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CONTROL OF BIOFOULING OF INDUSTRIAL SURFACES USING MICROPARTICLES CARRYING A BIOCIDE

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ABSTRACT

This study presents a new technological approach to minimize the use of antimicrobial agents, based on the principle of drug-delivery systems where the antimicrobials are transported on microparticles. The efficacy of microparticles (average size of 4 μm) carrying the quaternary ammonium compound benzyldimethyldodecyl-ammonium chloride (BDMDAC) was assessed against *Pseudomonas fluorescens* in planktonic and biofilm states. The microparticles were prepared using the layer-by-layer self-assembly (LBL) technique. Oppositely charged molecules of polyethyleneimine (PEI), sodium polystyrene sulfonate (PSS) and BDMDAC were assembled on polystyrene (PS) cores. BDMDAC coated particles were observed by CryoSEM and their composition analyzed by X-ray microanalysis. Zeta potential measurements indicating a change in the charge compatible with the BDMDAC/particle interaction. This antimicrobial carrier structure had a significant stability verified by the release of only 15% of BDMDAC when immersed in water for 18 months. The evaluation of biocide carrier activity was carried out through the determination of the survival ratio (CFU/CFU in saline solution; CFU – colony formation units) of *P. fluorescens* planktonic and biofilm cells after different exposure periods to BDMDAC coated particles. An efficient biocidal effect (minimum bactericidal concentration) was found for a concentration of 9.2 mg/L of BDMDAC in coated particles after incubation for 30 min and 6.5 mg/L of BDMDAC in coated particles after 60 min. The biofilm exposure to PS-PEI/PSS/BDMDAC resulted in a viability decrease of 60.5% and 66.5% of the total biofilm population for a 30 and 60 min exposure time, respectively. The overall results indicate that this novel antimicrobial strategy based on drug-delivery systems has potential application on the control of microbial growth of planktonic cells and biofouling. Moreover, this technique allows the reuse of the antimicrobial molecules, minimizing environmental risks associated with abusive use of antimicrobial agents with real benefits to public health.

INTRODUCTION

Layers of microorganisms and their extracellular polymers (biofouling) grow very easily on industrial cooling water tubes and heat exchanger channels, causing increased pressure drop and reduced heat transfer efficiency. These problems lead, ultimately, to an increase in the costs of the production and maintenance, as well as to public health problems and environmental impacts (Pereira et al, 2007). Often the layers build up in a non-uniform manner, with localized spots where thicker biofilms appear. Biofilm growth on surfaces is prevented by using biocidal products and dispersants in the water stream in considerable large amounts. Such toxic chemicals are not totally consumed by the interaction with the biological structures and their discharge into the environment or wastewater treatment plants is a source of serious problems (Rasmussen et al., 1999).

Modern strategies to minimize biofouling in industrial equipment (pipes, heat exchangers, etc) focus on optimizing process conditions and equipment design, developing new surfaces to reduce adhesion and applying efficient surface cleaning/disinfection methodologies, supported by on-line monitoring techniques. A considerable amount of work has been reported on the effect of process conditions, such as hydrodynamics, on biofilm growth and process performance (Characklis *et al.*, 1990; Melo and Vieira, 1999). Although further advances are still possible on this aspect, the improvements will be generally marginal. The use of liquid velocities around 2 m/s inside tubes is recommended in order to take advantage of the stronger shear stresses, reducing biofilm growth. In fact, high velocities have some drawbacks as they produce more compact deposits that are more difficult to remove from the surfaces by both mechanical and chemical methods (Melo and Vieira, 1999; Simões *et al.*, 2005). More recently, new low energy surfaces produced by surface bombardment to implant ions such as Mo and F, plasma sputtering and coatings with thin Ni-P-PTFE layers have been developed with an interesting potential for reducing deposit adhesion. However, their application is much dependent on the relative costs of such expensive materials comparatively to the costs associated with fouling (Rosmaninho and Melo, 2006; Santos *et al.*, 2004). Additionally, it was shown that the major advantage of such surfaces is that they allow the production of deposits that are easier to clean (Santos *et al.*, 2004). Therefore, cleaning has increasingly become the crucial step in the optimization of these systems. There are, here, two interconnected issues: a) biofilms and other deposits do not attach uniformly along the surfaces; b) chemicals used to remove biofilms are carried as solutes by the bulk liquid and only a minor fraction does actually take part in the cleaning process, leaving a large amount in the discharged waters. In fact, the current cleaning procedures are still highly inefficient processes, consuming large amounts of water, chemicals and time. More efficient cleaning techniques are needed, allowing the consumption of less water, chemicals,

energy and time, and, simultaneously, are able to efficiently control biofouling. This will reduce the use of toxic chemicals and will minimize health and environmental risks of antimicrobial chemicals (Gilbert and McBain, 2003).

The goal of this study was to: develop and characterize microparticles with functionalized surfaces that act as carriers of antimicrobial molecules; assess the effect of microparticles carrying BDMDAC against *P. fluorescens* planktonic cells and biofilms.

MATERIAL AND METHODS

Reagents

Polyethyleneimine (PEI – molecular weight of 750 000) 50% (w/v) in water, Poly(sodium 4-styrenesulfonate) (PSS - molecular weight of 70 000) and boric acid were obtained from Sigma-Aldrich. Benzyldimethyldodecylammonium chloride (BDMDAC - molecular weight of 339.9) was obtained from Fluka. All chemicals were used without further purification. Poly(styrene) (PS)-core particles 4.37 μm ± 0.07 μm 10% (w/v) aqueous solution were obtained from Microparticles GmbH.

Particles Production Process

The particles were prepared using the layer-by-layer self-assembly (LBL) technique. The original method was introduced in 1991 by Decher and co-workers for the construction of pure polymer multilayer films on planar supports (Caruso, 2001, Decher 1997). This technique uses electrostatic attraction and complex formation between polyanions and polycations to form supramolecular multilayer assemblies of polyelectrolytes. The first stage of shell fabrication involves step-wise deposition of polyelectrolytes from aqueous solutions. The polyelectrolyte multilayer film is formed by the alternate adsorption of oppositely charged layers on to the particle. After each adsorption step, the non- adsorbed polyelectrolyte in solution is removed by repeated centrifugation or filtration and washing (Donath *et al*., 1998).

The oppositely charged electrolytes, PEI, PSS and BDMDAC, were assembled on PS cores, in a process that comprises 3 steps (Fig. 1). PS particles were allowed to interact with the PEI solution (1 mg/mL in borate buffer solution) for 20 min, and then washed in 0.1 M borate buffer solution pH 9 to remove the excess polymer. After this procedure the core positively charged was used for the deposition of the polyanion PSS, followed by the BDMDAC, both solutions of 1 mg/mL in borate buffer pH 9. The adsorption steps were carried by adding the polymer solution to the cores for 20 min, centrifuging at 4000 rpm for 4 min and resuspending them in borate buffer pH 9. This step was repeated twice.

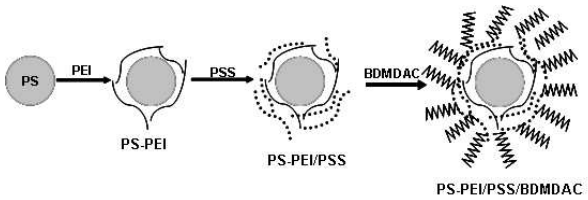


Fig. 1 Schematic representation of the particles production process.

The solvent used in the whole process was borate buffer solution at pH 9. It was selected as the ionic strength of the solution as well as the pH value that allows a better layer-by-layer process by promoting the right superficial charge for the different molecules intervening in the process.

Quantification of the Amount of Biocide in Particles

For direct quantification of BDMDAC in surface of coated particles a high performance liquid chromatography (HPLC) method was developed. The HPLC system consisted of a JASCO PU-2080 plus ternary pump, a manual injector equipped with a 20 μL sample loop and a JASCO MD-2015 plus diode array detector. A Jasco ChromPass Chromatography data system (version 1.8.6.1). allowed to control the equipment and the data processing The analytical column was CC 250/4 Nucleosil 100-5 C18. The mobile phase consisted of methanol/water pH 1 with ortho-phosphoric acid 85% (v/v). The flow rate was 1.0 mL/min and the detector was set at 210 nm. A calibration curve standards was assessed using different BDMDAC concentrations (50, 70, 100, 200, 250, 300 mg/L) in 0.1 M borate buffer solution pH 9. All the samples were injected in 0.1 M borate buffer solution pH 9. The concentration of BDMDAC adhered to the particles, was assessed as the difference between the BDMDAC solution concentration before (1000 mg/L) and after contact with the particles.

Particles Characterization - CryoSEM and X-ray Microanalysis

Cryo scanning electron microscopy (CryoSEM) and X-ray microanalysis were performed to analyze the coated particles integrity/morphological characteristics and surface composition, respectively. The samples were analyzed by CryoSEM (Model Gatan ALTO 2500) at CEMUP (Centre for materials characterization from the University of Porto).

Particles Characterization - Number and Volume Size Distribution

The size distribution of the particles was determined in a Coulter Particle Size Analyzer (model LS 230 – small volume module plus) by Laser Diffraction. The analysis of the particle size was considered as volume and number distribution.

Particles Characterization - Zeta Potential.

The zeta potential of the particles was determined using a Nano Zetasizer (Malvern instruments, UK). The zeta potential was measured by applying an electric field across the particles solution. Particles within the dispersion with

non-zero zeta potential migrated toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential.

Microorganism

Pseudomonas fluorescens was used throughout this work. This bacterium is a good biofilm producer and one of the major microorganisms found in industry (Simões *et al.*, 2005). The optimal growth conditions were 27 ± 3 ºC, pH 7, and glucose as the main carbon source. The *P. fluorescens* strain was cryopreserved in a refrigerated chamber at –80 ºC, in a mixture of nutrient broth and 15% (v/v) of glycerol. Bacteria propagation was obtained by removing an inoculum from the cryovial. The bacteria were then distributed evenly over the surface of solid medium of Plate Count Agar (PCA) and incubated for 24 h at 27 ± 3 ºC.

Antimicrobial tests with planktonic cells

The bactericidal effect of the BDMDAC coated particles (PS-PEI/PSS/BDMDAC) was tested by comparison with the effect of the non-coated particles. Planktonic cells were obtained from solid medium and resuspended in sterile saline solution (0.85% NaCl) to an optical density (610 nm) of approximately 0.220. The suspension was serially diluted to 10^{-5}. An aliquot of 1.0 mL was collected and used to test antimicrobial effect of the coated particles. BDMDAC coated particles were tested at different concentrations and compared with control samples (bacteria in saline solution and in contact with PS-PEI/PSS particles). The antimicrobial effect of the different systems was evaluated at different incubation times (0, 30 and 60 min). After each incubation time, 100 μL of sample were spread on PCA and incubated for 24 h at 27 ± 3 ºC. The viable cells were counted to assess the antimicrobial effect.

The evaluation of the minimum amount of BDMDAC needed for effective microbial reduction was carried out through the determination of the survival ratio (ratio between the CFU antimicrobial test/ CFU control; CFU – colony formation units) of the microbial population after different periods of exposure to BDMDAC coated particles.

Biofilm Set-up

Biofilms were developed in a well stirred continuous reactor at 27 ± 3 °C. *P. fluorescens* was grown in a 4 L polymethyl methacrylate (Perspex) fermenter, suitably aerated and magnetically agitated. The fermenter was continuously fed with 0.40 L/h of a sterile nutrient solution consisting of 50 mg/L glucose, 25 mg/L peptone and 12.5 mg/L yeast extract, in 0.2 M phosphate buffer at pH 7. The bacteria were grown in the fermenter by adding 500 mL of bacterial suspension (optical density 610 nm = 1.0) to 3.5 L of saline solution for approximately 3 h before the beginning of the continuous feeding process. The dilution rate of 0.1 h^{-1} ensured that biofilm activity predominated over that of planktonic cells (Simões *et al.*, 2003). Twelve slides (1.5 cm x 2 cm and 1 mm thick) of polyvinylchloride (PVC) were vertically placed within the bacterial suspension for 7 days for biofilm growth. The slides were degreased, rinsed twice with water and sterilised before they were suspended in the fermenter using a device that enabled their removal for biofilm sampling. The selection of initial growth conditions and system parameters were based on previous experiments (Simões *et al.*, 2003).

Biofilm Treatment

After biofilm development the biofilm-covered slides were carefully transferred to a closed flask that contained the BDMDAC coated particles solution (8×10^7 particles/mL). The flask was placed in an orbital shaker throughout the chemical treatment, to ensure the same temperature and agitation conditions as in the fermenter. Some biofilm-covered slides were placed in saline solution for control assays and in solution of particles at the same concentration but whitout BDMDAC. The biofilms were exposed to BDMDAC coated particles at independent periods of 30 and 60 min. Afterwards, the PVC slides with accumulated biofilm were carefully removed from the solution of BDMDAC-coated particles. Biofilm control action was measured as the variation in the mass deposited during the treatment period and in the colony forming counts (CFU).

Antimicrobial Tests with Biofilms

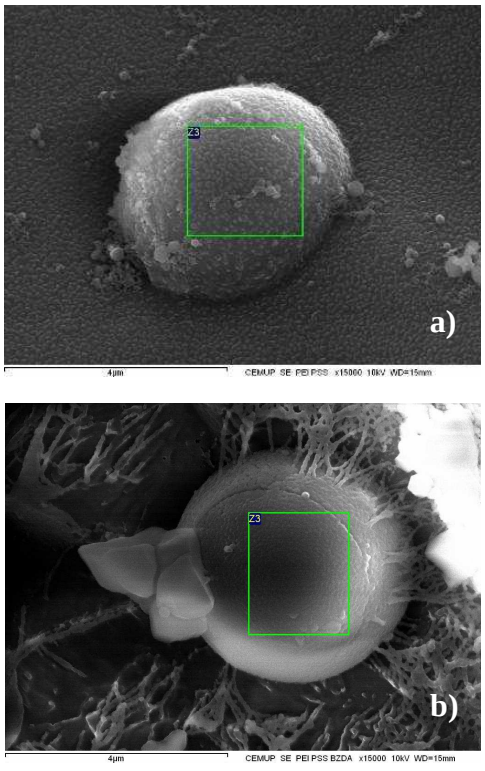
In each experiment and immediately after the 30 or 60 min of BDMDAC coated particles treatment, two PVC slides were sampled and the biofilms that covered the slides were completely scraped off using a metal device, exposed to a ultrasonic bath at 35 kHz for 15 min (Transsonic 420, Elma) and resuspended in 25 mL of saline solution. The biofilm suspensions were vortexed (IKA TTS2) for 30 s with 100% input and immediately used to assess the biofilm bacteria culturability. The bacterial samples were diluted to the adequate cellular concentration in sterile saline solution. A volume of 100 μL of the bacterial diluted suspension were transferred onto PCA plates. Colony enumeration was carried out after 24 h at 27 ± 3 ºC and the survival ratio was assessed as referred to above for the planktonic tests.

RESULTS and DISCUSSION

This study aims to show the potential of a new antimicrobial strategy to control planktonic cells and biofouling layers based on the principle of drug-delivery systems. The selected antimicrobial chemical is a QAC (quartenary ammonium compound) that is normally used as an industrial cleaner, such as in re-circulating cooling water systems (Bull *et al.*, 1998). BDMDAC has detergent and antimicrobial properties, acting on multiple biochemical targets of the bacterial cells. BDMDAC can disrupt cell membranes, interrupt proteins functions, stimulate the release of intracellular constituents and induce cell autolysis (Ferrer and Furlong., 2001; Ishikawa et al., 2002).

The particles (with and without BDMDAC coating) were characterized in order to assess potential changes induced by the antimicrobial coating process. CryoSEM was used to visualize the morphology of the particles as well as the presence of an external layer (BDMDAC and/or PSS). The selection of this technique was mainly related to the fact that

it allows the analysis of hydrated samples, conversely to the traditional SEM. X-ray microanalysis coupled with CryoSEM was used to confirm the elemental constitution of the particles surface. Cryo-SEM images of PS-PEI/PSS and PS-PEI/PSS/BDMDAC particles show that they are spherical and have a rough surface in both presence and absence of BDMDAC (Fig. 2). X-ray microanalysis indicates that the external layer was mainly composed by carbon and its content is considerably higher in PS-PEI/PSS/BDMDAC particles when compared with PS-PEI/PSS particles. This result indicates that the carbon content in the particles surface increases significantly when coated with BDMDAC. In fact, this is an expected phenomenon due to the long carbon chain of BDMDAC (C_{14}). This conclusion is reinforced by the HPLC results and by the zeta potential of the particles. HPLC experiments demonstrated that each particle carried 5.4×10^{-8} mg/L of BDMDAC. The zeta potential of PS-PEI/PSS was -34 ± 4 mV and of PS-PEI/PSS/BDMDAC was -21 ± 5 mV. It was expected that the interaction between BDMDAC and the particles induced a shift in the zeta potential from negative to positive values. However, this shift was not very pronounced. PSS is a polyanion inducing high negative zeta potential values (Tedeschi et al. 2003; Dmitry et al. 2003), while each BDMDAC molecule only provides one positive charge to link to the particle. This fact could be justified by hidrofobic interactions between the carbon chains of BDMDAC and water that block the access of more BDMDAC molecules to bond electrostatically to the free negative charges of PSS.



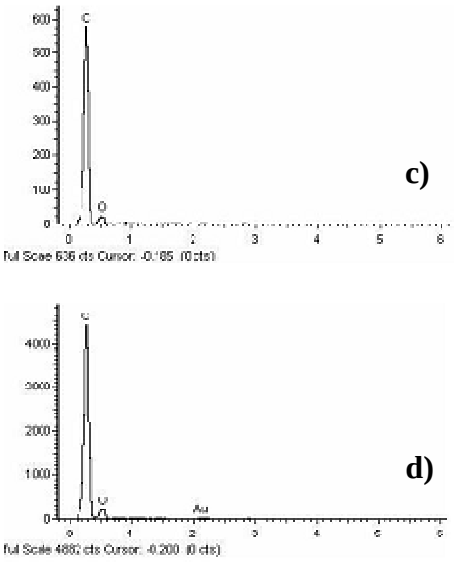
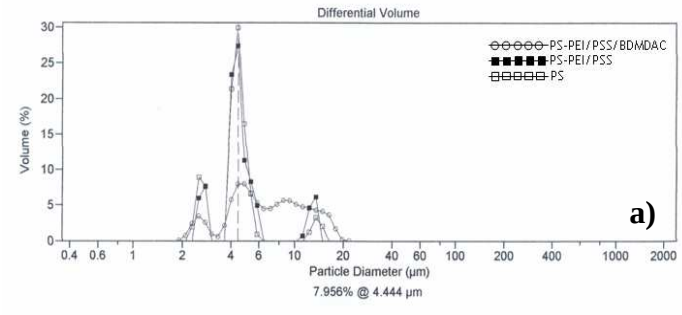


Fig. 2 CryoSEM images (×15000 magnification; 10 kV) of: (a) PS-PEI/PSS; (b) and PS-PEI/PSS/BDMDAC particles; (c) X-ray microanalysis of PS-PEI/PSS; (d) PS-PEI/PSS/BDMDAC particles.

In order to assess the effect of the BDMDAC coating process on the physical characteristics of the particles, the size distribution of BDMDAC coated and uncoated particles was assessed (Fig. 3). Three particles size distributions could be found for PS-PEI/PSS particles with sizes of 3.0, 4.4 and 15.0 µm. In the case of PS-PEI/PSS/BDMDAC, again three populations were observed, but here two of them are well defined (3.0 and 4.4 µm). A wider distribution could be seen between 6 µm and 20 µm (Fig. 3). This can be related to the particle aggregation due to hydrophobic interactions between the BDMDAC carbon chains. However, when considering the size distribution in number, only the two populations of smaller diameters (3.0 and 4.4 µm) are significant, indicating that the number of particles with a diameter of 20 µm is not significant, for the tested particles (Fig. 3).



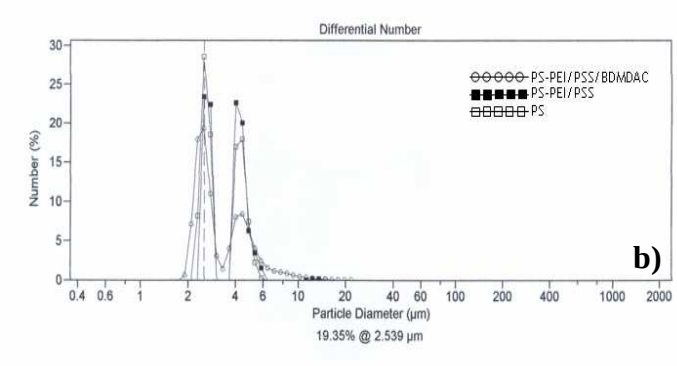


Fig. 3 Volume distribution (a) of particles (PS, PS-PEI/PSS and PS-PEI/PSS/BDMDAC); (b) Size distribution of the particles in number (PS, PS-PEI/PSS and PS-PEI/PSS/BDMDAC).

The effect of the QAC on the bacterial cells was first investigated in planktonic cells. The effect was determined as a survival ratio between the CFU for the antimicrobial test and the CFU in the saline solution (Fig. 4). The results obtained for the mixture of PS-PEI/PSS particles with the suspension of the *P. fluorescens* showed that they have no bactericidal effect (survival ratio around 1). The number of bacteria in the presence of these particles was approximately the same as for the control (saline solution) experiment. Also, the concentration of viable cells was invariable over time (1.10×10^3 CFU/mL). The particles coated with BDMDAC (PS-PEI/PSS/BDMDAC) showed antimicrobial effect (survival ratio = 0). The minimum bactericidal concentration (MBC) for a 30 min contact time was 9.2 mg/L, while the MBC for a 60 min contact time was 6.5 mg/L. The planktonic tests indicate a strong antimicrobial effect of BDMDAC when associated with the particles. In fact, this QAC has a strong antimicrobial activity comparatively to other QAC`s (Méchin *et al.*, 1999; Ishikawa *et al.*, 2002; Simões *et al.*, 2006). Moreover, the BDMDAC particles coating process seems to potentiate the antimicrobial activity. A previous report (Méchin *et al.*, 1999) demonstrated that the MBC of BDMDAC for *P. aeruginosa* in suspension was 20 mg/L.

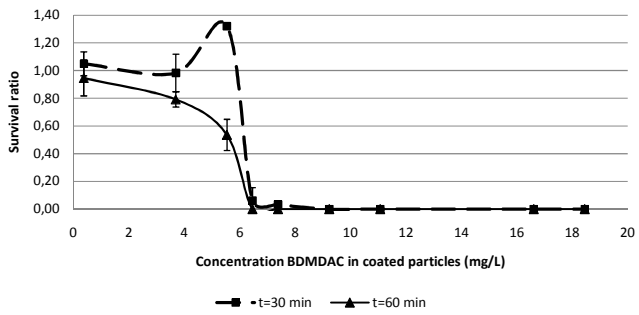


Fig. 4 Survival ratio of planktonic *P. fluorescens* exposed to different concentrations of the BDMDAC adsorbed in particles for two different exposure periods.

The effects of the application of BDMDAC at 0.87 mg/L for 30 and 60 min against biofilms formed on PVC slides were assessed by determining the biofilm population survival ratio (Fig. 5). The biofilm exposure to PS-PEI/PSS/BDMDAC resulted in a viability decrease of 60.5% and 66.5% of the total biofilm population for 30 and 60 min exposure time, respectively. This demonstrates that with the tested concentration, total biofilm inactivation was not achieved. It is expectable that, increasing the BDMDAC concentration to the levels applied to planktonic cells will increase the inactivation effects. By comparing the application of equivalent concentrations and exposure periods of free QAC, it was verified that the free BDMDAC had a moderately higher antimicrobial activity than the BDMDAC coated particles. However, the difference observed were not statistically significant (results not showed).

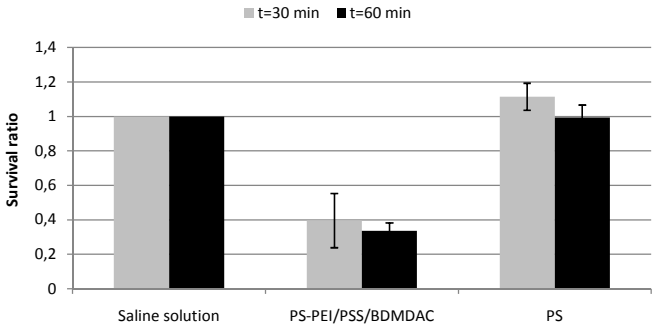


Fig. 5 Survival ratio of *P. fluorescens* biofilms exposed to saline solution (control experiment), 0.87 mg/L of BDMDAC in coated particles and PS for two different exposure periods.

The environmental aspects of the current use of antimicrobials are of severe concern because of their residual presence in surface and ground waters and the consequent propagation to the food-chain, with consequences on public health. The controlled application and reuse of antimicrobials based on highly efficient strategies might avoid the dissemination of antimicrobial resistance. In fact, the prolonged exposure of microorganisms to sub-lethal concentrations arise the existence of antimicrobial resistance and cross-resistance events (McDonnell and Russell, 1999; White and McDermott, 2001). There are several reports indicating the ability of bacteria to acquire resistance to QAC`s (Méchin *et al.*, 1999; Ishikawa *et al.*, 2002). Also, the economical costs associated with the continuous application of antimicrobial chemicals should be considered. The strategy used in this study to control planktonic and biofilm cells will allow the rational use and reuse of antimicrobials. After 18 months in borate buffer pH 9 (keep at 4ºC), the particles coated with BDMDAC only released 15% of the QAC. Antimicrobial strategies based on this methodology will have potential public health, environmental and economical benefits.

The main limitations associated with this antimicrobial strategy are associated with the possible transport limitations within the biofilm, due to the particles size, and with the

current particles cost. In fact, the selected concentration applied against biofilms was based on the particles cost (400 € per 15 mL of particles solution). The use of this technology with low cost particles such as clays and magnetites will make possible the large scale application of this process against both planktonic and biofilm cells.

CONCLUSIONS

The goal of this work was to develop and characterize microparticles with functionalized surfaces that act as carriers of antimicrobial molecules and test them against planktonic cells and biofilm formed by *P. fluorescens*. It was possible to conclude that the BDMDAC coated particles are significantly active against *P. fluorescens* planktonic and biofilm cells. The present work is the beginning of a study that will hopefully have, in the future, a potential impact in reducing environmental costs, health risks associated with the intensified use of antimicrobial chemicals and the cleaning and disinfection costs in many industrial plants.

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