A Functional DNase I Coating to Prevent Adhesion of Bacteria and the Formation of Biofilm

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Biofilms are detrimental in many industrial and biomedical applications and prevention of biofilm formation has been a prime challenge for decades. Biofilms consist of communities of adhering bacteria, supported and protected by extracellular-polymeric-substances (EPS), the so-called "house of biofilm organisms". EPS consists of water, proteins, polysaccharides and extracellular-DNA (eDNA). eDNA, being the longest molecule in EPS, connects the different EPS components and therewith holds an adhering biofilm together. eDNA is associated with bacterial cell surfaces by specific and non-specific mechanisms, mediating binding of other biopolymers in EPS. eDNA therewith assists in facilitating adhesion, aggregation and maintenance of biofilm structure. Here, a new method is described to prevent biofilm formation on surfaces by applying a DNase I enzyme coating to polymethylmethacrylate, using dopamine as an intermediate. The intermediate coupling layer and final DNase I coating are characterized by water-contact-angle measurements and X-ray photoelectron-spectroscopy. The DNase I coating strongly reduces adhesion of *Staphylococcus aureus* (95%) and *Pseudomonas aeruginosa* (99%) and prevents biofilm formation up to 14 h, without affecting mammalian cell adhesion and proliferation. Also agarose-gel-electrophoresis indicates loss of enzyme activity between 8 and 24 h. This duration however, is similar to many local antibiotic-delivery devices, which makes it an ideal coating for biomaterial implants and devices, known to fail due to biofilm formation with disastrous consequences for patients and high costs to the healthcare system. With threatening increases in antibiotic resistance, the DNase I coating may provide a timely, potent new approach to biofilm prevention on biomaterial implants and devices.

1. Introduction

Bacteria exist in nature on nearly all surfaces and have a strong potential to form biofilms that has been beyond prevention for centuries.[1,2] Bacterial biofilms are defined as groups or clusters of bacteria, embedded in a self-produced matrix of extracellular polymeric substances (EPS) forming a three-dimensional structure.[3–5] Despite extensive studies over many decades, the mechanisms involved in biofilm formation are still not fully understood and as a consequence, prevention of biofilm formation remains a prime challenge in many industrial and biomedical applications. In industrial applications, biofilms inflict major damage when formed on processing equipment or in pipes used to transport resources. In the biomedical field, infections that arise after implantation of a biomaterial implant (e.g., prosthetic hips and knees) or device (pace makers) are known to be very persistent and difficult to treat due to the formation of biofilms by adhering bacteria and in worst cases cause death of the patient. Moreover dental caries, the number one infectious disease in the world, is due to biofilms.[6]

Biofilm formation is a cyclic and step-by-step process, starting when bacteria adhere to a substratum surface, which is first and foremost a reversible process.[1] Initial adhesion however, is rapidly followed by a transition to irreversible adhesion through removal of interfacial water and adjustment of bacterial cell surface adhesion sites, generally occurring within several minutes. Next, aggregation, co-adhesion and growth lead to microcolony formation while continued production of EPS ultimately results in the construction of the "house of biofilm organisms",[7] providing mechanical stability to the biofilm.[6,8] At later stages after biofilm maturation, biofilms may release single bacteria for colonization of new surfaces.[9]

EPS can be associated with the bacterial cell surface or excreted in the environment and is involved in all stages of biofilm formation including initial adhesion of planktonic bacteria.[10,11] EPS is primarily composed of proteins, polysaccharides and extracellular DNA (eDNA) and is often referred to as the glue or cement that holds a biofilm together in its three-dimensional housing.[7] eDNA, being the longest molecule in EPS and shown to be associated with proteins and

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Table 1. Different roles proposed in the literature for extracellular DNA in biofilm formation.

Bacterial species	Role of eDNA in biofilm formation	References
Listeriamon ocytogenes, Bacillusc ereus, S.ep idermidis, S.a ureus, S.m utans	Increased initial adhesion	[19,29,30]
S.mu tans, S.ep idermidis, P.a eruginosa, E.c oli, S.a ureus	Increasedag gregation	[3,14,25,27]
P.a eruginosa, E.f aecalis, S.a ureus,	Enhanced mechanical stability of biofilm	[2,21,31]
Caulobactercr escentus	Inhibition of hold-fast adhesion	[32]

polysaccharides,[12,13] could well be the most important component of EPS keeping all its constituents together. Goodman and co-workers recently showed that *Escherichia coli* strain U91 produces two types of proteins (HU and IHF, both belonging to the DNABII family) that are responsible for arrangement of eDNA and also help eDNA in maintaining the integrity of the EPS in a biofilm.[14] eDNA is generally considered to originate either from the chromosomes of lysed cells in a biofilm or to be actively released via vesicles by metabolically active bacteria.[15–17] These bacterial membrane-derived vesicles have long been known to contain DNA,[18] but only recently their presence in bacterial biofilms has been shown.[19]

The essential role of eDNA as a component of EPS was first reported by Whitchurch et al. for *Pseudomonas aeruginosa* biofilms.[20] They showed that presence of DNase I, an enzyme which non-specifically cleaves DNA by breaking phosphodiester bonds of the phosphate backbone through hydrolysis, in growth medium could prevent biofilm formation and also rinsing a biofilm with DNase I could effectively disintegrate 60 h old biofilms.[20] Similar effects of DNase I treatment on whole biofilms have been later reported for Gram-positive and Gram-negative bacterial species,[21] including *Enterococcus faecalis* and *Staphylococcus aureus*.[22,23]

The important role of eDNA in biofilms (see overview in **Table 1**) can be attributed to its supporting function in adhesion and aggregation of bacteria, both crucial in the primary events leading to biofilm formation. Several studies reported that eDNA is involved in initial adhesion of bacteria to substratum surfaces. For instance, in *S. aureus* eDNA release due to cell lysis during initial stages of biofilm formation is essential for bacterial adhesion to the surface, and removal of eDNA or blocking of eDNA production at any time during biofilm formation results in a decrease in bacterial adhesion.[24] Similarly, initial adhesion of *Staphylococcus epidermidis, S. aureus* and *Streptococcus mutans* to a glass surface was inhibited by removal of eDNA.[25] Besides adhesion, removal of eDNA also reduced aggregation in *S. mutans, S. epidermidis, P. aeruginosa*[26] and *E. coli*.[27] Similar as observed for adhesion, aggregation of DNase I treated bacteria could not be fully restored by the addition of purified DNA.[12,28]

eDNA can interact non-specifically with bacterial cell surfaces, but different bacterial species also possess DNA binding proteins that specifically interact with eDNA in addition to their non-specific interaction.[13] However, in many bacterial species, non-specific mechanisms are involved in eDNA-mediated adhesion and aggregation. Liu et al. indicated that aggregation was independent of the source of the DNA and therefore concluded that DNA enhances aggregation of *E. coli* through a non-specific mechanism.[27] In support of this, it was shown that DNA-mediated aggregation is due to a combination of attractive Lifshitz-Van der Waals forces and attractive acid-base interactions.[26]

Since eDNA is essential in bacterial adhesion and biofilm formation, attacking this pivotal component of EPS as the cement that holds a biofilm together by DNase I, has been considered as a possible approach to prevent biofilm formation. Hitherto, the effectiveness of DNase I in inhibiting biofilm formation has been shown either by pre-treating bacteria prior to their adhesion or post-treating biofilms, neither of which would be a strategy to prevent biofilm formation in nature. Pre-treatment of bacteria requires continued exposure of new generations to DNase I, while post-treatment involves penetration of the DNase I into a biofilm, which can be a cumbersome process depending on the strains and environmental conditions for growth involved. Rather, a better and completely new strategy would be to have DNase I present at the time and place that bacteria first come in contact with a substratum surface.

In this study, we created a functional DNase I coating on a surface, while maintaining its biological activity. A DNase I coating on substratum surfaces was achieved through a dopamine coupling agent and we established this DNase I to be capable of inhibiting bacterial adhesion and biofilm formation by disrupting eDNA in EPS. The DNase I coating strongly reduced bacterial adhesion and prevented biofilm formation up to 14 h, without affecting mammalian cell adhesion and proliferation.

2. Results

Polymethylmethacrylate (PMMA), a commonly used biomaterial, was coated with dopamine as an intermediate coupling layer (see **Figure 1**), reducing the water contact angle on PMMA from

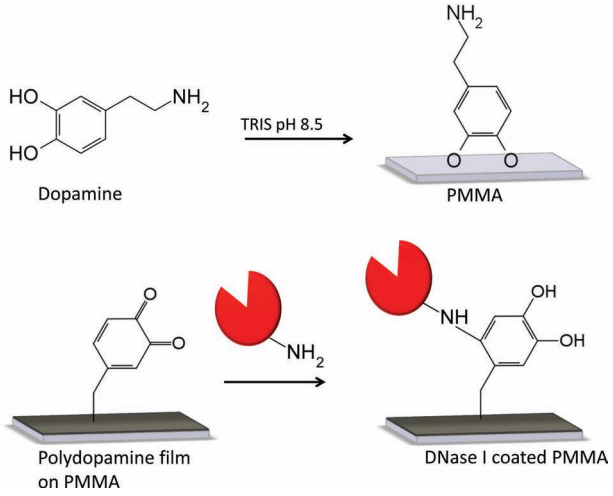

Figure 1. Adhesion of dopamine to PMMA by dip coating and subsequent DNase I coupling to the polydopamine film.

Table 2. Decomposition of the C_{1s} photo-electron peaks (binding energies given in eV) obtained in the process of coating PMMA with a DNase I layer, into four components, due to different chemical functionalities.

Sample	$C_{284.8}$ (C—C)	$C_{286.1}$ (C—N)	$C_{288.0}$ (O = C—O)	$C_{289.2}$ (O = C—O)
PMMA	67.9%	18.4%	13.8%	0.0%
Dopamine coating	59.8%	33.5%	4.5%	2.2%
DNase I overcoat	49.0%	36.7%	10.6%	3.7%

65° ± 6° to 48° ± 4°. Determination of the elemental surface composition of the coatings by X-ray electron spectroscopy (XPS) showed that the% nitrogen upon coating the PMMA surface with dopamine increased to 7.7%. Concurrently, the (C-N) component at 286.1 eV of the C_{1s} peak increased (see **Table 2**), attesting an increase in amine content as related to the presence of a dopamine layer. Next, dopamine coated PMMA surfaces were immersed in a DNase I solution for 6–8 h at room temperature. This caused a further decrease in water contact angle to 25° ± 10° and a reduction of the% nitrogen to 4.1%, while the (O=C-N) component at 288.0 eV of the C_{1s} peak increased, due to an increase in the number of peptide bonds in the DNase I overcoat. Note that full chemical analysis of the coating is impossible, since all elements other than nitrogen appear in PMMA, as well as in the coating components.

The activity of freshly prepared and heat inactivated DNase I coatings was established by studying the hydrolysis of plasmid DNA in solution droplets placed on the coatings. Full hydrolysis of plasmid DNA on freshly prepared DNase I coated PMMA occurred within 30 min (see **Figure 2**A), while in contrast no hydrolysis of plasmid DNA occurred within 60 min on coatings of heat inactivated DNase I. Note that immobilized DNase I and soluble DNase I showed complete hydrolysis of plasmid DNA, indicating that the covalent immobilization did not affect the activity of DNase I (Figure 2B).

In order to evaluate the stability of the DNase I coating, phosphate buffered saline (PBS) droplets were left on the coating for 1, 4, 8, or 24 h (Figure 2B), after which plasmid DNA was placed on the coating on the same spot as the PBS droplet. Hydrolysis of plasmid DNA on the surface was observed up to 8 h, while loss of activity of the DNAse I coating was indicated between 8 and 24 h (Figure 2B). The PBS droplet removed was mixed with DNA in order to detect DNase I possibly released from the coating, no activity in the PBS droplet was observed (see also Figure 2B).

The initial adhesion of bacteria to the DNase I coating on PMMA was strongly decreased compared to substrata without an active coating (see **Figure 3** A). After 60 min, the number of bacteria that adhered to DNase I coated PMMA was reduced by 99% for *P. aeruginosa* PAO1 and 95% for *S. aureus* ATCC 12600 compared to PMMA, dopamine coated PMMA and heat inactivated DNase I coated PMMA. Confocal laser scanning microscopic (CLSM) images show biofilms of *P. aeruginosa* PAO1 on all control substrata that fully cover the surface, whereas on DNase I coated PMMA only few adhering bacteria were observed, most of which were dead (Figure 3B). Similar observations were made for *S. aureus* ATCC 12600 biofilms. Quantitative analysis of CLSM images of *P. aeruginosa* PAO1 and *S. aureus* ATCC 12600 biofilms after 14 h on control substrata showed average biofilm thicknesses of approximately 10 and 18 µm, respectively, whereas on PMMA with DNase I

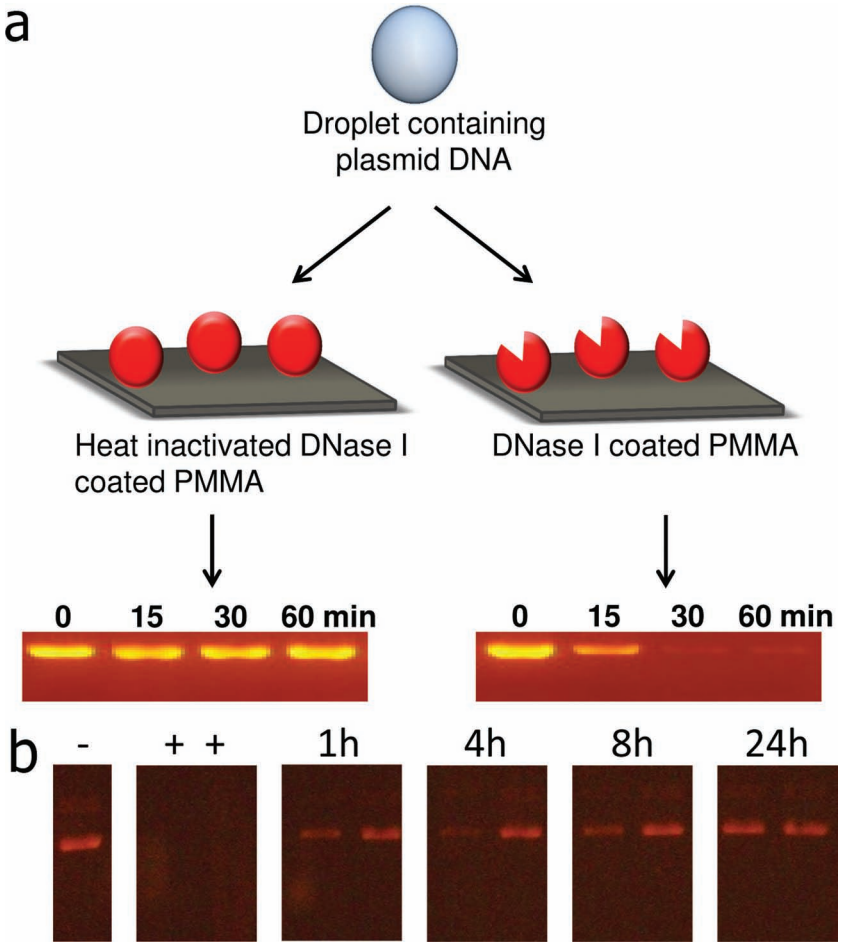

Figure 2. A) Agarose gel showing the degradation of plasmid DNA in a droplet on PMMA coated with DNase I with and without heat inactivation. The plasmid DNA is hydrolyzed within 30 min when placed on PMMA coated with active DNAse I. B) Agarose gel, showing the result of the DNase I release from the coating and the activity of remaining DNase I coating after exposure to PBS for up to one day. Plasmid DNA is shown as a negative control (–), whereas plasmid DNA after mixing with DNase I in PBS (+, left lane) and plasmid DNA on freshly coated DNase I (+, right lane) are shown as positive controls. Time-indicated gels (1 h to 24 h) show hydrolysis of plasmid DNA on the DNase I coating after exposure to PBS (left lanes) and hydrolysis of plasmid DNA with PBS droplets containing the released DNase I from the coating (right lanes).

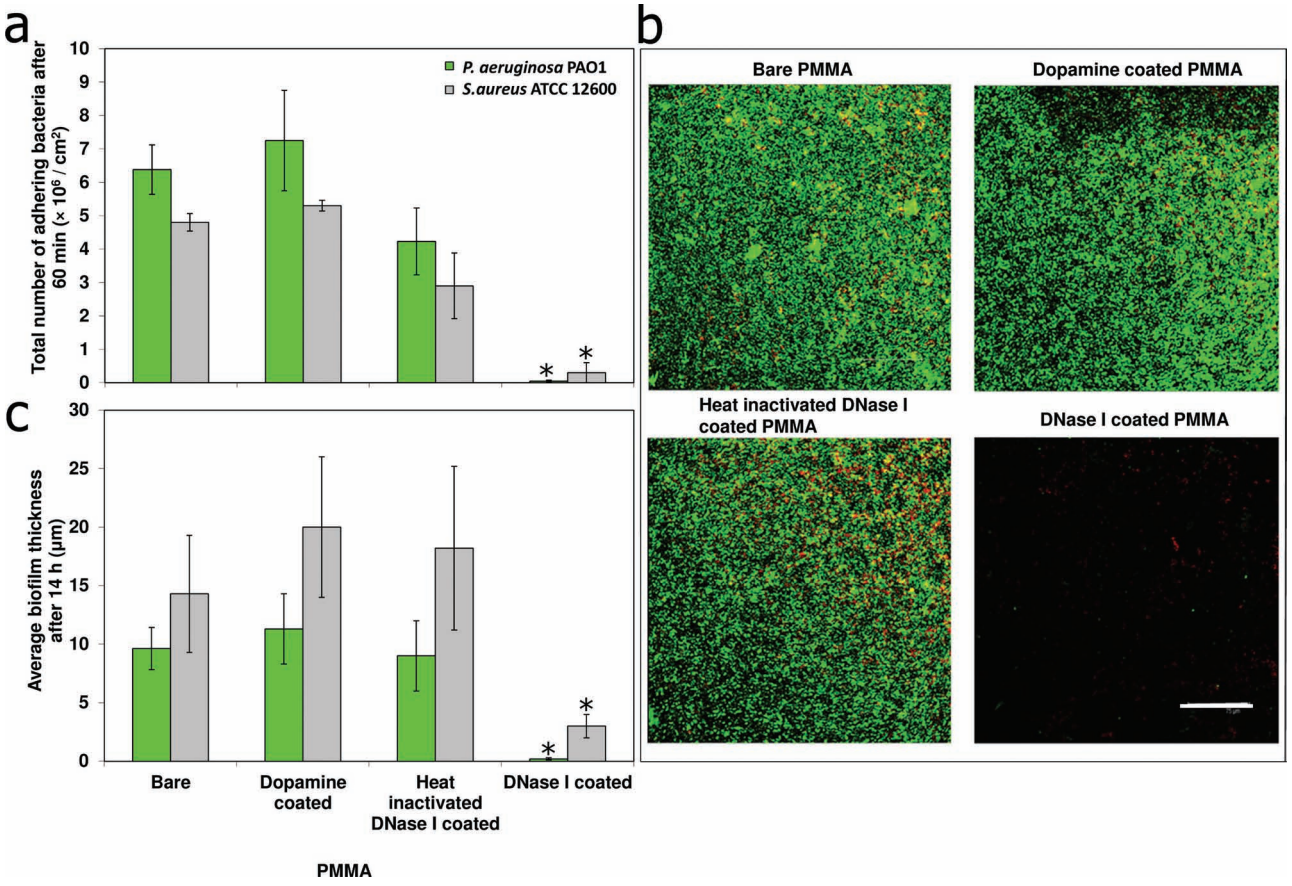

Figure 3. A) Initial adhesion of *P. aeruginosa* PAO1 and *S. aureus* ATCC 12600 after 60 min on various substrata. Error bars represent the standard deviations over three experiments with separately grown bacteria. B) CLSM images of 14 h old *P. aeruginosa* PAO1 biofilms on different coatings, scale bar represents 100 µm. Green and red dots represent live and dead bacteria, respectively. C) Average thickness of 14 h old *P. aeruginosa* PAO1 (green) and *S. aureus* ATCC 12600 (grey) biofilms. Error bars represent the standard deviations over three experiments with separately grown bacteria and three spots taken per sample. *indicates significant decrease ($p < 0.05$) in initial bacterial adhesion (A) and biofilm thickness (C) on DNase I coated on PMMA substratum in comparison to all other substrata.

coating average biofilm thickness was reduced to 0.2 and 3 µm, respectively (Figure 3**C**).

For biomedical application of the coating, it is necessary to demonstrate the absence of adverse effects on the interaction with mammalian cells. Therefore the effect of the DNase I coating on adhesion and proliferation of human osteosarcoma U2OS cells was studied. Coating of PMMA with dopamine, or DNase I with and without heat inactivation showed no effect on the adhesion and proliferation of U2OS cells (see **Figure 4**). Cells were able to adhere to all substrata and proliferate to form a confluent layer within 24 h. Furthermore, no significant differences were observed between the various substrata with respect to the total number of adhering U2OS cells and the area covered by cells after 24 h of growth (Figure 4).

3. Discussion

DNA is associated with the bacterial cell surface by both specific and non-specific interactions. Present at the bacterial cell surface, eDNA acts as a bridge between the bacterial cell wall and EPS by binding biopolymers in EPS, most likely polysaccharides and proteins, through attractive, short-range acid-base interactions. Therewith eDNA is not only responsible for the presence of structurally intact EPS on bacterial cell surfaces, but is also a crucial component in constructing the "house of biofilm" that holds the organisms together and on a surface during biofilm formation (**Figure 5**A). Degradation of eDNA by our DNase I coating disrupts the EPS glue of adhering bacteria, interfering with the initial step of biofilm formation and preventing the construction of the "house of biofilm organisms" (Figure 5B). Degradation of eDNA upon contact with the DNase I coating prevents bacteria from adhering. In case where a bacterium does manage to adhere to the coated surface, degradation of the eDNA leads to disintegration of the EPS formed during growth of the adhering bacteria, leaving it unable to develop into a structurally stable house.[24] Additionally, the presence of DNase I at the bacterial-substratum interface interferes with the aggregation of bacteria and cell-cell interactions at the substratum surface, since these processes are both mediated by eDNA.[25,26]

In this study it was demonstrated that a DNase I coating is effective against biofilm formation for up to a minimum of 14 h,

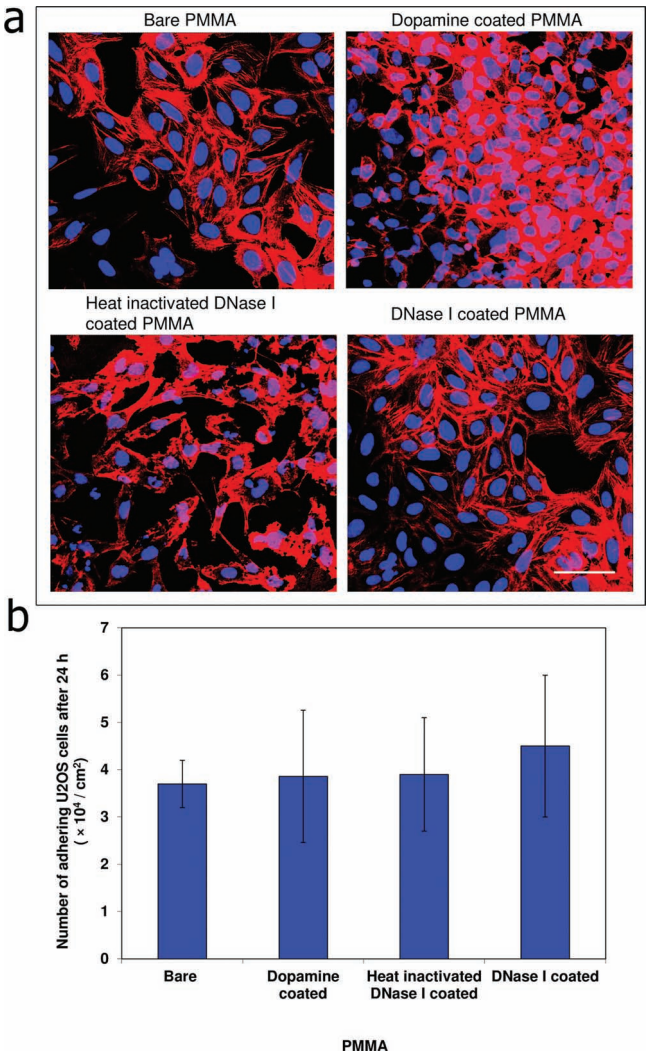

Figure 4. A) CLSM images of U2OS cell adhesion and proliferation on different substrata. Scale bar represents 100 µm. B) The total number of U2OS cells adhering after 24 h incubation on various substrata. Error bars represent the standard deviations over three experiments with separately grown U2OS cells. No significant difference in cell adhesion and proliferation was observed between DNase I coated on the PMMA substratum in comparison to other substrata.

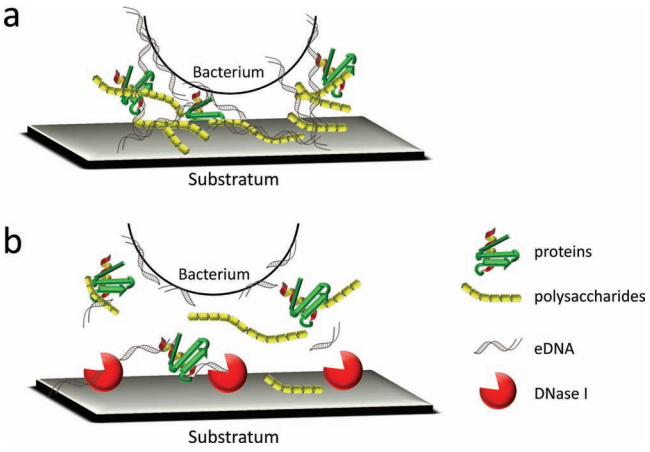

Figure 5. A) eDNA acting as a bridge between a bacterial cell surface and various biopolymers in EPS, like proteins and polysaccharides, therewith playing an important role in bacterial adhesion. B) Disruption of EPS by DNase I coating attacking the eDNA component of the EPS, preventing bacterial adhesion to the substratum surface.

while hydrolysis of DNA by the coating did not occur anymore after 24 h. No release of active DNase I into PBS was observed within 24 h, suggesting that either the coated enzyme became inactive after 8 h or release from the coating caused DNase I to become inactive after that period. Since it has been shown that DNase I is amongst the most stable enzymes known,[33] we believe it is likely that desorption of DNase I will occur.

The loss of activity of our DNase I coating within 24 h does not necessarily limit its applicability. Application in industrial processes, running 24 h per day for months, will not be possible when activity is lost within 24 h, but in the biomedical field many prophylactic measures with respect to the development of biomaterial-associated infection, i.e., infections associated with the surgical implantation of biomaterial implants and devices (prosthetic hips and knee, pace makers, etc.) are only active for 24 h. Currently, bacteria that are inevitably introduced during implantation of biomaterial implants or devices are eliminated through systemic antibiotic administration and local-antibiotic delivery materials. Local antibiotic delivery materials, such as gentamicin-loaded bone cements in orthopedics for the fixation of hip and knee prostheses, are also known to be active for a maximum of 24 h,[34] similar to the period during which our current DNase I coating is antimicrobially active. Therefore, in an era where the efficacy of many antibiotics is fading with respect to many pathogens involved in biomaterial-associated infection, a DNase I coating is a badly needed addendum to the antimicrobial armamentarium in modern medicine. Important for the further downstream translation of the DNase I coating toward clinical application is that DNase I is naturally produced in the human body by the pancreas, kidneys, liver and subsequently released into body fluids.[35] Moreover, aerosolized DNase I is often used via an inhaler for reducing the viscosity of the sputum of cystic fibrosis patients through hydrolysis of DNA.[36,37]

In conclusion, our biocompatible DNase I coating on PMMA resulted in unprecedented reductions of initial bacterial adhesion for 60 min and further biofilm inhibition for up to 14 h by a *Pseudomonas* and *Staphylococcus* strain. Although optimization of the coating might extend this time period, prevention of biofilm formation over a time span of 14 h is sufficient to prevent biomaterial-associated infection due to contamination of a biomaterial implant or device during surgery.

4. Experimental Section

DNase I Coating on PMMA: PMMA was coated with DNase I (Fermentas life sciences, Roosendaal, The Netherlands) using dopamine as an intermediate coupling layer. DNase I is a non-toxic enzyme and no report has been published showing bactericidal effects of DNase I, since it only degrades eDNA on the outside of a bacterium.[38] Dopamine was chosen as a coupling agent, because it has been shown not to interfere with mammalian cell adhesion and proliferation.[39,40] Adhesion of

dopamine to organic surfaces involves oxidation of catechol to quinone, which then reacts with the surface, and other catechols or quinones to form a polymer film.[41–43] Although the resulting polymer film is chemically heterogeneous and its composition is not precisely known, quinone functional groups are believed to be present at the interface and capable of covalently binding nucleophiles by Michael addition reactions[41,44].

PMMA (Vink, Didam, The Netherlands) substrata (1.5 cm × 1.5 cm) were cleaned by sonication for 3 min in 2% RBS35 (Omnilabo International BV, The Netherlands) followed by rinsing with tap water, methanol, tap water and finally with sterile ultrapure water. The PMMA substrata were then immersed in 8 mL of dopamine (Sigma-Aldrich, St. Louis, USA) solution (6 mg mL^{-1} dopamine-HCl in 10 mM Tris-HCl (Merck KGaA, Darmstadt, Germany) at pH 8.5) for 48 h at room temperature under static conditions. After 48 h, the PMMA substrata were dried in an oven at 40 °C for 6 h, followed by rinsing with sterile ultrapure water, drying again for 2 h at 40 °C and stored at 4 °C. To coat with active DNase I or heat inactivated (at 65 °C for 10 min) DNase I, dopamine coated PMMA surfaces were immersed in 5 mL (20 units mL^{-1}) of a DNase I solution in PBS (150 mM NaCl, 10 mM potassium phosphate, pH 6.8) in the presence of 10 mM $MgCl_2$ for 6–8 h at room temperature under static conditions. A schematic description of the coating procedure is shown in Figure 1. After coating, the samples were rinsed with sterile ultrapure water, stored at –20 °C and used within 14 h.

Water contact angles were measured on bare PMMA, dopamine coated PMMA, and on heat inactivated and active DNase I coatings using the sessile drop method and a home-made contour monitor. The elemental composition of PMMA, the dopamine coupling layer and the entire DNase I coating were determined using XPS (S-probe, Surface Science Instruments, Mountain View, CA, USA). First, samples were placed in the pre-vacuum chamber of the XPS, and then subjected to a vacuum of 10^{-7} Pa. X-rays (10 kV, 22 mA), at a spot size of 250 × 1000 µm, were produced using an aluminum anode. Scans of the overall spectrum in the binding energy range of 1–1100 eV were made at low resolution (pass energy 150 eV). The area under each peak was used to yield elemental surface concentrations for C, O, N, and other minor elements occurring after correction with sensitivity factors provided by the manufacturer. The C_{1s} peaks were decomposed in four components, i.e. for carbon involved in alkyl bonds (C-(C,H); 284.8 eV), amine bonds (C-N); 286.1 eV), amide groups (O=C-N; 288.0 eV) and carbon arising from carboxylic acid (O=C-OH; 289.2).

Analysis of DNase I Activity and Stability: The activity of DNase I coatings was determined by placing a 40 µL droplet of ultrapure water containing 90 ng $µL^{-1}$ plasmid DNA on DNase I coated PMMA samples for 60 min at 37 °C under static conditions. For control, PMMA samples were employed that were coated with DNase I with or without heat inactivation. After every 15 min time intervals up till 60 min, 5 µL aliquots of the DNA solution were removed and analyzed for hydrolysis following electrophoresis on a 1.5% agarose gel. 5 µL samples were loaded using 1 µL 6× loading buffer (Roti load DNA, Carl Roth GmbH, Karlsruhe, Germany), after which the gel was left running for approximately 1.5 h at a constant potential of 85 V. Images were taken directly after running the gels, under UV light using a digital camera.

To assess the stability of the coatings, release of DNase I from PMMA samples coated with active DNase I into the surrounding environment was measured by placing a droplet of 100 µL PBS on five coupons at 37 °C for 1, 4, 8, and 24 h respectively. At the end of this time period, 25 µL was taken from the droplet, mixed with 25 µL of plasmid DNA (10 ng $µL^{-1}$) and incubated for 1 h at 37 °C, followed by agarose gel electrophoresis (see above). The activity of DNAse I remaining in the coating was measured by removing the droplets of PBS completely and placing a new 50 µL droplet containing plasmid DNA (10 ng $µL^{-1}$) on the same five spots of the coating. After incubating at 37 °C for 1 h, hydrolysis of plasmid DNA in this 50 µL droplet was determined using agarose gel electrophoresis.

Bacterial Species and Culture Conditions: *P. aeruginosa* PAO1 and *S. aureus* ATCC 12600 were stored in 7% DMSO at –80 °C. Strains were inoculated onto blood agar plates and incubated overnight under aerobic conditions at 37 °C. Single colonies from the agar plates were used to inoculate 10 mL pre-cultures in tryptone soya broth (TSB, OXOID, Basingstoke, UK) and cultured for 24 h. This culture was in turn used to inoculate a 200 mL main culture in TSB, which was grown for 16 h prior to harvesting. Bacteria were harvested by centrifugation at 5000 × *g* for 5 min at 10 °C, and washed twice with PBS. *S. aureus* ATCC 12600 aggregates were disrupted by sonication on ice for 3 × 10 s at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, Connecticut, USA). The bacterial densities of the suspensions were determined using a Bürker-Türk counting chamber and bacteria were diluted in 50 mL PBS to a final density of 3×10^8 bacteria per mL for adhesion and biofilm experiments.

Initial Bacterial Adhesion and Biofilm Growth: Both initial adhesion of bacteria and biofilm growth were studied on bare PMMA, dopamine coated PMMA and PMMA coated with active and heat inactivated DNAse I. For initial adhesion, 5 mL of a bacterial suspension was added to each substratum, inserted in 6 wells plates and bacteria were allowed to adhere at 37 °C at 60 rpm. Bacterial suspensions were removed after 60 min and the substrata were gently washed with PBS. The total number of adhering bacteria were then counted after staining with Live/Dead stain (*Bac*Light, Invitrogen, Breda, The Netherlands) for 15 min in the dark using fluorescence microscopy (Leica DM4000B, Leica Microsystems GmbH, Heidelberg, Germany).

Biofilm growth was initiated after 60 min of initial bacterial adhesion. The bacterial suspension in the wells was replaced by 5 mL of TSB and wells were incubated for 14 h at 37 °C at 60 rpm. After 14 h, growth medium was decanted and the biofilms were gently rinsed with PBS in order to remove any planktonic bacteria followed by staining with Live/Dead stain for 15 min in the dark. Subsequently, optical cross-sections of the biofilms were made using CLSM (Leica DMRXE TCS-SP2; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). Optical cross-sections were further examined for the average biofilm thickness employing COMSTAT, a Matlab (The Mathworks, Inc.) based analysis program.

Human Osteosarcoma U2OS Cell Culturing and Harvesting: In order to determine possible adverse effects of the DNase I coating on mammalian cells, U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagle's low glucose medium, supplemented with 10% fetal calf serum, 0.2 mM ascorbic acid-2-phosphate containing antibiotics (penicillin/streptomycin). U2OS cells were maintained in a T-75 cell culture flask at 37 °C in a humidified, 5% CO_2 atmosphere and harvested at 90% confluency using trypsin/ethylenediaminetetraacetic acid. The harvested cells were counted using a Bürker-Türk counting chamber and subsequently, diluted with growth medium to a density of 6×10^5 cells per mL. Cell adhesion was initiated by addition of 5 mL of cell suspension to each substratum, inserted in a 6-well plate and cells were allowed to grow for 24 h at 37 °C in a humidified, 5% CO_2 atmosphere. To assess the morphology of U2OS cells adhering on different substrata after 24 h, samples were prepared for immuno-cytochemical staining. For fixation, 2 mL of 3.7% formaldehyde in cytoskeleton stabilization buffer (0.1 M Pipes, 1 mM ethylene glycol tetra-acetic acid, 4% (w/v) polyethylene glycol 8000, pH 6.9) was added to the substrata. Subsequently, cells were incubated in 0.5% Triton X-100 for 3 min, rinsed with PBS and stained for 30 min with 1 mL PBS containing 10 µL DAPI and 2 µg mL^{-1} of TRITC-Phalloidin. The substrata were washed 4 times in PBS and examined with CLSM and the number of adhering cells per unit area was determined.

Statistical Analysis: The effects of functional DNase I coated PMMA on initial bacterial adhesion and on biofilm thickness were analyzed using a two-tailed Student's t-test. Differences were considered significant if $p < 0.05$.

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