Full length article

Self-defensive antibiotic-loaded layer-by-layer coatings: Imaging of localized bacterial acidification and pH-triggering of antibiotic release

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Abstract

Self-defensive antibiotic-loaded coatings have shown promise in inhibiting growth of pathogenic bacteria adhering to biomaterial implants and devices, but direct proof that their antibacterial release is triggered by bacterially-induced acidification of the immediate environment under buffered conditions remained elusive. Here, we demonstrate that Staphylococcus aureus and Escherichia coli adhering to such coatings generate highly localized acidification, even in buffered conditions, to activate pH-triggered, self-defensive antibiotic release. To this end, we utilized chemically crosslinked layer-by-layer hydrogel coatings of poly(methacrylic acid) with a covalently attached pH-sensitive SNARF-1 fluorescent label for imaging, and unlabeled-antibiotic (gentamicin or polymyxin B) loaded coatings for antibacterial studies. Local acidification of the coatings induced by S. aureus and E. coli adhering to the coatings was demonstrated by confocal-laser-scanning-microscopy via wavelength-resolved imaging. pH-triggered antibiotic release under static, small volume conditions yielded high bacterial killing efficiencies for S. aureus and E. coli. Gentamicin-loaded films retained their antibacterial activity against S. aureus under fluid flow in buffered conditions. Antibacterial activity increased with the number of polymer layers in the films. Altogether, pH-triggered, self-defensive antibiotic-loaded coatings become activated by highly localized acidification in the immediate environment of an adhering bacterium, offering potential for clinical application with minimized side-effects.

Statement of significance

Polymeric coatings were created that are able to uptake and selectively release antibiotics upon stimulus by adhering bacteria in order to understand the fundamental mechanisms behind pH-triggered antibiotic release as a potential way to prevent biomaterial-associated infections. Through fluorescent imaging studies, this work importantly shows that adhering bacteria produce highly localized pH changes even in buffer. Accordingly such coatings only demonstrate antibacterial activity by antibiotic release in the presence of adhering bacteria. This is clinically important, because ad libitum releasing antibiotic coatings usually show a burst release and have often lost their antibiotic content when bacteria adhere.

1. Introduction

Bacterial infection associated with biomaterials implants and devices is a well-known, rapidly growing problem [1]. The traditional treatment of biomaterial-associated infections with systemic antibiotics is often inefficient because of the formation of bacterial biofilms [2,3] in which bacteria are poorly responsive to treatment [4-6]. Prevention of bacterial colonization of surfaces at early stages, i.e. upon adhesion of first few bacteria to the implant or device surface, is therefore crucial.

Polymer coatings designed with various molecular architectures have been broadly explored to prevent surfaces from colonization with bacteria. Often, these coatings contain highly hydrated polymers, such as polymer brushes [7] or thin-film hydrogels [8]. The hydrated, open molecular architecture of such
coatings can provide them with anti-adhesive properties, and also enable hosting of antibiotics. Several types of antibacterial polymer coatings exist that prevent bacterial adhesion [9], or kill bacteria by either direct contact [10] or through release of antibiotics [11-15]. Yet, ad libitum antibiotic-releasing films often exhibit a burst release followed by a long tail release with sub-lethal concentrations, leading to the development of antibiotic resistance in bacterial strains [16]. To avoid this, coatings that respond to an external stimulus such as light [17], and electrical pulses [12] have been designed. These coatings release antimicrobials on-demand and therefore are expected to slow down the increasing emergence of antibiotic resistance [16]. One attractive type of such coatings responds to the presence of adhering bacteria to release antibacterial content.

Recently, a series of such “self-defensive”, bacterially-triggered coatings has been developed, responding to bacterial presence only when and where needed [18-21]. Both enzymes [18,22] and acids [23,24] excreted by bacteria have been used as triggers for antimicrobial release to combat adhering bacteria. Recently, our group has developed several types of self-defensive coatings, which take advantage of the layer-by-layer (LbL) polymer deposition technique [19,20,25]. Among these pH-responsive coatings are LbL coatings assembled from montmorillonite clay nanoplatelets and polyacrylic acid and subsequently loaded with antibiotics [20], as well as coatings produced by the direct assembly of tannic acid and antibiotics [19]. Of specific relevance to this work are antibiotic-hosting [26] and antibiotic-free thin-film hydrogels [8], which both demonstrated significant pH-triggered antibacterial activity. In all cases, the pH-responsive ‘activation’ of the coatings was clearly demonstrated when the coating was exposed to the bacterial strains of Staphylococcus aureus or Escherichia coli, which are known to acidify the medium in which they grow as a result of secretion of lactic and acetic acid, respectively [23,24].

Although several types of pH- and otherwise bacterially-triggered polymer coatings have been developed, a direct link between bacterial presence and pH activation through localized acidification has never been demonstrated. One outstanding question is whether bacteria adhering to such coatings produce acidification and trigger antibiotic release at early stages of surface colonization, i.e. under physiological conditions when the bulk pH of the surrounding medium remains constant because of its buffering capability, and often while being exposed to fluid flow. Here, localized interfacial acidification induced by adhering bacteria is demonstrated and correlated with pH-triggered release of gentamicin and polymyxin B, by making use of antibiotic-loaded poly(methacrylic acid) (PMAA) hydrogel coatings [26-28]. PMAA hydrogel coatings contain primary amino groups as a result of one-end attachment of a diamine crosslinker [29], that enable covalent attachment of a pH-sensitive fluorescent dye for imaging of local acidification from adhering bacteria. Efficacy of the hydrogel coatings is evaluated against a Gram-positive S. aureus and a Gram-negative E. coli strain, both known to form biofilms on biomaterial implants and devices [2,3,30].

2. Experimental

2.1. Materials

Poly(glycidyl methacrylate) (PGMA, Mw ~ 20,000), sodium phosphate monobasic, and ethylene diamine (EDA) were obtained from Sigma Aldrich. Branched poly(ethyleneimine) (BPEI, 50% qa, M ~ 750,000) was obtained from Aldrich Chemical Company, Inc., poly(vinyl caprolactam) (PVCL, Mw ~ 1800) from Polymer Source, Inc., and poly(methacrylic acid) (PMAA) (Mn/reumer ~ 80,000) from Scientific Polymer Products, Inc. (3-Dimethylaminopropyl)-3-etacyloxy carbodiimide hydrochloride (ACT), hydrochloric acid, sodium hydroxide and sulfuric acid were obtained from Alfa Aesar. SNARF-1 carboxylic acid, acetate, succinimidyl ester (SNARF-1) was obtained from Thermo Fisher Scientific. All chemicals were used without further modification. Millipore (Milli-Q system) filtered water with a resistivity of 18.2 MΩ·cm was used. Phosphate buffered saline (PBS) was prepared from 0.01 M sodium phosphate monobasic with 0.2 M NaCl for all experiments unless otherwise noted. Fisher Chemically™ Permount™ Mounting Medium was used for mounting all samples for imaging.

2.2. Preparation of hydrogel-like coatings

Silicon wafers were cleaned as described previously [26]. A prime layer of PGMA, BPEI, and PMAA was deposited following a modified version of the procedure described by Wang et al. [31]. Briefly, the wafer was soaked for 15 min in 0.5 mg/mL PGMA in acetone, baked at 110 °C for 1 h, soaked in 1.0 mg/mL BPEI in methanol for 15 min, baked at 70 °C for 3 h, soaked in 1.0 mg/mL PMAA in methanol for 15 min and baked at 125 °C for 1 h. After deposition of the prime layer, alternating layers of PVCL and PMAA were added via spin-assisted deposition from 0.2 mg/mL methanol solutions at 3000 rpm to the desired layer number, varying from 6 to 18 bilayers. PMAA/PVCL LbL films assembled in a linear manner with an increase of 2.7 ± 0.1 nm per bilayer as measured by dry ellipsometry. In order to create hydrogel-like thin films, after assembly, the LbL films were crosslinked via ethylenediamine using carbodiimide chemistry, i.e. by activation of PMAA carboxylic groups with 9 mg/mL of ACT for 15 min and crosslinking with 3 mg/mL EDA for 1 h both in 0.01 M phosphate buffer at pH 5.2. After crosslinking, PVCL was released by slowly adjusting the pH of the solution to 8, and exposing the LbL films to 0.01 M phosphate buffer solutions at pH 8 overnight to completely release PVCL and form PMAA hydrogel-like coatings. After crosslinking and PVCL release, the dry thickness of all films decreased by ~40%, which is similar with the data on release of a neutral hydrogen-bonding polymer from previously reported PMAA hydrogel-like coatings [26].

2.3. Physicochemical coating properties

2.3.1. Film thickness and wettability

All thickness measurements, dry and in situ, were taken using a custom-made phase modulated ellipsometer, which enabled the simultaneous determination of refractive index and film thickness. [32] For ellipsometry measurements, a single layer model was used to fit the data. For dry measurements, the refractive index for all coatings was fixed at 1.5 while for wet measurements, the refractive index was measured and decreased to values below 1.5 due to water uptake in the film. All static contact angle measurements were taken using an Optical Contact Angle and Surface Tension Meter CAM 101 (KVS Instrument Inc.). For each dry pH specific measurement, the sample was rinsed with PBS of the corresponding pH and then dried under a flow of nitrogen before the measurement was taken. For in situ swelling measurements, each sample was soaked in PBS at the specified pH for 10 min at which point the samples were fully equilibrated.

2.3.2. Loading and release of antibiotics

Two common antibiotics were chosen: (1), gentamicin, a broad spectrum antibiotic aminoglycoside, which is a class of drugs that binds to the 30 S subunit of ribosomes thus preventing bacterial protein synthesis [33] and (2) polymyxin B, a cyclic cationic polypeptide, which is commonly used against Gram-negative bacteria, that works by disrupting the bacterial cell membrane [34]. Gentamicin and polymyxin B were loaded into the hydrogel by...
allowing the film to soak in a 0.1 mg/mL of the bioactive molecule in 0.01 M phosphate buffer at pH 7.5 for 2 h then washed three times in 0.01 M phosphate buffer at pH 7.5 to remove excess antibiotic bound to the surface. For testing the release of antibiotics as a function of pH, each sample was soaked in PBS solution of that pH for 2 h. The sample was then rinsed with phosphate buffer at the same pH, dried, and thickness measured by ellipsometry.

2.4. Bacterially-Induced pH changes and killing assays

2.4.1. Bacterial culturing

S. aureus ATCC 12600 and E. coli O2K2 were inoculated on blood agar plates from frozen stock, incubated at 37 °C overnight, and stored in a refrigerator for a maximum of 2 weeks. Single colonies from the blood agar plates were inoculated in 10 mL of growth medium (tryptic soy broth (TSB, OXOID, Basingstoke, UK) for S. aureus, and brain heart infusion (BHI, OXOID) for E. coli, and grown overnight at 37 °C, as the preculture. For the main culture, the preculture was inoculated in 200 mL of the appropriate growth medium, and grown overnight.

2.4.2. Minimal inhibitory concentration determination

To determine the MIC of gentamicin for S. aureus ATCC 12600 and of polymyxin B for E. coli O2K2, diluted bacterial precultures were added to 150 μL of serially diluted antibiotic concentrations (256 μg/mL to 0.0078 μg/mL) in TSB or BHI for S. aureus and E. coli, respectively, in a 96-well plates and incubated aerobically at 37 °C for 24 h. The MIC-value was taken as the lowest antibiotic concentration at which bacterial growth was visually absent.

2.4.3. Bacterial killing assays

For Petrifilm (3 M Petrifilm Aerobic Count Plates, Nelson-Jameson, Marshfield, WI, USA) experiments, bacterial suspension from the preculture were used in different concentrations. Petrifilms were treated with 1 mL of sterilized demineralized water for 30 min to hydrate the nutrient loaded gel layer. Silicon wafers with different numbers (6, 12, and 18) of Lbl layers and loaded with or without gentamicin or polymyxin B were placed face up on hydrated Petrifilms and 10 μL of bacterial suspension was placed onto the hydrogel coated wafers and incubated at 37 °C for 48 h. After 48 h, the bacterial colonies were counted on the Petrifilms. The antibiotic loaded Lbl layers were also tested in multiple cycles in the Petrifilm in order to determine the bacterial efficacy experiments of the Lbl layers when fresh bacteria were added to used Lbl layers.

For bacterial adhesion experiments, bacteria from the main culture were harvested by centrifugation at 5000g for 5 min at 10 °C, and washed twice with PBS (0.01 M potassium phosphate and 0.15 M NaCl, pH 7.0). Bacteria were counted and diluted to a concentration of 3 × 10^9 CFU (colony forming units)/mL in PBS. Bacterial suspensions of S. aureus ATCC 12600 and E. coli O2K2 were flown through a parallel plate flow chamber as previously described [35]. Briefly, the hydrogel coated silicon samples with different numbers of bilayers and both antibiotics were inserted in the poly(methyl methacrylate) bottom plate of the flow chamber which had a glass plate on top. The flow chamber was then rinsed with PBS to remove all air bubbles from the system, before the start of the adhesion experiment. The bacterial suspension was flown through the flow chamber for 2 h at a rate of 1 mL/min. After 2 h, fresh PBS buffer was run through the flow chamber to remove non-adhered bacteria and then live/dead stain was injected into the flow chamber to image the adhering bacteria. Live/dead stain of SYTO®9 nucleic acid (green fluorescent) and propidium iodide (red fluorescent) with a volume ratio of 1:1 was used (BacLight, Invitrogen, Breda, The Netherlands) with 3 μL of stain diluted in 1 mL of demineralized water. After allowing the stain to react with the sample for 15 min in the dark, the samples were imaged using a fluorescence microscope (Leica DM4000B, Leica Microsystems GmbH, Heidelberg, Germany) with a 40× water lens and GFP and N21 filters. Three fluorescent images were taken at different spots on each sample. The total number of adhering live and dead bacteria as well as the percent surface coverage was quantified using ImageJ software.

2.4.4. Visualization of local, bacterially-Induced acidification

For visualization studies, SNARF-1 was covalently bound to PMAA coatings by soaking the films in a solution of SNARF-1 dye dissolved in 0.01 M phosphate buffer for 2 h. After loading the dye, each sample was thoroughly washed with 0.01 M phosphate buffer to remove any dye that was not covalently attached. For calibration measurements of SNARF-1 labeled hydrogel-like coatings, samples were placed into PBS at the desired pH for 60 min and then gently dried with a flow of nitrogen and imaged with a confocal scanning laser microscope (5 PASCAL laser scanning microscope, Zeiss, Germany) with a C-Apochromat 60X/1.4 oil immersion objective. Samples were excited by a laser at λ = 543 nm (except for a control sample, which was excited at 488 nm) and emission intensities were collected using a LP 560 filter. All imaging parameters, such as exposure time, pinhole size, color contrast and color balance were held constant during data collection. Specifically, for generation of the calibration curve (Fig. 3), imaging of local acidification (Fig. 4) and comparison of emission intensity of the background hydrogel versus SNARF-1 emission (Fig. 5), all parameters were held constant at the following values: exposure time = 1.28 μs, laser power = 1 mW, transmission% = 25, pinhole = 106 μm, detector = 957, amplifier gain = 1.00 and amplifier offset = -0.24 V. For comparison of emission intensities profiles of bacteria residing on SNARF-free hydrogels at two different excitation wavelengths (Fig. S1), all parameters were held constant at the aforementioned values except for the following: exposure time = 1.60 μs, laser power = 25 mW for 488 nm excitation, and transmission% = 10 for 488 nm excitation. The average intensities of each image of the SNARF-1 labeled hydrogel-like coatings at each pH were determined using ImageJ [36,37].

To image local acidification, a colony from a tryptic soy broth agar plate that was prepared as described above was placed into 2 mL of TSB and grown overnight. After incubation, bacteria were counted and were diluted in TSB to a concentration of ~10^7 CFU/mL. 5 mL of this mixture was placed into a well-plate containing a hydrogel-like coating, which was sterilized in a 70% ethanol/water mixture, and then incubated for 4 h. To image the local bacterially-induced acidification, the same CSLM that was used for generating the calibration curve was used under the same conditions. Auto-fluorescence of bacteria was measured by exciting the bacteria at λ = 543 nm and 488 nm and comparing the emission intensities collected by a LP 560 filter. The average intensities of each image of the bacteria and SNARF-1 labeled hydrogel-like coatings were determined using ImageJ. To assess the local pH accurately, the autofluorescence of bacteria (λ = 543 nm) was subtracted from the fluorescence intensities of SNARF-1 (λ = 543 nm) at the locations of bacteria.

2.5. Statistical analysis

All data points indicate the mean over at least three measurements per sample (unless described otherwise below) while error bars reflect the standard deviation of the sample as calculated using STDEV.S in Excel. To generate the calibration curve in Fig. 3, intensity measurements were taken at three locations on a sample with each location providing 100 data points. For surface coverage and live/dead analysis (Fig. 5), four experiments with
different bacterial cultures and separately prepared coatings were run per each sample. Differences in percentage dead bacteria adhering to unloaded and gentamicin-loaded PMAA coatings were assessed with a paired Student’s t test and were significant with a p-value <0.05 using Minitab 18 software.

3. Results and discussion

To explore the ability of hydrogel-like thin polymer coatings to uptake and release antibiotics in response to a pH trigger, coatings were constructed from PMAA, which has a high content of carboxylic groups that can act as negative charges and thus be used as binding centers for positively charged antibiotics. Importantly, these thin-film hydrogels also enable facile modification with a pH-sensitive fluorescent label for imaging of bacteria-induced local acidification.

3.1. Coating construction, characterization, and pH-dependent antibiotic release

To form PMAA LbL coatings, hydrogen-bonded PMAA/poly-caprolactam (PVCL) multilayers were deposited via spin-assisted deposition and chemically crosslinked followed by the release of PVCL using a procedure similar to that described previously [26]. Fig. 1A shows dry thicknesses of PMAA LbL coatings, which increase linearly as a function of number of layers, with the thickness of priming layers (9.6 ± 3.4 nm) subtracted from all the data. To load antibiotics into the coatings, the negatively charged coatings were soaked in antibiotic solutions at pH 7.5 for 2 h. After exposure to positively charged gentamicin and polymyxin B (Scheme 1), the dry thickness of the antibiotic-loaded coatings increased, indicating antibiotic uptake within the entire thickness of the coatings (Fig. 1A). Calculation of film charge balance (data not shown) suggested that the inclusion of both positively-charged antibiotics resulted in charge neutralization in the film, therefore suggesting that binding mainly occurs through electrostatic interactions. Assuming a density of 1 g/cm³ for the antibiotic-loaded hydrogel coating, an 18-layer PMAA film was able to load 10 ± 4 µg/mm³ of gentamicin. As has been observed earlier [20,36] and can also be seen in Fig. 1A, increasing the number of layers deposited increased the amount of antibiotic that can be hosted in the film. Therefore, the dosage provided by this coating system is highly tunable by simple variation of the number of polymer layers used to create the film.

In addition to the amount of antibiotics absorbed within the coating, wettability and swelling are important factors that may affect antibacterial performance of the coatings. Before antibiotic loading, PMAA films were hydrophilic (Fig. 1B), with its water contact angle decreasing from 32 degrees to 12 degrees between pH 4.5 and 7.5. Upon incorporation of antibiotics, the coating became more hydrophobic. Consistent with the previous observation that drug hydrophobicity affects the wettability of weak polyacid hydrogels [37], polymyxin B, which is more hydrophobic than gentamicin [19], caused a larger increase in water contact angle of the films (Fig. 1B).

Fig. 2A shows in situ, ellipsometrically measured swelling of hydrogel coatings in PBS after an equilibration period. Importantly, exposure of unloaded PMAA coatings to pHs from 4.5 to 7.5 overnight does not result in a change in the dry film thickness and thus indicates the coatings are stable over this pH range as observed previously in our earlier works [26,28,38]. From the measured swelling of the films, the crosslinking density was calculated using a previously reported procedure [27], applying 0.985 cm³/g for the specific volume, 0.598 [27] as χ and 80,000 Da as the molecular weight before crosslinking. The volume fraction of polymer in the swollen gel was 0.42 as calculated from the swelling ratio at pH 4.5. Based on these assumptions, Mw was calculated to be 498 Da or ~5.8 monomer units between crosslinks for PMAA coatings. Such a crosslinking degree was sufficiently low to allow inclusion of antibiotics within the entire bulk of the hydrogel films. Loading antibiotics that carry positive charge into the negatively charged coatings decreased the LbL film swelling degree because of charge compensation and ion pairing of antibiotics with the carboxylate groups in the coatings [26].

To monitor the pH responsive release of gentamicin from the coatings, the thickness of the coatings after exposure to PBS solutions at decreasing pH values was measured via ellipsometry, under the assumption that loss of film thickness is due to the release of gentamicin from the coating. Gentamicin and polymyxin B contain 4 to 5 positive charges at pH 7.5 [19] and form ionic pairs
with MAA units, resulting in release that is solely dependent on the ionization degree of PMAA. Importantly, no release of antibiotics was observed when hydrogels containing antibiotics were copiously rinsed with a buffer at a constant pH. The percentage gentamicin released was calculated as $\frac{\text{tpH} - \text{t}_{10}}{\text{t}_{0} - \text{t}_{10}} \times 100\%$ where $\text{tpH}$ and $\text{t}_{10}$ are dry thicknesses of the antibiotic-loaded coatings after exposure to PBS at a specific pH and immediately post-loading at pH 7.5 (low salt), respectively, and $\text{t}_{0}$ is the dry thickness of antibiotic-free coatings. Fig. 2B shows that when the pH of the PBS surrounding a film is lowered, the percentage of antibiotic released increases as measured for release of gentamicin from an 18 layer PMAA film. pH-triggered release profiles were similar for gentamicin from coatings of 6 and 12 layers as well as polymyxin B from coatings of all thicknesses (data not shown). In agreement with our prior findings [19], pH-triggered antibiotic release from the coating was fast, and no long-term release occurred at a constant pH (data not shown). This is consistent with electrostatic retention of antibiotics, and absence of diffusional constraints to drug release from the coatings.

3.2. Imaging of local acidification induced by bacteria

After demonstrating pH-responsive antibiotic release from the coatings, we aimed to verify that bacteria adhering to the hydrogel coatings were able to locally acidify the medium sufficiently to ‘send’ a release signal to the coating. S. aureus and E. coli are both known to acidify growth medium as a result of secretion of lactic and acetic acid, respectively [23,24]. Mature biofilms formed by several bacterial strains [39,40], including S. aureus [41] have been demonstrated to be acidic, but acidity of the environment around adhering individual bacteria has never been shown. To visualize the areas of acidification on the coatings during bacterial adhesion, a pH sensitive, ratiometric fluorescent probe (see Scheme 1) was covalently bound to PMAA films. This probe enables the imaging of local pH changes on the hydrogel coating surrounding adhering bacteria via fluorescence emission selectively emerging in response to a decreased pH. Specifically, a reactive SNARF-1 label (SNARF-1 carboxylic acid, acetate, succinimidyl ester, Molecular Probes [42,43], see Experimental Section) was covalently attached to the amino groups of the ethylenediamine (EDA) crosslinker, which are approximately 50% one-end attached after crosslinking using carbodiimide chemistry [29]. Unlike exposure to antibiotics, SNARF-tagging did not result in a dry thickness change of the film. SNARF-1 has a pK$_a$ of ~7.5, and emits red fluorescence ($\lambda_{\text{em, max}} = 640$ nm) in its deprotonated form at high pH, and green fluorescence ($\lambda_{\text{em, max}} = 580$ nm) in its protonated form in an acidic environment. Fig. 3 shows combined emission at wavelengths higher than 560 nm (using excitation at 543 nm), as detected during CLSM imaging of SNARF-1-labeled PMAA hydrogel soaked in PBS at varied pH for 1 h in a well plate with no bacteria. The fluorescence was strongly pH dependent, with an almost fivefold increase in green fluorescence between pH 7.5 and 4. Cycling the pH of the solution of the SNARF-1-labeled PMAA hydrogel between pH 4.5 and pH 7.5 showed complete reversibility and fast adjustment of intensity to pH (data not shown) and thus provided evidence that SNARF-1 was covalently attached to the film.
emission intensity profiles show that the intensity of SNARF-1-tagged hydrogels are shown in Fig. 5. The cross-sectional groups caused by proximity of the charged bacterial wall. However, precise pH profile determination was prevented due to a high level of noise in the imaging experiments.

3.3. Antibacterial efficacy of self-defensive films in static, small volume and fluid flow conditions

We then aimed to explore how efficient the demonstrated bacterially-triggered acidification was to stimulate antibiotic release and kill adhering Gram-positive, S. aureus ATCC 12600, and Gram-negative, E. coli O2K2 under static, small volume conditions and under fluid flow, i.e., between Petrifilm Aerobic Count Plates and in flow chamber experiments, respectively. PMAA coatings with varying thicknesses (6, 12, or 18 layers) were loaded with either gentamicin or polymyxin B to be evaluated against adhering S. aureus or E. coli, respectively, while unloaded films of matched thicknesses were used as controls.

In Petrifilm assays, antibiotic-free coatings had no effect on the growth of bacteria, whereas the coating became highly efficient in bacterial killing when loaded with corresponding antibiotics. Fig. 6 shows that PMAA hydrogel films of varied thicknesses loaded with gentamicin were able to completely inhibit bacterial growth at challenge numbers of up to 10^7 S. aureus per 1 cm^2 coated sample surface. At a challenge of 10^7 bacteria per cm^2 sample surface, staphylococcal survival was only seen for 6 layer films. The difference in bacterial survival between 6 and 12 layer films can be explained simply from their gentamicin content – about 6.5 and 12.1 µg per cm^2 sample for 6 and 12 layer films, respectively (assuming a density of dry gentamicin of 1 g/cm^3). After release from the coatings in 1.01 mL of liquid employed in Petrifilm assays (1.0 mL to swell the agar plate and 10 µL of bacterial suspension), antibiotic concentrations of about 6.4 and 12.8 µg/mL were established between the Petrifilm Plates for the 6 and 12-layer coatings, respectively. These values are below and above the minimum inhibitory concentration (MIC) of gentamicin (12.5 µg/mL for this S. aureus strain), thus explaining the results in Fig. 6.

Polymyxin B-loaded PMAA coatings demonstrated even higher antibacterial efficacy in the Petrifilm experiments when tested with E. coli O2K2. Specifically, the coatings completely inhibited bacterial growth for all film thicknesses even when exposed to the highest bacterial challenge of 10^7 bacteria per cm^2 sample surface (data not shown). This is likely due to the much lower MIC of polymyxin B against E. coli O2K2 (0.25 µg/mL). After complete release of polymyxin B from the smallest coatings in 1.01 mL volume of buffer between the Petrifilm plates, polymyxin B concentration reached 14 µg/mL, i.e., well above the MIC of polymyxin B against E. coli O2K2.

Because of the high efficacy of polymyxin B-loaded films against E. coli, it was examined whether polymyxin B-loaded films could be repeatedly used against E. coli, while preserving their high killing efficacy. To investigate this, after exposure to 10^7 bacteria, films were removed from Petrifilm plates and directly placed into fresh Petrifilm plates and re-exposed to a fresh E. coli suspension. Fig. 7 shows that the high efficacy of the films was retained for two complete cycles of exposure to a challenge of 10^7 bacteria per cm^2 sample surface.

Finally, the efficacy of antibiotic-loaded films was explored in buffer under flow. The presence of flow of buffer through the chamber assures no changes in bulk pH during experiments, and antibiotic-release becomes completely dependent on local acidification by adhering bacteria. In these experiments, a bacterial sus-
pension in PBS was flown over samples at a volumetric flow rate of 1 mL/min for 2 h. E. coli could not be used in these experiments because the strain adhered in very low numbers, as previously observed for E. coli O2K2 onto negatively charged surfaces [48].
S. aureus ATCC 12600, suspended in PBS at a concentration of $3 \times 10^8$ CFU/mL, adhered very well to the coatings regardless of the number of film layers (Fig. 8). In spite of similar bacterial adhesion, gentamicin-loaded PMAA films were highly efficient in killing adhering staphylococci when compared to un-loaded PMAA films ($p < 0.05$ Student’s $t$-test), with a slight dependence on the number of layers of the film. Further testing with thicker films would need to be conducted to confirm the layer dependence. Nevertheless, this finding confirms that self-defensive coatings remain active even under fluid flow and when the bulk pH is maintained at a constant neutral value.

4. Conclusions

In this work, we have demonstrated that coatings composed of a weak polyacid were capable of loading large amounts of positively charged antibiotics primarily through an electrostatic mechanism and released those antibiotics in response to a pH trigger provided by adhering bacteria. Most importantly, our experiments showed that the pH- and bacterially-triggered antibiotic release is highly localized. The LbL technique used to prepare the coatings in this work is attractive for constructing antimicrobial-hosting coatings because of its ability to deposit conformal coatings on a variety of substrates and the ease of control of the antibiotic payload by the number of deposited layers. Combined with the self-defensive mechanism of release of antimicrobials, this approach enables enhancing the antibacterial efficacy of a coating by a simple increase of the number of assembled polymer layers. The simple electrostatics-based mechanism of retention of antimicrobials within the coatings enables their use to host and on-demand deliver, in a highly localized way, a range of positively charged antibiotics or antimicrobials, including those recently developed to which bacteria are not likely to develop resistance [49].

Acknowledgments

The authors would like to thank Professor Cattabani for his assistance with confocal imaging. In addition, the authors would like to thank Professor Libera for allowing us to use his bacterial culture lab as well as his graduate students Jing Liang and Yong Wu for their assistance. The authors would also like to thank Tjitske Boekema for performing Petrifilm and flow experiments. All authors have approved the final version of the manuscript and declare no competing financial interest. H.J.B. is also director-owner of SASA BV. The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organizations or their respective employers.

Funding sources

The authors would like to acknowledge financial support from the Healthcare Scholars Program from the Center for Healthcare Innovation, the Stevens Scholars Summer Research Program and the National Science Foundation under Grant No. NSF DGE-0742462. This research was also funded by the University Medical Center Groningen, Groningen, The Netherlands, and Texas A&M University, USA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2017.08.012.

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