Drug Release from Thermo-Responsive Polymer Brush Coatings to Control Bacterial Colonization and Biofilm Growth on Titanium Implants

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Despite decades of biomedical advances, the colonization of implant devices with bacterial biofilms is still a leading cause of implant failure. Clearly, new strategies and materials that suppress both initial and later stage bacterial colonization are required in this context. Ideal would be the implementation of a bactericidal functionality in the implant that is temporally and spatially triggered in an autonomous fashion at the infection site. Herein, the fabrication and validation of functional titanium-based implants with triggered antibiotic release function afforded via an intelligent polymer coating is reported. In particular, thermo-responsive poly(di(ethylene glycol) methyl ether methacrylate) (PDEGMA) brushes on titanium implants synthesized via a surface-initiated atom transfer radical polymerization with activators regenerated through the electron transfer technique (ARGET ATRP) allows for a controlled and thermally triggered release of the antibiotic levofloxacin at the wound site. Antibiotic loaded brushes are investigated as a function of thickness, loading capacity for antibiotics, and temperature. At temperatures of the infection site >37 °C the lower critical solution temperature behavior of the brushes afforded the triggered release. Hence, in addition to the known antifouling effects, the PDEGMA coating ensured enhanced bactericidal effects, as demonstrated in initial in vivo tests with rodents infected with Staphylococcus aureus.

1. Introduction

Biomedical device-associated infections (BAIs) are among the leading causes for failure in implantation therapy.[1–5] Despite continued efforts and also recent promising developments in implantable biomedical devices, consequences, such as severe pain, illness, loss of implant function, and sometimes even death of patients are still major issues associated with BAIs.[6,7] These issues are mainly related to implant failure caused by loosening and bacterial infections, which can be explained in terms of the “race for the surface”. [8] After surgical insertion of the implant devices, there is competition between adverse bacterial biofilm colonization and the targeted tissue integration. Thus, successful implantation will only occur, when tissue integration exceeds bacterial biofilm colonization, to induce the ingrowth of osteoblast cells on the implant device surface. Unfortunately, the immune response may be severely compromised by foreign body responses in the tissue, resulting in the survival of bacteria on and around the implant device.[9] This causes adhesion of planktonic bacteria on the surfaces of implant devices, referred to as “surface blanketing”. Bacteria residing inside the biofilm can hide from the hosts immune response and antibiotic treatment and may be even dispersed in the surrounding tissue.[10–12]

To overcome these challenges, different types of implant devices have been fabricated and passivating coatings with additional releasing features for antimicrobials were extensively studied over the past decades. For instance, Desai et al. reported on the non-adhesive behavior of different bacterial species on polyethylene oxide (PEO)-modified polyethylene terephthalate films.[13] Similarly, Kingshott et al.[14] studied polyethylene glycol and PEO coatings, considering their chain lengths along with surface coverage to provide full resistance against bioadhesion. The antibacterial characteristics of silver nanoparticles incorporated into titanium were studied on implant surfaces, where large amounts of reactive oxygen species were produced, which resulted in bacterial death.[15] Additionally, electrode surfaces made of Ag–NP with integrated ZnO nanowires showed in a self-powered water sterilization system both instant and sustainable sterilization effects against Gram-negative bacteria strains,

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which would be applicable to implantable self-powered biomedical devices.\textsuperscript{16} Furthermore, an antibiotic-release approach using polycaprolactone (PCL) coatings on catheters was investigated by Dave et al.\textsuperscript{17} To prevent colonization and biofilm growth, the release of ion-paired antibiotics from the self-degrading enzyme-embedded polymer coating was examined. A vancomycin-eluting polyethylene-coated joint implant device was fabricated against periprosthetic joint infection (PJI) and effective elimination of bacteria was studied in a rabbit model by Suhardli et al.\textsuperscript{18} Moreover, controlled release of antimicrobial peptides (SAAP) from titanium implants coated with a polymer–lipid encapsulation matrix was shown to be effective for up to 30 days against Staphylococcus aureus in a mouse model for BAI.\textsuperscript{19} Further, Kim et al. fabricated a biodegradable poly(c-caprolactone) (PCL) coated hydroxyapatite (HyA) scaffolds and their composition dependent tetracycline hydrochloride release behavior was studied.\textsuperscript{20}

On the other hand, biodegradable coatings that possess considerable thickness (\(\mu m\) scale) and release antibiotics may not be suitable with titanium implant devices due to their weak and decreased mechanical strength and durability with implantation time, which can potentially induce loosening at implantation site in moist environments.\textsuperscript{21–23} More importantly, since these approaches elude the antibiotics with fluctuating concentration in the later stage derived by the degradation of polymer, they may contribute to the undesirable and ineffective treatment, resulting in build-up of antibiotic resistance, or toxicity.\textsuperscript{24,25} A targeted, that is, temporally and spatially triggered release would be a much more preferred mode of action of functional coatings. Since a significant temperature increase is observed locally at the infected implant site,\textsuperscript{26} we propose that a temperature-triggered polymer-coated device with antimicrobial releasing features would be effective in preventing bacterial infections on implant devices. Hitherto this effect has been investigated in various contexts with thermo-responsive hydrogels, albeit only on polymeric and not stiff metallic implants. For example, Ross et al. developed a temperature-sensitive drug release system on polyethylene surfaces by functionalizing implant devices with poly(N-isopropylacrylamide) (PNIPAM)-co-poly(acrylic acid) (AAc).\textsuperscript{27} Sustained release of R-hirudin as a model drug to prevent restenosis from the functionalized surface was accomplished by employing the lower critical solution temperature (LCST) behavior. In addition, thermo-responsive implantable PNIPAM based hydrogels were applied to endotracheal tubes by Jones et al.\textsuperscript{28} The faster release of metronidazole at temperature above the LCST was employed to reduce mortality related to ventilator associated pneumonia. Recently, Perez-Köhler et al. developed a thermo-responsive rifampicin-loaded PNIPAM hyaluronan derivative (HApN) hydrogel to coat polypropylene mesh materials.\textsuperscript{29} At 37 °C, an effective drug release showed strong anti-staphylococcal activity both in vitro and in vivo. However, high fatigue strength implant materials such as titanium or stainless steel with covalently attached thermo-responsive polymer coating as an antimicrobial implant device have not yet studied to the best of our knowledge.

As an alternative to PNIPAM, thermo-responsive poly(di(ethylene glycol) methyl ether methacrylate) (PDEGMA) brushes were employed in this current study. PDEGMA is a promising coating material for implant devices as it is a nontoxic, biocompatible, and protein-resistant polymer, which also shows potential for post-modification with antimicrobial agents.\textsuperscript{30,31} Vasani et al. investigated the effect of temperature on levofloxacin release from post-modified random copolymer brushes of PDEGMA and poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) on biosilica microcapsules.\textsuperscript{32} A recent report by Jiang et al. describes the biocompatibility of PDEGMA brushes along with adhesion of human induced pluripotent stem cells (iPS cell), their growth into colonies, and the thermally triggered release behavior in an ambient culture environment.\textsuperscript{33} Moreover, Wassel et al. and Voss et al. studied the thickness-dependent thermo-responsive LCST characteristics of PDEGMA brushes\textsuperscript{34} and the passivation against proteins above 37 °C.\textsuperscript{35}

Here we report on the fabrication and in vitro test of titanium implant functionalized with thermo-responsive PDEGMA brushes (Scheme 1). The thermally triggered release of the loaded antibiotic levofloxacin from the up to 1.0 \(\mu m\) thick brush coatings in swollen state in Milli-Q water at 37 °C\textsuperscript{36} was investigated as a function of brush thickness, antibiotic loading, and temperature. The concomitant antimicrobial effects against Staphylococcus aureus were analyzed in vitro and in vivo to estimate the efficacy of the strategy in combating bacterial colonization of titanium-based implants.

2. Result and Discussion

The strategy summarized in Scheme 1 relies on a thick coating of a thermally responsive PDEGMA brushes, loaded with the fluorescent antibiotic levofloxacin, on the established implant material titanium, which exhibits high mechanical strength, biocompatibility, and corrosion resistance.\textsuperscript{37} Levofloxacin is a hydrophilic fluoroquinolone antibacterial agent acting against different types of Gram-positive and Gram-negative bacteria.\textsuperscript{38,39} To ensure a sufficiently thick reservoir for the antibiotic, the treatment and the bactericidal effects of the coatings via in vivo surgical procedure on rat in the intramuscular region with tight closure of the fascia and the skin.
brushes were synthesized through the oxygen-tolerant variant of surface-initiated atom transfer radical polymerization (SI-ATRP) called the activator regeneration by electron transfer (ARGET) technique, which affords brush thicknesses larger than 100 nm.\textsuperscript{[40]} As a comparison, POEGMA brushes were also investigated, which possess a similar chemical composition, but a much higher LCST of 90 °C.\textsuperscript{[41]}

2.1. Brush Synthesis and Characterization

To obtain thick thermo-responsive PDEGMA brushes on titanium substrates through surface-initiated activator regeneration by electron transfer atom transfer radical polymerization (SI-ARGET ATRP), the ATRP initiator attachment was achieved by a first oxidative polymerization of dopamine on titanium, followed by the covalent coupling of α-bromoisobutyryl bromide (BiBB) (Scheme 2). The titanium substrates were placed vertically in an aqueous solution of dopamine to avoid the attachment of sedimented aggregates on the substrates during the reaction. A 10 nm-thick polydopamine (PDA) layer was grown during 2 h of reaction, consistent with the results reported by Lee et al.\textsuperscript{[42]} This layer exhibited a granular but very smooth morphology with a rms roughness of 1.2 nm, as assessed with atomic force microscopy (AFM) (Figure S1, Supporting Information). Then BiBB was attached to the exposed amino functionalities. According to Hafner et al., the PDA–BiBB complex does not affect the polymerization compared to the typical initiator (3-aminopropyl)triethoxysilane used for SI-ATRP.\textsuperscript{[43]} Finally, polymer brushes were synthesized through SI-ARGET ATRP with di(ethylene glycol) methyl ether methacrylate (DEGMA) and oligo(ethylene glycol) methyl ether methacrylate (OEGMA) monomers.

The dry thickness of the brushes was measured ex situ after selected polymerization times through variable angle spectroscopic ellipsometry (Figure 1). Polymerization proceeded rapidly and a dry thickness of nearly 400 nm was obtained in 2 h reaction time. Up to a polymerization time of 2 h, the dry ellipsometric thickness of PDEGMA brushes increased linearly with time, at a rate of 3.6 nm ± 0.1 nm per minute. Ascorbic acid as a reducing agent was used in excess to regenerate Cu(I) from Cu(II), which is a process that generally does not occur during conventional ATRP reactions. In conventional SI-ATRP, merely 35 nm dry thickness were observed for the same monomer concentration after 2 h of polymerization (Figure S2, Supporting Information). Static water contact angle measurements afforded water contact angles of 63° ± 2° on the PDEGMA surfaces (Figure S3, Supporting Information). The values were independent of the polymerization time.\textsuperscript{[35]}

X-Ray photoelectron spectroscopy (XPS) was applied to characterize the elemental composition after each reaction step of the coating process. According the XPS survey scans, the dopamine-functionalized titanium (Ti–PDA) shows the expected C1s, O1s, and N1s signals at 285.0, 532.0, and 400.0 eV, respectively (Figure 2a). The experimentally determined N/C ratio in Ti–PDA was 0.10 ± 0.01, which is close to the theoretical value for the dopamine molecule (0.125).\textsuperscript{[44,45]} Titanium signals from substrates were not observed owing to the thickness of the dopamine layer (ellipsometric thickness: 10 nm, which exceeds the escape depth of the corresponding photoelectrons generated). After the immobilization of the ATRP initiator (Ti–PDA–Br), the Br3d signal appears at 69.6 eV. For PDEGMA brushes (Ti–PDEGMA), the C1s and O1s signals were obtained, while the N1s and Br3d signals vanished owing to the large thickness of the PDEGMA brushes (ellipsometric thickness: 400 nm). The experimentally determined C/O ratio for the PDEGMA brushes was 2.0 ± 0.3, which is consistent with the theoretically calculated value (2.3).\textsuperscript{[46]}

The high-resolution XPS scans in the N1s region (Figure 2b) were fitted with three peaks: nitrogen in N=C at 398.4 eV, C–NH–C at 400.0 eV, and H2N–C at 401.7 eV, which are all similar to those reported in earlier studies.\textsuperscript{[44,45]} The high-resolution Br3d scan corresponding to Br in C–Br (Figure 2c) comprises two peaks: 3d_{5/2} at 69.7 eV and 3d_{3/2} at 71.5 eV.\textsuperscript{[46]} In addition, the chemical structure of PDEGMA brushes was also confirmed in the high-resolution XPS scan of the C1s region, which can be convoluted into three peaks: C–C at 285.0 eV, C–O at 286.5 eV, and O–C=O at 288.7 eV, which all agree with previous results.\textsuperscript{[47]}

![Scheme 2](https://example.com/scheme2.png)

**Scheme 2.** Schematic showing the synthesis of PDEGMA brushes on titanium substrates: Step 1, dopamine deposition; Step 2, ATRP initiator attachment; Step 3, SI-ARGET ATRP with DEGMA or OEGMA monomers.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Dry ellipsometric thicknesses of PDEGMA brushes synthesized via SI-ARGET ATRP versus polymerization times (arithmetic mean ± σ, n = 3). The thicknesses of the PDA (10 nm) and ATRP initiator layers were not subtracted.
2.2. Release Study

Clinically, early onset infections are often treated with systematic antibiotics and indeed measurement long after 6 h will be necessary in the future. A release time of 6 h was chosen in this initial study, because the focus was the antifouling and antimicrobial properties from drug-loaded PDEGMA brushes against early stages of bacterial biofilm formation on implant devices (within a few minute to hours). To develop PDEGMA brushes as implant coating material with drug-releasing features, drug loading experiments were conducted by immersing the PDEGMA brushes in a levofloxacin solution for 10 h at room temperature (Figure S4, Supporting Information). The drug loading could be optimized by storing the PDEGMA brushes in a low-pressure environment in a vacuum chamber overnight before the loading experiments (pressure lower than 5 mbar). In addition, PDEGMA brushes are known to swell significantly in Milli-Q water at room temperature, which facilitates drug uptake.[36] Levofloxacin-loaded PDEGMA brushes (denote in the following as Ti-PDEGMA-LVF) were rinsed by dipping them into fresh Milli-Q water several times to remove nonspecifically bound drug molecules. Afterward, a series of drug release experiments were conducted in a six-well plate. Drug-loaded PDEGMA brushes were completely immersed in fresh Milli-Q water, which was preheated beforehand, if necessary. The released levofloxacin solution was removed from the six-well plate and the six-well plates were refilled with fresh Milli-Q water at selected time intervals. The concentrations of the collected levofloxacin solutions were determined using fluorescence spectroscopy. The values obtained were then converted into the mass of levofloxacin released from a given PDEGMA brush of a given thickness and constant lateral dimensions.

The amount of levofloxacin released from 400 nm thick PDEGMA brushes versus the drug release times at temperatures below and above the LCST are plotted in Figure 3a. As plotted in the bar graph, the mass of the released levofloxacin per nominal polymer brush-covered area over time was determined at below and above the LCST. Red, blue, and green colors in the graph represent each different levofloxacin release conditions at temperatures of 37, 25 and 25 °C to 37 °C, respectively. With the exception of the very first and second data points, which were acquired after 5 and 25 min release time, time intervals of 0.5 h were chosen until 4 h release time, followed by two final 1 h intervals, whereas time zero implies the state before the immersion of the substrates in Milli-Q water. From Figure 3a it is observed that a burst release of levofloxacin occurred within 5 min release time from the drug loaded PDEGMA brushes at both 25 and 37 °C owing to the gradual attainment of equilibrium swelling state of polymer brushes. At 37 °C, a faster drug release rate and sustained drug release up to 6 h compared to that at 25 °C was observed, similar to that reported in the work by Vasani et al.[32] In addition, a sudden increase in drug release was noticed after the solution temperature was increased from 25 to 37 °C at the second time point (green bar). Both observations can be attributed to the thermo-responsive behavior of the PDEGMA brushes, that is, the collapse of the brushes at the LCST.

Moreover, the cumulative levofloxacin release from PDEGMA brushes with different thicknesses at 37 °C was plotted (Figure 3b). The cumulative mass of the released levofloxacin per nominal polymer brush-covered area was determined with 50 nm (blue), 250 nm (red), and 400 nm (black) PDEGMA brushes over release time up to 6 h. A similar burst release of levofloxacin was observed, as shown in Figure 3a, from three different kinds of thick polymer brushes after 5 min release time. In addition, the progressive increasing trend of drug release up to 4 h was found in the case of 50 and 250 nm PDEGMA brushes, while up to 6 h it could be only observed for the thick 400 nm PDEGMA brushes. This indicates that the rate of the levofloxacin release was faster and more sustained in case of 400 nm thick brushes compared to that of 50 and 250 nm. Since thicker polymer brushes contain higher amounts of levofloxacin per unit area, this behavior is consistent with the higher loading capacities (Figure S4, Supporting Information).

To investigate and clarify the controlled thermo-responsive release behavior, the cumulative levofloxacin release from 400 nm thick PDEGMA and POEGMA brushes over release time up to 6 h was plotted at temperatures below and above the LCST of PDEGMA (Figure 4). PDEGMA and POEGMA brushes possess very different LCST values, namely 32 and 90 °C, respectively.[48,49] Temperatures of 25 °C (below the LCST, blue), 37 °C (slightly-above the LCST, red), and 45 °C (well-above the LCST, black) as drug release conditions were chosen considering the collapse of the swollen PDEGMA brushes in Milli-Q water with increasing temperature above the LCST.[36] Despite identical levofloxacin release conditions a significantly higher cumulative concentration of released drugs up to 6 h was observed only from PDEGMA brushes at temperatures above the LCST (37 and 45 °C) compared to that from POEGMA brushes. At the same time, the cumulative released amount of levofloxacin at
25 °C from both types of polymer brushes is comparable with each other. This implies that thermally triggered release of the loaded levofloxacin is possible using PDEGMA brushes in the temperature range of 30–45 °C, corresponding to temperatures at infection sites. This result also indicates that faster drug release is caused by the collapse of PDEGMA brushes at and above the LCST, leading to the expulsion of a higher amount of levofloxacin from PDEGMA brushes.

To assess the suppression of bacterial colonization and bactericidal effects of the Ti-PDEGMA-LVF at elevated infection site temperatures, it is necessary to compare the concentrations of the released levofloxacin with the corresponding minimal inhibitory concentration (MIC) values. Considering that the first stage of bacterial biofilm formation involving the introduction of free-floating bacteria on the implant surfaces is driven by Brownian motion and gravitational forces,[50,51] the estimation of the concentration gradient of the released levofloxacin from an implant device is very important. When the released drug diffuses further away from the implant device, a low local concentration will be obtained (Figure 5a).

To compare with MIC values of *S. aureus* ATCC 13709 obtained from literature (*S. aureus*: 0.25 µg mL⁻¹),[52] the mean concentrations of the released levofloxacin up to a distance of 1 mm from the implant device was estimated, which is a very conservative choice for free-floating bacteria to reach the implant device surface. Considering that the antibiotic diffused ≈1 mm away from the substrate over the time of the release, the concentration of levofloxacin was thus calculated considering the corresponding volume of the medium (size of the Ti disc × 1 mm) and the released amount (compare Figure 3b). Figure 5b shows a plot of the cumulative concentrations of the levofloxacin released up to 1 mm away from the implant surfaces for PDEGMA brushes of different thicknesses versus release time (Figure 5b). Similar to the results in Figure 3b, higher concentrations of released levofloxacin were obtained by release from 400 nm thick PDEGMA brushes compared to 50 and 250 nm thick brushes. With PDEGMA brushes
thicker than 20 nm\cite{35}, antifouling behavior of free-floating bacteria on the implant surface is expected. At the same time, the amount of levofloxacin released at 37 °C, that is at temperatures above the LCST, is high enough to inhibit bacterial growth during the first time point of release at all thicknesses of PDEGMA brushes.

According to the minimum bactericidal concentration (MBC)/MIC ratio for *S. aureus* with levofloxacin, which is ≈2–3\cite{53,54}, the levofloxacin-loaded PDEGMA brushes possess bactericidal effects on *S. aureus* ATCC 13709. On the other hand, certain bacterial strains, such as *S. aureus* MB5 (single mutation in gyrA), have relatively high MIC values (2 µg mL$^{-1}$, green-colored dashed horizontal line).\cite{55} In this case, only implant surfaces coated with 400 nm thick PDEGMA brushes are effective regarding a growth inhibitory effect, although not yet reaching a bactericidal effect for *S. aureus* MB5, as the released levofloxacin exceeds these MIC values after 1.5 h. This behavior can be explained via their high encapsulation capacity and more sustained release of levofloxacin compared to PDEGMA brushes with lower thicknesses. In addition, the cumulative concentrations of levofloxacin released from 400 nm thick PDEGMA and POEGMA brushes up to 1 mm from the implant device were plotted at 37 °C versus release time (Figure 5c). The amount of levofloxacin released from 400 nm thick POEGMA brushes at 37 °C is above the MIC value of *S. aureus* MB5 after 4 h of release time, whereas it takes only 1.5 h to reach the MIC value in case of 400 nm thick PDEGMA brushes. It is worth noting that it takes only a few minutes for planktonic free-floating bacteria to adhere on the implant surface and subsequently to start to form bacterial colonies within a few hours.\cite{36–38} Hence, it is very important to reach the MIC value as soon as possible to prevent early bacterial colonization and provide effective and complete protection to the implant surface against biofilm formation. Based on the above results, the MIC value for *S. aureus* MB5 was reached much faster with thermal triggering. This suggests that thick PDEGMA brushes are excellent implant coating materials offering enhanced antifouling and bactericidal effects at elevated infection site temperatures for coating implant devices under these conditions owing to their unique LCST behavior.

2.3. In Vitro Antibacterial Activity

Among the different bacterial species, *S. aureus* is the main cause of BAIs.\cite{59} Thus, in vitro studies were conducted to assess the antifouling and bactericidal effects of levofloxacin-loaded PDEGMA brushes against *S. aureus*. First, to evaluate the antifouling behavior on the bare titanium plates, Ti-PDEGMA, and Ti-PDEGMA-LVF, the adhesion of *S. aureus* ATCC 29213 on the surface of each group was assessed through scanning electron microscopy (SEM) analysis after 72 h of incubation (Figure 6a). According to the micrographs, *S. aureus* ATCC 29213 adheres and spreads as spherical cells only on the bare titanium plates, while the PDEGMA covered plates showed no traces of bacterial biofilm. This behavior is not surprising, as titanium does not exhibit antifouling properties. The colonizing pattern of *S. aureus* observed in the SEM images are similar to those reported by Harris et al.\cite{60} All the six bare titanium plates in the control group showed distinct bacterial biofilm formation, as indicated by the density of the feed bacteria (10$^7$ CFU mL$^{-1}$, colony forming units). By contrast, the other two groups show bacteria-free surfaces, whose morphology resembles bare titanium plates as well as PDEGMA-coated titanium with or without levofloxacin before and after bacteria incubation (compare also Figure S5, Supporting Information). This antifouling is attributed to the presence of the very thick polymer brushes (400 nm). Hence PDEGMA functionalized implant surfaces can lower the risk of infection owing to the strong and efficient antifouling properties of the PDEGMA brushes.
To evaluate the antimicrobial effect of levofloxacin released from Ti-PDEGMA-LVF, further antimicrobial tests have been performed via determination of the concentration of living bacteria in supernatant after 24 h incubation via CFU counting in comparison with bare titanium plates and Ti-PDEGMA. According to Figure 6d, the bare titanium and Ti-PDEGMA showed no antibacterial ability. Upon incubation of highly concentrated bacteria suspensions (≈10^9 CFU mL^{-1}), only Ti-PDEGMA-LVF showed a more than 90% reduction of living bacteria after 24 h incubation and thus a clear antibacterial effect, which is attributed to the presence of levofloxacin released from the brushes. In comparison, an in vitro study with gentamicin sulfate (GS) releasing titania nanotubes coated with a biodegradable thin polymer film demonstrated not only reduced bacterial adhesion, but also strong initial antibacterial activity against S. aureus (nearly 100% antibacterial rate).[61]

### 2.4. In Vivo Study

To study the in vivo antifouling and antibacterial effects of PDEGMA brushes on implant devices, surgical experiments with rats were conducted (Figure 7a). As for the surgical intervention, the infection model was prepared using the back of a rodent, as described by Ueno et al. Minor modifications were made to the model for use in the current study.[62] As in precedent in vitro experiments, the bare titanium plates were used as the control group. Five rats died during the tests (four during surgery and one after a day), with nine, six, and seven rats remaining for evaluation with the bare titanium, Ti-PDEGMA, and Ti-PDEGMA-LVF, respectively. At the time of euthanasia, 16 rats showed gross infection, appearing as local swelling (12), prominent redness (3), or abscess discharge (1) at the injection site. To validate the in vivo infection, the implant devices with different surface conditions from each group were isolated from rats after 7 days of implantation and bioluminescence images of the S. aureus Xen29 on the implants were captured and interpreted using an IVIS (in vivo imaging system) camera system (Figure 7b). Based on the emission during bioluminescent imaging, which reached ≈10^7 photons/s/ROI, bacterial colonization on the bare titanium plates was significantly higher, whereas the other two groups showed at
PMNLs are granulated white blood cells, that exhibit a nucleus clear leukocytes (PMNL) after 7 days of implantation (Figure 8b).

The dark blue staining of cell nuclei illustrates a strong infiltration of polymorphonuclear leukocytes (PMNL). For further details, see Figure S6, Supporting Information. Further Gram staining data supporting the observations are shown in Figure S7, Supporting Information.

To verify the antifouling properties of PDEGMA brushes on titanium plates with different surface characteristics after 7 days of implantation, in vivo SEM images of the S. aureus Xen29 biofilm were recorded (Figure 8a). The SEM images of only the bare titanium plates show significantly higher S. aureus adhesion to the surfaces, whereas clear surfaces are observed on the plates in the other two groups. In addition, an antibacterial effect, that is proved by a more than 99.9% reduction of luminescence intensity, which is proportional to the presence of living bacteria was obtained only from Ti-PDEGMA-LVF groups due to the presence of levofloxacin from the brushes.

According to Gristina et al., bacterial biofilm maturation caused by irreversible bacterial cell aggregations could occur either through random particle settlement or cell colony growth; this could lead to further severe infection from the bacterial biofilms on the substrates by dispersion and expansion of the biofilms to surrounding tissue. Nevertheless, the in vivo SEM results confirm that the PDEGMA brush system shows excellent antifouling properties. Furthermore, histological analysis was conducted to evaluate in vivo soft tissue inflammation in the course of substrate implantation. The soft tissue near the implant plates was studied with H & E staining focusing on presence of cells that show typical hallmarks of polymorphonuclear leukocytes (PMNL) after 7 days of implantation (Figure 8b). PMNLs are granulated white blood cells, that exhibit a nucleus with a multilobulated shape (black arrows in Figure S6, Supporting Information) and play a mature role in bacterial clearance in the cause of bacterial infections. In all specimens, the PMNLs spread throughout the soft tissue. On the bare and PDEGMA-coated titanium plates, PMNLs in the fascia of the back muscle of the rats were found and they had spread in the soft tissue, indicating prominent acute inflammatory reaction and strong immune response against bacteria. On the other hand, possibly owing to the bactericidal properties of levofloxacin followed by wound healing-associated tissue remodeling, three out of seven PDEGMA-coated titanium plates with levofloxacin showed some prominent fibroblasts (red arrows in Figure S6c, Supporting Information), with the infiltrated PMNLs still being present. The presence of prominent fibroblasts suggests hyperproliferation of fibroblast and thus fibrosis of soft tissue typically observed in the proliferation stage of wound healing or during maturation of the abscess, reflecting the potential bactericidal effects of levofloxacin.

In summary, functionalized surfaces of PDEGMA brushes with levofloxacin could possess in vivo antifouling and bactericidal properties, making them promising candidates for BAlS models.

Histological analysis indicated that the infiltrated PMNLs still existed in the presence of levofloxacin. This might be attributed to the insufficient amount of released levofloxacin from the particular PDEGMA brush system. Although the concentration of levofloxacin was enough to reach the MIC value and sustain the antifouling effect on the surface, it was not adequate to kill the bacteria in the soft tissue, which is a few millimeters away from the implant device. To address this problem, PDEGMA brushes thicker than 400 nm could be used to load higher amounts of levofloxacin and ensure its prolonged release from the brushes. This can be achieved by prolonged the polymerization time with the ARGET ATRP technique. Moreover, antimicrobial effects could also be enhanced by combining this approach with other methods, such as covalently linked antimicrobials on the surfaces of the polymer brushes. Such a combined method, which appears to be feasible using block copolymer brushes, could prove beneficial, if antimicrobial exhaustion is addressed, while...
simultaneously considering surface biofouling caused by dead bacterial cells and bacteriostatic effects on the implant surfaces. Further, utilizing such implants can be beneficial in situations, in which there is a risk of developing periprosthetic infection as in open fractures or in patients with comorbidities. According to the results of the current study, the developed coating system will prevent the adherence of bacteria on the implant surface, which will ultimately prevent the development of bacterial biofilms. In addition, the anti fouling properties of the PDEGMA coating may also inhibit adherence of osteoblasts and may adversely affect the osteogenesis around the implant devices. Nevertheless, implants containing the described coating system will be highly beneficial when applied as temporal fixation devices or as a spacer in cases with active infection or high risks of infection development.

3. Conclusion

PDEGMA brushes with dry thicknesses up to 400 nm were synthesized via SI-ARGET ATRP on titanium implant substrates. These brushes were successfully applied in vitro as coating materials for implant devices for protection against S. aureus infection because of their excellent anti fouling properties and unique LCST behavior. The release of the fluorescent antibiotic levofloxacin from the brush coating was shown to be temperature- and brush thickness-dependent. In particular, at elevated temperatures, the antimicrobial molecules were effectively released from the collapsed 400 nm thick PDEGMA brushes and reached MIC and MBC values of levofloxacin for S. aureus ATCC 13709 in the vicinity of the implant surface. The in vitro and in vivo assessment for determining the application potential of the PDEGMA brush system to surgical implant devices revealed a strong suppression of bacterial colonization and antimicrobial effects of the brush system on titanium implant surfaces. This brush system is hence suitable as a coating material for implants used as temporal fixation devices or spacers in cases with active infection or high risks of infection development from the bacterial biofilm considering the absence of osteoblast adhesion.

4. Experimental Section

Materials: 2-propanol (≥99.6, Acros Organics, Germany), titanium granules < 6 mm (99.8%, Chempur, Germany), dopamine hydrochloride (98%, Sigma-Aldrich, Germany), trizma hydrochloride (tris-HCl) (≥99.0%, Sigma-Aldrich, Germany), triethylamine (TEA, ≥99.5%, Sigma-Aldrich, Germany), dichloromethane (DCM, ≥99.5%, VWR, Germany), α-bromoisobutyryl bromide (BiBB, 98%, Sigma-Aldrich, Germany), methanol (≥99.9%, Merck, Germany), 2,2′-bipyridine (99%, Sigma-Aldrich, Germany), neutral aluminum oxide (Macherey-Nagel, Germany), di(ethyleneglycol) methyl ether methacrylate (DEGMA, number average molar mass Mn = 300 g mol⁻¹, 95%, Sigma-Aldrich, Germany), poly(ethylene glycol) methyl ether methacrylate (Mn = 475 g mol⁻¹, Sigma-Aldrich, Germany), copper (II) chloride, anhydrous (≥98%, VWR, Germany), l-ascorbic acid (≥99%, Sigma-Aldrich, Germany), levofloxacin (98–102%, Sigma-Aldrich, Germany), isofurane (Sigma-Aldrich, South Korea), Luria–Bertani (LB) broth (Invitrogen, South Korea), and phosphate-buffered saline (PBS) (Sigma-Aldrich, Germany) were used as received.

Bacteria: Bioluminescent S. aureus strain Xen29 (Perkin Elmer, USA) exhibiting a stable copy of the photonhabus luminescens lux operon; S. aureus Rosenbach 1884 (ATCC 29213, purchased from DSMZ, Braunschweig, Germany).

Preparation of Titanium Substrates: Glass substrates, cut to 1.5 cm × 2.5 cm pieces using a diamond tip glass cutter beforehand, were sonicated in soap water twice for 10 min, followed by cleaning with Milli-Q water. Then the substrates were washed in boiling isopropanol. Following drying, the cleaned substrates were coated with 100 nm of titanium (Titanium granules < 6 mm, 99.8%) through electron beam evaporation under high vacuum (Edwards E306 coating system, Moorfield, UK). Titanium disks for in vivo experiments (Ø: 15 mm, thickness: 1 mm, GRS ELI) were prepared with two 1 mm diameter holes to tag the threads used to identify the disk locations inside the rats. The disks belonged to one of the following categories: 1) unprocessed (control) disks, 2) PDEGMA-brush coated disks, and 3) PDEGMA-brush coated disks with loaded levofloxacin.

Deposition of Polydopamine Layer: The titanium substrates were rinsed with ethanol and Milli-Q water, followed by drying in a stream of nitrogen. The cleaned substrates were placed in a Bioforce UV/Ozone Pro Cleaner (Bioforce Nanoscience, Ames, USA) for 20 min. Subsequently, they were immersed in an aqueous solution of dopamine (2 mg of dopamine hydrochloride per mL of 10 mm tris-HCl, pH 8.5) and placed on a shaker for 2 h at room temperature. Then the substrates were sonicated for 15 min, followed by rinsing with Milli-Q water and drying in a stream of nitrogen, respectively.

Attachment of ATRP Initiator: The amino-functionalized substrates were immersed in 30 mL of dried DCM under an argon atmosphere, which was cooled in an ice bath beforehand. TEA (500 µL) was added dropwise to the reaction mixture, followed by the dropwise addition of diluted BiBB solution (250 µL, 2 mmol in 3 mL DCM) as the initiator for the ATRP reaction. The flask was sealed with a parafilm and the reaction was conducted for 1 h. Afterward, the initiator-functionalized substrates were washed with DCM, ethyl acetate, ethanol, and blown dry in a stream of nitrogen.

Fabrication of PDEGMA brushes via SI-ARGET ATRP: To fabricate PDEGMA brushes on the initiator-functionalized substrates, DEGMA monomer was first purified by passing through a column containing neutral aluminum oxide to remove the inhibitors (hydroquinone monomethyl ether and butylated hydroxytoluene). Next, Milli-Q water (19.20 mL), methanol (20 mL), and 2,2′-bipyridine (50 mg) were added to a 100 mL round-bottom flask with a stir bar. After the mixture was stirred under argon flow for 30 min, purified DEGMA (7.34 mL) and a solution of copper(II) chloride (0.40 mL, 0.04 M) were added. The resulting light-blue solution was stirred under argon flow for another 15 min. A solution of l-ascorbic acid (0.40 mL, 0.4 M) was added to the mixture, and after 1 min, the resulting red-colored solution was transferred via a glass syringe to the reaction flask containing the initiator-functionalized substrates under an argon atmosphere at room temperature. The polymerization was stopped after selected time intervals by removing the substrates from the reaction mixture, followed by rinsing with ethanol and Milli-Q water, and drying in a stream of nitrogen.

Ellipsometry: The dry thicknesses of the PDEGMA brushes on the titanium substrates were measured using an alpha-SE ellipsometer (J.A. Woollam Co., Inc., Lincoln, USA) at different angles of incidence (65°, 70°, and 75°) using wavelengths ranging from 380 to 900 nm. The measurements were obtained at least thrice at different locations on the PDEGMA layer to determine the values of the arithmetic mean and corresponding standard deviations. The titanium background was subtracted in all measurements. The Sellmeier model[47] with a refractive index of 1.52 at 632.8 nm for DEGMA[48] was used to determine the thickness.

Static Water Contact Angle Measurements: Static water contact angle measurements were conducted with an OCA-15 contact angle microscope (Dataphysics, Filderstadt, Germany) at room temperature. The static water contact angle of 2 µL drops of Milli-Q water was measured at three different spots on each substrate and the mean values and standard deviations were determined.

X-Ray Photoelectron Spectroscopy (XPS): XPS spectra were obtained with a photoelectron/ESCA spectrometer (SSX 100 S-probe, Surfaces science instruments, Mountain View, USA) with 200 W of Al Kα X-ray radiation. The spectra were recorded from 0 to 1200 eV with a resolution of 1 eV. High-resolution scans for each element were conducted with a pass energy of 20 eV and resolution of 0.1 eV. The XPS spectra were analyzed using the Casa XPS processing software (version of 2.3.16 PR 1.6).
Loading of Antibiotics into PDEGMA Brushes: PDEGMA brushes, which were completely dried beforehand, were immersed into 3 mL of 5 mm levofloxacin in Milli-Q water at room temperature. After 10 h of loading, the substrates were dipped into Milli-Q water 10 times at room temperature and then dried in a stream of nitrogen.

Release of Antibiotics from PDEGMA Brushes: Fluorescence spectrometry (Varian Cary Eclipse spectrometer, Varian, Mulgrave, Victoria, Australia) was used to monitor the amount of released levofloxacin from PDEGMA brushes at excitation and emission wavelengths of 292 and 540 nm, respectively. An emission slit width of 2.5 nm was chosen and the temperature was maintained constant at 25 ± 1 °C throughout the measurement. A Peltier element. The concentration of the released levofloxacin was calculated using the previously acquired calibration curve (serial dilution of levofloxacin in Milli-Q water).

Determination of In Vitro Antibacterial Effect: Before bacterial testing with PDEGMA brushes, the substrates were sterilized by washing with ethanol and drying with nitrogen gas. Afterward, the substrates were immersed in 70% ethanol for 30 min, followed by washing with PBS twice. The substrates were stored in PBS until further use. One colony of S. aureus ATCC 29213 was transferred from agar plates to a bacteria culture tube with 5 mL LB and incubated at 37 °C and shaken at 200 rpm for 18 h in a MAXQ 6000 stackable shaking incubator (Thermo Scientific, Manheta, OH, USA). Then the bacterial suspensions were diluted with LB to OD600nm 0.5 which was measured via the Spectrophotometer Helios Eppendorf system software (PerkinElmer). The total photon emission was measured using the Living Image 4.5.4 system software (PerkinElmer) in the IVIS imaging system. The light emission measured using this system correlated with the CFU count. SEM was used to confirm the formation of the S. aureus biofilm on the disks. The disks were fixed with 4% formaldehyde for 10 min and air-dried. The samples were dehydrated in an ethanol mixture with increasing ethanol concentrations (65%, 75%, 85%, 95%, and 100%) and finally air-dried overnight. The dehydrated titanium plates were coated with gold and palladium. The formation of the S. aureus biofilms was observed under 5.0 and 10.0 K magnifications using the S-4800 field emission SEM, Hitachi, Japan.

Antibacterial Effect on Surrounding Tissue: Histologic analysis was performed to evaluate the antibacterial effect of the released levofloxacin on the surrounding tissue. The muscle tissue immediately adjacent to the titanium disk (less than 5 mm) was harvested and fixed with 4% formaldehyde for 48 h. The collected tissue samples were cut into 4 µm thick sections, embedded in paraffin blocks, and stained with hematoxylin and eosin (H & E) to identify the ongoing infection of the surrounding tissue. The degree of infection was determined based on the presence of PMNLs in a high-power field using Olympus BX51 microscope.

Statistical Analysis: Ellipsometry data, release (mass or cumulative mass release) data: arithmetic mean ± σ; n = 3. CFU counting/log reduction factors: Experiments were performed in triplicates (independent substrates and sub Cultures) affording arithmetic mean values ± σ, error bars were then calculated based on Gaussian error propagation. Statistical analysis of the log reduction was performed using the one-way ANOVA test (triplet experiment) with a Bonferroni post hoc test (OriginPro, version 9.0). In case of p-values < 0.05, the log reduction was statistically significant. Luminescence: Boxes show the interquartile range, lower and upper bars indicate the lowest and largest data point excluding any outliers (cross shapes), lines and squares inside the boxes show median and mean values, respectively. n as stated in the Figure captions.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
H.C. did most of the experiments and wrote down the manuscript. M.M. performed the in vitro bacteria test. A.S. conducted the XPS measurements and analyzed the data. S.J. and M.P. carried out the in vivo rat study. S.J. and H.S. conceptualized the study, secured funding, supervised the research, and perfected the manuscript. All authors checked the manuscript and have given approval to the final version of the manuscript.

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Research data are not shared.

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