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BIOFILM CONTROL WITH NEW MICROPARTICLES WITH IMMOBILIZED BIOCIDE

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ABSTRACT

Biofouling of heat exchangers, pipes and valves is an unavoidable hazard in industrial plants. Operators have to face the problems of cleaning the fouled equipment, treating the wastewater from the discharge of antimicrobial cleaning agents (biocides, surfactants, etc) and protecting the consumer from contamination risks. To tackle these problems, a targeted cleaning technique is here proposed that unites efficient removal of the biological deposits (specifically targeting the places where deposits grow) with low cleaning agents consumption. The goal is to develop microparticles with functionalized surfaces that act as carriers of biocidal molecules, attach to biofilm surfaces and deliver the biocide on the desired sites. This novel biofilm control technology may provide cost effective, environmental and health friendly strategies that will improve operational efficacy. Previously, our group had performed studies with PS (polystyrene) microparticles functionalized with the biocide benzyldimethyldodecylammonium chloride (BDMDAC) against biofilms of *Pseudomonas fluorescens*. BDMDAC functionalized microparticles were prepared using the layer-by-layer self-assembly (LBL) technique. Since PS particles are very expensive, our group tested calcium carbonate microparticles ($CaCO_3$) (diameter: 3 µm), produced by the same LBL assembly technique. These microparticles have the advantage of being cheaper, porous and highly abundant in nature. In this study, *P. fluorescens* biofilms were exposed to 6.33 mg/L and 11.75 mg/L of BDMDAC-coated $CaCO_3$ microparticles for 60 min. This strategy promoted inactivations of 81.9% (6.33 mg/L) and 93.3 % (11.75 mg/L mg/L) of the total population. As control, free BDMDAC was tested against *P. fluorescens* biofilms, demonstrating similar inactivation ($P > 0.05$) to the new strategy.

INTRODUCTION

In industrial systems, biofilms frequently grow on cooling water tubes and heat exchanger channels. They cause increased pressure drop and reduced heat transfer efficiency, which ultimately lead to an increase in the costs of production and maintenance, as well as to public health concerns and environmental impacts (Ferreira et al. 2010).

Biocides (chemical agents with antiseptic, disinfectant or preservative actions), with their broad spectrum of usage, appear to be a good way to control or prevent undesirable biofilm formation. The applications of these chemical require a considerable economic effort and expensive infrastructures (Shannon et al. 2008), but they are also responsible for the production of harmful disinfection by-products (DBP). Chemicals like free chlorine, chloramines and ozone can react with various natural water constituents thus forming DBPs, many of which are toxic and/or carcinogenic (Li et al. 2008; Shannon et al. 2008). For these reasons more efficient cleaning procedures are needed in order to reduce the consumption of chemicals and energy, as well as to minimize the health and environmental risks of these chemical disinfectants.

Recent advances in the micro-nanotechnology field have gained significant interest in its environmental and biological applications. In fact nano and microtechnology presents a unique alternative to biofilm control and elimination (Ferreira et al. 2010). Nano and microparticles are excellent adsorbents, catalysts and sensors due to their large surface to volume ratio (optimized for loading and carrying antimicrobial agents, for example), are highly reactive and present unique interactions with biological systems (Li et al. 2008; Weir et al. 2008; Taylor and Webster 2009). These particles are also non-toxic, economical to produce and stable, once made (Weir et al. 2008). Antimicrobials can be loaded into these particles by physical encapsulation, adsorption or chemical conjugation. This can present several advantages such as significant improvement of the agents activity, in contrast to the free product, and release of the antimicrobial in a sustained and controlled manner (Zhang et al. 2008; Zhang et al. 2010).
Following previous results obtained with polystyrene micro-particles, the aim of the present work was to develop new functionalized calcium carbonate microparticles nanocoated with biocide, and assess their efficacy against biofilm microbial cells. The layer-by-layer assembly was used to produce these microparticles and the chosen biocide was benzyldimethyldodecylammonium chloride (BDMDAC), a surface-active agent and low-cytotoxic disinfectant that is often used in industrial applications (concentrations around 50 mg/L) (Ferreira et al. 2010).

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 purchased from Sigma-Aldrich (Portugal). Benzyldimethyldodecylammonium chloride (BDMDAC - molecular weight of 339.9) was purchased from Fluka (Portugal). Poly(styrene) (PS)-core particles 4.37 µm ± 0.07 µm 10% (w/v) aqueous solution were obtained from Microparticles GmbH. Calcium carbonate microparticles

($CaCO_3$) with a diameter of 2-4 µm were obtained from PlasmaChem GmbH. All chemicals were used without further purification.

Particles Manufacture Process

Both particles were prepared using the layer-by-layer self-assembly (LBL) technique. This method was first introduced by Decher and co-workers for the assembly of pure polymer multilayer films on planar supports (Decher et al. 1992; Decher 1997; Caruso 2001). This technique relies on the electrostatic attraction and the 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 both calcium carbonate and PS cores, in a process that comprises 3 steps (Figure 1). Both particles were allowed to interact with a 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, now positively charged, was used for the deposition of the polyanion PSS, followed by the BDMDAC, both solutions at 1 mg/mL in borate buffer pH 9. The adsorption steps were carried by adding the polymer solution to the PS cores for 20 min, centrifuging at 2880 g for 4 min and resuspending them in borate buffer pH 9. This wash step was repeated twice.

The borate buffer (0.1 M) at was used in the whole process was selected due to its ionic strength. The pH 9.0 was chosen since it allows a better layer-by-layer process by promoting the right superficial charge for the different molecules intervening in the process.

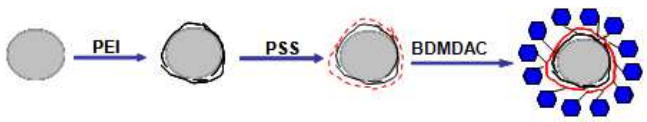


Figure 1- Schematic representation of the layer-by-layer assembly used to produce the microparticles coated with the biocide.

Quantification of the Amount of Biocide in Particles

To quantify BDMDAC on the surface of coated particles, a high performance liquid chromatography (HPLC) method was assembled. 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 the control of the equipment and the data processing. The analytical column was a 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 done using different BDMDAC concentrations (50, 70, 100, 200, 250, 300 mg/L) in a 0.1 M borate buffer solution, pH 9. All the samples were injected in this buffer solution. The concentration of BDMDAC adhered to the particles was determined as the difference between the BDMDAC solution concentration before (1000 mg/L) and after contact with the particles.

CryoSEM Analysis of Particles

Cryo-scanning electron microscopy (CryoSEM) was performed to analyze the coated particles integrity and morphological characteristics. CryoSEM analyses were performed in a Gatan ALTO 2500 Model, at the Center for Materials Characterization from the University of Porto (CEMUP).

Zeta Potential of Particles

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 in the aqueous 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 chosen for this work, as this bacterium is a good biofilm producer and is one of the major microorganisms found in industrial systems (Simões *et al*. 2005). The optimal growth conditions were 27 ± 3 ºC, pH 7, with glucose as the main carbon source. The *P. fluorescens* strain was cryopreserved in a –80 ºC refrigerated chamber, 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 Plate Count Agar (PCA) and incubated for 24 h at 27 ± 3 ºC.

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. Bacteria were grown in the fermenter by adding 500 mL of bacterial suspension ($O.D._{610}$ nm = 1.0) to 3.5 L of a 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).

Antimicrobial Test with Biofilm

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 a solution of particles at the same concentration but without BDMDAC. For comparison, additional experiments were performed with free BDMDAC at the same concentration as in the coated particles. The biofilms were exposed to BDMDAC coated particles at different concentrations (8.33 mg/L and 11.75 mg/L) and at independent periods of 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 colony forming counts (CFU).

Biofilms Analysis

In each experiment and immediately after 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 an 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 number of CFU. 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. The evaluation of the microbial reduction was carried out through the determination of the survival ratio (ratio between CFUs in the antimicrobial test and CFUs in the control; CFU- colony formation units) of the microbial population after different periods of exposure to BDMDAC coated particles.

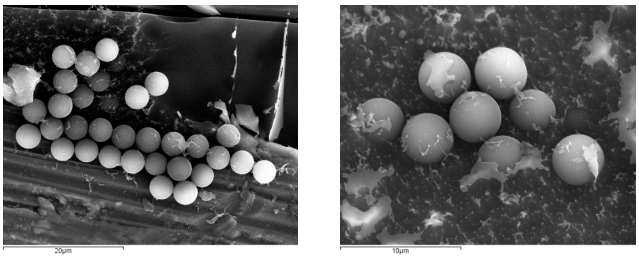
Statistical Analysis

The data were analysed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low samples numbers contributed to uneven variation, nonparametric Wilcoxon test procedure was used to compare the different conditions tested. Statistical calculations were based on confidence level equal or higher than 95 % ($P < 0.05$ was considered statistically significant).

RESULTS and DISCUSSION

With the aim of making industrial cleaning/disinfectant methods more efficient, we produced PS and $CaCO_3$ microparticles coated with BDMDAC, a quaternary ammonium compound frequently used as an industrial cleaner/disinfectant (Ferreira et al. 2010). *P. fluorescens* was used in this study as a model bacterium not only because it is a well studied, Gram-negative bacterium ubiquitous in nature, medical and industrial environments, but also because it has a strong ability to form disinfectant-resistant biofilms (Simões et al. 2008). Thus, *P. fluorescens* has a strong potential to cause serious problems in a wide range of areas in its planktonic and biofilm states (Hsueh et al. 1998; Tuttlebee et al. 2002; Simões et al. 2010).

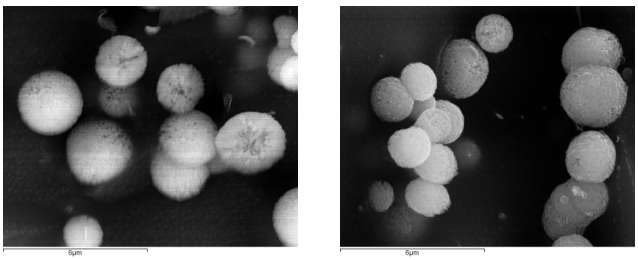
The characterization of the particles surface with and without BDMDAC coating was done by CryoSEM (Figure 2). This technique allows the analysis of hydrated samples, with the advantage, of the freezing process being so fast, that the samples structures preserve their integrity.



(a) (b)

Figure 2 - CryoSEM images (×2500 magnification; 15 kV) of PS particles (a) and PS-PEI/PSS/BDMDAC particles (×5000 magnification; 15 kV).

CryoSEM images of PS and PS-PEI/PSS/BDMDAC particles show that they are spherical and have a rough surface in both the presence and absence of BDMDAC.

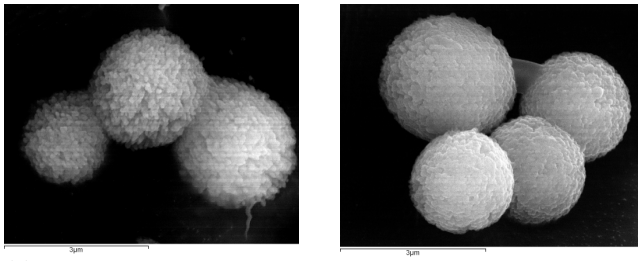


(a) (b)

Figure 3 - CryoSEM images (×10000 magnification; 15 kV) of $CaCO_3$ particles (a) and $CaCO_3$-PEI/PSS/BDMDAC particles (b).

CryoSEM images of $CaCO_3$ particles and $CaCO_3$-PEI/PSS/BDMDAC particles show that these particles are spongier than the PS ones (Figure 3).

Higher amplifications of CryoSEM images of $CaCO_3$ particles with and without biocide allow the observation that the particles surfaces with biocide are smoother than the surfaces of particles without biocide (Figure 4). In fact, this is an expected phenomenon since we are adding layers that stay strongly attached to the particles surfaces and will cover up those referred surfaces. Consequently, the biocide is bounded to the microparticles by ionic interactions, using the layer-by-layer technique, and therefore it will not be released from the particles when interacting with the biological structures. In fact, preliminary experiments were developed on the reuse of particles showing promising results on biofilm control, even after 3-times reprocess (results not shown).



(a) (b)

Figure 4 - CryoSEM images (×20000 magnification; 15 kV) of $CaCO_3$ particles (a) and $CaCO_3$-PEI/PSS/BDMDAC particles.

This conclusion is reinforced by the HPLC results and by the zeta potential of the particles. HPLC experiments demonstrated that each particle of PS carried 5.4×10^{-8} mg/L of BDMDAC and each particle of $CaCO_3$ carried 1.3×10^{-7} mg/L of BDMDAC. This is expected, since the surface area of $CaCO_3$ is larger.

The zeta potential of PS-PEI/PSS was -34 ± 4 mV and of PS-PEI/PSS/BDMDAC was -21 ± 5 mV. For the particles $CaCO_3$-PEI/PSS and $CaCO_3$-PEI/PSS/BDMDAC the values of zeta were -46±4 mV and -18±3 mV, respectively. Once again, due to their large surface, it is possible to incorporate more PSS in the $CaCO_3$ particles, which is a polyanion capable of inducing high negative zeta potential values (Tedeschi et al. 2004; Volodkin et al. 2004). It was expected that the particles would shift positive values of their zeta potential when coated with BDMDAC (a positively charged QAC). However, this does not happen, probably due to the hydrophobic interactions between the carbon chains of BDMDAC and water. These will block the access of more BDMDAC molecules that would bond electrostatically to the free negative charges of PSS. Still, as expected, this shift was more pronounced in $CaCO_3$ particles. This proposes that the antimicrobial action of coated microparticles is promoted by the long carbon chains that are turned out on the particles and the biocide can act on membrane cells. This was evidence in a previous study with BDMDAC (Ferreira et al. 2011).

The effects of the application of BDMDAC at different concentrations against biofilms formed in PVC coupons were assessed by determining the biofilm population survival ratio between the CFUs from the biocides and the CFUs from the assays with saline solution (control). The assessment of culturability to characterize the antimicrobial activity of a biocide was already used in a previous work (Ferreira et al. 2011).

Biofilms were exposed to two different concentrations of BDMDAC (free and coated on PS particles): 6.33 mg/L and 11.7 mg/L (Figure 5).

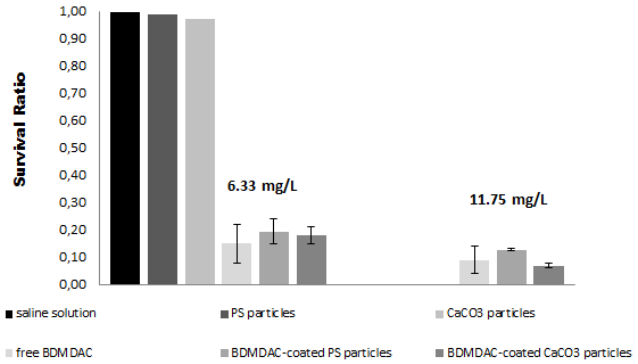


Figure 5 – Survival ratio of *P. fluorescens* after 1 hour of exposure to free biocide, PS particles coated with BDMDAC and $CaCO_3$ particles coated with the same biocide.

These treatments decreased the number of biofilm viable cells in 80.6 % (6.33 mg/L) and 87.2 % (11.7 mg/L).

The same tests were performed for the application of $CaCO_3$ particles coated with BDMDAC. Preliminary results are promising comparatively to the polystyrene microparticles carrying the antimicrobial agent, with the same amount of biocide promoting higher percentage of biofilm inactivation: 81.9% for 6.33 mg/L and 93.3% for 11.75 mg/L of BDMDAC. The particles acted on biofilm control without apparent destruction or biodegradation.

The effects of the application of free BDMDAC had a moderately higher effect on number of viable cells 84.9% (6.33 mg/L) and 91.3% (11.75 mg/L). However, the differences observed were not statistically significant ($P > 0.05$).

In a previous report (Simões et al. 2005), it was verified that the exposure of *P. fluorescens* biofilms during 30 min to the QAC cetyl trimethyl ammonium bromide at 328 mg/L, only promoted the inactivation of 65% of the biofilm activity. Moore and his co-workers (2008) applied QAC`s at concentrations higher than 100 mg/L to control domestic drain biofilms.

Overall, the results obtained in the present study clearly demonstrate that this novel biofilm control strategy may have potential public health, environmental and economical benefits by effectively limiting the levels of biocides used in cleaning and disinfection practices.

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 biofilm formed by *P. fluorescens*. It was possible to conclude that the BDMDAC coated particles of PS and $CaCO_3$, even if only partial inactivation was achieved (reduction of viable counts of about 90%), are significantly active against *P. fluorescens* biofilms. $CaCO_3$ coated particles seem to be more active than those of PS. This promising technology will have a potential impact in reducing environmental costs, health risks associated with the intensified use of antimicrobial chemicals and cleaning and disinfection costs in many industrial plants. Studies are in progress in order to assess the life span and the reuse of those active particles.

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