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Enzyme multilayer coatings inhibit *Pseudomonas aeruginosa* biofilm formation on urinary catheters

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Abstract Bacteria use a signaling mechanism called quorum sensing (QS) to form complex communities of surface-attached cells known as biofilms. This protective mode of growth allows them to resist antibiotic treatment and originates the majority of hospital-acquired infections. Emerging alternatives to control biofilm-associated infections and multidrug resistance development interfere with bacterial QS pathways, exerting less selective pressure on bacterial population. In this study, biologically stable coatings comprising the QS disrupting enzyme acylase were built on silicone urinary catheters using a layer-by-layer technique. This was achieved by the alternate deposition of negatively charged enzyme and positively charged polyethylenimine. The acylase-coated catheters efficiently quenched the QS in the biosensor strain *Chromobacterium violaceum* CECT 5999, demonstrated by approximately 50 % inhibition of violacein production. These enzyme multilayer coatings significantly reduced the *Pseudomonas aeruginosa* ATCC 10145 biofilm formation under static and dynamic conditions in an in vitro catheterized bladder model. The quorum quenching enzyme coatings did not affect the viability of the human fibroblasts (BJ-5ta) over 7 days, corresponding to the extended useful life of urinary catheters. Such enzyme-based approach could be an alternative to the conventional antibiotic treatment for prevention of biofilm-associated urinary tract infections.

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Introduction

The discovery of antibiotics in the early 20th century allowed the treatment of life-threatening microbial infections. However, the emergence of multidrug resistance bacteria overwhelming the effect of the conventional antibacterial agents poses a great concern in medical settings (Fernandes et al. 2014). The formation of biofilms, a complex community of surface-adherent cells embedded in a self-produced extracellular polymeric matrix, is one of the most efficient protective behaviors of bacteria (Donlan 2001). Biofilms can be formed either on living tissues, including lungs, wounds, and teeth, or non-living surfaces, such as medical implants and indwelling medical devices causing more than 60 % of the hospital-acquired infections (Darouiche 2004; Richards 2009). The urinary tract infections (UTIs) caused by biofilm occurrence on urinary catheters have a large share among the nosocomial infections (Jacobsen et al. 2008). They are responsible for increased time of hospitalization, health care costs, morbidity, and mortality (Rajakaruna and Harber 2014).

To reduce the incidence of biofilm-associated infections, a variety of strategies, such as replacement of the contaminated catheters, utilization of novel materials for catheter construction, and their surface functionalization with antibacterial agents, have been suggested (Evliyaoğlu et al. 2011; Lee et al. 2004; Lellouche et al. 2012; Li et al. 2014; Trautner 2010). Nowadays, the material of choice for production of urinary catheters is the silicone due to its intrinsic hydrophobicity and resistance to bacterial adherence. Nevertheless, the silicone materials are still prone to bacterial colonization, especially after prolonged usage (Lawrence and Turner 2005). Therefore, the effective control of the formation of bacterial

biofilms on silicone urinary catheters is still an issue. More recently, strategies targeting the molecular mechanisms involved in biofilm development, such as quorum sensing (QS), have been investigated (Chen et al. 2013; Richards 2009). The QS process allows bacteria to communicate through specific signals called autoinducers (AIs) and further coordinates their group behavior in order to form biofilms (Ivanova et al. 2013). Following surface attachment, the bacteria secrete in the extracellular environment AIs that in threshold concentrations bind to and activate the specific receptor inducing QS-regulated gene expression and consequent biofilm development. The disruption of this process provides novel antibiofilm approaches, which are expected to reduce the risk of resistance development (Ivanova et al. 2013). Whereas the conventional antibiotics exert substantial evolutionary stress on bacterial population by targeting different intracellular components, the QS inhibitors use other bactericidal mechanisms, which induce less selective pressure and therefore decrease the emergence of drug resistance (Rasko and Sperandio 2010).

Inactivation of the AIs in the extracellular environment could provide control of the QS-regulated behaviors and eliminate the need for internalization of the active agents into the bacterial cells. Acylase has been reported as a quorum quenching (QQ) enzyme that cleaves the amide bond of acyl-homoserine lactones (AHLs), the QS signals produced by Gram-negative bacteria, in the extracellular environment. This enzyme was reported to prevent the formation of *Pseudomonas aeruginosa* biofilm on polystyrene surfaces, interrupting the bacterial communication (Hong et al. 2012; Lin et al. 2003; Xu et al. 2003).

Despite the advances in QQ enzymes for antibacterial purposes, these have never been applied to prevent the biofilm formation on indwelling medical devices. Layer-by-layer (LbL) deposition technique has been demonstrated as an efficient method for enzyme immobilization on a variety of materials with the advantage of utilizing mild conditions (e.g., aqueous solutions) favorable to preserve the protein folding and activity (Onda et al. 1999; Pavlukhina et al. 2012; Sakr and Borchard 2013). These multilayer coatings comprised of oppositely charged polyelectrolytes are easy to fabricate and can be built on various substrates with different chemistry and geometrical shape (Hammond 2004). In contrast to the surfaces functionalized with enzyme proteins through covalent binding and physical adsorption, LbL assembly enables the deposition of larger amounts of bioactive compounds on the surfaces that are usually protected from proteolytic activities (Pavlukhina et al. 2012; Sakr and Borchard 2013). Previous studies showed the potential of the multilayer coatings to maintain the stability and activity of the bioactive molecules entrapped within the polyelectrolyte layers. Moreover, the amount of the active agents at the surface might be controlled through the thickness of the LbL coatings, normally determined by the number of deposited layers (Pavlukhina et al. 2012; Hammond 2004).

The goal of the present study is to develop multilayer acylase coatings on silicone urinary catheters able to interfere with bacterial QS phenomenon in the extracellular space and thereby inhibit the formation of biofilms. In contrast to the conventional antibacterial treatments targeting intracellular components and mechanisms, this enzyme has extracellular targets and would not kill bacteria thus reducing the risk of resistance development. The quorum quenching activity of an LbL immobilized enzyme will be studied in the signal molecule reporter strain, *Chromobacterium violaceum* CECT 5999. The ability of the coatings to prevent the *P. aeruginosa* ATCC 10145 biofilm formation on urinary catheters will be assessed also in dynamic conditions using an in vitro model of a catheterized human bladder.

Materials and methods

Enzymes, AHLs, and bacterium

Acylase from *Aspergillus melleus* (Sigma-Aldrich, Madrid, Spain) with a protein content of 4.5 % (*w*/*w*) and specific activity of 0.05 U mg^{-1} was used as a QQ enzyme. AHLs were also purchased from Sigma-Aldrich and used as model QS signaling compounds produced by Gram-negative bacteria. *P. aeruginosa* ATCC 10145, a bacterium proficient in biofilm formation, was obtained from ATCC (Barcelona, Spain). Polydimethyl/vinylmethyl-siloxane (PDMS) material, in the form of Foley urinary catheters and sheets designated according to ASTM D 1418, was provided by Degania Silicone, Ltd. (Emek Hayarden, Israel).

Enzymatic degradation of AHLs

The degradation of *N*-butyryl-DL-homoserine lactone (C4-DL-HL), *N*-hexanoyl-DL-homoserine lactone (C6-DL-HL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-L-HL), and *N*-(3-oxododecanoyl)-L-homoserine (3-oxo-C12-L-HL) lactone model compounds by acylase was carried out as follows: 30 μg mL^{-1} of each AHL compound and 2.16 U mL^{-1} of the enzyme were incubated in tricine buffer (100 mM, pH 8) for 15 min at 37 °C in a thermoshaker bath. The resulting degradation products were analyzed by a fluorescamine fluorimetric assay and *C. violaceum* CECT 5999 (CECT, Valencia, Spain) bioassay. In the fluorimetric quantification assay, 150 μL of the reaction mixture containing homoserine lactone was added to 50 μL of fluorescamine (Sigma-Aldrich) reagent (3 mg mL^{-1} in dimethyl sulfoxide (Sigma-Aldrich)), and after incubation at room temperature (RT) for 3 min, the fluorescence at 390/470 nm was measured. The amount of homoserine lactone released in the enzymatic

reaction was calculated from the calibration curve built with α-amino-γ-butyrolactone hydrobromide (Sigma-Aldrich) as a standard.

In the *C. violaceum* CECT 5999 bioassay, only the degradation of C6-DL-HL by acylase was tested. The bacterial strain *C. violaceum* CECT 5999 is a mutant that produces a purple pigment in the presence of AHLs. The most easily detectable AHL is C6-DL-HL, whereas the sensitivity to the other model compounds is different depending on their structure (Steindler and Venturi 2007). Before performing the bioassay, the products from the reaction mixture were extracted for three times with ethyl acetate (Sigma-Aldrich) as previously described (Ravn et al. 2001). The organic phase was then evaporated to dryness and the sample reconstituted in 300 μL methanol. Thereafter, 60 μL of the reconstructed sample was tested for the presence of AHLs by *C. violaceum* CECT 5999 bioassay. The sample solutions were pipetted into wells (Ø= 7 mm) punched in the Luria Bertani broth (LB) agar (supplemented with 25 μg mL^{-1} kanamycin (Sigma-Aldrich)), and the plates were incubated at 26 °C for 48 h. Then, the degradation of the C6-DL-HL signal by acylase was confirmed by the decrease of the purple zones surrounding the wells. C6-DL-HL extracts without acylase served as a control.

Silicone functionalization

Prior to coating, silicone catheter samples (3.2×9 cm) were washed for 30 min with 0.5 % (*w*/*v*) sodium dodecyl sulfate (SDS, Sigma-Aldrich). Afterward, the silicone stripes were rinsed alternately with distilled H_2O and EtOH (96 %, Scharlau, Barcelona, Spain) and dried with nitrogen. The silicone surface was pretreated using 5 % (*v*/*v*) amino-propyl-triethoxy-silane solution (APTES, Sigma-Aldrich) in order to introduce amino groups on the surface for better attachment of the first enzyme layer. The presence of amino groups on the surface was confirmed by immersing the silicone material in ninhydrin solution (2 % (*w*/*v*), Sigma-Aldrich). Thereafter, the APTES-treated silicone stripes were coated in an LbL fashion (Scheme 1). Acylase, having isoelectric point between pH 5 and 6 (negatively charged at a pH higher than 6), was used as polyanion, whereas linear polyethyleneimine (PEI, M_w= 25 kDa, Polysciences, Inc., Salamanca, Spain) was used as a polycation. A bilayer was constructed by dipping the silicone stripes for 10 min in 1 mg mL^{-1} acylase solution in tricine buffer pH 8, followed by dipping in 1 mg mL^{-1} PEI solution. After each polyelectrolyte deposition, a 10-min rinsing step in 0.15 M sodium chloride (NaCl, Sigma-Aldrich) solution pH 8 was performed. This procedure was repeated for ten times, and the coated silicone samples were finally dried with a continuous flow of nitrogen. LbL coating with a bilayer architecture of $(Acy/PEI)_{10}$/Acy was built, with the outermost layer being acylase (LbL Acylase). The same procedure was performed to coat silicone Foley urinary catheters.

Attenuated total reflection Fourier transform infrared spectroscopy (FTIR-ATR) analysis

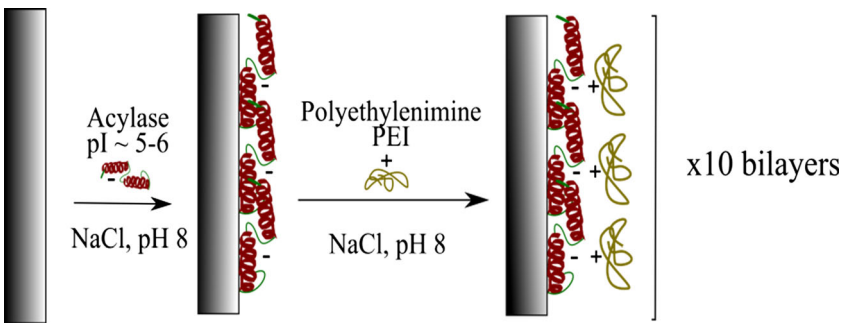
The deposition of enzyme proteins on the silicone surface was analyzed by FTIR-ATR using a Spectrum 100 FTIR spectrometer (PerkinElmer, MA, USA). The FTIR-ATR spectra of non-treated silicone and LbL Acylase were collected in the range over 4000 to 625 cm^{-1}. All the spectra were obtained after 64 scans at 4 cm^{-1} resolution, and the data were analyzed using essential eFTIR - 3.00.019 software.

Water contact angle measurements

The hydrophilicity of the enzyme coatings was assessed measuring the static contact angle (sessile drop method) of 5-μL ultrapure water droplet on the material, using drop shape analysis system DSA 100 (Krüss, Hamburg, Germany), equipped with ½ CCD camera. Three independent measurements were performed and analyzed using Krüss DSA3 v1.0.1.3-02 software for each sample.

Atomic force microscopy

The morphology of the different surfaces was studied by atomic force microscopy (AFM) using a Dimension 3100 AFM from Veeco. The AFM images were analyzed and post-processed using the Nanotec WSxM software (Horcas et al. 2007).



Scheme 1 LbL assembly of acylase on the silicone catheters

Enzyme activity in multilayer coatings

The enzyme-coated silicone samples were evaluated for acylase activity. The activity was measured by a colorimetric assay using *N*-acetyl-L-methionine (NAMET, Sigma-Aldrich) as a substrate. Briefly, 2 mL of tricine buffer (100 mM, pH 8.0) and 1 mL of 0.5-mM cobalt chloride solution (Sigma-Aldrich) were added to the samples and equilibrated at 37 °C. Then, 1 mL of 100 mM NAMET in tricine buffer (100 mM, pH 8.0) was added, and the test tubes were incubated for 30 min. The reaction was stopped by rising the temperature to 100 °C for 4 min. The product of the enzymatic reaction was determined using the colorimetric ninhydrin assay as follows: 1 mL of the sample was mixed with 2 mL of 2 % (*w*/*v*) ninhydrin solution (prepared in 200-mM citrate buffer pH 5 and ethylene glycol monomethyl ether (Sigma-Aldrich) (1:1)) and 0.1 mL 1.6 % (*w*/*v*) stannous chloride solution (Sigma-Aldrich). The samples were incubated for 20 min at 100 °C and cooled down before measuring their absorbance at 570 nm. Non-treated silicone material was used as a control in all experiments. The immobilization efficiency of acylase on silicone material was evaluated as a percentage of the enzyme specific activity retained after immobilization on the surface.

Acylase activity on quorum sensing signaling molecules

C. violaceum CECT 5999 was used to study the ability of the acylase coatings to inhibit the QS process via degradation of QS signaling molecules. Untreated silicone sample (negative control) and acylase-coated silicone stripes were cut into 2×2-cm pieces and placed in sterile test tubes. The silicone pieces were inoculated with 5-mL overnight bacterial culture of *C. violaceum* CECT 5999 (grown at 26 °C) diluted to optical density $(OD)_{660}=0.002$ in LB (Sigma-Aldrich), supplemented with 25 μg mL^{-1} kanamycin and 5 μM C6-HSL. Thereafter, the tubes were incubated at 26 °C with shaking (230 rpm) for 18 h. After the incubation period, 200 μL of the cell suspension was transferred into Eppendorf tubes and mixed with 200 μL 10 % SDS (*w*/*v*) (Blosser and Gray 2000). The samples were mixed and vortexed for 5 s, and the violacein extraction was performed by addition of 500 μL 1-butanol (Sigma-Aldrich) followed by centrifugation for 5 min at 13,000 rpm. After phase separation, the butanol (upper phase) was collected and the absorbance measured at 584 nm. Non-immobilized acylase (at the same concentration as on the silicone surface) was used to evaluate the QQ activity loss after immobilization.

Initial bacterial adhesion

Initial bacterial adhesion was studied on enzyme-coated and non-treated silicone sheets. The samples were cut into small pieces (1×1 cm) and placed in a cell culture 24-well plate (Costar 3337, Corning CellBIND Surface). Then, the samples were inoculated with 1 mL of bacterial culture of *P. aeruginosa* ATCC 10145 in tryptic soy broth (TSB, Sigma-Aldrich) in which the concentration of glucose was 0.25 % (*w*/*v*). The bacterial inoculum was prepared from overnight culture grown in TSB and diluted to $OD_{600}=0.01$. The culture plate was incubated for 3 h at 37 °C to allow bacterial adhesion. Thereafter, the samples were washed for three times with sterile phosphate-buffered saline (PBS, Sigma-Aldrich) and left in 4 % (*v*/*v*) formaldehyde (Sigma-Aldrich) at 4 °C overnight to allow fixation of the bacteria. Then, the samples were washed with sterile PBS and 25, 50, 75, and 96 % ethanol for 10 min. The initial bacterial attachment was studied in a bright-field mode using Eclipse Ti-S microscope (Nikon Instruments, Inc., Amsterdam, the Netherlands).

Total biomass assessment

Antibiofilm activity of the developed materials in static conditions was assessed against *P. aeruginosa* ATCC 10145 using crystal violet assay for total biomass determination. *P. aeruginosa* ATCC 10145 inoculum was prepared form overnight-grown culture in TSB diluted to $OD_{600}=0.01$. The enzyme-coated samples were cut into small pieces (1×1 cm) and placed in a cell culture 24-well plate. Thereafter, 1 mL of bacteria was inoculated in each well, and the plate was incubated for 24 h at 37 °C. The liquid medium was removed and the biofilm washed with distilled H_2O for three times to eliminate non-adhered bacteria. The biofilms were further fixed by heat (60 °C for 60 min) and the biofilm mass quantified using crystal violet assay. Briefly, 700 μL of 0.1 % (*w*/*v* in distilled H_2O) crystal violet (Sigma-Aldrich) was added to each well and incubated for 10 min at RT. Then, crystal violet was discarded and samples washed with sterile distilled H_2O for three times. The remaining stain was eluted with 1 mL 30 % (*v*/*v*) acetic acid (Sigma-Aldrich), and the total biomass was determined as the amount of crystal violet bound to each sample by measuring the absorbance at 595 nm using TECAN Infinite M200 (Tecan Austria GmbH, Grödig, Austria). Untreated silicone samples served as negative control (no biofilm inhibition) in all experiments.

Bacterial viability in biofilm

P. aeruginosa ATCC 10145 biofilms were formed as described above and analyzed using Live/Dead BacLight kit (Molecular probes L7012, Invitrogen, Barcelona, Spain) according to the instructions of the manufacturer. The Live/Dead kit contains two nucleic acid stains—green fluorescent Syto 9 and red-fluorescent propidium iodide. Syto 9 labels all viable bacteria in the population in green, whereas the propidium iodide only stains the nucleic acid of the dead bacteria. After

24-h incubation, the biofilms were washed with 1 mL 0.9 % NaCl solution, pH 6.5, and stained for 15 min in the dark with a mixture of both stains (1:1). Non-reacted stains were further washed with the same NaCl solution, and the biofilm was observed using fluorescence microscopy at 480/500 nm for Syto 9 and at 490/635 nm for propidium iodide.

P. aeruginosa growth inhibition

The effect of enzyme-coated silicone material on *P. aeruginosa* ATCC 10145 planktonic growth was further evaluated. Overnight culture of *P. aeruginosa* ATCC 10145 in TSB was diluted to OD_{600}=0.01, and then, 1 mL of freshly prepared bacterial inoculum was cultured with treated and non-treated silicone for 24 h at 37 °C. Afterward, 200 μL of bacterial suspension was placed into 96-well plates and the inhibitory activity assessed by measuring the bacteria growth at OD_{600} in the presence of acylase-coated silicone samples.

Functional stability of the coatings

The stability of acylase-comprising coatings was studied after incubation in artificial urine, prepared according to UNE EN 1616, for 1 and 7 days (UNE-EN 1997). The samples were cut, placed in a test tube containing 2-mL artificial urine, and incubated at 37 °C with 20-rpm shaking. Afterward, the silicone pieces were washed with distilled H_2O, and the antibiofilm activity was determined in static conditions against *P. aeruginosa* ATCC 10145, as already described.

Dynamic biofilm inhibition tests

Dynamic biofilm tests were performed in a physical model of catheterized bladder, previously described by Stickler et al. (1999). This model consists of a glass bladder model maintained at 37 °C by a water jacket. After sterilization of the glass bladder model by autoclaving (121 °C for 15 min), acylase-coated silicone Foley catheter was inserted into the dynamic system and the catheter balloon inflated with 10 mL distilled H_2O. Then, the bladder was filled with 60-mL sterile artificial urine containing 1 mg mL^{-1} TSB and inoculated with *P. aeruginosa* ATCC 10145 (OD_{600}=0.01). The catheterized bladder model was left for 1 h without urine supply to allow the organisms to adapt, and then, the supply was started with 1 mL min^{-1} flow rate. After 7 days, the catheter was removed and washed with sterile distilled H_2O. Then, different sections of the catheter-balloon (1×1 cm) and catheter shaft (1 cm) (referred in this work as urethra) were cut and subjected to analysis for biofilm formation using crystal violet and Live/Dead kit as described above. The same parts of pristine silicone Foley catheter were used as controls for biofilm formation.

Cell culture

The BJ-5ta cells (human foreskin fibroblasts, ATCC-CRL-4001) were maintained in 4 parts Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 4 mM L-glutamine, 4500 mg L^{-1} glucose, 1500 mg L^{-1} sodium bicarbonate, 1 mM sodium pyruvate, and 1 part of Medium 199, supplemented with 10 % (*v*/*v*) of fetal bovine serum (FBS, Sigma-Aldrich), and 10 g L^{-1} hygromycin B (Sigma-Aldrich) at 37 °C, in a humidified atmosphere with 5 % CO_2, according to the recommendations of the manufacturer. The culture medium was replaced every 2 days. At pre-confluence, cells were harvested using trypsin-EDTA (ATCC-30-2101, 0.25 % (*w*/*v*) trypsin/0.53-mM EDTA solution in Hank's BSS without calcium or magnesium) and seeded at a density of 4.5×10^4 cells per well on a 96-well tissue-culture-treated polystyrene plate (Nunc).

Cytotoxicity

For biocompatibility evaluation, the BJ-5ta cells were previously seeded at density of 4.5×10^4 cells per well on 24-well tissue-culture-treated polystyrene plate (Nunc). For the cytotoxicity assessment, the silicone (control) and acylase-coated silicone were cut into small pieces (1×1 cm) and placed in contact with the cells. Then, 0.75 mL of complete growth medium (DMEM) was added, and the tested samples were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 1 and 7 days. At the end of each time point, the cells were examined for signs of toxicity using an alamarBlue assay kit (alamarBlue®, Invitrogen). A sample of DMEM subjected to the same conditions was used as a negative control, whereas a hydrogen peroxide solution (30 % (*v*/*v*), Sigma-Aldrich) prepared in fresh culture medium was used as a toxicity positive control.

Resazurin, the active blue ingredient of the kit, is a nontoxic, cell-permeable compound that, once in a viable cell, is reduced to red-colored resorufin. AlamarBlue® reagent was diluted in culture medium (1:10) and added to each well after aspirating the culture medium containing the samples in contact with cells. After 4 h of incubation at 37 °C, the absorbance at 570 nm was measured in a microplate reader, using 600 nm as a reference wavelength. The quantity of resorufin formed is directly proportional to the number of viable cells. The error bars for each datum were the standard deviation of three independent measurements.

Results

Acylase-induced degradation of QS signals

The ability of acylase to act upon different model AHLs was evaluated in order to confirm the quorum quenching concept

subjacent to this study. Acylase was able to degrade several AHL model compounds, including C4-DL-HL, C6-DL-HL, 3-oxo-C10-L-HL, and 3-oxo-C12-L-HL (Fig. 1a), through amide bond cleavage, producing homoserine lactone and the corresponding fatty acid. Among them, the acylase-induced degradation of 3-oxo-C12-L-HL was of main importance as this signal was found to play a key role in *P. aeruginosa* biofilm formation, one of the main pathogens causing UTIs in catheterized patients in hospitals (Cole et al. 2014; Davies et al. 1998). The enzyme showed higher activity on 3-oxo-C10-L-HL and 3-oxo-C12-L-HL (more than 50 and 40 % conversion, respectively) when compared to C4-DL-HL and C6-DL-HL (up to 10 and 15 % conversion, respectively) (Fig. 1a). Acylase I from *A. melleus* is highly enantioselective in its interaction with the substrate mainly promoting the degradation of the L-configuration of amide bond, which suggests that the lower conversion of C4-DL-HL and C6-DL-HL was due to the presence of both D- and L-configurations in the substrates (Youshko et al. 2004). This factor may play a significant role in quenching the QS, as bacteria recognize and respond to these configurations (Kalia 2013).

The bioassay using *C. violaceum* CECT 5999 also revealed the decreasing concentration of the QS molecule C6-DL-HL upon contact with acylase (smaller purple area in the presence of acylase) accounting for the quorum quenching activity of this enzyme (Fig. 1b) (Ravn et al. 2001). Based on the observed in vitro enzymatic inactivation of the QS molecules, we assumed that acylase could inactivate the AHLs of Gram-negative bacteria such as *P. aeruginosa* and thereby inhibit the signal accumulation in the extracellular environment and subsequent biofilm formation.

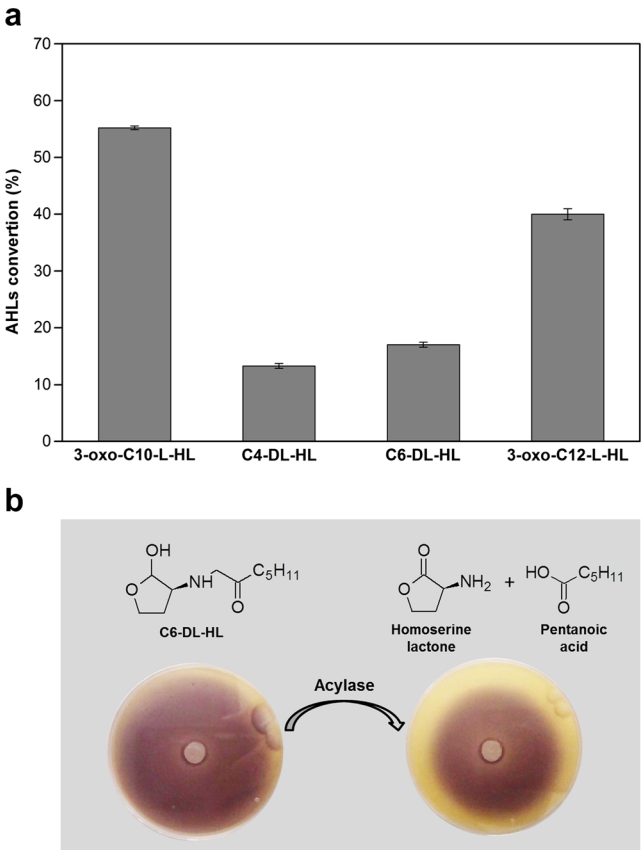


Fig. 1 **a** AHL degradation by acylase assessed with fluorescamine method and **b** C6-DL-HL degradation study using the *C. violaceum* CECT 5999

Acylase multilayer coatings build-up onto silicone and its characterization

In order to obtain a coating with quorum quenching activity, acylase was immobilized in an LbL fashion onto silicone sheets and urinary catheters. A pre-amination of the silicone surface with APTES was performed in order to provide amine groups for better deposition of the first enzyme layer. The amination reaction takes place with the hydroxyl groups presented on the surface of the PDMS (Gilles 2007). The presence of amino groups on silicone surface was then confirmed by the development of a characteristic purple color in the ninhydrin assay (results not shown).

After ten consecutive immersions of the silicone material into anionic enzyme solution and oppositely charged PEI typical protein bands, it was not observed on the untreated silicone control, appeared in the FTIR spectra of the acylase-coated samples (Fig. 2). The band with a maximum around 3300 cm^{-1} was assigned to N–H stretching vibrations, whereas the band at 1650 cm^{-1} and the broad region between 1490 and 1590 cm^{-1} were ascribed to amide I (C=O stretching) and amide II (mainly N–H bending) vibrations. The FTIR analysis demonstrated the development of the enzyme multilayer assemblies onto the catheter surface (Kong and Yu 2007).

The surface morphology and roughness of the acylase/PEI multilayer coatings were also studied by AFM. AFM two-

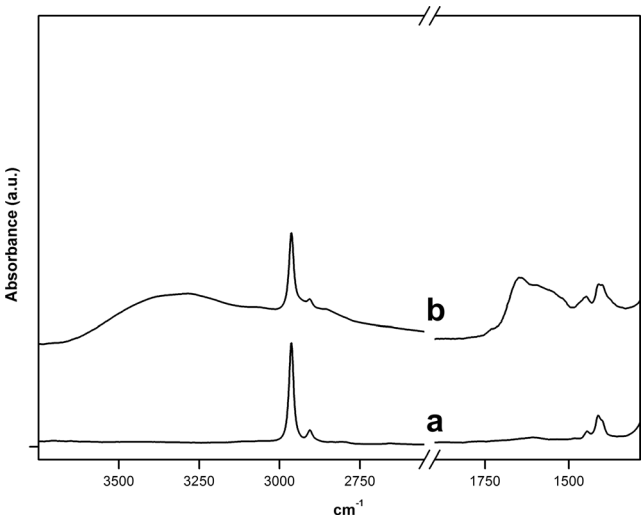


Fig. 2 Representative FTIR-ATR spectra of **a** untreated silicone and **b** acylase multilayer coatings

dimensional (2D) and three-dimensional (3D) analysis demonstrated nanostructural changes in the silicone surface topography after the multilayer coating assembly (Fig. 3a, b). After deposition of ten bilayers, the silicone surface was coated with relatively large clusters (Fig. 3b). The 2D AFM topographic profiles, analyzed using Nanotec WSxM software, showed that the surface roughness of acylase-coated silicone significantly increased when compared to non-treated silicone, with a peak height maximum about 35 nm (Fig. 3). According to the generally accepted polyelectrolyte multilayer buildup mechanism, the oppositely charged polyelectrolytes form small islets on hydrophobic surfaces, such as those of PDMS, at early deposition stages (Richert et al. 2003). As the number of deposited layers increases, the islets coalesce and become larger until a continuous coating is formed. However, in this study, the deposition of ten enzyme-PEI bilayers was not sufficient for a continuous and homogeneous coating formation. The number of bilayers required to completely cover a substrate surface would be lower when (i) the electrostatic interactions between the components of the coating are stronger and (ii) the conformations of the adsorbed components are adequate to favor the deposition (Follmann et al. 2012).

The differences in the surface coverage of the catheter material could be also observed in terms of wettability (Fig. 3). From AFM and water contact angle images, it is apparent that the wettability is directly proportional to the extent of silicone surface coverage with enzyme protein. The water contact angle of the untreated silicone specimen (136°±10°) decreased significantly after depositing the acylase multilayer coating (94°±9°) due to its hydrophilic character (Fig. 3).

The activity of the enzyme in the multilayer coating was further studied to analyze the functional properties of the modified silicone material. Acylase retained 80 % of its specific activity, when compared to the relative amount of protein immobilized on the silicone surface assessed by measuring the fluorescence of FITC-labeled acylase multilayer coatings (results not shown). The mild conditions used for LbL buildup, such as aqueous solution, may ensure activity preservation during the enzyme immobilization (Sakr and Borchard 2013).

In addition, the activity of acylase in the multilayered coatings and in solution was determined at physiological conditions by measuring the QS-regulated violacein production in reporter bacterial strain *C. violaceum* CECT 5999 (Sio et al. 2006). The degradation of C6-DL-HSL QS molecule by acylase was indicated by the decrease of violacein pigment production in the AHL containing liquid broth. Clear difference was observed between the positive control (non-treated silicone in the presence of QS molecules) and the samples containing immobilized/non-immobilized acylase together with the same concentration of QS molecule, where less violacein was synthesized (Fig. 4a), showing the ability of the acylase multilayer coatings to act upon the QS signals produced by *P. aeruginosa*.

The degradation of C6-DL-HSL in the liquid broth was also quantified spectrophotometrically after violacein extraction from the broth cultures of *C. violaceum* CECT 5999 using butanol (Blosser and Gray 2000). The C6-DL-HSL-inducible

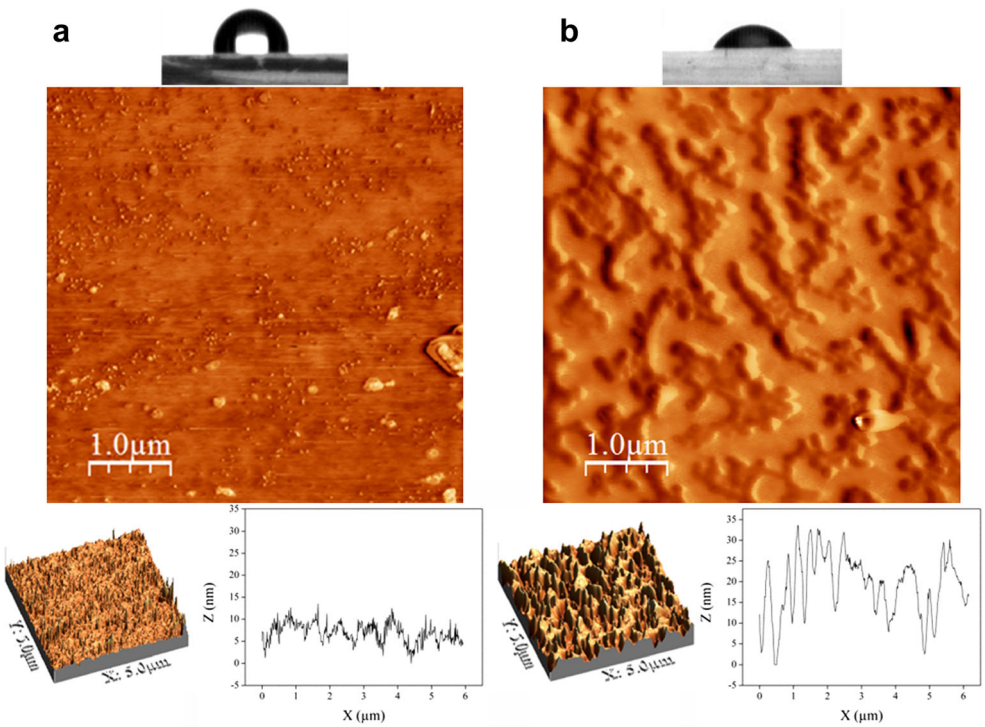


Fig. 3 AFM (2D and 3D topographic) and water contact angle images of **a** untreated silicone before APTES functionalization and **b** acylase multilayer coatings

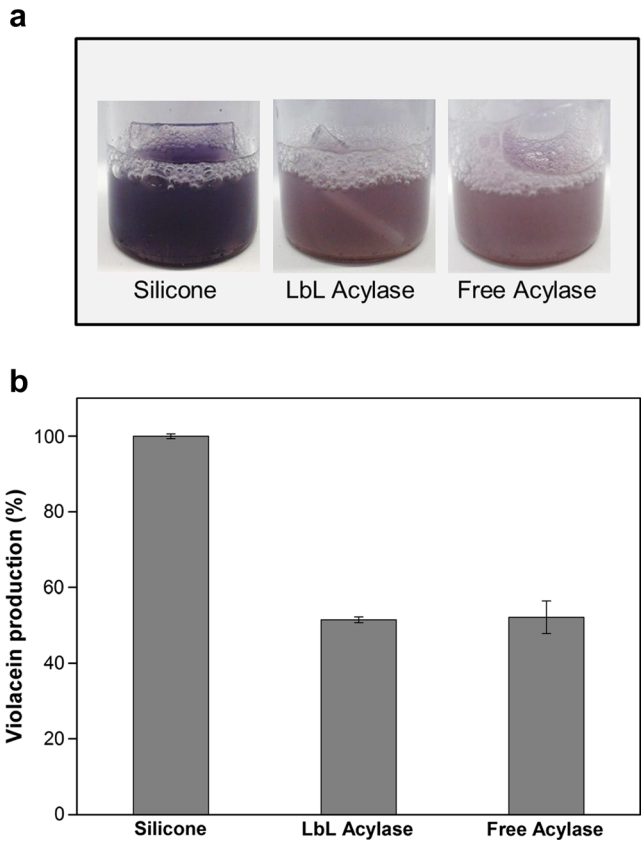


Fig. 4 Violacein production by *C. violaceum* CECT 5999 in the presence of free acylase solution and acylase multilayer coatings on silicone. The control—untreated silicone was set as 100 % violacein production

violacein production was inhibited by acylase up to 40 % in both free and immobilized forms of the enzyme (Fig. 4b). The reduced QS-regulated response of *C. violaceum* CECT 5999 by acylase-coated silicone catheters indicated their potential for inhibition of biofilm formation.

Functional properties of the acylase multilayer coatings

After proving the functionalization of silicone catheters with acylase using LbL technique as well as the activity of these multilayered coatings toward the *P. aeruginosa* QS signals, their functionality in terms of antibiofilm activity was also assessed. Since the initial bacterial adhesion is thought as a part of the biofilm development process on the surface, we studied whether the cell adherence would be delayed by the protein multilayer coatings. The initial attachment of *P. aeruginosa* ATCC 10145 (after 3 h of contact) was strongly inhibited by the acylase multilayered coating when compared to the untreated silicone material (Fig. 5a). Microscope images showed that *P. aeruginosa* ATCC 10145 formed bacterial clusters on untreated silicone as a first indicator for the initiation of biofilm formation, while the cells present on the acylase coatings were spread, and the clusters formed on the surface were considerably smaller (Fig. 5a). Hydrophobic interactions have been suggested as one of the important factors promoting bacterial cell adherence on surfaces (Palmer et al. 2007). The hydrophobic character of the silicone surface is believed to govern the primary adhesion of *P. aeruginosa* ATCC 10145, which possesses a thin layer of glycoprotein covered by a thick layer of lipoproteins and lipids facilitating the interaction of bacterial cells with various hydrophobic surfaces (Fu et al. 2005). The decrease of *P. aeruginosa* ATCC 10145 initial adhesion on the acylase-coated silicone surface could have been favored by the increased hydrophilicity of the enzyme-modified surface. These qualitative observations suggested that the process of mature biofilm formation could be delayed when silicone is coated with quorum quenching enzyme, e.g., acylase, corroborating the results obtained on the ability of acylase coatings to affect the QS process in the reporter strain *C. violaceum* CECT 5999 (Fig. 4).

Besides increasing the wettability of the silicone surface, the specific enzyme function in the coating is believed to have a major contribution to the inhibition of biofilm proliferation on the catheter material. To prove this assumption, the formation of *P. aeruginosa* ATCC 10145 biofilms after 24-h contact with pristine and coated silicone samples was studied in static conditions using crystal violet for total biomass assessment and Live/Dead cell viability kit for structural characterization of the developed biofilm. Fluorescence microscopy images after Live/Dead staining showed that the biofilm is well established on the untreated silicone material, with characteristic microcolonies of bacterial cells, while on the acylase-coated silicone, the biofilm was significantly reduced (Fig. 5b). A characteristic 3D structure was observed on the control with both viable (stained in green) and non-viable (stained in red) cells due to the repeatable pattern of death and lysis in biofilms, which corresponds to the normal course of biofilm development of *P. aeruginosa* (Webb et al. 2003). In contrast, only few bacteria spread individually on the silicone surface without the presence of typical bacterial clusters were observed for the acylase multilayer coatings (Fig. 5b). The total biomass assessment of the biofilms supported these results showing about 75±0.2 % inhibition of biofilm formation on silicone coated with quorum quenching enzyme, when compared to the untreated sample.

With the emergence of antibiotic-resistant bacteria, alternative approaches targeting different processes than bacteria killing mechanisms to control pathogenic biofilm formation on medical devices have been sought. While conventional antibiotics kill or inhibit bacteria by targeting their survival processes, interrupting the QS pathways through signal degradation efficiently quenched the QS process and inhibited *P. aeruginosa* ATCC 10145 biofilm formation in static conditions without affecting planktonic bacterial growth after 24-h contact (Fig. 6). Consequently, these multilayer coatings might prevent biofilm-associated urinary tract infections

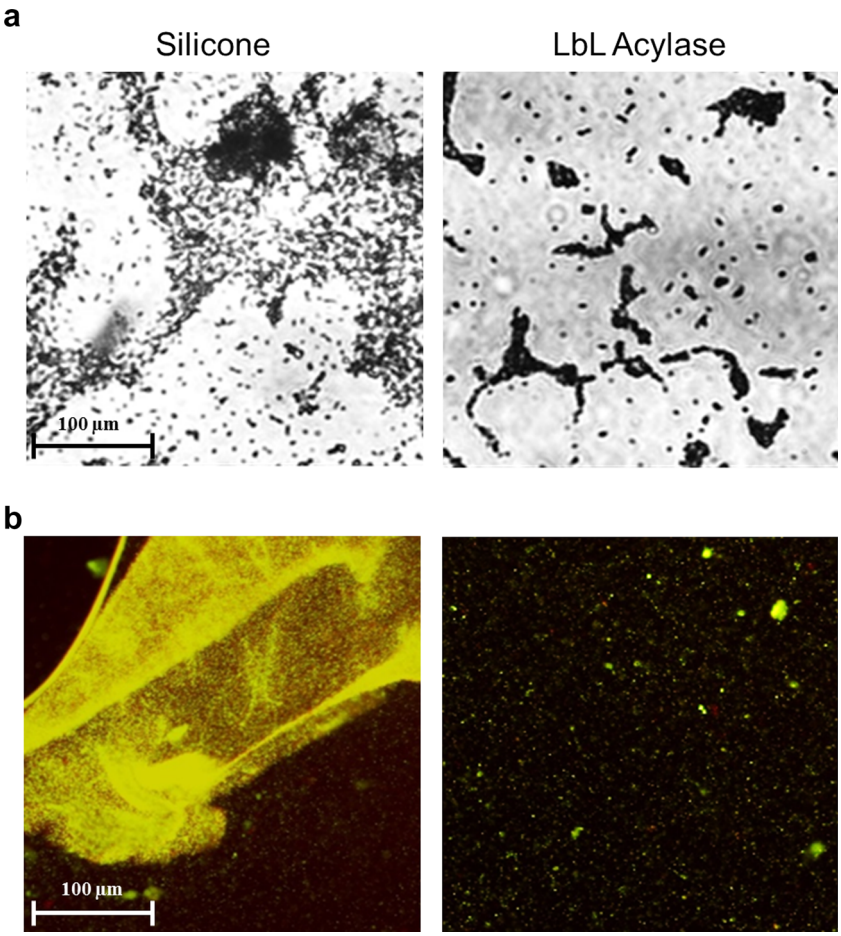


Fig. 5 **a** Initial adhesion of *P. aeruginosa* ATCC 10145 on untreated silicone and acylase multilayer coatings after 3-h contact (×40 magnification). **b** Fluorescence microscopy images of 24-h-old *P. aeruginosa* ATCC 10145 biofilms (formed in static conditions) on untreated silicone and acylase multilayer coatings analyzed after Live/Dead staining (×20 magnification)

exerting less selective pressure on the target bacteria and reducing the risk of resistance development.

However, aiming at developing enzymatic coatings for urinary catheters, the stability of the developed materials in artificial urine is a prerequisite due to the possible influence of the various compounds present in the human fluids on the enzyme activity. Therefore, the biofilm inhibiting activity of acylase-coated silicone samples (1 × 1 cm) was tested using crystal violet assay in static conditions after 1 and 7-day incubation in artificial urine. The results did not demonstrate any significant difference in the biofilm inhibiting capacity of acylase-based coatings and the freshly prepared ones (Fig. 7).

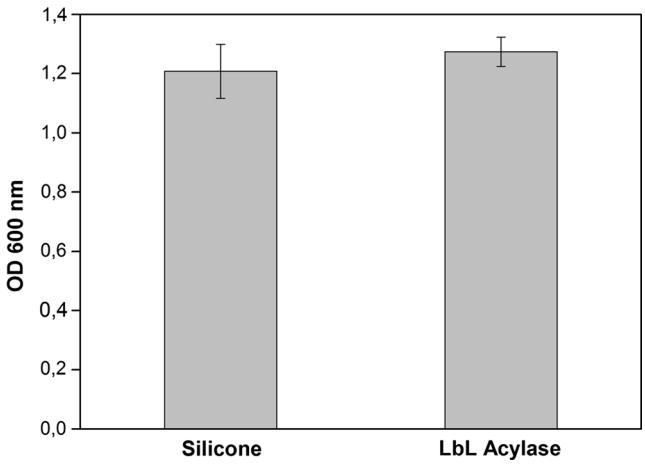


Fig. 6 *P. aeruginosa* ATCC 10145 planktonic growth inhibition in the presence of untreated silicone and acylase multilayer coatings

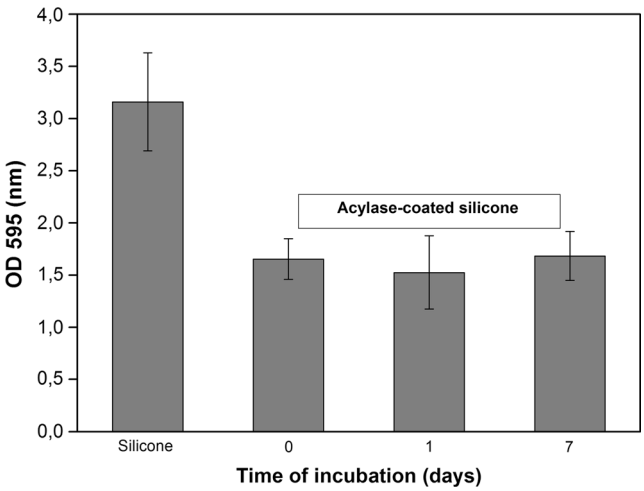


Fig. 7 Crystal violet assessment of biofilm formation of *P. aeruginosa* ATCC 10145 on untreated silicone and acylase multilayer coatings after incubation in artificial urine for 1 and 7 days

In vitro stability of acylase multilayer coatings under dynamic conditions in catheterized bladder model

Biofilm formation on pristine and acylase-coated urinary catheters was also evaluated in dynamic conditions using a catheterized bladder model, where the shear stress and flow rate vary similarly to the situation in the human bladder during catheterization. Biofilms were allowed to grow on the catheters for 7 days, and the biofilm inhibition by the acylase-based coating was analyzed using crystal violet to assess the total biomass on the catheter and Live/Dead cell viability kit for biofilm characterization (Fig. 8a, b). The fluorescence images after staining of live and dead bacteria attached to the balloon and urethra demonstrated decreased biofilm formation, when compared to pristine Foley catheters after 7-day catheterization (Fig. 8a). The bacterial biofilm formation was inhibited by 80 % in the balloon part, which was immersed over 7 days in the bladder model infected with *P. aeruginosa* ATCC 10145. The acylase-coated urethra part inserted into the model bladder was also able to inhibit the biofilm formation by 45 % compared to the untreated silicone Foley catheter (Fig. 8b). The results obtained with the dynamic system showed the same tendency in inhibiting biofilm formation as in static conditions. Therefore, the acylase-coated Foley catheters would have potential in preventing biofilm-associated urinary tract infections.

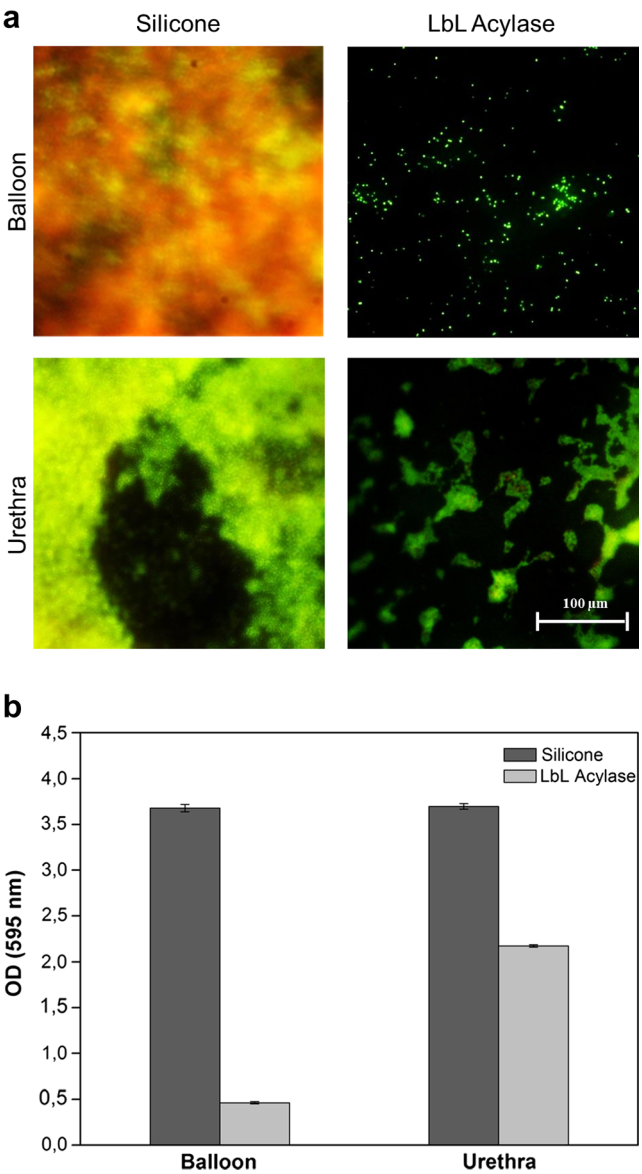


Fig. 8 **a** Microscopic images of untreated and acylase-coated Foley catheters after 7-day incubation in a dynamic bladder model system (×40 magnification). **b** Crystal violet assessment of total biomass formed on urinary catheters: untreated and acylase multilayer coatings on silicone Foley catheter

Biocompatibility evaluation

The biocompatibility of the multilayer coatings is an essential parameter for biomedical applications of the developed materials. It is important to ensure that they do not cause adverse effects upon interaction with human cells. Since PEI, regarded as cytotoxic, was the polycation used in the ten bilayers coating buildup, it was necessary to determine whether the combination of enzymes with PEI induced toxicity to cultured skin fibroblasts (Sarkar and Kundu 2012). The results of the direct contact study following fibroblast incubation with the silicone samples showed no cytotoxicity regardless of the contact time (Fig. 9). Either 24-h or 1-week contact did not induce cell toxicity, suggesting that the PEI present in the coating is in a rather low concentration to be toxic. Moreover, the interaction of PEI with the enzymes in the coating could attenuate its toxicity through "neutralization" of PEI positive charges (Lopez-Perez et al. 2010). Previous studies have shown that the toxicity of amine-terminated polymers is solely related to their positive charges. In particular, the acylation of the amine

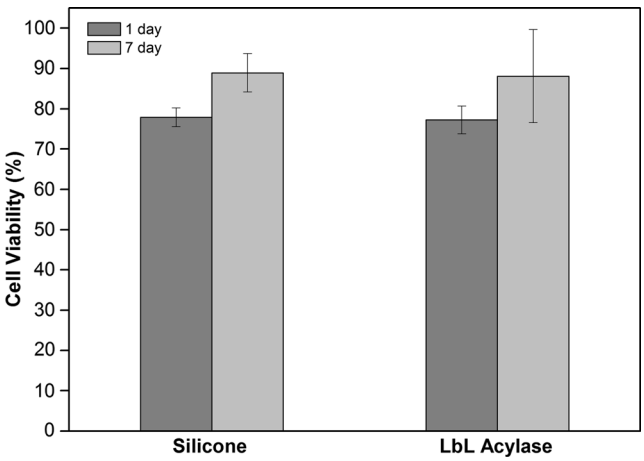


Fig. 9 Viability of fibroblasts (BJ-5ta) after being in contact with untreated silicone and acylase multilayer coatings: 1 and 7 days

groups of PEI to form neutralized and negatively charged materials has been shown to provide biocompatibility with mammalian cells (Hong et al. 2006).

Discussion

The emerging antibiotic resistance of bacteria encased in biofilms is the common reason for difficult-to-treat infections caused by colonized foreign bodies. This is a medical and public health issue that requires the application of novel approaches to counteract the biofilm growth on indwelling catheters. Strategies that target the bacterial molecular mechanisms involved in biofilm occurrence such as QS are reported to exert less evolutionary pressure on the bacterial population and thus could decrease the possibility of resistance development. Previous studies have demonstrated the key role of QS in biofilm differentiation of pathogenic *P. aeruginosa* into complex multicellular structures. This bacterium uses three types of QS systems: (i) *las*—sense 3-oxo-C12-HSL, (ii) *rhl* uses C4-HSL, and (iii) *pqs* is based on 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) that evidently regulates a number of factors important for biofilm development on various surfaces (Harmsen et al. 2010). The QS systems are hierarchically arranged, with the *las* system controlling the *rhl* and *pqs* QS systems. When these QS systems are blocked by mutation or by QS inhibitors, the bacteria lose ability to form well-defined surface-attached structures, i.e., biofilms (Christensen et al. 2007). Davies et al. (1998), for example, showed that mutants lacking the *las* QS system (incapable of using the 3-oxo-C12-HSL signal molecules) formed frail and unstructured biofilms sensitive to detergents. Because the *las* QS system is involved in the expression of numerous genes, it is believed that the biofilm-defective phenotype is multifactorial including defects in swarming and DNA release and defects in the production of other structural components of biofilm matrix (Sakuragi and Kolter 2007).

Based on the in vitro enzymatic inactivation of the model QS molecules observed in this study (Fig. 1), we assumed that acylase from *A. melleus* could inactivate the AHLs of *P. aeruginosa* ATCC 10145 and thereby inhibit the signal accumulation in the extracellular environment and subsequent biofilm formation. Xu et al. (2003) reported similar findings about acylase isolated from kidney, which efficiently degraded in vitro several model QS signals, C4-HSL and C8-HSL, and consequently inhibited *P. aeruginosa* biofilm formation on a polystyrene surface. Therefore, the ability of acylase to interrupt the cell-to-cell communication process that the Gram-negative *P. aeruginosa* uses to coordinate its biofilm-forming behavior suggested the enzyme application as antibiofilm coating on indwelling medical devices.

The biological activity and stability of the enzyme after immobilization was further evaluated. Despite that acylase in the free form was shown to efficiently degrade the QS signals and inhibit the *P. aeruginosa* biofilm formation (Xu et al. 2003), after immobilization this efficacy could be impaired due to the lower amounts of active protein attached onto silicone surface and loss of enzymatic activity. Nevertheless, only 20 % of acylase activity was lost during the deposition process, and the layers formed were shown to be antibiofilm active after 7-day contact with artificial urine (Fig. 7). In fact, the LbL technique used to construct these enzyme multilayer coatings is considered as a method that uses mild conditions (e.g., aqueous solutions) and could preserve the protein folding and activity. Most probably, the entrained water presented in between the polyelectrolyte layers is the reason why the proteins remained active on the surface (Sakr and Borchard 2013). Previous studies have shown that enzymes immobilized on different surfaces using the LbL approach retained high percentage of their catalytic activity and also were more stable than their free counterparts (Caseli et al. 2007; Kong et al. 1994; Onda et al. 1999). The enzyme-based assemblies developed in this work were also proven to inhibit the QS process through the degradation of C6-DL-HSL signal, similarly to what was observed for the free enzyme, and consequently decreased violacein production in the model bacterial strain *C. violaceum* CECT 5999 (Fig. 4). Moreover, the coatings counteracted QS-regulated biofilm formation of *P. aeruginosa* in static and dynamic conditions using an in vitro model of catheterized human bladder (Figs. 5 and 8).

Prior to the utilization of these enzyme multilayer coatings on urinary catheters in vivo, their potential cytotoxicity to human cells is an essential parameter that should be taken into account. Despite the presence of PEI, regarded as toxic, the acylase-coated silicone material did not cause any adverse effect upon interaction with human cells compared to the pristine silicone (Fig. 9). Neither 24 h nor 7 days of contact affected cell viability, suggesting that the PEI in the coatings is in a rather low concentration to affect the human cell viability (Sarkar and Kundu 2012). Moreover, the combination with negatively charged enzymes during the LbL assembly may reduce the availability of positively charged PEI amino groups thought to induce human cell cytotoxicity. Based on the results about the cytotoxicity and antibiofilm activity (Figs. 9 and 8, respectively), we assumed that the prolonged usage (over 7 days) of enzyme-modified catheters in vivo would not imply any concern for consumer's safety. Thus, the QQ active coatings could be a promising alternative to control biofilm-associated urinary tract infections.

The strategy used in this work to induce antibiofilm activity on indwelling medical devices is considered beyond the state of the art because it targets the inactivation of bacterial QS process, an approach that has received a great deal of attention

among researchers due to the antibiotic resistance issue. While conventional antibiotics kill or inhibit bacteria by targeting their structure or survival processes, interfering with QS pathways is thought to exert less selective pressure on bacterial population and could reduce the threat of resistance emergence. In fact, inactivation of AHL signals in the extracellular environment is a way of scrambling the wires of bacterial communication before it starts. As a consequence, bacteria will never know that they have achieved the threshold population density necessary to establish mature biofilm on silicone material.

In summary, we have developed coating on silicone catheter surface that significantly reduced the formation of *P. aeruginosa* biofilm due to the acylase-induced degradation of QS signals involved in bacterial cell-to-cell communication process. These findings suggested that the acylase-coated catheters could be a viable alternative to prevent QS-regulated biofilm occurrence on urinary catheters. Further studies regarding stability after sterilization and potential to counteract biofilm formation in vivo as a part of product development will reveal whether these novel coatings could be used to control biomaterial-associated tract infections caused by Gram-negative bacteria such as *P. aeruginosa*.

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Conflict of interest The authors declare no competing financial interests.

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