# Polydopamine-Mediated Immobilization of Alginate Lyase to Prevent *P. aeruginosa* Adhesion

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Given alginate's contribution to *Pseudomonas aeruginosa* virulence, it has long been considered a promising target for interventional therapies, which have been performed by using the enzyme alginate lyase. In this work, instead of treating pre-established mucoid biofilms, alginate lyase is immobilized onto a surface as a preventive measure against *P. aeruginosa* adhesion. A polydopamine dip-coating strategy is employed for functionalization of polycarbonate surfaces. Enzyme immobilization is confirmed by surface characterization. Surfaces functionalized with alginate lyase exhibit anti-adhesive properties, inhibiting the attachment of the mucoid strain. Moreover, surfaces modified with this enzyme also inhibit the adhesion of the tested non-mucoid strain. Unexpectedly, treatment with heat-inactivated enzyme also inhibits the attachment of mucoid and non-mucoid *P. aeruginosa* strains. These findings suggest that the antibacterial performance of alginate lyase functional coatings is catalysis-independent, highlighting the importance of further studies to better understand its mechanism of action against *P. aeruginosa* strains.
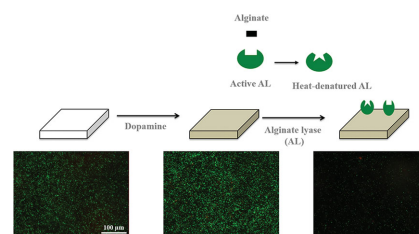
1. Introduction

Modern healthcare is strongly dependent on the use of biomaterials and medical devices to support or restore physiological functions after trauma or disease.[1] There are, however, some drawbacks associated with their extended use, as they constitute a primary avenue for nosocomial infections.[2] Bacteria are able to reach the biomaterial surface, adhere to it and form multicellular aggregates enclosed in a self-produced matrix of extracellular polymeric substances (EPS), with the resultant structures commonly referred as biofilms.[3] When biofilms are at the root of a bacterial infection, treatment becomes extremely difficult as bacteria within these sessile communities adopt special features that confer increased resistance to antimicrobial treatments and to the host immune system.[3,4] Biofilm formation is a cyclic and developmental process, initiated by adhesion of bacteria to the surface of a biomaterial, followed by proliferation, aggregation, and maturation.[5] Upon interfacing with a biomaterial surface, replicating adherent bacteria secrete mostly insoluble gelatinous exopolymers, which function as a "glue," holding bacterial cells together.[6] This extracellular matrix, comprising mainly of water, polysaccharides, proteins, and extracellular DNA, makes biofilms the most successful living structures on earth, providing mechanical support, mediating cell–cell and cell–surface interactions and acting as a protective barrier.[7]

Among the organisms most frequently isolated from infections associated with commonly used medical

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devices and implants, *Pseudomonas aeruginosa* stands out for its high incidence and remarkable ability to form strong biofilms in devices such as peritoneal catheters,[8] ventricular assist devices,[9] endoscopes,[10] and cochlear implants.[11] To establish an infection, *P. aeruginosa* relies on a number of unique virulence factors, including its extensive genetic regulatory networks,[12,13] secretion of enzymes and exopolysaccharides, as well as ability to adhere to various surfaces and form biofilms.[14] The production of the exopolysaccharide alginate is one of the most extensively studied virulence factors. Alginate is a linear polymer of β-D-mannuronic and α-L-guluronic acid residues and it is mainly associated with mucoid *P. aeruginosa* isolates recovered from the lungs of patients with cystic fibrosis.[15] The role of alginate in *P. aeruginosa* adhesion and subsequent biofilm formation has been the subject of some controversy. Several independent studies have shown that overproduction of this exopolysaccharide yields significant architectural and morphological changes in the biofilm[16–18] and contributes to the persistent nature of lung infections.[19] These findings have led to the assumption of alginate as an attractive target for interventional therapies which can be accomplished by the use of the enzyme alginate lyase. This enzyme is able to depolymerize alginate through a β-elimination reaction that releases unsaturated polysaccharides with C=C double bonds at their non-reducing terminal urinate residues.[20] In support of this role, alginate lyase has been shown to detach mucoid biofilms from abiotic surfaces,[21] to increase antibiotic susceptibility of mucoid *P. aeruginosa* biofilms,[22] to reduce viscosity in cultures of clinical isolates and in cystic fibrosis sputum,[23] and to enhance phagocytosis and killing of *P. aeruginosa* by human immune cells.[24] However, other studies demonstrated that alginate synthesis is not required for biofilm development[25,26] and it was reported that the exogenously added *A. vinelandii* alginate lyase was not able to remove mucoid *P. aeruginosa* biofilms, despite being active toward alginate surface.[27] The authors suggested that alginate did not contribute to the cohesiveness of biofilms or it was protected from enzymatic degradation in biofilms. In a recent study, it was shown that alginate lyase dispersion of *P. aeruginosa* biofilms and enzyme synergy with tobramycin is completely decoupled from catalytic activity, as equivalent results were obtained with an isogenic non-mucoid strain and the same anti-biofilm effects could be achieved with bovine serum albumin or simple amino acids.[28]

In the present study, a new approach for utilizing alginate lyase was investigated. Instead of using the enzyme for treatment of pre-established mucoid biofilms, the ability of alginate lyase to prevent *P. aeruginosa* adhesion to a surface was investigated. For that purpose, a polydopamine dip-coating strategy was applied for functionalization of polycarbonate surfaces with alginate lyase. Enzymes immobilization has contributed for the success of enzyme therapy approaches[29] and several approaches have been applied including non-covalent adsorption, immobilization via ionic interactions, covalent attachment, cross-linking and entrapment in a polymeric gel or capsule.[30] There are, however, some drawbacks associated to most of them as typical chemical conjugation methods require multistep, complicated, and time-consuming procedures and physical adsorption processes can cause enzyme aggregation and also an uneven distribution along the surfaces. An alternative approach, which has been attracting considerable interest in the last few years, is the use of dopamine. In this strategy, surfaces are simply immersed in an aqueous solution of dopamine and its self-polymerization leads to a thin adhesive film (polydopamine) on the surface of a wide range of materials.[31] This polydopamine layer can subsequently act as a versatile platform for immobilization of bioactive compounds,[32,33] including enzymes.

2. Experimental Section

2.1. Materials

All surface modifications were performed on square pieces of polycarbonate, PC (McMaster Carr, USA), measuring $1.3 \times 1.3 \times 0.3$ cm^3. Alginate lyase from *Sphingobacterium multivorum* (specific activity ≥ 10 000 units g^{-1} solid), bovine serum albumin (BSA), bicine buffer, dopamine-HCl, and NaCl were purchased from Sigma (St. Louis, MO). Absolute propanol was purchased from VWR International (West Chester, Pennsylvania). Ultrapure, deionized water (18.2 MΩ cm) was used to prepare all aqueous solutions. 12-well flat bottom tissue culture plates were purchased from BD Falcon (Franklin Lakes, NJ). 20 mL glass scintillation vials were purchased from Wheaton (Millville, NJ). Tryptic-soy broth (TSB) and Tryptic-soy agar (TSA) were purchased from BD Diagnostic Systems (Sparks, Maryland). Syto-9/Propidium-Iodide BacLight Bacterial Viability Kit and BacLight Mounting Oil were purchased from Invitrogen (Eugene, OR).

2.2. Strains and Bacterial Conditions

Two reference strains of *P. aeruginosa*, a mucoid strain (ATCC 39324) and a non-mucoid strain (ATCC 27853) were used throughout this study. Four *P. aeruginosa* clinical isolates (from peritoneal catheters), and internally coded as PD 64.8, 68.7, 50.2, and 96.4, were also used. The strains were first streaked on a TSA plate from a frozen stock solution and grown for 24 h at 37 °C under aerobic conditions. For each experiment, a few colonies from each strain were collected from the TSA plates and grown overnight in batches of TSB at 37 °C under agitation (120 rpm). Subsequently, cells were harvested by centrifugation (9000 *g*, 5 min, at room temperature) and washed in sterile saline solution (0.9% NaCl prepared in ultrapure water). The cellular suspension was adjusted to a final concentration of 1×10^8 CFU mL^{-1}, determined by optical

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density at 640 nm and calibrated in terms of CFU using spread plate method.
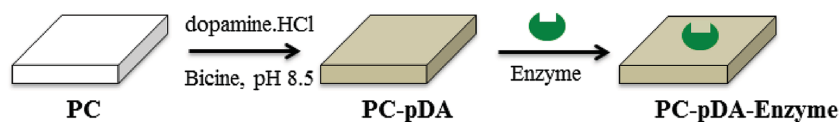
2.3. Alginate Lyase and BSA Coating on PC

Alginate lyase and BSA (a protein without catalytic activity used as control) coatings were prepared as illustrated in Figure 1, adapted from a two-step method previously developed for immobilizing enzyme onto magnetic nanoparticles.[33] Prior to surface modification, PC surfaces were cleaned by sonication for 20 min in 0.12 M HCl and 20 min in isopropanol, followed by rinsing with ultrapure water and finally air-dried overnight. PC surfaces were then immersed in 10 mL of freshly prepared dopamine solution (1 mg mL^{-1} dopamine-HCl in 10×10^{-3} M bicine buffer, pH 8.5) for 18 h at room temperature under agitation. The surfaces were then rinsed with ultrapure water and dried with nitrogen gas. To further coat with active or heat denatured (at 105 °C for 15 min) alginate lyase, polydopamine-coated PC surfaces (PC-pDA) were immersed in 5 mL of alginate lyase solution (1 mg mL^{-1} in bicine buffer supplemented with 600×10^{-3} M NaCl, pH 8.5) for 2 h at room temperature under agitation (PC-pDA-AL and PC-pDA-inactive AL). For BSA immobilization, polydopamine-coated PC surfaces were immersed in 5 mL of BSA solution (1 mg mL^{-1} in bicine buffer supplemented with 600×10^{-3} M NaCl, pH 8.5) for 2 h at room temperature, under agitation (PC-pDA-BSA). Simple adsorption of alginate lyase without the intermediate layer of polydopamine was also performed by immersing unmodified PC surfaces in 5 mL of active alginate lyase solution prepared in the same conditions (PC-AL).

2.4. Physicochemical Characterization of Surfaces and Cells

The hydrophobicity parameters of material surfaces and bacteria were determined using the sessile drop contact angle method, using an automated contact angle device (OCA 15 Plus, Dataphysics, Germany) that allows image acquisition and data analysis. Measurements were performed using 3 μL drops of liquid on cleaned and dried coupons of PC before and after polydopamine coating, as well as on bacterial layers deposited on membrane filters, as previously described.[34] Briefly, a bacterial suspension was adjusted to a concentration of ≈1 × 10^9 CFU mL^{-1} in sterile saline solution from an overnight culture and was deposited onto a 0.45 μm cellulose membrane filter, previously wetted with 10 mL of distilled water to obtain a thick lawn of cells. The filters with the resultant lawn of cells were afterward kept on petri dishes containing 1% (w/v) agar and 10% (v/v) glycerol for at least 3.5 h, until the so called "dried-plateau" was obtained. All measurements were performed at room temperature and water, formamide, and α-bromonaphthalene were used as reference liquids for standardized contact angles measurements. Contact angles were related to the surface hydrophobicity, using the van Oss approach.[35] According to van Oss,[36] hydrophobicity can be expressed in the form of the free energy of interfacial interaction (ΔG_{sws}^{TOT}) between the particles of a solid surface (s), in an aqueous environment (w). According to this model, when $\Delta G_{sws}^{TOT} > 0$, the surfaces are hydrophilic and for $\Delta G_{sws}^{TOT} < 0$, they are hydrophobic.

Figure 1. Schematic representation of coating strategy developed for alginate lyase immobilization onto polycarbonate (PC) surfaces.

From the physicochemical parameters of each adhesion entity (bacteria and surface), it was possible to determine the thermodynamic relation between both entities, namely, the free energy of adhesion (ΔG_{bsb}^{TOT}) between the bacteria (b) and the surfaces (s). According to the thermodynamic theory, adhesion will be favored if interaction leads to a decrease on free energy of adhesion.

2.5. Enzymatic Activity of Alginate Lyase-Coated Surfaces

The activity of alginate lyase immobilized onto PC surfaces using the different coating procedures aforementioned was determined by measuring the increase in absorbance at 235 nm.[37] Alginate lyase catalyzes depolymerization of alginate through cleavage of the 4-O-glycosidic bond via a β-elimination mechanism which leads to formation of a double bond between C-4 and C-5 and production of 4-deoxy-L-erythohex-4-ebe pyranosyluronate at the non-reducing end of the resulting oligomers which can be detected by measuring absorbance at 235 nm. Briefly, alginate lyase-coated surfaces were covered with 0.6 mL of 0.1% sodium alginate (Sigma) prepared in 0.2 M potassium phosphate buffer, pH 6.3. After incubation at 37 °C for 30 min, the absorbance of the reaction mixture was measured at 235 nm. As a control, the activity of alginate lyase heat-inactivated before its immobilization was also determined.

2.6. Surface Characterization

X-ray photoelectron spectroscopy (XPS) (Omicron ESCA Probe; Omicron, Taunusstein, Germany) was used to characterize the chemical composition of surfaces. The X-ray source operated at 300 W with a spot size of 1.5 mm and a constant sample deflection angle of 45°. An electron gun was used to minimize surface charging effects, operating with a beam current of 0.008 mA at 12.5 eV. High-resolution spectra of the C1s region were obtained by averaging three separate sweeps between 277.5 and 292.5 eV. The surface morphology of the surfaces was analyzed by scanning electron microscopy (SEM, S-4800 FE-SEM, Hitachi, Schaumburg, Illinois). SEM imaging was performed with the following parameters: 20 kV accelerating voltage, 10 μA beam current, 6 mm stage distance, and 22 K magnification. Surface wettability was investigated by measuring static water contact angle as aforementioned.

2.7. Bacterial Attachment Assay

Antibacterial performance of the generated surfaces against bacterial adhesion was evaluated using different *P. aeruginosa* strains. A bacterial suspension with 1 × 10^8 CFU mL^{-1} was prepared in sterile saline solution from an overnight culture of each strain. Polymer surfaces were placed into wells of a 12-well tissue culture plate and covered with 2 mL of bacterial suspension. The

samples were kept at 37 °C for 24 h with agitation at 120 rpm, stained with a live/dead stain, washed with saline, and observed using a fluorescent microscope (Leica, DM IRB, Leica Microsystems, Bannockburn, IL). At least three images per sample surface (a single grayscale image for each fluorescence channel) were collected and a total of three sample surfaces were evaluated in each independent experiment. ImageJ (Version 1.49 m, Wayne Rasband, National Institutes of Health, USA) software was used to subtract the image background and the threshold function was used to render each grayscale image into a binary translation with distinct areas identifying adhered bacteria. The threshold value supplied by ImageJ was used as default but when necessary the threshold value was manually adjusted until all visible cells were included within the threshold range. The area measurement function was used to quantify the area of the pixels above the threshold and to thereby quantify the area covered by bacteria discriminating, at the same time, the fraction of live and dead bacteria, depending on the channel being analyzed. Values were normalized to % coverage on PC control, as previously performed.[38]

2.8. Statistical Analysis

All the experiments were performed in triplicate and results were presented as mean ± standard deviation (SD). Statistical analysis was performed by Kolmogorov–Smirnov normality test using Graph Pad Prism 5.0 for Macintosh. After this analysis, parametric tests (one-way ANOVA followed by Tukey's test) or nonparametric (Kruskal–Wallis test) were used depending on whether the samples were from normally distributed populations or not, respectively.

3. Results and Discussion

3.1. Alginate Lyase Immobilization onto PC Surfaces

The potential of alginate lyase for the treatment of mucoid *P. aeruginosa* biofilms has been the subject of research for the past few decades.[18,19,22] Posttreatment of mucoid *P. aeruginosa* biofilms with alginate lyase and subsequent biofilm detachment has led to the assumption that alginate plays an important role on biofilm establishment. In the present work, it was hypothesized that the presence of alginate lyase during the first stages of biofilm establishment, namely, bacterial adhesion to a surface, could prevent its establishment. To confirm this hypothesis, alginate lyase was immobilized using a bio-inspired coating strategy.

The immobilization platform was performed on polycarbonate as it has been reported that there is an annual 6% demand for its use in medical devices such as filters cartridges for dialysis, blood oxygenators, reservoirs, filters, and connection components.[39] The first step of the coating strategy involved the deposition of a uniform polydopamine (pDA) coating from dopamine-HCl solution at a slightly alkaline pH. The pDA coatings were then used as a platform for enzyme immobilization by immersion in an alginate lyase solution (Figure 1). It is likely that the presence of residual quinones within the pDA coating present convenient sites for covalent grafting of nucleophilic groups, such as amino functional groups commonly found in enzymes, via Michael addition and/or Schiff base reactions.[32]

3.2. Surface Characterization

XPS analysis was employed to confirm each modification step (Figure 2). Polydopamine deposition was established

A)
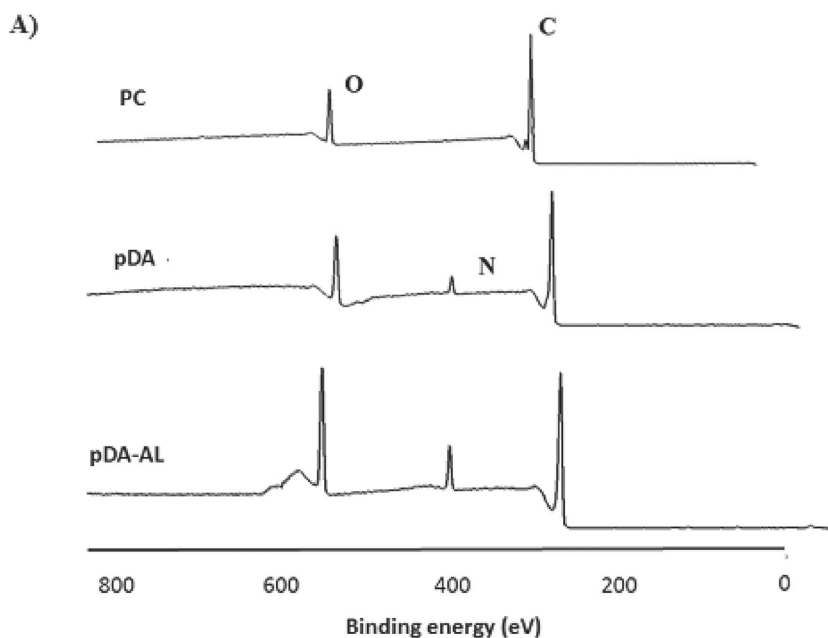
B)

Sample	C (%)	O (%)	N (%)	S (%)
PC	85.3	14.7	0.0	0.0
PC-pDA	70.13	21.18	8.69	0.0
Dopamine	72.7	18.2	9.1	0.0
pDA-AL	67.39	19.91	12.45	0.25
AL powder	65.4	26.8	5.7	2.1

Figure 2. XPS analysis of the polymer surfaces. A) XPS peaks of the unmodified polycarbonate surfaces (PC), polydopamine-coated PC surfaces (PC-pDA), and pDA-coated PC surfaces with immobilized alginate lyase (PC-pDA-AL). B) Quantification of atomic compositions on the polymer surfaces, alginate lyase in powder (AL), and the theoretical composition of dopamine based on its molecular composition.
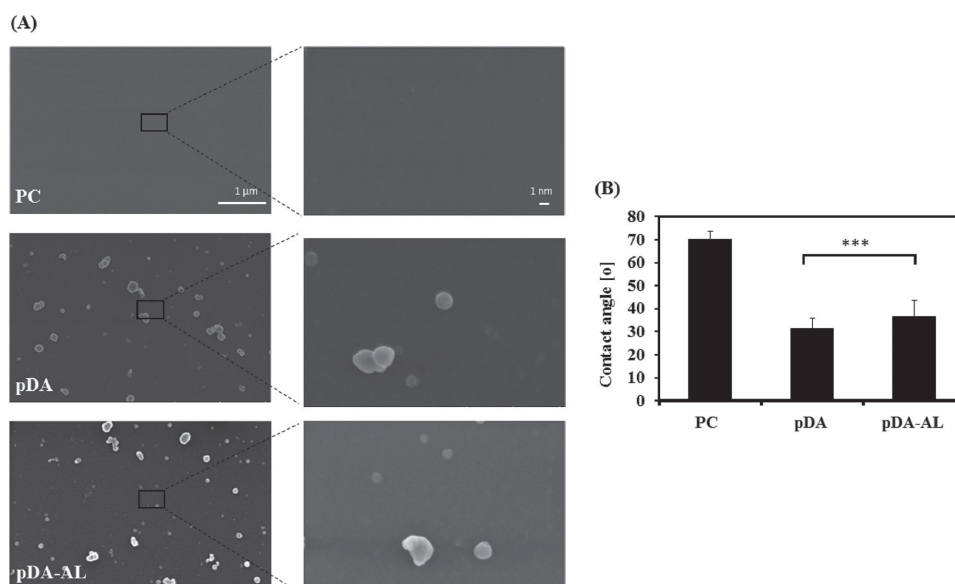
Figure 3. A) SEM images and B) measurement of the water contact angle of the unmodified polycarbonate surfaces (PC), polydopamine-coated PC surfaces (PC-pDA), and pDA-coated PC surfaces with immobilized alginate lyase (PC-pDA-AL). Significant differences were found for (***) $p < 0.001$, compared to PC control.

by the presence of the N1s (399 eV) peak in addition to the C1s (4. 284.7 eV) and O1s (531 eV) peaks present in unmodified PC. Furthermore, pDA-modified surfaces yielded surface chemical compositions similar to the theoretical ones of dopamine (Figure 2B). Alginate lyase immobilization was suggested by the slight decrease of carbon accompanied by an increase of nitrogen composition. Differences in chemical composition of enzyme powder before and after its immobilization may be attributed to the sampling depth achieved by XPS, which is ≈10 nm, which means the pDA layer may have contributed to the chemical signature detected by XPS analysis. Sulfur decrease on immobilized enzyme may be attributed to a reduced number of exposed thiol groups on the surface, which were necessary for grafting to the pDA-coated PC. Surface morphology of polydopamine-mediated surfaces was characterized using SEM analysis. The unmodified PC surfaces exhibited smooth surface morphology compared to the modified surfaces (Figure 3A). Polydopamine particles as a result of dopamine self-polymerization in solution could be observed on both pDA-coated surfaces and after further functionalization with alginate lyase, confirming the presence of pDA coating. For further characterization of the surfaces prepared under these conditions, water static contact angles of the surfaces before and after modification were measured (Figure 3B). After applying the polydopamine coating, PC surfaces became more hydrophilic with a significantly reduced contact angle (31°) which is in agreement with studies previously reported.[31] Further functionalization with alginate lyase had no significant effect on surface hydrophilicity.

3.3. Enzymatic Activity of Immobilized Alginate Lyase

The catalytic activity of the immobilized alginate lyase onto PC surfaces by simple adsorption or mediated by dopamine polymerization was assessed by measuring spectrophotometrically its ability to depolymerize sodium alginate. As a control, the activity of alginate lyase heat denatured before its immobilization was also determined. Results in Table 1 showed that alginate lyase retained its catalytic activity after being immobilized by both coating strategies. A higher activity was, however, obtained when alginate lyase immobilization was mediated by polydopamine (absorbance values of 0.21 and 0.13, respectively). These results also suggest that immobilization mediated by pDA yielded a better immobilization efficiency although the amount of immobilized enzyme could not be determined. Heating of alginate lyase before its immobilization caused enzyme denaturation as it was not able to act against sodium alginate.

3.4. Prediction of Adhesion

The evaluation of the hydrophobicity parameters for the reference strains and the PC surfaces before and after their modification with polydopamine is shown in Table 2A. Results show that the two reference strains of *P. aeruginosa* showed water contact angles lower than 65° and positive values of free energy of interaction (ΔG_{iwi}), which are indicative of a hydrophilic feature.[36,40] PC surfaces can be considered hydrophobic since the water contact angle values were higher than 65° and a negative value of free

Table 1. Intensities of absorbance measured at 235 nm corresponding to alginate lyase activity against sodium alginate. Values are means ± SD.

Sample	Abs$_{235\ nm}$
PC-AL	0.13 ± 0.04
PC-pDA-AL	0.21 ± 0.04
PC-pDA-inactive AL	0.03 ± 0.02

energy of interaction. After modification with polydopamine, their thermodynamic properties were altered which can be clearly shown in terms of the free energy of interaction (ΔG_{iwi}^{TOT}) for which a positive value was obtained. This means that, theoretically, the affinity of an organism for the unmodified PC surfaces is superior when compared to the polydopamine-coated PC. From the physicochemical parameters of each adhesion entity (bacteria and surface), it was possible to determine the thermodynamic relation between both entities, namely, the free energy of adhesion (Table 2B). Results suggested that adhesion to both unmodified PC and polydopamine-coated PC is more favored for the non-mucoid strain, as indicated by the lower values of free energy of adhesion.

3.5. Antibacterial Performance of Surfaces Functionalized with Alginate Lyase

To investigate the antibacterial performance of PC surfaces functionalized with alginate lyase, attachment assays

were performed in which bare and treated surfaces were exposed to bacteria and the remaining cells on the surfaces were imaged with fluorescence microscopy. These assays were performed under starvation conditions (saline solution) and for a long period of time, 24 h, in order to enhance alginate production from mucoid strains.[41] It was possible to measure the remaining cells on the modified surfaces and simultaneously discriminate between live and dead cells, or more specifically, evaluate bacterial membrane integrity. Two reference strains of *P. aeruginosa*, one mucoid (ATCC 39324) and the other non-mucoid (ATCC 27853), were first used to assess the antibacterial properties of the modified surfaces (Figures 4 and 5). Unmodified PC surfaces allowed the adhesion of both bacterial strains and most of them remained alive. The presence of polydopamine coating decreased mucoid strain attachment and enhanced the adhesion of the non-mucoid strain, as compared to the unmodified surfaces. These results may be attributed to the differences found on the hydrophobicity parameters of bacteria and surfaces aforementioned as they suggested a higher affinity of non-mucoid strain to PC surfaces before and after their modification with polydopamine. The fraction of dead cells found on pDA-coated surfaces slightly increased after 24 h of incubation, which can be attributed to a decrease in the pH of saline solution in contact with pDA-coated surfaces, as previously reported.[38] The fraction of mucoid bacterial cells found on surfaces functionalized with alginate lyase was significantly lower than on unmodified PC and PC coated with polydopamine. Interestingly, alginate

Table 2. (A) Values of contact angles (°) with water (θ_W), formamide (θ_F), α-bromonaphthalene (θ_B), surface tension parameters (mJ m^{-2}), and free energy of interaction (ΔG_{iwi}^{TOT}) (mJ m^{-2}) between the bacteria and the surfaces (i) when immersed in water (w). (B) Free energy of adhesion between bacteria (b) and the surfaces (s). Values are means ± SD.

(A)

Bacteria/surface	Contact angle [°]			Surface tension parameters [mJ m^{-2}]			Free energy of interaction [mJ m^{-2}]
	θ_W	θ_F	θ_B	γ_i^{LW}	γ_i^+	γ_i^-	ΔG_{iwi}^{TOT}
P. a ATCC 27853	30.8 ± 9.9	23.0 ± 4.7	53.9 ± 6.7	28.1	4.2	41.7	16.2
P. a ATCC 39324	35.8 ± 11.6	86.5 ± 15.9	26.9 ± 3.4	39.7	0	127.8	121.8
PC	76.2 ± 8.4	63.1 ± 5.7	12.8 ± 2.9	43.3	0	12.5	−37.8
PC-pDA	33.9 ± 5.2	10.2 ± 1.7	25.0 ± 5.3	40.3	2.0	34.7	6.6

(B)

	Free energy of adhesion [mJ m^{-2}]	
	ΔG_{bsb}^{TOT}	
Bacteria	*PC*	PC-pDA
P. a ATCC 27853	2.8	13.3
P. a ATCC 39324	41.7	48.4

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the attachment of both strains, alginate lyase polydopamine-based immobilization was more efficient in preventing bacterial attachment. As another control, alginate lyase heat-inactivated (105 °C for 15 min) as well as an irrelevant protein, BSA, were immobilized onto pDA-coated surfaces and their antibacterial performance against the two reference strains evaluated. Results (Figure 5) demonstrated that modified surfaces functionalized with heat-inactivated alginate lyase or BSA were also able to impair bacterial adhesion of all strains investigated, suggesting that alginate lyase effects on bacterial attachment were decoupled from its catalytic activity.

These results suggested that alginate lyase immobilized on pDA-coated surfaces is a promising approach to impair *P. aeruginosa* adhesion regardless of its mucoid phenotype, therefore qualifying the strategy to be applied in a different context than just cystic fibrosis, where the mucoid phenotype predominates. For instance, this enzyme could be used to develop functional coatings able to prevent *P. aeruginosa* infections associated with a variety of biomaterials. To confirm this hypothesis, the attachment of four clinical strains of *P. aeruginosa* isolated from peritoneal catheters was also evaluated and similar anti-adhesive properties were observed. Although the mucoid phenotype of these clinical isolates was not known, non-mucoid *P. aeruginosa* strains are the predominant clinical and environmental phenotype.[26] Results in Figure 6 showed that clinical isolates were allowed to attach to unmodified surfaces and most of them remained alive after a 24 h incubation under non-growing conditions. The presence of a polydopamine coating did not have a significant effect on bacterial attachment but caused a slight decrease in cell viability, similar to what was seen with the two reference strains. The immobilization of active or heat-denatured alginate lyase on the surfaces, caused a decrease on the attachment of clinical strains with the exception of PD 96.4, as its adhesion to the unmodified PC was already low.

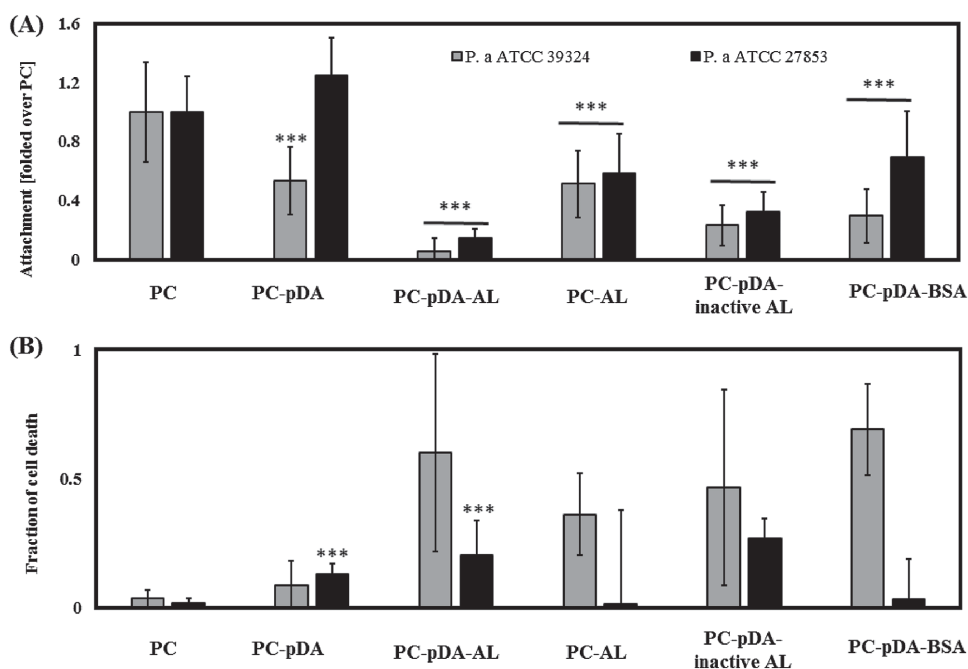These findings are consistent with a previous study[28] reporting that alginate lyase effects on *P. aeruginosa*

Figure 4. Representative fluorescent live/dead stain images obtained during *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 39324 adhesion for 24 h on unmodified surfaces (PC), polydopamine-coated surfaces (PC-pDA), and polydopamine-coated surfaces functionalized with active (PC-pDA-AL) and heat-inactivated alginate lyase (PC-pDA- inactive AL).

lyase immobilized on pDA-coated surfaces was also able to reduce the number of attached non-mucoid bacterial cells. Regarding cell viability, the presence of alginate lyase resulted, in general, in a higher fraction of dead bacteria, especially for the mucoid strain, which can be attributed to the antibacterial activity of lyase-depolymerized products of alginate previously reported.[42] As a control, alginate lyase was also immobilized onto PC surfaces using simple adsorption and, although it was able to prevent

Figure 5. A) Normalized attachment and B) fraction of dead cells of *P. aeruginosa* ATCC 27853 and *P. aeruginosa* ATCC 39324 on unmodified surfaces (PC), polydopamine-coated surfaces (PC-pDA), alginate lyase adsorbed onto PC (PC-AL), and polydopamine-coated surfaces functionalized with active alginate lyase (PC-pDA-AL), heat-inactivated alginate lyase (PC-pDA-inactive AL), and BSA (PC-pDA-BSA). Attachment values were normalized to PC control. Significant differences were found for (***) $p < 0.001$, compared to PC control.

biofilms are completely decoupled from its catalytic activity. The authors suggested that the anti-biofilm effects of alginate lyase enzymes could be related to

enzyme-mediated changes in cell physiology. They hypothesized that instead of actively degrading the biofilm matrix, alginate lyase enzymes act as a nutrient
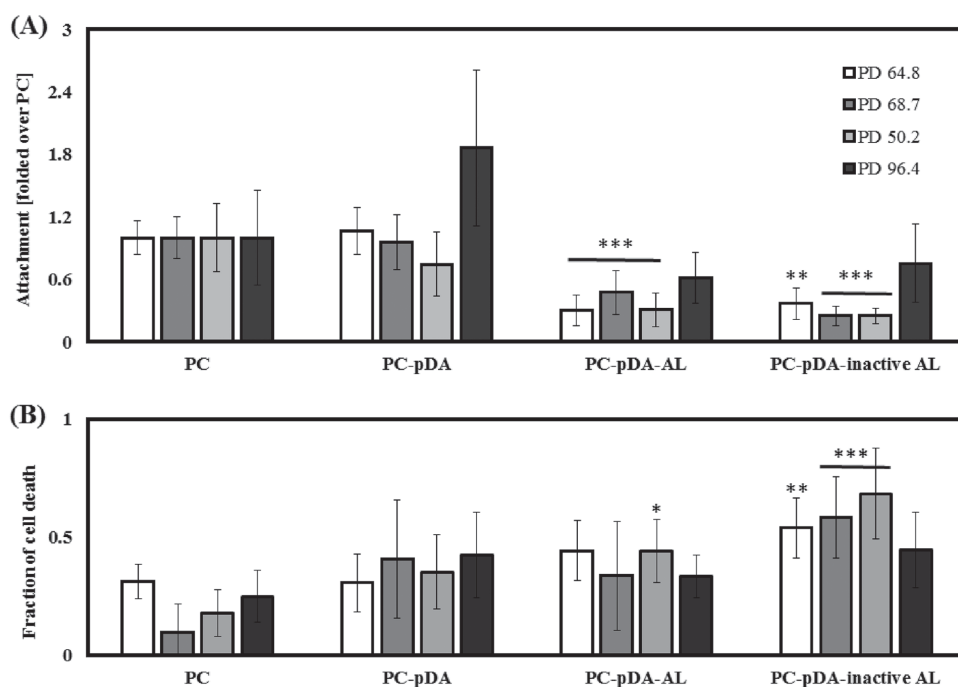

Figure 6. A) Normalized attachment and B) fraction of dead cells of clinical isolates of *P. aeruginosa* on unmodified surfaces (PC), polydopamine-coated surfaces (PC-pDA), and polydopamine-coated surfaces functionalized active alginate lyase (PC-pDA-AL) and heat-inactivated alginate lyase (PC-pDA-inactive AL). Attachment values were normalized to PC control. Significant differences were found for (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$, compared to PC control.

source, modulating cellular metabolism, and thus inducing biofilm detachment and enhancing antibiotic efficacy. In the present study, however, taking into account the enzyme's immobilization, it may not be available to act as a nutrient source and, therefore, a different mechanism may be at the root of alginate lyase effects on *P. aeruginosa* attachment. The enzyme may prevent nonspecific binding of bacteria in a similar way to BSA, which has been commonly used to inhibit nonspecific biomolecule and bacterial adhesion to surfaces in applications such as bacteria sensors and micro patterning.[43,44] Accordingly, polydopamine-based immobilization of BSA on PC was also able to prevent *P. aeruginosa* reference strains attachment. Immobilized alginate lyase proved to be more efficient than BSA to prevent the attachment of non-mucoid strain of *P. aeruginosa*. Such results may be attributed to the physicochemical properties of the mucoid strain, which makes it more susceptible to the hydrophilic character of both alginate lyase and BSA coatings. Given the similar hydrophilic characters of both alginate lyase and BSA coatings via polydopamine, results suggest that alginate lyase may have another underlying mechanism for preventing bacterial adhesion, beyond preventing nonspecific adhesion. The combination of this preventive approach with therapeutic approaches, namely, antibiotic therapies may hold great potential to fight biomaterial-associated infections, as it is expected that bacterial cells adhered to these modified surfaces will be more susceptible to antibiotic therapy in a similar way to bacteria that adhered more weakly to brush-coated silicone rubber, enhancing their susceptibility to gentamicin treatment.[45]

4. Conclusions

A simple and bio-inspired strategy was applied to develop an alginate lyase functional coating able to impair *P. aeruginosa* adhesion. Surface characterization confirmed the immobilization of alginate lyase onto polydopamine-coated PC surfaces without compromising its catalytic activity. It was confirmed, however, the ability of surfaces functionalized with alginate lyase to significantly impair *P. aeruginosa* adhesion, regardless of its mucoid or non-mucoid phenotype. It was hypothesized that alginate lyase could be applied in other contexts aside from cystic fibrosis. However, it was found that alginate lyase effects on *P. aeruginosa* adhesion were decoupled from its catalytic activity, as the heat-inactivated enzyme could also impair bacterial attachment. In conclusion, although the mechanism(s) of action of alginate lyase against *P. aeruginosa* strains as well as against other strains commonly associated to biomaterial-associated infections such as *Staphylococcus aureus* and *S. epidermidis*, needs

to be further explored, this work suggests that alginate lyase immobilization on biomaterials may have potential as a preventive approach to fight biomaterial-associated infections.

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