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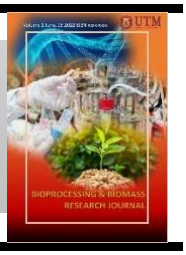

Research Article

Lateral detachment forces of *Bacillus niabensis* and *Alteromonas litorea* against antifouling paint additive

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ABSTRACT

Bacterial adhesion on various marine biotic or abiotic surfaces and subsequent biofilm formation paves the path of biofouling in marine environment. The aim of the present investigation was to determine the lateral detachment forces between coated antifouling paint additive and local isolated marine bacteria; *Bacillus niabensis* and *Alteromonas litorea* using atomic force microscopy (AFM). A paint additive was prepared by employing the active compounds, silver ion (Ag^+) and surfactant hexadecyltrimethylammonium (HDTMA) embedded in the kaolinite clay structure. An incubated 100 µL bacterial suspension loaded onto the coated antifouling paint additive were scanned using AFM with the scan rate of 40 µm/s and scan size of 10 × 10 μm^2. Lateral detachment force was measured from a lower set point value of 0.3V to a maximum set point 10.0V. A weak interaction was observed between the model bacteria and paint additive (Or-Ag-Kao) coated surface with the mean lateral detachment force of 139.4 nN (*B. niabensis*) and 146.2 nN (*A. litorea*). Major contact surface area reduction observed on paint additive (Or-Ag-Kao) coated surfaces with 0.275 μm^2 for *B. niabensis* and 0.391 μm^2 for *A. litorea* indicated that paint additive coating successfully minimized bacterial attachment on the surface. The antifouling paint additive shows a reduction in lateral forces and minimized its surface contact, which could further prevent the microfouling formation on marine structures.

INTRODUCTION

Maritime industries represent about 90% of the worldwide trade (Selim et al., 2017). Since mankind has used the sea for transportation, marine biofouling has been an issue to maritime industries. Marine biofouling refers to an undesirable colonization of marine organisms accumulated on the submerged surfaces (Clare & Aldred, 2009). Bacterial communities colonized on the submerged surface are recognized as a key factor in the formation of complex biofrajaseouling phenomenon in the marine environment (Caruso, 2020). Bacterial colonization on surface induced biofilm formation. Biofilm is described as the aggregation of multiple species of bacteria that assemble as a whole community and are attached together by the secretion of extracellular polymeric substances (EPS) on a foundation surface (Flemming, 2016). Formation of undesired biofilm on the surface may alter the local surface chemistry, provides a preferential substrate for the settlement of algal spores and the larvae of marine invertebrates. Within, two or three weeks later, these will finally evolve into a complex biological community and large-scale macrofoulers (e.g.: barnacles, mussels, polychaete worms, bryozoans, and

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seaweed that will settle and grow on the substratum surface in a few months later (Müller *et al.*, 2013; Dobretsov and Rittschof, 2020). Therefore, biofilm formation is identified as the precursor of marine biofouling (Agostini *et al.*, 2019; Heidarian *et al.*, 2019).

Marine biofouling is a big issue that leads to costly problems which impacts infrastructures and equipment served in maritime industries (Selim *et al.*, 2017). The adverse effects of marine biofouling are well known, and they can be either in form of economic, environmental or safety related (Hadfield, 2011; Salta *et al.*, 2013; Vinagre *et al.*, 2020). Starting from localized biofouling, without any precautions taken there are high chances that the biofouling is able to spread to the whole marine structure which later ruin the entire structure and system. Although the biofouling is a slow process until the predominant effects are observed but the effects are worst. Deterioration of the coating due to attachment of fouling organisms such as barnacles and other invertebrates may favor corrosion (Vinagre *et al.*, 2020). Long-term corrosion of metal and concrete structures decreases the efficiency of equipment and facilities and threatens the overall structure integrity (Blackwood *et al.*, 2017). As a result, developing an effective biofouling control system for maritime man-made structures is critical.

The primary strategy for combating marine biofouling is to use anti-fouling (AF) coating (Rajasekar *et al.*, 2007; Donnelly *et al.*, 2019). AF coating is a combination of paint and biocides substances serves as dual purpose: to keep the structures looking good and protect the structure from biofouling deterioration. AF coating usually containing varying concentrations and mixtures of biocides substances; however, due to environmental concerns and legislation issues, researchers are urgently sought for greener solution to deter marine biofouling (Wang *et al.*, 2017; McNeil, 2018). One of potential greener solution of biofouling control is to utilize biocidal-free coating to prevent biofilm formation. Biofilm formation was prevented by either deterring the microorganisms from attaching in the first place ('prevention is better than cure') or reducing the adhesion strength of microfouler attachment, so that they are easily removed by the shear forces generated by ship movement or mild mechanical cleaning process. Hence, this study offers a promising environmental-friendly solution to combat biofouling using biocide-free paint coatings emphasis on active compounds, silver ion (Ag^+) and hexadecyltrimethylammonium (HDTMA) embedded in kaolinite clay structure.

To design an effective biofouling control strategist that works from initial stage of bacterial adhesion and biofilm formation, an in-depth understanding of the cellular nanomechanical properties, including quantification of surface topography and adhesive forces with nanoscale resolution is needed. To meet such complex demand of biofilm structures, a sophisticated, versatile, robust, and highly sensitive analytic tools is requiring. Within the current inventory of analytic tools, atomic force microscopy (AFM) has proven to providing imaging and force measurement capabilities that can interrogate the nanoscale properties of surfaces. AFM has been used with great success to provide novel insight into the structure of biofilms and the interplay of interaction forces and mechanical properties that govern the behaviour of biofilms and their response to chemical and physical attack (Wright *et al.*, 2010; Grzeszczuk *et al.*, 2020). AFM can be used to study whole biofilms or the influence of their component parts, from bacterial surface proteins to extracellular polysaccharides (EPSs) and individual cells (Wang *et al.*, 2017; Boudjemaa *et al.*, 2020; Bhat *et al.*, 2021; Dufrêne *et al.*, 2021).

In this study, three types of AFM measurements were performed: 1) nanoscale surface topographical imaging and surface roughness measurement of surface coated with commercial paint; 2) the strength of the model bacterial attachment on paint surfaces were evaluated via interaction forces of cell-substrate surface interaction and 3) the total reduction of bacterial cells contact surface area after lateral detachment forces applied. The tested surfaces are glass coupons coated with (i) commercial paint, (ii) commercial paint supplemented with modified kaolinite: silver modified kaolinite (Ag-Kao) and HDTMA-silver modified kaolinite (Or-Ag-Kao). Two marine microfouler, bacteria, *B. niabensis* (Gram-positive strain) and *A. litorea* (Gram- negative strain) were used in this study to determine whether the modified kaolinite paint coating are species specific or more generally applicable to a larger spectrum of bacteria.

MATERIALS AND METHOD

Materials

Commercial kaolinite ($Al_2Si_2O_5(OH)^4$) was purchased from Sigma-Aldrich, UK used to prepare the paint additive in this study. The surfactant compound, Hexadecyltrimethylammonium bromide (HDTMA), ($(CH_3)_3N(CH_2)_{15}CH_3Br$)) and silver nitrate ($AgNO_3$) were purchased from Merck, USA. Marine agar (MA) and marine broth (MB) powder for bacterial culture were obtained from BD Difco™, USA. Phosphate Buffer Saline (PBS) solution and ethanol (99% and 95% purity) were purchased from Sigma-Aldrich, USA and Merck, USA, respectively. All chemical reagents were of analytical grade, and all aqueous solutions were prepared with sterile distilled water. The commercial paint used for coating is Nippon Paint: Economic Undercoat. Borosilicate glass slide (DURAN Group, Germany) with dimensions of 38 mm x 26 mm x 2 mm was used as the substratum for the paint deposition. Model bacteria, *A. litorea* and *B. niabensis* were isolated from Tanjung Balau, Desaru seashore, Kota Tinggi, Malaysia (Al Amin *et. al*, 2021). The strains were stored in glycerol stock at -80°C (Department of Biosciences, Universiti Teknologi Malaysia, Johor) for further used.

Preparation of Modified Kaolinite

The silver modified kaolinite (Ag-Kao) and HDTMA-silver modified kaolinite (Or-Ag-Kao) samples were prepared following the method by Muthoovaloo (2015). In brief, the silver kaolinite (Ag- Kao) sample was prepared by adding 20 g of kaolinite powder into 1000 mL of 0.016% (w/v) silver nitrite ($AgNO_3$) solution and mixed for 16-17h. The mixture was then separated using a vacuum filter. The liquid suspension was discharged, and solid part was dried at 80°C overnight. The retrieved dried solid was then crushed by using a mortar and pestle before being sieved. For the preparation of Or-Ag-Kao, 10 g of Ag-Kao was added into 1000 mL of 2.0 mM HDTMA solution. As

for the preparation of dry solid Or-Ag-Kao, the same method was conducted as described above.

Lateral Detachment Forces by AFM

An overnight culture of *A. litorea* and *B. niabensis* were grown in MB and shaked at 30°C, 120 rpm until the optical density OD_{600nm} reached ~1.0. Then, the bacterial suspensions were centrifuged at 3000 rpm for 10 min to obtain the bacteria cells biomass. The bacterial biomass was washed twice by suspending the biomass in sterile distilled water before 100 µL of the suspended biomass was transferred onto glass coupons coated with: (i) Commercial paint (control), (ii) 1% (w/v) Ag-Kao mixed with commercial paint and (iii) 1% (w/v) Or-Ag-Kao mixed with commercial paint. Each sample was incubated for 30 min before it was rinsed 3 times with sterile 0.1 M PBS to remove unattached bacterial cells prior observing under AFM contact mode imaging.

The bacterial biomass on the coated glass coupons were first scanned using contact mode at a lower set point, 0.3V to determine the quantity of bacteria cells present in the scanned region. Then, the force applied onto the cantilever tip was increased gradually from lower set point value of 0.3V to 3.0V, 5.0V and to maximum set point of 10.0V until the bacterial cells in the scanned region were removed if possible. The lateral detachment force of the bacterial cell removal was determined through repeated images of bacterial cell on the coated paint glass coupon by repeatedly scanning the same area with the increment of set point values applied on the cantilever tip with a 40 µm/s scan rate and 10 µm × 10 µm with 512 × 512 line/pixel of resolution were used for the measurements (Zhang et al., 2011). The sensitivity and spring constant of the cantilever was calibrated before each measurement was taken.

Analysis of Lateral Detachment Forces

Data collected were analyzed using JPK SPM processing software to determine the adhesion strength of the bacteria and the coating surfaces. The lateral detachment force, F_{lat} was calculated by summing up the $V_{setpoint}$ (0.3V, 3.0V, 5.0V and 10.0V) and the $V_{deflection}$ (Eqn.1). The $V_{deflection}$ was obtained by dragging a line profile on vertical deflection channel bacteria cell images. The line profile formed will generate a vertical deflection graph. The $V_{deflection}$ represented as maximum Z-Range (highest peak in the graph) in the vertical deflection graph.

After the $V_{deflection}$ values were obtained, the data were substituted in the equation below (Eqn. 3) to quantify the lateral detachment force of the bacterial cell:

$$V_{total} = V_{setpoint} + V_{deflection} \quad \text{(Eqn. 1)}$$

$$F_{lat} = kSV_{total} \sin(\vartheta + \emptyset) \quad \text{(Eqn. 2)}$$

$$\emptyset' = \emptyset + \theta - 2\tan^{-1}\left[\frac{L-\sqrt{(V_{total}S)^2 + (L\cos\emptyset)^2}}{V_{total}S + L\sin\emptyset}\right] \quad \text{(Eqn. 3)}$$

where:

k: cantilever spring constant (Nm/nm)
S: Sensitivity of applied cantilever (nm/V)
L: Cantilever length (100 µm)
ϑ and $\emptyset$: Angles of cantilever tip bending due to counterforce from bacterial cell to tip during detachment (10°)

The contact surface area of the bacterial cell on the paint surfaces were later analyzed using the ImageJ (version 1.50i) software. The vertical deflection channel bacteria cell images were uploaded in the software and the software analyzing tools were automated measured the contact surface area of the bacterial cell on the paint surface. For each sample, the measurement was performed in triplicates. Means and standard deviations of proportions were evaluated.

RESULTS AND DISCUSSION

Biofouling process in marine environment is preceded by marine biofilm-forming bacterial on the marine man-made surfaces. In the first stages of the biofilm formation, adhesion of primary colonizing bacteria cells on surface which is considered as microfouling frequently influenced by the bacterial species, the interaction medium, and physical properties of interaction surface (Chaumeil and Crapper, 2013; de Carvalho, 2018). Although the initial adhesion of bacterial on the surface is a reversible process, it is a critical primary step in marine biofouling process as it provides the prerequisite conditions for further attachment and increases the settlement of other macrofouling marine organisms (Donlan and Costerton, 2002).

The adhesive force strength of bacterial cells varies with different substrata, materials, topography, and chemo-mechanical properties of the surrounding environment (Zheng *et al.*, 2021). The potential for adhesion between a bacterial cell and a surface is governed by several factors, including the physio-chemical properties of the bacterium and substratum, and the environmental conditions under which the attachment takes place (Bos *et al.*, 1999; Boks et al., 2015). **Figure 1** depicts the mean lateral detachment force required to remove the *B. niabensis* and *A. litorea bacteria* cell from the coated surfaces. The mean lateral detachment force of *B. niabensis* on commercial paint coated surface increased from 15.7193 nN (0.3V) to 164.3606 nN (10.0V). Similar patterns of increment in the mean lateral force detachment were observed on *A. litorea bacterial* cell (17.3644 nN (0.3V) and 165.2754 nN (10.0V)). The same observation was reported by Zhang *et al.* (2011). Among the tested coating, a strong interaction between the bacterial cells and coated surface was observed on commercial paint coated surface compared to Ag-Kao + commercial paint and Or-Ag-Kao + commercial paint coated surfaces. Both bacteria showed lowest interaction on Or-Ag-Kao + commercial paint coated surface. *B. niabensis* recorded 139.4004 nN (10.0V), whilst *A. litorea* recorded 146.2251 nN (10.0V) on Or-Ag-Kao paint coated.

The statistical analysis of One-way ANOVA was performed on data sets of various setpoints of 0.3V, 3V, 5V and 10V for both tested bacteria. Paint and modified paint with increasing setpoint values do not significantly differ in *B. niabensis* and *A.litorea*. For *B. niabensis* and *A. litorea*, student t-test analysis revealed a significant difference for each type of paint coatings.

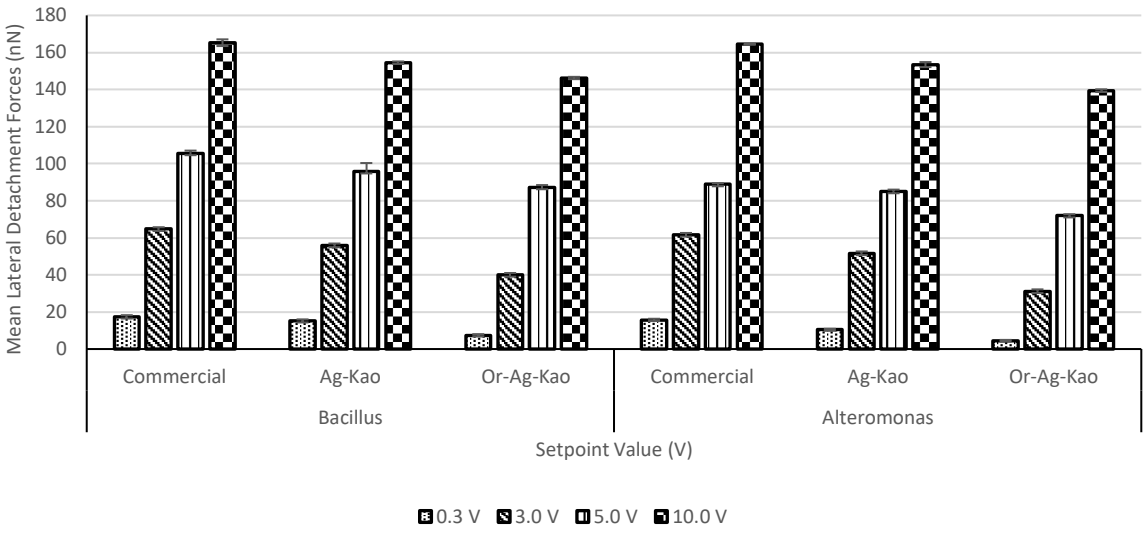

Figure 1 Mean lateral detachment forces of *B. niabensis* and *A. litorea*

The lateral detachment forces demonstrated that Gram-positive bacteria, *B. niabensis* attached loosely on the tested coated surfaces, hence it was easier to be detached from the coated surfaces. Meanwhile, Gram-negative bacteria, *A. litorea* possess stronger adhesion force on the tested coated surface, therefore higher forces was needed to remove the bacteria from the coated surface. Among tested surfaces, surface coated with Or-Ag-Kao + commercial paint recorded the lowest means lateral detachment. This result indicates that surface coated with Or-Ag-Kao + commercial paint has successfully minimized the adhesion on the tested bacteria, resulting less forces needed to remove the bacteria from the surface. Or-Ag-Kao + commercial paint coated surface was found to be effective in inhibiting biofilm formation for both strains of tested bacteria, which could be explained by synergically antibacterial effect of Ag^+ and HDTMA. The efficacy of Or-Ag-Kao + commercial paint is slightly lower in the case of *B. niabensis*, which is primarily attributed to the difference in the nature of its cell wall (Jubeh et al., 2020). In addition to the presence of the cell membrane, the Gram-negative *A. litorea* further supported by a thicker outer membrane comprising lipopolysaccharide. This outer membrane provides Gram- negative bacteria extra protection against chemical attacks; thus, they are less susceptible to the Ag^+ and HDTMA present in the Or-Ag-Kao + commercial paint coated surface.

Figure 2 shows the height image of *B. niabensis* bacterial cell in four consecutive AFM scan at different $V_{setpoint}$. Based on the Figure 2 (a to d), it clearly showed that the size of the bacterial cells decreased with the increment of the setpoint from 0.3V to 10.0V. Deterioration of the bacterial cell structure was observed on *B. niabensis* bacterial cell adherence on the Or-Ag-Kao + commercial paint coated surfaces. The outcome was likely due to the presence of Ag^+ and HDTMA in the paint sample. Possible mechanisms of the bacterial cell deterioration were proposed herein. Firstly, the charged HDTMA could be perturbing the bacterial cell wall membrane through electrostatic interactions. Secondly, the released of Ag^+ could penetrate the bacteria cell membrane, before interacting with sulphur and phosphate containing compound such as DNA and then killing the cell through the traditional lysis mechanism, ultimately resulting in cell deterioration and subsequently cell death (Malachova *et al.*, 2011). Figure 2 (d) shows that the *B. niabensis* was totally detached from the Or-Ag-Kao + commercial paint coated surface after scanning at maximum setpoint value (10.0V). To confirm that the lateral detachment force successfully removed the adhered bacterial cell from the surface the sample was rescanned again under lower setpoint 0.3V. Hence, the lateral detachment process is considered as successful as no bacterial cells were observed after the rescanning under lower setpoint (Figure 2 (e)). As for *A. litorea*, similar height micrograph also revealed that the bacterial cells decreased in size with the increasing setpoint of 0.3V to 10.0V before total detachment was observed after scanning at maximum setpoint value (10.0V).

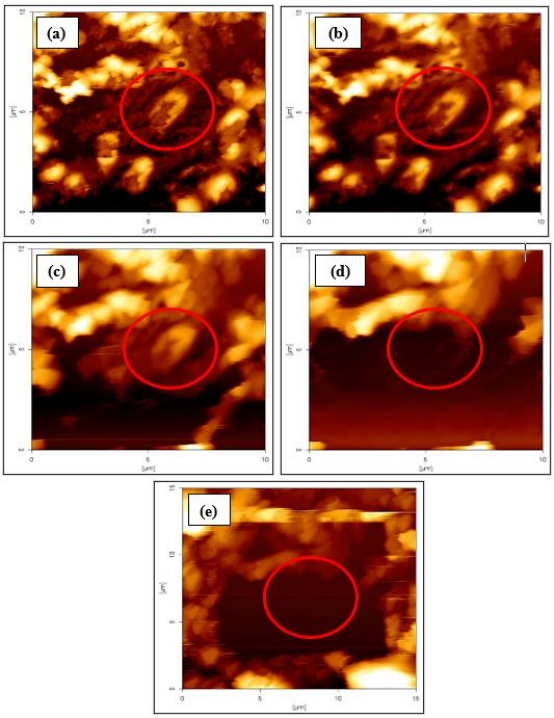

Figure 2 Two-dimensional height micrograph of *B. niabensis* bacterial cell (position showed in red circle) on Or-Ag-Kao + commercial paint coated surface in AFM scan at $V_{setpoint}$ of (a) 0.3V, (b) 3.0V, (c) 5.0V, (d) 10.0V and (e) Full scan after applied minimum setpoint (0.3V) (512 x 512 line/pixel of resolution).

The total contact surface area of bacterial cell was determined from the AFM micrographs and analysed using ImageJ software. Figure 3 and Figure 4, revealed that the mean contact surface area of the both bacterial cells were reduced from commercial paint coated surface to Or-Ag-Kao + commercial paint coated surface. Surface contact area of *B. niabensis* bacterial cells on Or-Ag-Kao + commercial paint coated surface (0.275 μm^2) at maximum setpoint value was significantly smaller than the commercial paint (0.581 μm^2, $P<0.05$) and Ag-Kao + commercial paint coated surface (0.314 μm^2, $P<0.05$). *A. litorea* also shows a reduction in bacterial cells contact surface area at the maximum setpoint value (10.0V). The bacterial cells contact surface area reduced from 0.554 μm^2 on the commercial paint coated surface to 0.452 μm^2 on Ag-Kao + commercial paint coated surface. Major contact surface area reduction of *A. litorea* bacterial cells was observed on Or-Ag-Kao + commercial paint coating with 0.391 μm^2. The reduction on contact surface area of the bacteria after lateral forces applied to the bacteria adhered on the surface represent the force applied during any cleaning process. More contact surface area of the cell detached/ removed after forces applied indicated that the surface successfully minimized the adhesion of the bacteria and the coated surface. Lowest total contact surface area recorded on Or-Ag-Kao + commercial paint coated surface after maximum setpoint value applied indicated that synergically anti-bacterial adhesion possessed by Ag^+ and HDTMA manage to reduce the adhesion strength of the tested strains on the coated surface.

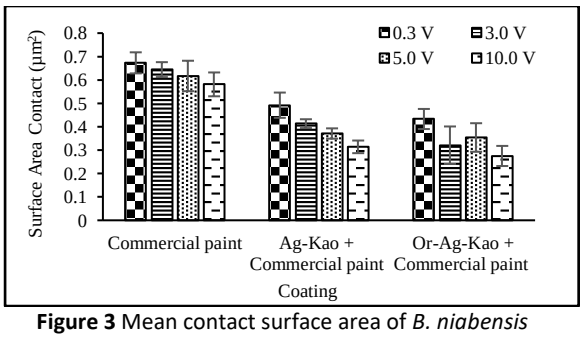

Figure 3 Mean contact surface area of *B. niabensis*

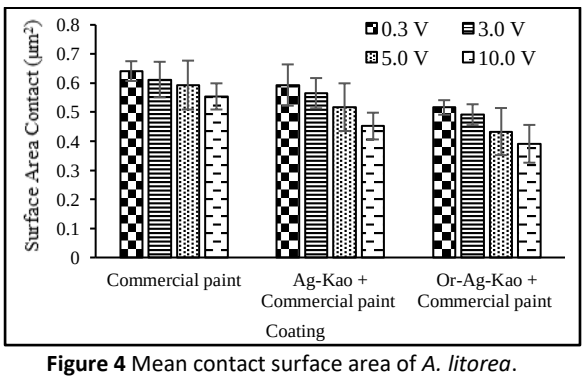

Figure 4 Mean contact surface area of *A. litorea*.

The data collected in the AFM lateral detachment force and surface contact area measurements, shows the lowest interaction occurred for the bacterial cell and the Or-Ag-Kao + commercial paint coating. This might be the reason that Or-Ag-Kao + commercial paint coating is the most effective mixture to reduced bacterial attachment thus minimizing biofilm formation on the surface. The findings of this study proved that both Ag^+ and HDTMA incorporated in the kaolinite framework possess synergic anti-bacterial adhesion and anti-biofilm effects on both Gram-positive and Gram-negative biofouling bacteria. Both Ag^+ and HDTMA in the Organo-silver-kaolinite (Or-Ag-Kao) act as dual effects contact-killing coating by hindering bacterial adhesion on the surface and at the same time destruction of the marine fouling bacteria. The effectiveness of both Ag^+ and HDTMA in the developed anti-biofouling paint additive reduced the adhesion strength of the biofouling bacteria to surface, thus eradicated the marine biofilm could be the offer the best solution to prevent biofouling on marine structures. The current findings on Or-Ag-Kao + commercial paint coating in preventing biofilm formation is expected to have immediate implications in their design and utility for commercial applications, especially within maritime industry.

CONCLUSION

Lateral detachment force of isolated local marine bacteria; *B. niabensis* and *A. litorea* on the coated surfaces was successfully investigated via AFM. Both strains showed lowest interaction and major contact surface area reduction on Or-Ag-Kao + commercial paint coating. These results indicated that Or-Ag-Kao + commercial paint coating is the most effective mixture to reduce bacterial attachment thus minimized biofilm formation on the surface.

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