physicochemical properties of conditioning layer [17,18]. It is a linear polysaccharide [19,20], with the capability of forming bonds with most divalent cations, such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Pb^{2+} [21–24], and an egg-box interaction model for calcium alginate was already proposed [25–27]. In this study, alginate was selected as the model polysaccharide to build a simplified conditioning layer. The conditioning layer formed by the marine polysaccharide and its derivative copper alginate was examined by PeakForce quantitative nanonechanical mapping atomic force microscopy (AFM). Negative-staining electron microscopy technique was employed in this research to further characterize the conformation evolvement of copper alginate. The interaction fits the egg-box model and an interaction regime between the biomacromolecule and Cu^{2+} was proposed. Influence of copper and copper alginate on adhesion of Gram-positive bacteria (*Bacillus* sp.), diatoms (*Phaeodactylum tricornutum*), and green algae (*Chlorella pyrenoidosa*) were also investigated and elucidated.

2. Materials and methods

2.1. Sample preparation

Sodium alginate (Aladdi ustrial Corp., China) was used as received without any further purification. The M/G ratio of alginate is ~0.8 as calculated from its NMR spectrum [28]. For examination of the conditioning layer formed by alginate, appropriately diluted suspensions of sodium alginate were prepared by being dissolved in deionized water. Conditioning layer was prepared by soaking glow-discharged silicon wafers (1.0 cm × 1.0 cm) in 1 mL aqueous alginate suspension for 10 min. The samples were subsequently rinsed twice in deionized water for 5 min to remove unabsorbed alginate and then dried by flowing air at 37 °C. The alginate solution with a final concentration of 0.2 mg/mL was used for microscopy analyses. To investigate the conditioning layer formed by alginate in the presence of copper ions in the solution, the solution containing 0.1 mg/mL $CuCl_2$ and 0.2 mg/mL alginate was prepared for further experiments.

2.2. Preparation of bacterial and algal strains

Artificial seawater (ASW) was prepared according to ASTM D1141-98. All the reagents and solvents were used as received without any further purification. Gram-positive *Bacillus* sp. (MCCC1A00791, Marine Culture Collection of China) were chosen for the adhesion testing and the culturing was conducted in CM 0471-2216E media. The media were prepared by dissolving 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 25 °C at 120 rpm for 24 h. The bacteria were washed with sterile ASW for 3 times through centrifugation with a rotational speed of 2500 rpm for 5 min and then re-suspended in sterile ASW. In this work, marine strains *Chlorella pyrenoidosa* (NMBluh015-1) and *Phaeodactylum tricornutum* (NMBguh001) (Ningbo University, China) were typically chosen for the adhesion testing. *Chlorella pyrenoidosa* was cultured in filtered sterilized seawater enriched with Guillard's F/2 growth media, while *Phaeodactylum tricornutum* was cultured in sterilized seawater with silicate-enriched Guillard's F/2 growth media. The algae were cultured in an incubator with a 12 h:12 h light/dark cycle at 22 °C. The testing was carried out as they were in the exponential phase of growth.

2.3. Electron microscopy characterization

Morphology of alginate was characterized by transmission electron microscopy (TEM, FEI Tecnai F20) operated at 200 kV. For acquiring the EM images, low dose conditions (10–15 e/Å^2) were used. To investigate the interaction of Cu^{2+} with alginate, the solution containing 0.001 mg/mL $CuCl_2$ and 0.002 mg/mL alginate was prepared and excess ions were removed by dialysis. Subsequently, these solutions were used to prepare negatively stained samples by the drop by drop protocol for following TEM characterization [29]. Briefly, a 7 μL drop of prepared solution was applied to a thin carbon-coated 300-mesh copper grid. After incubating for 15 min at room temperature, the excess solution was removed by blotting with filter paper. Two consecutive drops of 7 μL 2% (w/v) uranyl acetate solution were then applied on the grid for staining. Excess stain was removed by blotting and the grid was quickly air dried at room temperature after final blotting.

2.4. AFM imaging

PeakForce quantitative nanonechanical mapping (PeakForce QNM) was performed to characterize the morphology of alginate after adsorption by using the Bruker Dimension FastScan™ AFM. The microscope was covered with an acoustic hood to minimize vibrational noise. 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 with the resolution of 1024 × 1024 pixel. All images were flattened and plane fitted by using the NanoScope Analysis software (Bruker).

2.5. Bacterial/algal adhesion testing

Bacillus sp. suspension with the bacterial concentration of 10^7 mL^{-1} was prepared in sterile ASW. Silicon wafers with three specimens for each testing group were put into 24-well plates after being ultrasonically washed with ethanol and subsequent deionized water and then dried under a flow of dried air at 37 °C. 2 mL of the *Bacillus* sp. suspension was added into each well for soaking in a shaker at 25 °C at 120 rpm for 3 days. After the incubation, the samples were washed with ASW for three times to remove the bacteria that did not adhere onto the samples and then fixed by 2.5% glutaraldehyde in ASW. Morphological features were characterized by field emission scanning electron microscopy (FESEM, FEI Quanta FEG 250). For FESEM observation, dehydration of the samples was carried out through the critical point drying using 25%, 50%, 75%, 90%, and 100% ethanol solution in turn. To clarify the synergistic influence of Cu^{2+} and alginate on the bacterial adhesion, the adhesion testing was conducted under various solution conditions, i.e. Cu^{2+}-alginate-free media, Cu^{2+}-containing media, alginate-containing media, and Cu^{2+}/alginate-containing media. The Cu^{2+}-containing media were prepared by adding 10 mM $CuCl_2$ into ASW. Concentration of alginate was 1 mg/mL in the alginate-containing ASW. The Cu^{2+}/alginate-free media were used as the control group.

2 mL algal suspension (Cu^{2+}-alginate-free media, Cu^{2+}-containing media, alginate-containing media, and Cu^{2+}/alginate-containing media) with the algal concentration of 10^6 mL^{-1} was used for the adhesion testing. Silicon wafers with 3 specimens for each testing group in 24-well plate were soaked by algal suspension in shaker for 7 days with a 12 h:12 h light/dark cycle at 22 °C. After the incubation, the wafers were washed with sterile seawater to remove the algae that did not adhere and then fixed by 2.5% glutaraldehyde in ASW for 2 h. The samples were observed by confocal laser scanning microscopy (CLSM, Leica TCS SP5, Germany).