International Journal of Pharmaceutics 558 (2019) 72–81

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Immobilization of antimicrobial and anti-quorum sensing enzymes onto GMA-grafted poly(vinyl chloride) catheters

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ARTICLE INFO

Keywords:
Poly(vinyl chloride) catheters
Glycidyl methacrylate
Lysozyme
Acylase
Quorum sensing
Quorum quenching
Antimicrobial medical device

ABSTRACT

Catheter-associated infections still represent a challenging thread because of the likelihood of biofilm formation. The aim of this work was the surface modification of catheters to immobilize lysozyme and acylase under mild conditions while preserving antimicrobial and anti-*quorum sensing* performances. Glycidyl methacrylate (GMA) was grafted onto poly(vinyl chloride) (PVC) catheters by a pre-irradiation method. The effects of monomer concentration, pre-irradiation dose, reaction time, monomer concentration and reaction temperature were investigated. The grafting process was monitored using FTIR-ATR spectroscopy, differential scanning calorimetry, thermogravimetric analysis, and swelling data. Lysozyme was directly immobilized onto PVC-g-GMA maintaining the hydrolytic activity, which hindered *Staphylococcus aureus* adhesion. For acylase immobilization, the PVC-g-GMA catheters were reacted with ethylenediamine and glutaraldehyde in order to facilitate acylase covalent binding. Free acylase in solution demonstrated notably capability to act as *quorum sensing* inhibitor, as observed using *Chromobacterium violaceum* as biosensor, by degrading a wide variety of acylated homoserine lactones (AHLs), including those produced by *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Acylase-immobilized PVC-g-GMA catheters were challenged against degradation of AHLs and the activity monitored using both the biosensor and HPLC-MS. Relevantly, the functionalized catheters completely degraded all tested AHL signals, opening new ways of preventing biofilm formation on medical devices.

1. Introduction

Implantable or insertable biomedical devices have become indispensable tools in healthcare steps from diseases diagnosis to the physical replacement of organs or their functions (European Commission, 2010). Nevertheless, the presence of a medical device usually compromises immunologically to the adjacent zone increasing the risk of infection (Rochford et al., 2012). Device surfaces are prone to sorb proteins that facilitate the adhesion of microorganisms that enter into contact with the device accidentally from the skin of the patient or that migrate from a previous focus of infection in the patient (Chouirfa et al., 2018). If the host immune response is compromised, pathogenic agents cannot be cleared from the device surface via phagocytosis. Bacterial colonization may lead to biofilm formation, which results in clinical failure of the medical device and may compromise the life of the patient (Rochford et al., 2012; Alvarez-Lorenzo et al., 2016; Schierholz and Beuth, 2001). Healthcare-associated infections affect to a large number of patients both outside and inside intensive care units, and nearly 1 out of 10 patients with healthcare-associated infections dies (Centers for Disease Control and Prevention, 2016).

Relevantly, urinary catheters are the most common indwelling devices, being used in approx. 20% hospitalized patients in Europe and the USA (Magill et al., 2014; Nicolle, 2014). Insertion maneuver may drag urethral organisms into the bladder, which then remains directly connected to the external part of the catheter facilitating the access of further microorganisms (Warren, 2001). Bacteria adhered to the catheters multiply rapidly, and thus the risk of an urinary tract infection increased with the duration of the catheterization at a rate of 3–7%/day

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https://doi.org/10.1016/j.ijpharm.2018.12.075
Received 5 October 2018; Received in revised form 4 December 2018; Accepted 29 December 2018
Available online 09 January 2019
0378-5173/

(Nicolle, 2014). Overall, nearly 80% of urinary tract infections are associated to the use of catheters. The biofilm protects the bacteria from the host defenses and the systemically or locally applied antibiotics. Further growth of the microorganisms may lead to incrustations until complete blockage of catheter and also to migration through the damaged urinary epithelium causing a systemic infection (Warren, 2001; Jones et al., 2005). High susceptibility and difficulties to prevent/treat catheter-associated infections are prompting a strong research on the development of anti-infective biomaterials (Rochford et al., 2012; Campoccia et al., 2013; Singha et al., 2017).

Incorporation of antibiotics in the bulk or in the coating of the biomaterials has been extensively tested as way to achieve effective local concentration for an extended time period minimizing toxicity risks derived from systemic administration (Campoccia et al., 2013; Alvarez-Lorenzo et al., 2010). Nevertheless, the regular use of antibiotic-loaded medical devices for prophylaxis may increase the occurrence of antibiotic-resistant bacteria, which is even more likely for sessile bacteria embedded as communities into the biofilm (Campoccia et al., 2013; Chen et al., 2013; Boo et al., 2015). As a result, alternative bio-based strategies relying on enzymes that can inhibit cell growth or disrupt biofilm forms has gained broad interest (Chen et al., 2013; Glinel et al., 2012; Thallinger et al., 2013). The cell-to-cell communication system of bacteria, called *quorum sensing* (QS), allows them to coordinate their behavior to form the biofilm and to synchronize gene expression, especially of genes responsible of virulence (Dong and Zhang, 2005; Kalia, 2013). QS in Gram-negative bacteria is mediated by diffusible signaling molecules such as the *N*-acyl-homoserine lactones (AHLs) that bacteria release, detect and respond to in a concentration-dependent manner (Whitehead et al., 2001). The interference of bacterial communication, so called *quorum quenching* (QQ), disrupts the ability of the pathogen to sense its cell density through the trapping or the enzymatic degradation of the signal. In that way, QQ provides an opportunity for disease control blocking certain gene expression, including the genes required for biofilm formation (Chen et al., 2013; Huang et al., 2016; Morohoshi et al., 2013; Brackman et al., 2016).

In this regard, acylases are AHL-degrading enzymes that hydrolyze the amide linkage between the lactone and the side acyl chains in an irreversible manner leading to the inactivation of the signaling molecule (Huang et al., 2016; Hong et al., 2012). The acylase I from *Aspergillus melleus* (ACY1) has been immobilized onto membranes to mitigate biofouling in nanofiltration process (Kim et al., 2011) and on silicone catheters using a layer-by-layer method (Ivanova et al., 2015a,b), or incorporated to the bulk of polyurethane coatings (Grover et al., 2016) to inhibit *Pseudomonas aeruginosa* biofilm. Also recently, combination of an acylase with other antimicrobial enzymes with a different mechanism of action, such as esterases (Kisch et al., 2014) or amylases (Ivanova et al., 2015b), has been explored regarding synergic effects. Nevertheless, acylase covalent immobilization is quite challenging because the most reactive lysine amino acids are close to the active site, which may compromise the access of the AHL substrates (Scaramozzino et al., 2005). Moreover, differently to other antimicrobial enzymes such as lysozyme (pI of 11.35), the highly negatively charged structure of acylase at neutral pH (pI is 5.1) (Ivanova et al., 2015a,b) hinders the reaction with common immobilizing agents (Scaramozzino et al., 2005).

The aim of this work was to implement a procedure for the immobilization of antimicrobial enzymes, namely lysozyme and acylase, onto poly(vinyl chloride) (PVC) urinary catheters using glycidyl methacrylate (GMA) as an intermediate for multipoint covalent binding. The work relies on the hypothesis of that an adequate number of glycidyl mers on the surface of the catheters and mild reaction conditions may allow the binding of the enzymes while preserving their antimicrobial and quorum quenching activities, thus minimizing the risk of healthcare-associated infections. To carry out the work, the catheters were first decorated with poly(GMA) using gamma-ray radiation grafting; a technique commonly used for surface functionalization of medical devices that does not require initiators or catalyzers and operates at radiation doses similar to those used for sterilization (Alvarez-Lorenzo et al., 2016). Dependence of the grafting yield on absorbed dose, monomer concentration and reaction time and temperature was evaluated in detail. Then, direct immobilization of lysozyme and acylase was investigated in separate because of their different structural properties. Lysozyme (molecular weight of 14.3 kDa) is positively charged at neutral pH and its mechanism of action relies on their ability to degrade polysaccharides of Gram-positive and Gram-negative cell walls (Thallinger et al., 2013). As *quorum quencher* we chose acylase I from porcine kidney because it exhibits high specific activity and is does not require pre-activation unlike other aminoacylases (Chibata et al., 1976). Its molecular weight is 86 kDa (two subunits of 43 kDa) and the dimerization is crucial for the catalytic activity since the active site lies between its two zinc binding domains. Differently to lysozyme that can directly react with poly(GMA), the highly negatively charged structure of acylase may obligate to use intermediate linkers; in the present work ethylenediamine was investigated as a second intermediate. Finally, antimicrobial and *quorum quenching* performance of the enzyme-immobilized catheters was evaluated. Since each acylase is active against different AHL molecules, to fully elucidate the therapeutic value of the functionalized catheters they were challenged against a large battery of AHLs bearing short and long, oxo- and non-substituted, acyl chains.

2. Materials and methods

2.1. Materials

Poly(vinyl chloride) (PVC) urinary catheters (diameter 3 mm, thickness 0.5 mm; Biçakcilar, Turkey) were washed with ethanol for 24 h and then dried under reduced pressure. Glycidyl methacrylate (GMA) was from Sigma Aldrich (St. Louis, MO, USA) and purified by vacuum distillation. Acylase I from porcine kidney (500–1500 units/mg protein) (EC Number 3.5.1.14), L-methionine, *N*-acetyl-L-methionine, acyl-homoserine lactones (AHLs), and *Micrococcus lysodeikticus* (ATCC 4698) were from Sigma Aldrich (St. Louis, MO, USA). Ethylenediamine (EDA; 99.5%, absolute) was from Fluka Chemika (Buchs, Switzerland). Glutaraldehyde (50% pure solution) and ethyl acetate (HPLC grade) were from Scharlau Chemicals (Barcelona, Spain). Ethanol from Baker Co. (ME, USA) was used as received. Distilled water was used for all experiments.

Biosensor strains *Chromobacterium violaceum* CV026 CECT5999 (McClean et al., 1997) and VIR07 ATCC12472 (Morohoshi et al., 2008) were used for AHLs degradation assay. These strains were cultured on Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/mL) at 30 °C. The AHLs *N*-hexanoyl (C6-HSL), *N*-oxohexanoyl (OC6-HSL), *N*-octanoyl (C8-HSL), *N*-oxooctanoyl (OC8-HSL), *N*-decanoyl (C10-HSL), *N*-oxodecanoyl (OC10-HSL), *N*-dodecanoyl (C12-HSL), *N*-oxododecanoyl (OC12-HSL), *N*-oxotridecanoyl (OC13-HSL), *N*-tetradecanoyl (C14-HSL), *N*-oxotetradecanoyl (OC14-HSL) and *N*-hydroxydodecanoyl (OHC12-HSL) were from Sigma Aldrich (St. Louis, MO, USA).

2.2. Grafting GMA onto PVC catheters

PVC catheters were irradiated with a ^{60}Co gamma source (Gammabeam 651PT, Nordion International from Ottawa, Canada, placed at UNAM), in the presence of air at room temperature, dose rate of 9.9 kGy h^{-1} and absorbed dose from 5 to 70 kGy. The irradiated PVC catheters were placed in glass ampoules containing GMA solutions in ethanol/water medium (75/25 vol%). GMA concentration varied from 10 to 60% vol/vol. The ampoules were sealed in vacuum after degassing by repeated freeze/thaw cycles. Reaction was carried out at temperature ranging from 40 to 80 °C for 1 and 6 h. The grafting reaction was conducted by placing the ampoules in a water bath set at fix

temperature. Then, the grafted catheters were taken out from the monomer solution and washed with ethanol during 24 h to remove the unreacted monomer and the formed homopolymer. The obtained catheters were referred as PVC-g-GMA.

The grafting percentage (Yg) was calculated as follows:

$$Y_g(\%) = \frac{W_g - W_0}{W_0} \times 100 \quad (1)$$

where W_0 and W_g represent the weights of the initial and the grafted catheter, respectively.

PVC-g-GMA catheters were characterized by FTIR-ATR (attenuated total reflection) using Perkin-Elmer Spectrum 100 spectrometer (Perkin Elmer Cetus Instruments, Norwalk, CT, USA). Differential scanning calorimetry scans were recorded at heating rate of 10 °C min^{-1} using a DSC 2010 calorimeter (TA Instruments, USA). Thermogravimetric analysis were performed with a TGA Q50 (TA Instruments, New Castle, DE, USA) at heating rate of 10 °C min^{-1} under nitrogen atmosphere. For determination of water uptake equilibrium, catheter pieces were immersed into distilled water and the weight of the catheters was recorded every 15 min for 3 h. The excess of water on the PVC surface was wiped with paper, and the swollen catheters were weighed.

Swelling % was calculated as follows:

$$Swelling(\%) = \frac{W_s - W_d}{W_d} \times 100 \quad (2)$$

where W_s and W_d are the weights of the swollen and the dried grafted, respectively.

SEM images of previously swollen catheters and subsequently freeze-dried were recorded in a scanning electron microscope (JEOL JSM-7401F).

2.3. Lysozyme immobilization and enzymatic activity assay

Catheters were hydrated in ultrapure water for 24 h at 37 °C. Then, pieces (1 cm length) of the catheters were introduced in vials containing 5 mL of a lysozyme solution (2.5 mg/mL in PBS pH 8) and kept under agitation (100 rpm orbital shaker) for 48 h. Finally, functionalized samples were washed in PBS pH 7.4 to remove the non-attached lysozyme and kept at 5 °C until use.

Enzymatic activity of lysozyme immobilized on the catheters was evaluated against *Micrococcus lysodeikticus* (*M. luteus*) (Ollis and Datta, 1976). Lysozyme-functionalized catheters and controls (bare catheters) were incubated in a suspension of *M. lysodeikticus* in PBS (pH 6.22) (previously adjusted to an absorbance of 1.22) and the absorbance, which was inversely proportional to bioactivity, was recorded at 450 nm as a function of time. Lysozyme activity was expressed in *M. lysodeikticus* units, which were defined as ΔA450 of −0.001/min and normalized against that showed by cultures in presence of bare catheters (controls).

2.4. Bacterial adhesion on lysozyme-immobilized catheters

Staphylococcus aureus (ATCC 25923) was cultured in trypticase soy agar overnight at 37 °C. Portions (1 cm length) of the PVC-g-GMA catheters with and without lysozyme immobilized were placed in vials, and 2 mL of a bacterial suspension (in TSB; 3.2×10^9 CFU/mL) was added to each vial and incubated at 37 °C for 2 h. Then, catheters were washed three times in PBS to eliminate the non-adherent bacteria. 2 mL of PBS was added to each vial and sonicated (Branson Sonifier 250; Branson Ultrasonics, Danbury, CT USA) for 5 min to resuspend the attached bacteria. Bacterial suspensions were serially diluted and spread on TSA plates and cultured for 24 h at 37 °C to determine the number of bacterial cells (CFUs). Bare catheters were used as controls.

2.5. Functionalization of epoxy groups of PVC-g-GMA catheters with EDA

The epoxy groups of catheters were functionalized with EDA adapting a procedure previously reported (Kim et al., 1996). Pieces (1 cm) of pristine PVC (control) and PVC-g-GMA catheters with different graft percentages were washed in ultrapure water during 3 h. After that, catheters were dried at 50 °C overnight. Four replicates of each PVC-g-GMA catheter with different graft percentage were tested.

Dried PVC-g-GMA catheters and controls were individually immersed in 4 mL of EDA at 30 °C and kept under stirring for 4 h. Then, catheters were removed and immediately washed three times with abundant water and finally dried at 50 °C in an oven for 5 h. The obtained catheters were referred as (PVC-g-GMA)-EDA.

Conversion degree of the epoxy groups into aminated groups was calculated, as follows:

$$Conversion(\%) = \frac{(W_2 - W_1)/60}{(W_1 - W_0)/142} \quad (3)$$

In this equation, W_2 represents the weight of PVC-g-GMA catheter after functionalization with EDA, W_1 the initial weight of PVC-g-GMA, and W_0 the initial weight of PVC catheter whereas 60 and 142 are the molecular masses of EDA and GMA, respectively.

2.6. Acylase immobilization

Firstly, aminated catheters and controls were individually placed in vials and covered with a 10% (v/v) glutaraldehyde solution (4 mL) for approx. 10 min at room temperature. Then, the catheters were rinsed with ultrapure water three times to eliminate the unreacted glutaraldehyde, and immediately immersed in separate in an acylase solution (1 mg/mL) prepared in ultrapure water. Catheters were incubated at 25 °C in an oscillatory shaker for 24 h. After immobilization, catheters were removed from the incubation medium and rinsed with ultrapure water for removal of non-covalently immobilized enzyme. Finally, samples were stored in ultrapure water at 4 °C for subsequent activity assays.

2.7. Acylase activity assay

Activity of acylase both in solution and after immobilization onto the catheters was measured adapting the standard hydrolytic assay of the substrate *N*-acetyl-L-methionine (Bakker et al., 2000). The enzyme activity was referred as the amount of enzyme necessary to produce 1 μmol of L-methionine per hour at 37 °C. Catheters (1 cm) were individually immersed in *N*-acetyl-L-methionine (20 mM; 5 mL) solution prepared in a Tris buffer (50 mM; pH 7.4). For soluble acylase, 0.5 mL of solution (0.1 or 0.5 mg/mL) in phosphate buffer pH 7.4 was added to 5 mL of 15 mM *N*-acetyl-L-methionine solution. Systems containing either soluble or immobilized acylase were incubated at 37 °C in an oscillatory shaker (200 osc/min). Periodically, aliquots (0.5 mL) were withdrawn for measuring of amount of L-methionine formed by hydrolysis reaction using HPLC. The enzymatic reaction of soluble acylase aliquots was stopped by the addition of HCl 1 M. Experiments were carried out in duplicate.

For the HPLC method, stock solutions of L-methionine and *N*-acetyl-L-methionine (50 mM) were individually prepared. Aliquots of these solutions were mixed to prepare standard solutions having both L-methionine and *N*-acetyl-L-methionine at the same concentration. The selected concentration range was 1–20 mM. Before HPLC analysis, each sample or standard solution was filtered through 0.2 μm GHP membrane filters (Acrodisc, Waters, USA). Quantification of the substrate *N*-acetyl-L-methionine and the product L-methionine was performed using a Waters HPLC system (Controller 600) equipped with a C18 reversed phase column (3.9 mm × 150 mm, 5 μm from Simmetry®, Waters, USA) and a photodiode array detector (996, Waters, USA). Samples and standard solutions were eluted isocratically using a mobile phase of

12.5 mM aqueous phosphoric acid and acetonitrile in a ratio of 80:20 (v/v). The flow rate was set to 0.8 mL/min and the detector to 210 nm. The retention times were 2.7 and 1.5 min for *N*-acetyl-L-methionine and L-methionine respectively.

2.7.1. Determination of kinetic constants

Kinetic studies were performed to estimate the effect of substrate concentration on initial rates of enzymatic hydrolysis. The Michaelis-Menten kinetic model was applied using Origin 8.0 (OriginLab Corporation, USA) and kinetic parameters, Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were calculated using the following equation

$$v_0 = \frac{V_{max} \times [S]}{K_m + [S]} \quad (4)$$

In this equation, v_0 is the initial rate of reaction and [S] is the concentration of substrate. Both acylase solutions (0.5 mg/mL) and catheters with immobilized acylase were incubated in *N*-acetyl-L-methionine solutions (5 mL; 3–20 mM) at 37 °C, and the initial rate of reaction was calculated as μmoles of L-methionine obtained per hour. Kinetic studies were analyzed by plotting the reaction rate versus substrate concentration.

2.8. AHLs degradation by acylase in C. violaceum bioassay

Soluble Acylase I was first screened against a variety of AHLs. The AHLs stock solutions (1 mg/mL) were prepared in acetonitrile and diluted in sterile PBS to prepare the working solutions. AHLs were individually added to the acylase solution (1 mg/mL in 0.2 M phosphate buffer pH 7.5) for a final AHL concentration of 2 μg/mL. Controls were prepared in PBS at the final same concentration. Samples and controls were incubated for 24 h at room temperature in a shaker (200 rpm).

C. violaceum CV026 and VIR07 strains were used as biosensors for short and long acyl chain AHLs respectively. These biosensors produce the purple pigment violacein as a response to exogenous added AHL. After incubation, aliquots (90 μL) of the controls and reaction mixtures were placed in wells (6 mm) on LB agar plates covered with a mixture of 4 mL of soft LB (0.8% agar) and 1 mL of an overnight culture of the selected *C. violaceum* strain. Plates were incubated for 24 h at 30 °C, and examined for the presence of violacein in order to verify AHL degradation.

The degradation activity of acylase immobilized onto catheters against OC12-HSL and C12-HSL was also evaluated. Pieces (0.5 cm) of control and acylase-immobilized catheters with different graft percentage were individually incubated in AHLs solutions (5 μg/mL; 0.5 mL final reaction volume) in sterile PBS. As controls, AHLs solutions (5 μg/mL) in sterile PBS were used. Samples were incubated for 24 h at room temperature in a shaker. The remaining AHL activity in controls and reaction mixtures was checked using the *C. violaceum* agar plate bioassay described above.

2.9. Determination of AHLs degradation by HPLC-MS

Degradation of C12-HSL, OC12-HSL and OHC12-HSL by soluble acylase or acylase immobilized onto catheters was also evaluated and quantified using HPLC-MS. Pieces (1 cm) of control and acylase-immobilized catheters with different grafting percentage were individually incubated in AHLs solutions (2 μg/mL; 0.5 mL) in sterile PBS. Acylase solutions (1 mg/mL in 0.2 phosphate buffer pH 7.5; 0.5 mL) were also incubated with AHLs individually. As controls, AHLs solutions (2 μg/mL; 0.5 mL) in sterile PBS were used. For AHLs degradation, both samples and controls were incubated for 24 h at room temperature in a shaker (200 rpm). Experiments were carried out in triplicate.

The remaining AHLs were extracted from aliquots (400 μL) of the reaction mixtures and controls twice with the same volume of ethyl acetate and then evaporated under nitrogen flux. Dried extracts were resuspended in 1 mL of acetonitrile for further HPLC-MS analysis and quantification. The HPLC system used was an Agilent 1100 series (Agilent Technologies, USA) equipped with a Zorbax Eclipse XBD-C18 column (4.6 mm × 150 mm, 5 μm particle size, Agilent, USA). The mobile phase was 0.1% formic acid in water (A) and methanol (B) and the flow rate was 0.4 mL/min. Gradient HPLC method was performed and the elution conditions were as follows: 50% B from 0 to 10 min followed by a linear gradient from 50 to 90% over 15 min, and finally 90% B for 25 min. The column was re-equilibrated for a total of 4 min. The MS experiments were carried out by an API4000 triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems, USA) used in positive ion electrospray mode. Remaining AHLs were quantified comparing with a calibration curve of the appropriate AHL synthetic standards.

3. Results and discussion

3.1. Preparation of PVC-g-GMA

PVC is widely used as component of medical devices but also very prone to bacteria adhesion. Owing to its inert nature, numerous methods have been tested to endow PVC with antimicrobial properties (Herrero et al., 2016; Islas et al., 2016). Surface functionalization has been extensively investigated as a way to incorporate antimicrobial agents to polymers that per se exhibit poor affinity for bioactive compounds, while allowing the polymer-based medical devices to retain their mechanical properties for their primary mode of action (Alvarez-Lorenzo et al., 2010; Goddard and Hotchkiss, 2007). In the present work, a pre-irradiation oxidative method was evaluated for the grafting of GMA from PVC catheters. Grafting sites were generated on the carbon backbone by replacement of chlorine atoms with peroxy and hydroperoxy intermediates (Arenas et al., 2007). The activated catheters were then immersed in GMA solutions and subsequently heated in order that the intermediates decomposed into oxyradicals able to react with the GMA double bond. The formed macroradicals propagated the grafting onto PVC (Zuñiga-Zamorano et al., 2018).

Effect of irradiation dose, GMA concentration (in ethanol/water mixture) and reaction time and temperature on the grafting yield was first evaluated. For a pre-irradiation dose of 40 kGy, the grafting percentage progressively increased with the reaction time until 6 h, attaining a maximum close to 120% (Fig. 1A). Beyond this time, grafting percentage remained nearly constant. The effect of absorbed dose on GMA grafting was evaluated keeping constant the dose rate (9.9 kGy h^{-1}), the reaction time (5 h), the reaction temperature (60 °C) and the monomer concentration (50% in ethanol/water) (Fig. 1B). As irradiation dose increased, higher amounts of free radicals were generated and consequently graft polymerization increased. Grafting percentages were around 15 and 155% at 20 and 70 kGy, respectively. When irradiation dose exceeded 70 kGy, homopolymerization of GMA occurred at a large extent and the homopolymer was difficult to remove from the grafted PVC. Grafting yield leveling off was not reached in the range of doses studied.

The effect of GMA concentration on the grafting yield at 60 °C for a reaction time of 5 h was examined (Fig. 1C). The grafting percentage became higher as the GMA concentration increased, which is likely due to the direct effect of monomer concentration on the polymerization rate. Above 60% GMA concentration, a gel-like material covered the catheter. This effect results from a slowing of the termination step owing to the lack of mobility of the growing chains, increasing the yield of grafting and the crosslinking of GMA. However, higher concentration of GMA also favored homopolymerization (Bhattacharya and Misra, 2004). The grafting percentage ranged from a minimal value of 5% at 10% monomer concentration, to a saturation value close to 90% at 60% monomer concentration.

The influence of the reaction temperature on grafting percentage is shown in Fig. 1D, for catheters pre-irradiated at a dose of 40 kGy and

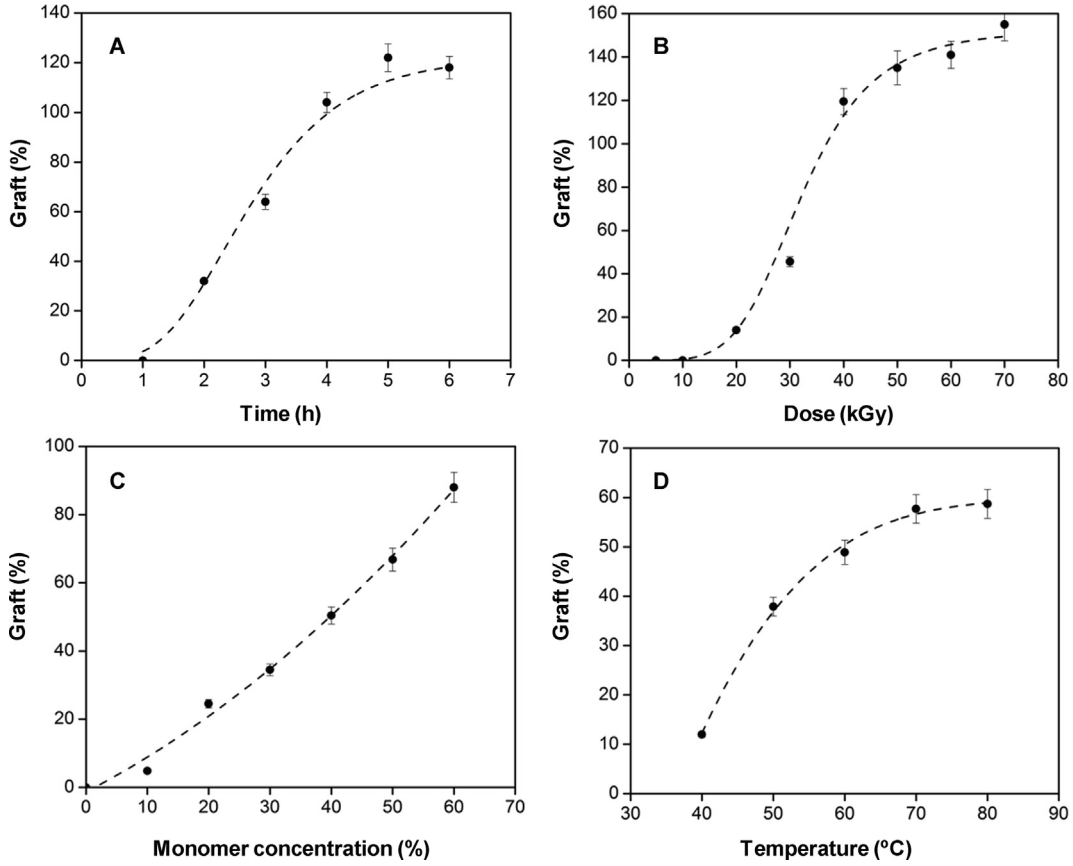

Fig. 1. Dependence of poly(GMA) grafting percentage onto pre-irradiated PVC catheters on (A) reaction time at 60 °C for a monomer concentration 50% and a pre-irradiation dose of 40 kGy; (B) pre-irradiation dose at monomer concentrations 50%, t = 5 h, and T = 60 °C; (C) monomer concentration, D = 40 kGy, t = 5 h, and T = 60 °C; and (D) reaction temperature at t = 5 h, monomer concentration 50%, and a pre-irradiation dose of 40 kGy.

5 h reaction in GMA 50% vol/vol. Grafting percentage augmented progressively as temperature increased from 40 to 70 °C. The increase of temperature allows faster diffusion of the monomer towards the catheter surface and facilitates the decomposition of hydroperoxides and peroxides providing higher amount of free-radicals for graft-polymerization (Bhattacharya and Misra, 2004). However, grafting ability decreased as temperature increased, as observed at 70 °C or above. This could be due to molecular motion and radical decay with higher temperatures; even temperature above *Tg* may decrease radicals causing lower grafting yields (Bhattacharya and Misra, 2004).

3.2. Characterization of PVC-g-GMA

FTIR-ATR spectra of pristine PVC, PVC-g-GMA and poly(GMA) formed during grafting reaction are shown in Fig. 2. The spectrum of pristine PVC catheter (Fig. 2a) showed a broad band in the 2850–3000 cm^{-1} region due to the symmetric and asymmetric stretching vibrations of CH_2 groups. Characteristic bands ascribed to C-Cl bonding were observed at 742 and 690 cm^{-1}. Owing to presence of plasticizer, a band at 1721 cm^{-1} from carboxylic groups was observed. The spectrum of PVC-g-GMA showed distinctive bands from GMA confirming the graft polymerization onto PVC catheters (Fig. 2b). Characteristic absorption bands of ester group at 1724, 1254 and 1145 cm^{-1} and bands ascribed to epoxy group at 905 and 843 cm^{-1} were observed, as for poly(GMA) homopolymer (Fig. 2c) (Kimmins et al., 2014). Furthermore, graft polymerization produced disappearance/reduction of bands from C-Cl bond in PVC-g-GMA spectrum.

The DSC thermograms of pristine PVC, poly(GMA) and GMA-grafted PVC catheter are shown in Fig. S1 (Supporting Information). Pristine

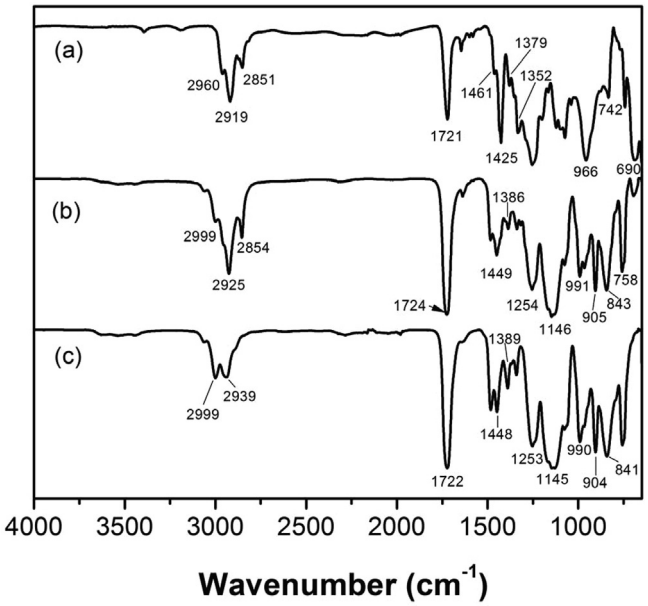

Fig. 2. FTIR-ATR spectra of a) PVC catheter, b) PVC-g-GMA 156% catheter, and c) poly(GMA).

PVC and poly(GMA) exhibited *Tg* at 103 °C and 75 °C respectively (Fig. S1 a and c). The DSC thermogram of PVC-*g*-GMA showed an exothermic peak at 144 °C (Fig. S1 b), probably due to a crystalline structural rearrangement of grafted GMA during heating (Xu et al., 2015). Regarding TGA scans (Fig. S2, Supporting Information), the temperature

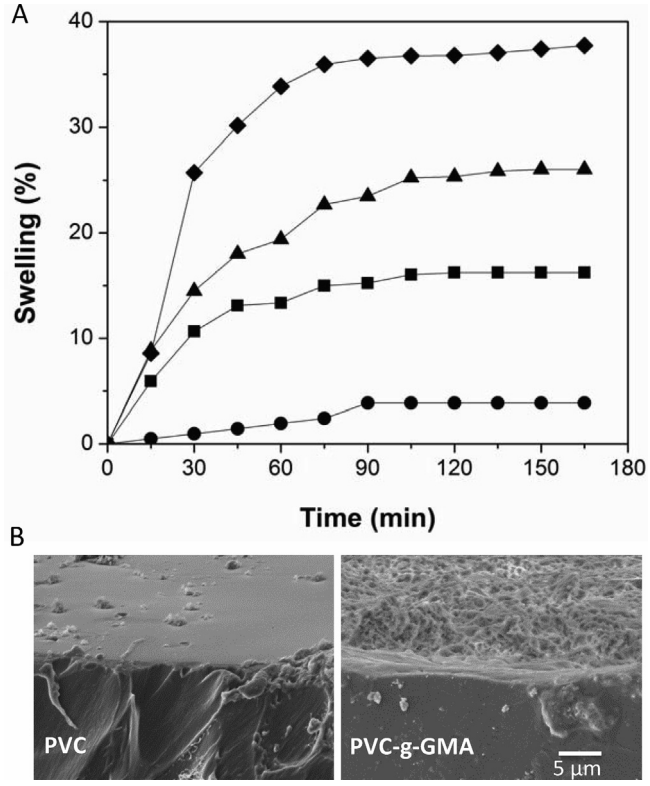

Fig. 3. (A) Swelling kinetics of PVC catheters with poly(GMA) graft contents of 14% (●), 46% (■), 120% (▲), and 156% (◆) in water at room temperature; and (B) SEM images of pristine PVC and of PVC-g-GMA 156% catheter (transversal cut partially showing the outer surface of the catheter).

at which 10% weight loss occurred was 248.7 °C for pristine PVC, 233.3 °C for poly(GMA), and 305.7 °C for PVC-g-GMA, which indicates that the grafted catheters exhibit good thermal stability.

Differently to PVC catheters that do not uptake water, catheters grafted with GMA exhibited certain ability to swell (Fig. 3A). Swelling equilibrium was reached in ≈100 min and the swelling percentage increased with the content in GMA. The hydrophilic character of PVC-g-GMA catheters increased with the length of poly(GMA) chains onto the PVC catheters. SEM images of freeze-dried (previously swollen) catheters prepared with the highest graft percentage revealed the homogeneous coating of the catheters surface with a spongy hydrogel layer of few microns thickness (Fig. 3B).

3.3. Lysozyme immobilization

Epoxy-activated supports are commonly used for enzyme immobilization through covalent linkages under mild conditions (Datta et al., 2013; Krishnamoorthi et al., 2015). Thereby, enzymes could maintain their enzymatic activity for long-terms (Barbosa et al., 2013; Mateo et al., 2000a,b), which may be particularly useful to endow medical devices with antimicrobial performance. Lysozyme readily reacted with PVC-g-GMA, which may be favored by the predominance of basic amino acids arginine and lysine. Antimicrobial activity of PVC-g-GMA catheters with and without lysozyme immobilized was tested against *M. lysodeikticus* at pH 6.22. The human urine may have a wide variety of pH values, but acidic values are more common. Enzymatic activity was observed in all catheters functionalized with lysozyme (Fig. 4), but not in PVC-g-GMA without the enzyme. Catheter activity was related to the graft percentage; the catheters with the highest grafting tested (27.7%) exhibited the highest bioactivity.

Lysozyme-immobilized catheters were then challenged against the Gram-positive bacteria *S. aureus* by incubation in a highly concentrated

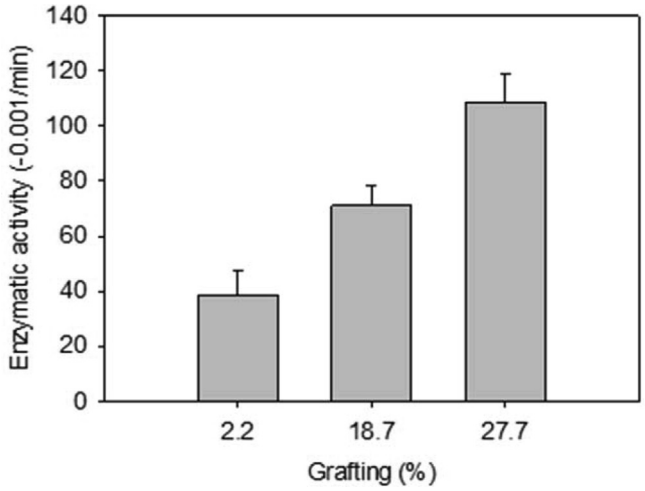

Fig. 4. Hydrolytic activity of the lysozyme-functionalized PVC-g-GMA catheters against *M. lysodeikticus*.

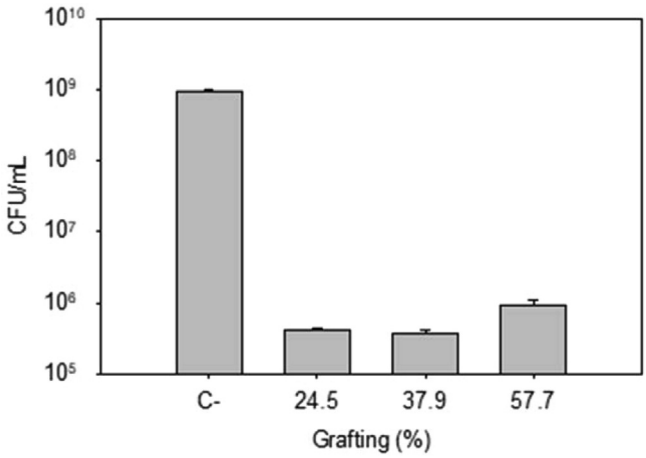

Fig. 5. Viable *S. aureus* adhered to pristine PVC and lysozyme-immobilized PVC-g-GMA catheters.

bacterial suspension (3.2×10^9 CFU/mL) for two hours. Viable bacteria adhesion levels were compared to negative control (bare catheter) (Fig. 5). Catheters functionalized with lysozyme resulted in a 2–3 orders of magnitude decrease in bacterial attachment to the surface, compared to the pristine catheter or PVC-g-GMA without lysozyme, which may be useful to prevent biofilm formation. These results are in accordance with the outstanding activity showed *in vitro* (Fig. 4). The 100- to 1000-fold decrease in *S. aureus* adhesion is significantly larger than the 11- to 12-fold decrease previously reported for lysozyme-immobilized onto woven and knitted forms of crimped polyethylene terephthalate cardiovascular grafts (Al Meslmani et al., 2016).

3.4. Functionalization of epoxy groups of PVC-g-GMA catheters with EDA

Attempts to immobilize acylase onto PVC-g-GMA under the same conditions as for lysozyme failed. Several strategies were tested to facilitate the binding of acylase to the epoxy-containing catheters in order to obtain covalent attachment. Acylase was dissolved in buffers with different salt concentrations and pH values to elucidate the effect of ionic strength on the immobilization. The use of a medium of high ionic strength usually promotes enzyme-support hydrophobic interactions, which facilitate the subsequent covalent linkages between epoxide groups and nucleophilic groups of enzyme (Barbosa et al., 2013; Mateo et al., 2000b; Wheatley and Schmidt, 1999). Catheters with different grafting percentage incubated in acylase solution in 0.2 or 1 M

Table 1
Conversion of epoxy groups of PVC-g-GMA by EDA functionalization after 4 h incubation in EDA. Percentages of conversion were gravimetrically quantified. Mean values and, in parenthesis, standard deviations.

Catheters	% Conversion
PVC-g-GMA (15%)	98.4 (11.5)
PVC-g-GMA (55%)	157.4 (26.9)
PVC-g-GMA (92%)	178.7 (77.5)

phosphate buffer or 1 M ammonium sulfate did not show immobilization.

Since acylase (pI 5.1) is highly negatively charged at neutral pH, PVC-g-GMA catheters were functionalized first with EDA to provide cationic charges (Mateo et al., 2007) and subsequently treated with glutaraldehyde to endow the catheters with terminal moieties that are more reactive than epoxy groups. In such a way, we aimed to electrostatically attract acylase to the catheters surface and then trigger the multiple point covalent immobilization. Reaction of GMA with EDA was only successfully achieved when the catheters were immersed in a 100% EDA medium. The time of reaction was limited to 4 h to avoid degradation of PVC. Conversion degree of epoxy groups into aminated groups is summarized in Table 1. Catheters with a high percentage of GMA graft attained values above 100%, which suggests certain adsorption of EDA onto the catheter through ionic interaction.

3.5. Acylase immobilization

(PVC-g-GMA)-EDA catheters were functionalized with glutaraldehyde in order to form stable covalent bonds (imines) with amine-activated supports (Marques et al., 2013). Due to its bifunctional nature, glutaraldehyde is widely used for enzyme immobilization in a variety of supports, and usually the immobilized enzymes become more resistant to denaturation and more stable for prolonged use (Marques et al., 2013; Walt and Agayn, 1994). (PVC-g-GMA)-EDA catheters functionalized with glutaraldehyde were incubated with acylase solution prepared in ultrapure water to avoid the interference of salts in the immobilization process. The glutaraldehyde pre-activated catheters were expected to react mainly with non-ionized primary amino groups of acylase, providing multipoint covalent attachment (Barbosa et al., 2013; López-Gallego et al., 2005), but forming relatively long bridges from the catheter surface (compared to the initial GMA mers) to facilitate the access of the substrates to the active site.

Enzymatic activity of the covalently immobilized acylase was first evaluated in terms of capability to hydrolyze *N*-acyl-L-methionine to L-methionine. The enzymatic activity (U) was referred as the amount of enzyme that produces 1 μmol of L-methionine per hour at 37 °C. As a reference, the activity of free acylase in aqueous medium was calculated to be 0.015 U/mg for 0.1 mg/mL and 0.053 U/mg for 0.5 mg/mL; therefore it was clearly dependent on enzyme concentration as reported for other enzymes (Ozyilmaz et al., 2005). The effect of substrate concentration on the activity of soluble and immobilized acylase was also investigated. Kinetics studies were performed plotting the initial reaction rate versus substrate concentration (Fig. S3, Supporting Information). The kinetic parameters of free acylase in solution were 5769 ± 505 μmol $[L.h]^{-1}$ for V_{max} and 3902 ± 624 μmol.L^{-1} for Michaelis constant (K_m). At low substrate concentration, reaction rate was almost linear. However, higher substrate concentrations saturated the reaction; in fact initial rate of reaction even decreased.

3.6. Acylase-mediated AHLs degradation

In Gram-negative species, *quorum sensing* is mediated by AHLs. At high cell densities, the accumulated AHLs bind to regulatory proteins forming a complex that then triggers the expression of target genes for production of *quorum sensing* response (Kalia, 2013; Safari et al., 2014). Thus, before testing the acylase-immobilized catheters, the ability of free acylase in solution as *quorum sensing* inhibitor was investigated in detail in terms of capability to degrade different AHL signals and was monitored using *C. violaceum* biosensors that produce violacein in response to the presence of remnant AHLs.

A screening with different AHL was first carried out to verify whether acylase I can degrade them through disruption of amide bond producing homoserine lactone and the corresponding fatty acid. As mentioned above, two strains of *C. violaceum* were used depending on the AHL signals that induce the violacein production. The capacity to degrade AHLs with short acyl side chains is shown in Fig. 6, whereas

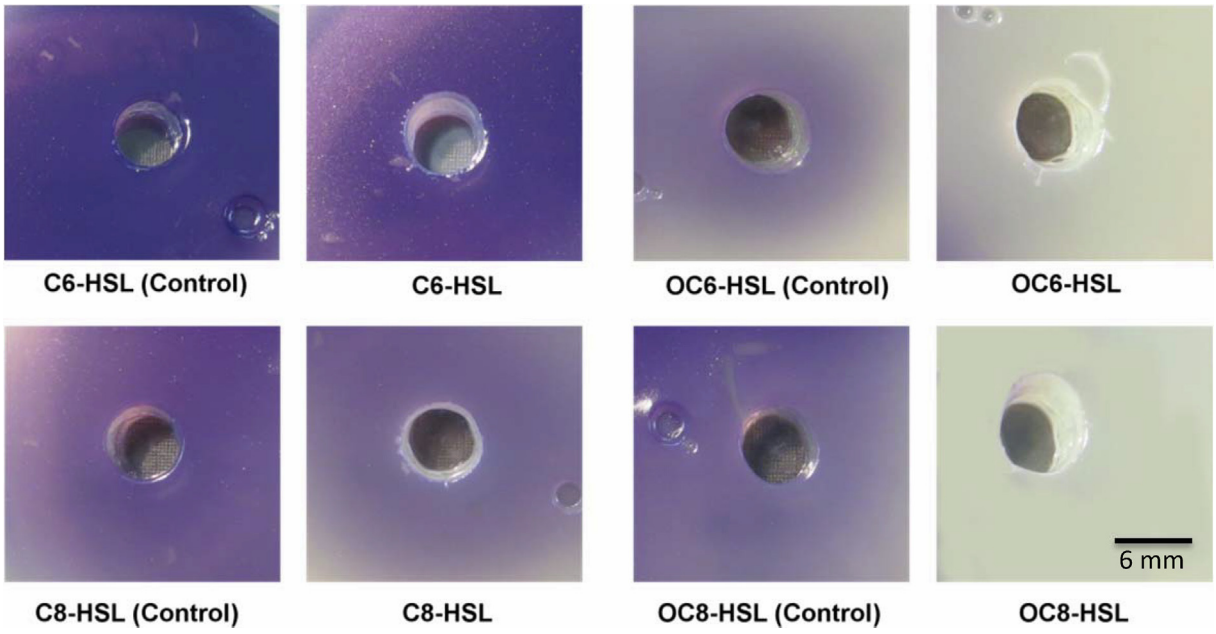

Fig. 6. Degradation of short acyl chain AHLs oxo-substituted (OC6-HSL; OC8-HSL) as well as non-substituted (C6-HSL; C8-HSL) by acylase I from porcine kidney as detected by the *C. violaceum* CV026 strain solid-plate bioassay. Capability of acylase to degrade AHL after incubation at 30 °C for 24 h was evidenced as a lack of violacein production. AHLs incubated without acylase (Control, first column panels) were used as positive controls.

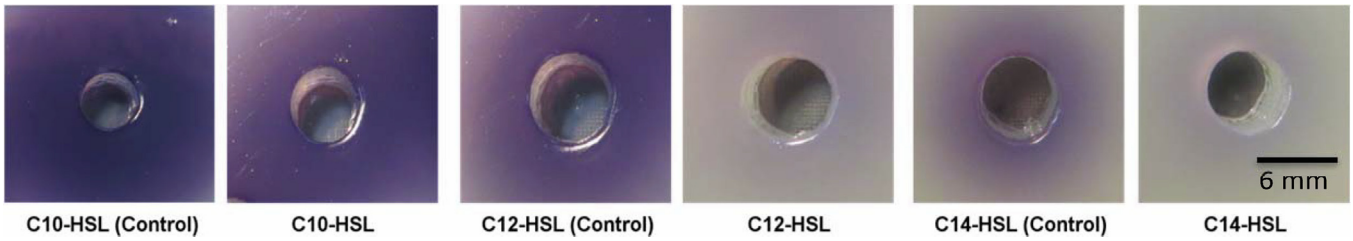

Fig. 7. Degradation of non-substituted long acyl chain AHLs by acylase I from porcine kidney as detected by a solid plate assay using the *C. violaceum* VIR07 ATCC12472 strain as biosensor. Capability of acylase to degrade AHL after incubation at 30 °C for 24 h, was evidenced as a lack of violacein production. AHLs incubated without acylase were used as positive controls.

the degradation of AHLs with unsubstituted and substituted long acyl side chains are depicted in Figs. 7 and S4, respectively.

Short acyl chain AHLs with or without oxo-substitution were detected with *C. violaceum* CV026 strain, although oxo-substituted AHLs triggered violacein production at a lower extent (Fig. 6). Acylase did not degrade non-substituted AHLs. Differently, degradation of oxo-substituted AHLs by acylase was effective, since the solutions did not trigger the production of violacein.

Regarding unsubstituted long acyl side chain AHLs, C10-HSL was hardly degraded as compared to the violacein production caused by the controls (Fig. 7). On the contrary, the effect of C12-HSL was almost completely eliminated after incubation with the acylase, indicating an intense degradation of this signal. C14-HSL (control) caused violacein production at lower extent than C12-HSL (control) in *C. violaceum* VIR07; nevertheless, incubation with acylase I significantly degraded C14-HSL as observed in the attenuated production of violacein.

The ability of acylase to interfere the *quorum sensing* mediated by substituted long chain AHLs was also tested (Fig. S4). In case of oxo-substitution, all AHLs were clearly detected by *C. violaceum* VIR07 and significantly degraded by acylase I. *C. violaceum* was especially able to recognize OC10 resulting in high gene expression for pigment production. *C. violaceum* VIR07 did not detect C12-HSL substituted with hydroxyl group (OHC12-HSL); therefore, it was not possible to demonstrate its degradation with acylase through changes in violacein production. Overall, the results obtained with free acylase suggest that all oxo-substituted and various unsubstituted AHLs may be susceptible for degradation by acylase-immobilized catheters. This means that the chosen aminoacylase shows a wide spectrum of activity against AHLs in spite these are not the main substrate (Muras et al., 2018). Moreover, the grafting procedure could be applied to other more specific *quorum quenching* enzymes that may degrade the signals even faster (Mayer et al., 2015).

3.7. Acylase-immobilized catheters

Many bacteria species produce more than one type of AHL and each one is responsible of expression of a certain gene that triggers a physiological response. Thus, *quorum quenching* has detrimental effects on bacterial pathogenicity and biofilm formation (safari et al., 2014; Davies et al., 1998; Watson et al., 2002). It is well known that OC12-HSL is involved in *P. aeruginosa* biofilm formation (Davies et al., 1998; Pearson et al., 1994). *P. aeruginosa* may grow as biofilm either on natural (lungs) or artificial (e.g. catheter) surfaces causing persistent infections. Surfaces functionalized with acylases have been shown able to inhibit biofilm formation in this pathogen (Ivanova et al., 2015a; Kisch et al., 2014; Lee et al., 2017; Utari et al., 2018).

Acylase-immobilized catheters prepared with different GMA-graft percentage were tested for degradation of C12- and OC12-HSL (Fig. 8). AHL solutions were also incubated in the plate to verify violacein production. Pristine PVC catheters (i.e., non-functionalized) did not show degradation ability against any AHLs, as demonstrated by the induction of violacein production after incubation. No improvement was observed when the pristine catheters were soaked in the acylase solution under the same conditions as for the immobilization onto the grafted catheters. Differently, the acylase-immobilized catheters prevented the production of violacein, which clearly indicates that the immobilized enzyme was active and readily degraded both C12-HSL and OC12-HSL. Relevantly, the acylase-immobilized catheters with low GMA content (15% of grafting percentage) showed complete inhibition of violacein production, meaning that the amount of enzyme immobilized was sufficient for the degradation of the *quorum sensing* signals.

Quantitative analysis was performed in order to confirm the decay of AHLs concentration caused by acylase as detected in the bioassay. Soluble acylase and acylase-immobilized catheters were incubated with C12-HSL, OC12-HSL and OHC12-HSL, and after 24 h remaining signal molecules were quantified using HPLC-MS. The degradation of OHC12-HSL could not be detected with the *C. violaceum* bioassay since this molecule cannot activate the QS system of this sensor. OHC12-HSL is the major QS signal of the important nosocomial pathogen *Acinetobacter baumannii* and is involved in biofilm formation in this pathogen (Niu et al., 2008; Chow et al., 2014; Mayer et al., 2018). This bacterial pathogen is responsible for surgical wound infections, bacteremia and urinary tract infections. Its facility to acquire antibiotic-resistance genes has caused an increase of multidrug-resistant strains, limiting the therapeutic options (Niu et al., 2008; Castillo-Juarez et al., 2017; Mayer et al., 2018). The HPLC-MS analysis confirmed that C12-, OC12- and OHC12-HSL were degraded by acylase in solution as well as by acylase immobilized onto catheters (Fig. 9). Acylase-containing catheters with 32% and 62% GMA percentages showed 100% degradation against all the AHLs after 24 h incubation.

Catheters with 0% graft (control) and soaked in acylase solution showed high AHLs remaining percentages. The small decay in AHL concentration may be not enough to suppress *quorum sensing*, as it was observed in the bioassay (the violacein production was indeed quite intense). This decrease could be due to minor amounts of acylase physically adsorbed onto the pristine catheter when processed in the same way as for acylase immobilization onto PVC-g-GMA catheters.

Free acylase in solution was able to degrade the signaling molecules; degradation levels were above 50% for C12 and OHC12, and almost 100% for OC12-HSL. The advantages of having acylase immobilized onto catheters were clearly seen as an improvement in the hydrolytic activity probably due to the enzyme stabilization; the acylase-immobilized catheters were able to degrade the entire amount of all AHLs also including C12-HSL and OHC12-HSL. Since *quorum quenching* enzymes have been previously demonstrated to efficiently inhibit biofilm formation in important Gram-negative pathogens such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Kisch et al., 2014; Ivanova et al., 2015a,b; Castillo-Juarez et al., 2017; Utari et al., 2018; Mayer et al., 2018) our immobilization technique that enhances the activity of the acylase may constitute an interesting approach for the development of new medical materials with antibiofilm activity.

4. Conclusions

GMA-graft polymerization onto catheters was successfully achieved

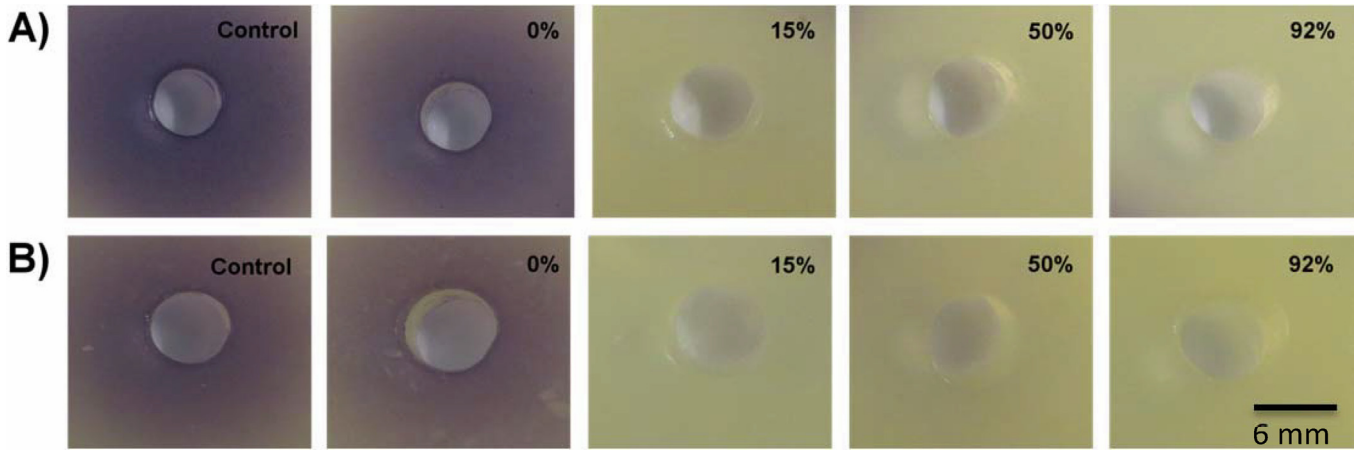

Fig. 8. Bioassay with *C. violaceum* VIR07 ATCC12472 strain as biosensor to detect degradation of C12-HSL (A) or OC12-HSL (B) in solutions (5 μg/mL) exposed to control catheters (0% graft) or acylase-immobilized PVP-g-GMA catheters (15, 50 and 92% graft) at 25 °C for 24 h. C12-HSL (A) and OC12-HSL (B) solutions without any treatment were set in the plates as positive control of *quorum sensing* which was expressed by violacein pigment formation.

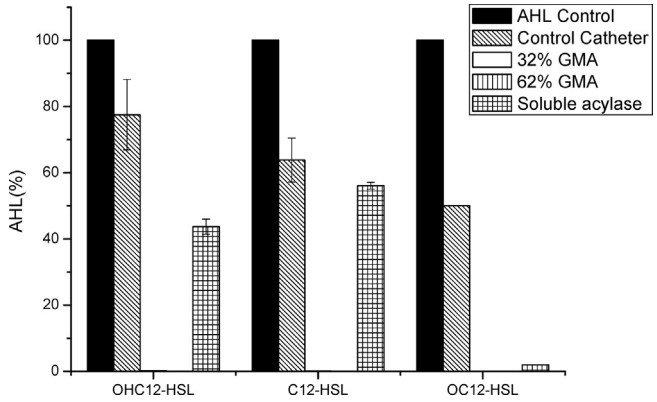

Fig. 9. Percentages of OHC12-HSL, C12-HSL and OC12-HSL remaining, as measured by HPLC-MS, after exposition for 24 h to acylase in solution and acylase-immobilized PVP-*g*-GMA catheters with different graft percentages.

applying a gamma ray pre-irradiation method. Lysozyme was readily immobilized onto PVC-g-GMA under mild conditions and significantly prevented *Staphylococcus aureus* attachment. Alternatively, acylase immobilization required further modification of PVC-g-GMA catheters. Reaction of epoxy groups with EDA and subsequent activation with glutaraldehyde allowed for acylase covalent binding, while the enzyme retained its hydrolytic activity. Acylase-immobilized on PVC-g-GMA catheters can degrade a variety of AHL signals responsible of *quorum sensing*, as demonstrated by inhibition of production of violacein pigment regulated by AHLs in *C. violaceum* and confirmed using HPLC-MS. In sum, lysozyme and acylase can be successfully immobilized onto PVC catheters through covalent linkages enabling inhibition of biofilm formation through their respective mechanism of action.

Conflicts of interest

There are no conflicts of interest

Acknowledgments

Work funded by MINECO (SAF2014-52632-R; SAF2017-83118-R), Agencia Estatal de Investigación (AEI) Spain, Xunta de Galicia (AEMAT ED431E 2018/08), FEDER, and DGAPA-UNAM Grant IN201617 Mexico. L. Diaz-Gomez acknowledges Consellería de Cultura, Educación e Ordenación Universitaria for a Posdoctoral fellowship (Xunta de Galicia, ED481B 2017/063). H.I. Meléndez-Ortiz is grateful to the program Catedras-CONACyT for economic support. The authors thank to B. Leal, M. Cruz, and E. Palacios from ICN-UNAM, B. Magariños from USC for technical assistance, and Prof. Tomohiro Morohoshi from the Utsunomiya University for kindly providing us with *Chromobacterium violaceum* VIR07 biosensor strains.

Appendix A. Supplementary data

DSC curves of PVC catheter, PVC-g-GMA 156% catheter, and poly (GMA); Thermogravimetric analysis of PVC, poly(GMA), and PVC-g-GMA 156%; Michaelis-Menten kinetic model for free acylase in solution obtained plotting L-methionine conversion and substrate (N-acetyl-L-methionine) concentration; and degradation of substituted long acyl chain AHLs by acylase I from porcine kidney measured by solid plate assay using the *C. violaceum* VIR07 ATCC12472 strain as biosensor.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2018.12.075.

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