Innovative coatings for anti-bacterial surfaces
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CHAPTER 4

A PROTECTIVE, BIODEGRADABLE PLGA-COATING RELEASING INULIN-PACKAGED DNASE I TO PREVENT BACTERIAL ADHESION AND BIOFILM FORMATION
ABSTRACT

Enzyme coating of biomaterial surfaces has proven to be a promising in vitro concept to prevent biomaterial associated infections. However, coating stability and robustness can be a critical factor, depending on the site of the implant or device in the human body, especially enzymes can easily become inactive due to changes in their tertiary structure. By using poly-(lactic-co-glycolic)-acid (PLGA) as a biodegradable protective overcoat on titanium with inulin-packaged DNase I, bacterial adhesion of two *Staphylococcus aureus* strains was drastically reduced and the biovolume of a 20 h biofilm was significantly lowered with respect to bare titanium, a common orthopedic biomaterial. More importantly, the PLGA coating combined with inulin-packaging of DNase I withstood different storage and handling conditions, without negatively affecting antibacterial efficacy. With antibiotic treatment of infections becoming less effective due to increasing antibiotic resistance worldwide, a protective, biodegradable coating of PLGA with inulin-packaged DNase I could offer a potential new strategy to prevent the infection of medical devices and implants, possibly even without the need for post-operative antibiotic treatment.
INTRODUCTION

Driven by a high level of protection and increased survival rate, bacteria seek shelter on material surfaces in their biofilm mode of growth rather than by staying planktonic in suspension [1,2]. In these sophisticated biofilm communities, bacteria produce extracellular polymeric substances (EPS) that protect biofilm inhabitants against predators and penetrating antimicrobials [3,4]. Combined with a low metabolic state, bacteria in a biofilm mode of growth can easily become resistant to treatment. Accordingly, biofilm formation represents a major problem in many industrial and biomedical applications [5–7]. When biofilms form on biomaterial implants and devices, persistent infections develop and frequently removal of the colonized implant or device is the only successful treatment option [4].

EPS consists mainly of proteins, polysaccharides and extracellular DNA (eDNA). The exact roles of the different individual components of EPS are not exactly known, but collectively they provide mechanical stability to a biofilm [8,9]. Genetically, eDNA resembles chromosomal DNA and is therefore suspected to originate from cell lysis, although also active secretion of DNA containing vesicles has been observed [10,11]. The presence of eDNA favors acid-base mediated crosslinking between the various components of the EPS with a distinct role of Ca^{2+} ions [12]. The resulting network can trap bacteria to bring them close enough to each other to allow cell-cell communication and facilitate metabolic interactions [1,13,14].

eDNA is not only a key factor in providing stability to mature biofilms, but also plays an important role in the initial adhesion and aggregation of bacteria [15–18]. The recognition of the role of eDNA in maintaining the mechanical stability of biofilms, opens a new pathway to the disruption of biofilms, i.e. their EPS matrix using enzyme treatments. DNase I is an enzyme which catalyzes hydrolysis of the phosphodiester bonds in the phosphate backbone of DNA and was involved in the studies leading to the initial findings on the role of eDNA in bacterial adhesion, biofilm formation and stability [19,20]. Treatment of Pseudomonas aeruginosa biofilms with DNase I dispersed biofilms that were up to 60 h old, whilst on 84 h old biofilms only minor effects were seen [19]. Addition of DNase I to bacterial suspensions prior to adhesion drastically reduced adhesion of Staphylococcus epidermidis [21] and Listeria monocytogenes [22], while initial adhesion of an environmental Pseudomonas isolate in the presence of DNase I was decreased by more than 95% after 30 min of adhesion [17]. Recently, we attached DNase I to polymethylmethacrylate (PMMA) using dopamine as a coupling agent and showed that surface attached DNase I can decrease initial bacterial adhesion and lower biofilm formation during 14 h [9]. However, the use of a biomolecule to attach DNase I to surfaces leaves the enzyme unprotected and prone to detachment, while its surface exposure can also result in loss of activity. Poly-(lactic-co-glycolic)-acid (PLGA) has been used as a protective overcoat on titanium surfaces spray-coated with gentamicin [23] and as a bio-degradable, gentamicin-loaded coating on tibia nails [24] to prevent infection. The degradation time of PLGA can be adjusted to the application aimed for by varying the coating thickness, the ratio of poly-lactic to glycolic acid and the molecular weight. Formation of a PLGA coating however, requires the polymer to be dissolved in a volatile solvent that can be applied to the desired material, yielding a hard, protective layer of PLGA.
CHAPTER 4

after evaporation of the solvent [25]. Accordingly, PLGA could be an ideal candidate to protect DNase I on implant materials in order to prevent bacterial adhesion and biofilm formation, with the possibility to fine-tune its release.

However, the use of volatile solvents poses a major disadvantage of using PLGA for the protection of DNase I. Even though DNase I is one of the most stable enzymes known, when brought in solution its stability at 4°C is limited to a few days and mixing DNase I with PLGA dissolved in a volatile solvent will yield a severe reduction of activity [26]. The stability of DNase I can be increased by spray-drying DNase I with inulin [27], yielding inulin-packaged DNase I that retains more than 80% of enzyme activity after weeks of storage, even at 85°C.

In this study, we combine the clinically applied concept of using a protective, biodegradable PLGA [25] coating with inulin-packaging [27] to yield a stable DNase I releasing coating on titanium that prevents bacterial adhesion and biofilm formation. Titanium is a common biomaterial used in various biomaterial implants prone to bacterial colonization, such as hip and knee arthroplasties, dental implants and bone fixator pins. By using a degradable polymer coating, DNase I is released over an extended time period, providing breakdown of bacterial eDNA at the bacteria-biomaterial interface. In addition, inulin protects DNase I during coating preparation, while protection by inulin also increases the stability of DNase I during storage.

MATERIALS AND METHODS

Particle preparation
Inulin-DNase I mixed (weight ratio 4 : 1) and single powders of inulin and DNase I were produced by dissolving DNase I (from bovine pancreas, purity ≥ 86%, 400 Kunitz units/mg, Sigma Aldrich, St. Louis, MO, USA) and inulin (DP23, Sensus, Roosendaal, The Netherlands) in water up to a total concentration of 5 mg/ml. The solutions were spray-dried using a B-90 spray-dryer (Büchi Labortechnik AG, Flawil, Switzerland) in combination with a B-296 dehumidifier and a two-fluid nozzle. The inlet air temperature was set at 80°C, the aspirator at 150 l/min, liquid feed flow at 1 ml/min and atomizing air flow at 50 mm. Particle size was determined by laser diffraction (HELOS, Sympatec, Clausthal-Zellerfeld, Germany).

Surface coating
Titanium substrata (1.50 × 1.50 × 0.10 cm, Goodfellow Cambridge Ltd., Cambridge, United Kingdom) were cleaned by sonication for 3 min in 2% RBS35 (Omnilabo International BV, The Netherlands) followed by rinsing with water, methanol and water again. PLGA (PURASORB PDLG 5002, Corbion, Diemen, The Netherlands) was dissolved (10% w/v) in acetonitrile (Merck KGaA, Darmstadt, Germany) while stirring. Particle formulations consisting of either only inulin, only DNase I, or inulin-packaged DNase I particles were added (1% w/v) to the PLGA solution in acetonitrile and stirred for an additional 3 h. 100 µl of the suspension was applied to titanium substrata and left to dry overnight at room temperature, resulting in
a total of 4.4 mg PLGA/cm², containing 0.44 mg of particles/cm² (in the case of inulin-packaged DNase I particles, the ratio of inulin over DNase I was 4 to 1). Coated substrata were stored at room temperature and used within five days. The thickness of the PLGA layer was determined by making a cut through the coating, exposing the bare titanium, and measuring the depth using white light interferometry (Proscan 2000, Scantron Industrial Products Ltd, Taunton, England).

**Release kinetics**

After surface coating, titanium substrata were placed in 2 ml PBS (150 mM NaCl, 10 mM potassium phosphate, pH 7) and incubated at 37°C and 60 rpm, in order to determine the release kinetics of DNase I and inulin from the protective PLGA coating. Individual samples were removed from PBS at different time points (1, 4, 8, 24, 48, 72 and 96 h) after which the concentration DNase I and inulin was determined in PBS using photospectrometry.

The amount of inulin released was determined using an anthrone assay; a commonly used assay to determine carbohydrate concentrations [28,29]. Anthrone reagent (Sigma Aldrich) was freshly prepared for each measurement at a concentration of 1 mg/ml in sulfuric acid (H₂SO₄, 95-97%, Merck KGaA, Darmstadt, Germany). First, a calibration curve of the absorbance of inulin solutions with known concentrations was constructed, using 0, 20, 40, 60, 80 and 100 µg/ml inulin in PBS. To measure the absorbance, 1 ml of each solution was mixed with 2 ml anthrone reagent and vortexed. After letting the mixture cool down for 30 min, it was transferred to a plastic cuvette and the absorbance at 630 nm was measured (Smart Spec™ 3000, Bio-Rad, Hercules, Ca, USA). When necessary, samples were diluted 10 times in PBS and measured again. The calibration curve was used to determine the concentration of inulin released by the different samples.

The amount of DNase I released from the samples was measured by mixing 100 µl PBS containing DNase I released from samples with 100 µl Bradford reagent (Sigma Aldrich) and measuring the absorbance using a NanoDrop® spectrophotometer (ND-1000, NanoDrop Technologies, Inc, Wilmington, DE) at 595 nm. The absorbance was then compared to a calibration curve, prepared similarly as for inulin, to calculate the amount of DNase I released.

**Bacterial culture and harvesting**

Green fluorescent protein (GFP) expressing *Staphylococcus aureus* ATCC 12600 and *S. aureus* Newman D2C (also known as *S. aureus* ATCC 25904) were obtained by introduction of pMV158GFP [30] into competent bacteria by electroporation, as described by Li et al. [31]. Transformants were selected on tryptone soya broth (TSB, OXOID, Basingstoke, UK) agar (bactoagar, BD Le Pont de Clai, France) agar plates containing 10 µg/ml of tetracycline (hydrochloride, purity ≥ 95%, Sigma). *S. aureus* ATCC 12600GFP and *S. aureus* NewmanGFP were stored in 7% DMSO at -80 °C. Green fluorescent protein (GFP) expressing bacteria were used, as they allow easy quantification of adhesion numbers and biofilm formation without additional staining.
S. aureus ATCC 12600 is a common reference strain in biofilm research, while S. aureus Newman D2C is a highly virulent clinical isolate, known to cause highly persistent infections [32]. Strains were inoculated on TSB agar plates containing 10 µg/ml tetracycline and incubated overnight under aerobic conditions at 37°C. Single colonies were used to inoculate 10 ml pre-cultures in TSB containing 10 µg/ml tetracycline and cultured for 24 h. Pre-cultures were used to inoculate 200 ml main cultures in TSB in absence of antibiotics, which were grown for 16 h. Cells were harvested by centrifugation at 5000 x g for 5 min at 10°C and washed twice with PBS, before being sonicated at 30 W (Vibra Cell model 375, Sonics and Materials Inc., Danbury, CT, USA) for 3 x 10 s on ice with 30 s breaks in between in order to break up bacterial aggregates. Bacterial densities were determined using a Bürker-Türk counting chamber and suspensions were diluted to 3 x 10^8 bacteria per ml in PBS for all experiments.

**Initial bacterial adhesion and biofilm growth**

For initial adhesion, 5 ml of bacterial suspension supplemented with 1 mM CaCl_2 and 10 mM MgCl_2 was added to the various substrata in 6-well plates and incubated at 37°C and 60 rpm. After 1 h, samples were rinsed in PBS and the total number of adhering bacteria was counted using fluorescence microscopy (Leica DM4000B, Leica Microsystems GmbH, Heidelberg, Germany).

Biofilm growth was initiated after 1 h of initial bacterial adhesion. Samples were removed from their suspensions and placed into new 6-well plates containing 5 ml TSB, supplemented with 1 mM CaCl_2 and 10 mM MgCl_2 and incubated for 20 h at 37°C and 60 rpm. After incubation, biofilm-covered samples were carefully transferred to 6-well plates containing PBS and examined by confocal laser scanning microscopy (CLSM) (Leica DMRXE TCS-SP2; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany). Optical cross-sections of the biofilms were taken at three different positions and their biovolumes calculated employing COMSTAT [33,34], a Matlab (The MathWorks, Natick, MA, USA ) based analysis program. Biofilms grown on bare titanium and protective PLGA coatings containing inulin-DNase I particles were also evaluated using optical coherence tomography (OCT) (Telesto-II 1300, Thorlabs, Newton, NJ, USA) in addition to CLSM, in order to rule out possible effects of DNase I on fluorescence.

**Stability of the PLGA-inulin/DNase I coating**

To determine the stability of the PLGA coatings containing DNase I and inulin-packaged DNase I, several conditions were applied that could affect the efficacy of the coating during storage or handling. In a first condition, coated substrata were placed in 6-well plates containing 5 ml PBS and incubated for 2 h at 37°C. In a second condition, the robustness of the coating and its resistance against physical handling was determined by pressing a finger on coated substrata with a force of 20 N for 10 s, resulting in a pressure of approximately 3.2 x 10^4 Pa, after which DNase I activity was determined. Finally, coated samples were stored at ambient conditions for 4 weeks prior to assessing DNase I activity. Efficacies of the coatings were evaluated by assessing bacterial adhesion and biofilm formation, as described above.
**Cell adhesion assay using U-2 OS**

The presence of possible adverse effects of the coatings on mammalian cells was determined using U-2 OS osteosarcoma cells. Cells were cultured in Dulbecco’s modified Eagle’s low glucose medium supplemented with 10% fetal bovine calf serum and 0.2 mM ascorbic acid-2-phosphate. Cells were maintained in T-75 cell culture flasks at 37°C in a humidified, 5% CO₂ atmosphere until 95% confluency. Cells were harvested using trypsin/ethylenediaminetetraacetic acid and counted using a Bürker-Türk counting chamber and diluted to 5.5 x 10⁵ cells per ml in growth medium. Cell adhesion assays were performed in 6-well plates containing each of the substrata and 5 ml of the cell suspension. After 24 h, substrata were prepared for immuno-cytochemical staining by fixation in 3.7% paraformaldehyde in cytoskeleton stabilization buffer (0.1 M Pipes, 1 mM ethylene glycol tetra acetic acid, 4% (w/v) polyethylene glycol 8000, pH 6.9) and subsequent treatment with 0.5% Triton-X100. After rinsing with PBS, substrata were incubated in 1 ml PBS containing 2 µg/ml TRITC-Phalloidin and 4 µg/ml DAPI for 30 min in the dark. Before being examined using fluorescence microscopy, substrata were washed 4 times with PBS to remove any excess stain, and the number of cells per cm² on each sample was determined by counting the number of blue-stained nuclei.

**Statistics**

Bacterial adhesion, biofilm formation and mammalian cell adhesion data followed a normal distribution (Shapiro-Wilk test, p < 0.05). Differences were analyzed using a one-way ANOVA and considered significant if p < 0.05.

**RESULTS**

**Surface coating thickness and release kinetics**

Spray-drying of inulin, DNase I or inulin-packaged DNase I, resulted in particles with a mean diameter of 1 μm. Resulting particles were added to a solution of PLGA in acetonitrile and used to coat titanium. The thickness of the applied PLGA layer on titanium substrata was approximately 6 μm in absence and 15 μm in presence of inulin-packaged DNase I particles, while appearing more rough in the presence of particles, as determined using white light interferometry (Fig.1).
FIGURE 1 Height map of a protective PLGA coating on titanium in absence and presence of inulin-packaged DNase I particles obtained interferometrically. The blue region represents a cut made throughout the PLGA layer, revealing the bare titanium surface. (A) PLGA coating only. (B) Coating of PLGA with inulin-packaged DNase I. Total area is 5 x 5 mm and colors are artificially generated to yield a height map.

PLGA-protected coatings containing inulin-packaged DNase I particles showed a burst release of DNase I and inulin within the first hour of placement in PBS, after which release continued at a slower rate (Fig. 2). While the burst release amounted approximately 25% of the total content of the coating, after 96 h more than half of the total amounts incorporated had been released (total inulin and DNase I contents were 800 and 200 µg, respectively). Note that the ratio of inulin over DNase I released was 4 : 1 on average, in line with the ratio of inulin over DNase I incorporated during particle preparation (see Materials and Methods section).

FIGURE 2 (A) Calibration curves of DNase I (595 nm) and inulin (630 nm) solutions as a function of their concentration
in solution obtained photospectrometrically. (B) Cumulative amounts of DNase I and inulin released from PLGA-protected coatings with inulin-packaged DNase I immersed in PBS for different periods of time obtained photospectrometrically, using the calibration curves in Fig. 2A. Note that the amount of released DNase I is shown on the left axis, while the released inulin is depicted on the right axis. Error bars indicate standard deviations over three experiments with separately prepared coatings.

**Initial bacterial adhesion and biofilm growth**

Fluorescent microscope images show that bacterial adhesion was greatly reduced by the release of DNase I from PLGA coatings for both *S. aureus* 12600<sup>GFP</sup> and *S. aureus* Newman D2C<sup>GFP</sup> (Fig. 3A). The number of initially adhering staphylococci on titanium substrata was not affected by applying a coating consisting of only PLGA, or PLGA containing inulin particles (see Fig. 3B). Addition of particles containing DNase I to the PLGA coating significantly reduced staphylococcal adhesion, regardless of whether particles consisted of only DNase I, or inulin-packaged DNase I. Interestingly, *S. aureus* Newman D2C<sup>GFP</sup> adhered in higher numbers than *S. aureus* ATCC 12600<sup>GFP</sup> to bare titanium and coatings consisting of PLGA or PLGA containing inulin particles, but on PLGA-protected coatings containing DNase I and inulin-packaged DNase I lower numbers of *S. aureus* Newman D2C<sup>GFP</sup> were found, indicating that this strain is more sensitive to DNase I treatment than *S. aureus* ATCC 12600<sup>GFP</sup>.

![Fluorescence microscopy images of staphylococcal adhesion after 1 h in PBS to titanium surfaces and various coatings. Scale bar denotes 75 µm. (B) Number of adhering *S. aureus* ATCC 12600<sup>GFP</sup> and *S. aureus* Newman D2C<sup>GFP</sup> after 1 h adhesion in PBS to titanium surfaces and various coatings. Error bars represent the standard deviations.](http://example.com/fig3.png)
over three experiments with separately grown bacteria. *Indicates a significant difference ($p < 0.05$) in the numbers of adhering bacteria, within the same strain, compared to titanium and coatings not containing DNase I.

Both CLSM (Fig. 4A) and non-fluorescence-based OCT (Fig. 5) images of staphylococcal biofilms grown for 20 h show abundant biofilm formation on bare titanium, while biofilm formation is nearly absent on PLGA coatings containing inulin-packaged DNase I particles. In line with effects on initial adhesion, coating of titanium with either only PLGA, or PLGA containing inulin particles in absence of DNase I did not significantly affect the biovolumes for both *S. aureus* strains (Fig. 4B). Importantly, *S. aureus* ATCC 12600<sup>GFP</sup> biovolume was hardly reduced on PLGA coatings containing DNase I over the growth period of 20 h, but *S. aureus* Newman D2C<sup>GFP</sup> showed a significant decrease in biovolume compared to titanium and titanium with either only PLGA, or PLGA containing inulin particles. Inulin-packaging of DNase I yielded much stronger reductions in biovolume for both strains, indicating that DNase I activity is better preserved by inulin-packaging than by solely protecting it in PLGA. Note that the amount of DNase I being five-fold lower in inulin-packaged DNase I coatings, compared to DNase I only coatings.

![Figure 4A](image1.png)

**FIGURE 4** (A). CLSM overlay images of 20 h old biofilms of *S. aureus* ATCC 12600<sup>GFP</sup> and *S. aureus* Newman D2C<sup>GFP</sup> on titanium surfaces and various coatings. Scale bar denotes 75 µm. (B) Average biovolumes of 20 h old biofilms of *S. aureus* ATCC 12600<sup>GFP</sup> and *S. aureus* Newman D2C<sup>GFP</sup> grown in TSB on titanium surfaces and various coatings. Error bars
represents standard deviations over three experiments with separately grown bacteria and different batches of coated samples. *Indicates a significant difference ($p < 0.05$) in biovolume, within the same strain, compared to titanium and coatings not containing DNase I.

**FIGURE 5** 3-D (top) and side (bottom) views of staphylococcal biofilms obtained using non-fluorescence-based, optical coherence tomography. (A) *S. aureus* Newman D2C<sup>GFP</sup> biofilms grown on bare titanium. (B) *S. aureus* Newman D2C<sup>GFP</sup> biofilms grown on titanium coated with PLGA containing inulin-packaged DNase I particles.

**Stability of the PLGA-inulin-packaged DNase I coating**

To assess the stability of the protective PLGA coating with unpackaged and inulin-packaged DNase I, staphylococcal adhesion and biofilm formation were also determined after different conditions of storage and handling (see Fig. 6) and compared with adhesion and biofilm formation on bare titanium and a previously described [9] dopamine-coupling of DNase I on PMMA.

High numbers of adhering staphylococci and large biovolumes were seen on bare titanium, regardless of storage and handling conditions for both staphylococcal strains. PLGA yielded effective protection when judged on the reductions achieved in initial adhesion numbers and when evaluated based on 20 h biofilm formation; no significant differences were seen after storage or handling. Benefits of inulin-packaging of DNase I become most evident with respect to 20 h biofilm formation. The necessity of protecting the DNase I with PLGA and inulin follows directly from the comparison with the efficacy of dopamine-coupled DNase I on PMMA, that is clearly far less than of our new protective, biodegradable PLGA-coating releasing inulin-packaged DNase I particles.
**FIGURE 6** Effects of different storage and handling conditions on the efficacy of different coatings on titanium, including immediate use, 2 h exposure to PBS, exposure to a pressure of $3.2 \times 10^4$ Pa for 10 s and 4 weeks of storage in ambient air. (A, B) Numbers of adhering staphylococci after 1 h adhesion in PBS for \textit{S. aureus} ATCC 12600$^{\text{GFP}}$ (A) and \textit{S. aureus} Newman D2C$^{\text{GFP}}$ (B). (C, D) Biovolumes of staphylococcal biofilms grown for 20 h, for \textit{S. aureus} ATCC 12600$^{\text{GFP}}$ (C) and \textit{S. aureus} Newman D2C$^{\text{GFP}}$ (D). Error bars represent standard deviations over three experiments with separately grown bacteria and different batches of coated samples. *Indicates a significant difference ($p < 0.05$) from titanium within the same storage and handling conditions. # indicates a significant difference ($p < 0.05$) between PLGA-DNase I and PLGA-inulin-packaged DNase I within the same storage and handling conditions.

**Cell adhesion assay using U-2 OS**

For use in biomedical applications, it is important to exclude harmful effects of the coating towards mammalian cells. The coating was therefore assessed for its influence on the adhesion and proliferation of human osteosarcoma U-2 OS cells. None of the coatings showed any negative effect on the adhesion and proliferation of U-2 OS cells after 24 h of incubation. Cells adhered to all substrata and formed a confluent layer within 24 h. No significant differences in the number of adhering cells were observed between any of the coatings (Fig. 7).
FIGURE 7 (A) Fluorescence microscopy images of adhering U-2 OS after 24 h of growth on titanium surfaces and various coatings showing complete coverage. Scale bar denotes 75 µm. (B) Number of adhering U-2 OS cells after 24 h of growth on titanium surfaces and various coatings. Error bars represent standard deviations over three experiments with separately grown U-2 OS cells and different batches of coated samples.

DISCUSSION

Previously, we reported on a biomaterials coating of DNase I coupled through dopamine PMMA, which resulted in the prevention of initial bacterial adhesion, as well as reduced biofilm formation over a timescale of 14 h [9]. Loss of enzyme activity occurred between 8 and 24 h. Combined, this indicates that the stability of this DNase I coating varied between 14 to 20 h. Whereas it is clear that this timescale will not be able to prevent late biomaterial-associated infection arising from the spreading of hematogenously introduced bacteria to the infection site, it is a matter of debate whether a timescale of 14-20 h is sufficiently long to prevent infection arising from peri-operatively introduced bacteria as is currently done with a dose of post-operatively administered antibiotics. Moreover, dopamine-coupled DNase I may not be able to withstand physical handling and exposure to fluids as common during insertion of a biomaterial implant or device. The conditions under which an orthopedic implant is inserted will inevitably lead to mechanical stress and exposure to fluids, such as for example PBS or blood. In the current study, we have developed a PLGA coating on titanium in which DNase I is embedded. DNase I is released upon degradation of the PLGA and disrupts the EPS matrix surrounding bacteria at the biomaterial-bacteria interface through hydrolysis of eDNA, similar as our previous dopamine-coupled DNase I on PMMA. The
hardened PLGA provides protection against storage and handling conditions. Importantly, even in the situation where an implant needs to be hammered into a tight bone junction and the integrity of the coating will be inevitably compromised, PLGA containing DNase I will remain to be accumulated at the implant-bone junction where protection is needed. Our coating allows for unhindered tissue integration (Fig. 7), which is a prerequisite for a successful outcome of biomaterial associated surgery in many applications and at the same time offers the best long-term protection against bacterial colonization [35].

To protect DNase I from the harsh volatile solvents used to dissolve and coat PLGA, we packaged DNase I in inulin. Inulin is a sugar glass which helps to prevents damage to the tertiary structure of enzymes and has been used for stabilizing DNase I in applications for patients suffering from cystic fibrosis [27]. Stabilization of DNase I using inulin increases the activity of coated DNase I by protecting it during the coating process, as well as it helps to increase the shelf life. The beneficial effect is indicated by the enhanced activity of inulin-packaged DNase I in this study. Even though coatings containing inulin-packaged DNase I harbored only one fifth of the amount of DNase I compared to coatings with DNase I only particles, their effectiveness against bacterial adhesion and biofilm formation was at least equal and in some cases even increased (Fig. 6). The choice of PLGA as a protective material above other possible degradable polymers has the advantage of already being approved by the U.S. Food and Drug Administration (FDA) for biomedical applications, including implantation [36]. Besides being approved for biomedical applications, the degradation rate of PLGA can be easily tuned by varying the ratio of lactic to glycolic acid, or the molecular mass, in order to comply with the desired application requirements [37].

The rise of bacterial strains resistant to antibiotics, together with the limited development of new antibiotics, calls for antibiotic independent mechanisms to prevent biomaterial associated infections [38,39]. The use of DNase I seems extremely suitable in this matter, as it leaves bacteria unable to adhere and form biofilms, causing them to stay planktonic, meaning they more easily eradicated by the host immune system. Additionally, the authors have no knowledge of any literature reporting bacterial resistance against enzymatic treatment. Compared to regular antibiotic treatment of biofilms on non-adhesive coatings and similar systems releasing antibiotics rather than enzymes, the reduction in biofilm formation by our coating is comparable [40,41]. This indicates that the results obtained using DNase I might not require the use of additional antibiotic treatment. Moreover, the presented method of protecting and packaging can be applied using other enzymes that are known to possess the ability to prevent bacterial adhesion and biofilm formation, such as lysozyme and dispersin B [42,43]. Not using antibiotics after biomaterial implant surgery, which is the premise of this coating, may be considered too much of a risk by treating physicians and therewith impede downward clinical translation. In this respect it is important to note that the coating can be used together with common post-operative antibiotic treatment and it may even be expected that the antibiotic efficacy will be increased as enzymatic disruption of the EPS matrix will prevent biofilm formation. Alternatively, antibiotics may be directly included in the biodegradable PLGA coating [36,44].
CONCLUSIONS

In this study, we studied the effect of a protective, biodegradable PLGA coating containing inulin-packaged DNase I on bacterial adhesion and biofilm formation. We demonstrated that coating of titanium drastically decreases initial bacterial adhesion and biofilm formation in vitro, without affecting the ability of tissue cells to adhere. By packaging DNase I in inulin, DNase I underwent less damage during the coating process and activity was not lost during exposure to several conditions aimed to mimic handling of an implant before surgery.

Infections associated with biomaterial implant and devices are accompanied by a large use of antibiotics, mostly in vain, and therewith contribute to the development of antibiotic resistant strains and species. Underlined by a recently published first report of the World Health Organization on antibiotic resistance [45], the rise of antibiotic resistant bacterial strains and the limited number of newly discovered antibiotics indicate the importance of finding alternatives to battle biomaterial associated infections. Our biodegradable PLGA coating containing inulin-packaged DNase I provides such an alternative, that is not based on antibiotics but rather on the disruption of the integrity of the EPS matrix in which biofilm organisms find shelter.

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