Integrated Masters in Chemical Engineering

Development of Microparticles with Biocidal Properties

Master's Thesis

Developed within the discipline of

Development Project in Academic Environment

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Abstract

Biofilm formation is a strategy that bacteria use in order to survive in hostile environments, causing serious problems in the food industry, cooling water systems, medical equipment, etc. The control and destruction of undesirable biofilms often include the use of chemical products with antimicrobial properties such as biocides and surfactants. However, these substances can be harmful to the environment and consequently they should be used in small quantities as possible.

The goal of this work was to develop microparticles with biocidal properties for biofilm control. In this study, the efficacy of biocide carriers against suspended cells of *Pseudomonas fluorescens* was assessed by quantitative cell-plating viability studies. The biocide benzyldimethyldodecylammonium chloride (BDMDAC), a benzalkonium chloride surfactant, was adsorbed on polystyrene (PS) particles of 4 μm diameter, pre-coated with a single layer of polyethyleneimine (PEI) and of sodium polystyrene sulfonate (PSS) by layer-by-layer self-assembly (LBL) technique.

The evaluation of biocide carrier activity was carried out through the determination of the survival ratio (CFU's/CFU's in saline solution) of the microbial population after different periods of exposure to BDMDAC coated particles. The assays were performed with a cell suspension in sterile saline solution (0.85% NaCl) containing 1.1×10^3 CFU/mL. After exposure to BDMDAC coated particles, the quantification of viable cells was done by spreading bacteria on Plate Count Agar, and incubation at 30 ºC for 24 h. A cell suspension without coated microparticles was used as control. An efficient biocidal effect (survival ratio = 0) was found at a coated particle concentration of 1.73×10^8 particles/mL after incubation for 30 minutes and 1.21×10^8 particles/mL after 60 minutes. The possibility of reusing BDMDAC coated particles to increase their life time and save biocide was also studied in order to optimize the industrial cleaning procedures. It is possible to conclude that the particles can be reutilized.

Keywords: microparticles, biocides, microorganism and biofilms

Resumo

A formação de biofilmes é uma estratégia que as bactérias utilizam para sobreviver em ambientes hostis. Estes biofilmes podem causar graves problemas na indústria alimentar, sistemas de refrigeração de água, equipamento médico, etc. O seu controlo e eliminação muitas vezes inclui o uso de produtos químicos com propriedades antimicrobianas como por exemplo, biocidas e surfactantes. No entanto, estas substâncias podem ser nocivas para o meio ambiente e consequentemente devem ser utilizados em quantidades tão pequenas quanto possível.

O objectivo deste trabalho foi desenvolver micropartículas com propriedades biocidas para controlo de biofilmes. A eficácia das micropartículas (diâmetro: 4 μm) foi testada numa suspensão de bactérias de *Pseudomonas fluorescens*. O biocida escolhido para ser transportado na superfície das partículas foi o cloreto benzildimetildodecilamónio (BDMDAC). Este biocida é um surfactante que pertence a família dos cloretos de benzalcónio. As micropartículas foram preparadas através da adsorção de polielectrólitos de cargas opostas à superfície de partículas de poliestireno, na seguinte ordem: polielectrólito PEI (Polietilenoimina) em seguida PSS (poli(4-estirenossulfonato de sódio)) e por último o biocida BDMDAC.

A avaliação da eficácia das micropartículas revestidas com biocida foi realizada através da determinação da razão de sobrevivência dos microorganismos (UFC's/UFC's na solução salina) após diferentes períodos de exposição às partículas revestidas. Utilizou-se uma suspensão de *Pseudomonas fluorescens* em solução salina (0.85% NaCl) contendo 1.10×10^3 UFC/ml. Após os diferentes tempos de exposição, a quantificação de células viáveis foi feita através de espalhamento em meio de cultura PCA e incubação a 30 ºC durante 24 h. Para controlo foi utilizado uma suspensão de bactérias sem as partículas. A eficiência das partículas revestidas com BDMDAC, razão de sobrevivência=0, foi registada para uma concentração de partículas de 1.73×10^8 partículas/mL após incubação durante 30 minutos e 1.21×10^8 partículas/mL após 60 minutos. A possibilidade de reutilização das partículas revestidas foi ainda estudada a fim de aumentar o seu tempo de vida e assim poupar biocida. Os resultados mostram que é possível reutilizar as partículas revestidas com biocida.

Palavras-chave: micropartículas, biocidas, microrganismos, biofilmes

List of contents

Nomenclature

LbL	Layer-by-layer
BDMDAC	Benzyldimethyldodecylammonium Chloride
QAC	Quaternary Ammonium Compound
BAC	Benzalkonium Chloride
MBC	Concentration of the Bacterial Effect
PEI	Polyethyleneimine
PSS	Poly(sodium 4-stryrenesulfonate)
SEM	Scanning Electron Microscope
PCA	Plate Count Agar
CFU	Colony-forming Unit
PAH	Poly (allylamine hydrochloride)
EDTA	Ethylenediamine Tetraacetic Acid
M_w	Molecular Weight

1 Introduction

1.1 Framework and Presentation of the Project

Layers of microorganisms and their extracellular polymers ("biofilms") 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 biocides (e.g., chlorine) and detergents in the water stream in considerable large amounts. According to the Directive 98/8/EC, biocides are chemicals with an active and in general toxic effect on living organisms, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism (Rasmussen et al, 1999). Such toxic chemicals are not totally consumed and their discharge into the environment or wastewater treatment plants is a source of serious problems.

The goal of this project was to develop and characterize microparticles with functionalized surfaces that act as carriers of biocide molecules. This will probably reduce the use of toxic chemicals and will minimize health and environmental risks of biocides.

The foreseen advantages of this approach are that polymeric microstructures can be functionalized to be adsorbed at the surface layer of biofilms and to penetrate deeply enough to release the biocide agent in the inner layers. This methodology has not yet been developed for industrial systems and can be used either as a cleaning or preventive technique.

1.2 Work Contributions

Nanotechnology permits to create artificial systems with enormous potential for numerous applications. The purpose of the project is to develop micro- and nanoparticles with functionalized surfaces that carried the biocide. This will save significant amounts of biocide, prevent undesired reactions with other components that can lead sometimes to the formation of organochlorinated compounds (potentially carcinogenic), and finally will reduce

the costs of wastewater treatment due to the presence of residual concentrations of biocides in the effluent. The mitigation of biofilm build up on industrial surfaces will also reduce pressure drop and thermal resistance in pipes and heat exchangers.

Therefore, beneficial impacts are expected with this project in terms of:

- Reduction of environmental costs;
- Better control of the health risks associated with the use of biocides (toxic compounds);
- Savings in energy consumption (reduced pumping costs and higher heat transfer efficiency);
- Reduction of the biocide costs in a large number of industrial plants.

Furthermore, the project will contribute with advancements on the fields of surface science, biofilm science and engineering, because it will: i) allow a better control of the micro- and nanoparticles stability in aqueous solutions; ii) optimize surface interactions in biological systems (particle-biofilms); and iii) study the effects of a controlled release of biocides into the biofilm matrix.

1.3 Thesis Organization

This thesis is organized in five chapters. The first one is the general introduction to the subjects developed along the dissertation as well as some theoretical aspects. In this chapter the context and motivations of the work are presented, as well as the main goal of the project and thesis organization.

The second chapter encloses an overview of layer-by-layer technique, biocides and microorganism that were used in the study. The third chapter fully describes the materials and methodologies used to perform all the experimental work. The main results are presented and discussed in chapter four. The last chapter summarizes the main conclusions of the thesis.

2 State of the Art

Modern strategies to minimize fouling in industrial equipment (pipes, heat exchangers, etc.) focus on optimizing equipment design, developing new surfaces to reduce adhesion and applying efficient surface cleaning/disinfection methodologies, supported by on-line monitoring techniques. During the last 3 decades, a considerable amount of work has been reported on the effect of hydrodynamics on equipment performance and biofilm growth (Characklis *et al.*, 1990; Melo and Vieira, 1999) and although further advances are still possible on this aspect, the improvements will be generally marginal. The present state-of-the-art recommends the use of liquid velocities around 2 m/s inside tubes in order to take advantage of the stronger shear stresses to reduce biofilm growth. However, high velocities have some drawbacks since, at the same time, they were shown to produce more compact deposits that are more difficult to remove from the surface (Melo and Vieira, 1999) both by mechanical actions and chemical methods (biocides find a higher resistance to diffusion in such compact structures). 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 (Rosmaninho and Melo, 2006; Santos *et al.*, 2004) have been developed with an interesting potential for reducing deposit adhesion, but their application is much dependent on the relative costs of such expensive materials as compared to the costs associated to fouling. Applications to specific cases, such as tubing in medical purpose equipment, can be cost-effective, but this is not the case at industrial equipments. 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 (such as biocides and dispersants) are carried as solutes by the bulk liquid and only a minor part does actually take part in the cleaning process, leaving a large amount in the discharge waters.

Nowadays, cleaning procedures are still highly inefficient processes that consume great amounts of water, chemicals and time. Large quantities of biocides are applied everyday to remove biofilms from power plant condensers (water flow rates above 2000 m^3/hour). Localized biofouling layers appear in different places of shell-and-tube heat exchangers and in plate heat exchangers, such as in the cooling section of milk pasteurizers (both in the process and in the water side). More efficient cleaning techniques are needed, that consume less water, fewer chemicals, less energy, less time and, simultaneously, are able to target

the sites where the fouling layers develop. This will reduce the environmental burden (wastewater treatment) and minimize production losses caused by frequent stopping for cleaning.

To reach such goals, a new approach that combines new knowledge-based multifunctional materials surface science and controlled flow patterns was proposed, based on the use of polymeric functionalized microstructures (i.e., particles) that will carry the cleaning/disinfection agents and will deliver them at the appropriate sites. The thesis addresses only the first phase of the project (particle development).

2.1 Layer-by-Layer Thecnique

The Layer-by-Layer (LbL) self-assembly of oppositely charged polyelectrolytes onto colloidal particles has been used to create novel nano- and microparticles with well controlled size and shape, finely tuned wall thickness and variable wall compositions (Decher, 1997; Donath *et al.*, 1998; Caruso *et al.*, 1998; Cordeiro *et al.* 2004). 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).

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 (figure 1) (Donath *et al.*, 1998).

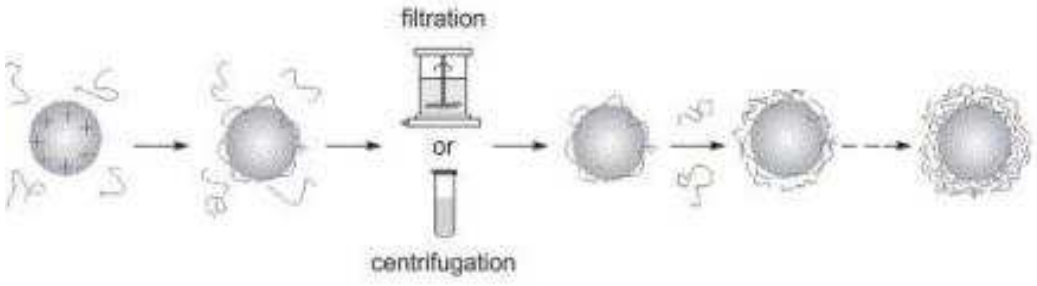


Figure 1: Schematic representation of LbL technique. Polyelectrolyte added into a system adsorbs onto the template leading to the charge reversion. After removal of polyelectrolyte excess (by washing of flat substrate or filtration or centrifugation of colloidal cores), oppositely charged polyelectrolyte is added. The cycle is repeated to obtain a multilayer film or shell (adapted from Antipov *et al.*, 2004).

At present, there are two general approaches to encapsulate macromolecules into polyelectrolyte capsules using the LbL technique. The first method consists of formation of particles out of molecules subjected to encapsulation. Dye and drug nanocrystals were used to template LbL assembly leading to encapsulation. The second approach for encapsulation of macromolecules exploits preformed hollow capsules and incorporates the macromolecules from the surrounding medium by switching the permeability of the hollow capsule shell (Volodkin *et al.*, 2004) (figure 2 and 3).

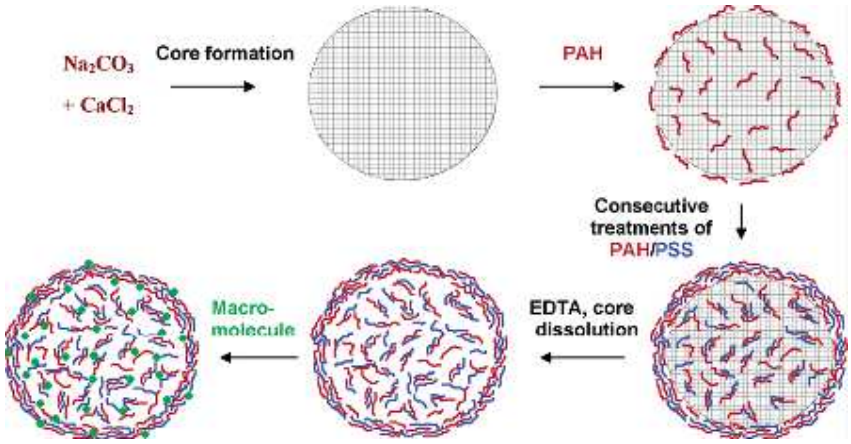


Figure 2: Scheme of capsules fabrication and encapsulation of macromolecules into capsules (Volodkin *et al.*, 2004).

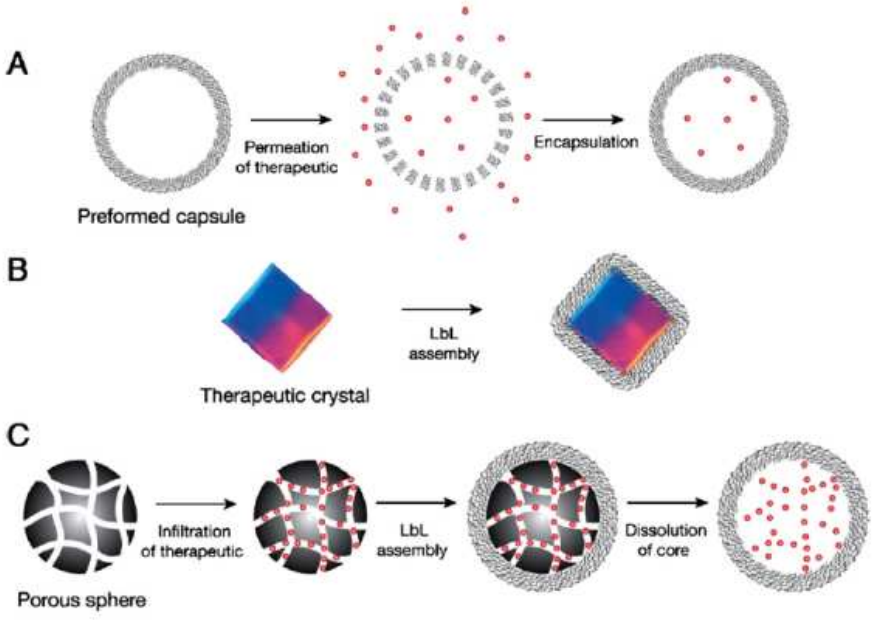


Figure 3: Different methods for encapsulating therapeutics: (A) loading preformed capsules, (B) encapsulation of crystalline particles and (C) incorporation in porous materials (Johnston *et al*, 2006).

2.2 Biocide

The cleaning agent benzyldimethyldodecylammonium chloride (BDMDAC) used in this work is a quaternary ammonium compound (QAC) and a component of benzalkonium chloride (BAC) that is normally used in re-circulating cooling water systems (figure 3). BAC consists of a mixture of three alkyldimethylbenzlylammonium chlorides which differ only in the length of the alkyl side chains (C_{12}, C_{14} or C_{16}) (Bull *et al.,* 1998). BACs are surfactants with detergent and antimicrobial properties that are produced as industrial cleaners (Ferrer and Furlong, 2002) and are useful antiseptics and disinfectants.

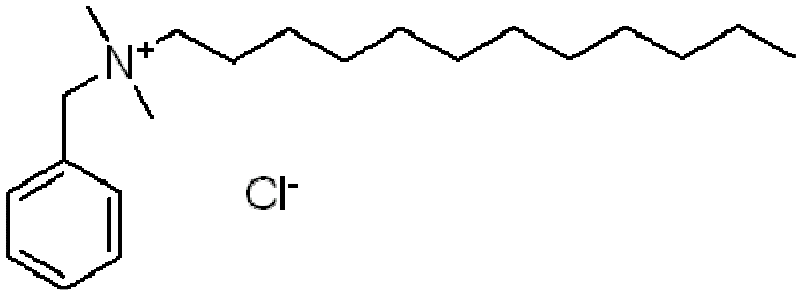


Figure 3: Chemical structure of the biocide BDMDAC.

Surfactants have two regions in their molecular structure: a hydrocarbon water-repellent (hydrophobic) group and a water-attracting group (hydrophilic or polar) group. They can be classified as cationic, anionic, non-ionic and ampholytic, depending on the nature of their hydrophilic group (McDonnell and Russell, 1999).

Quaternary ammonium compounds such as BDMDAC are cationic act by disrupting cell membranes and, depending on the their concentration, they can be either bacteriostactic or bactericidal (Ferrer and Furlong., 2001).

QACs are membrane active agents with a target site predominantly at the cytoplasmatic membrane in bacteria and at the plasma membrane in yeast (figure 4).

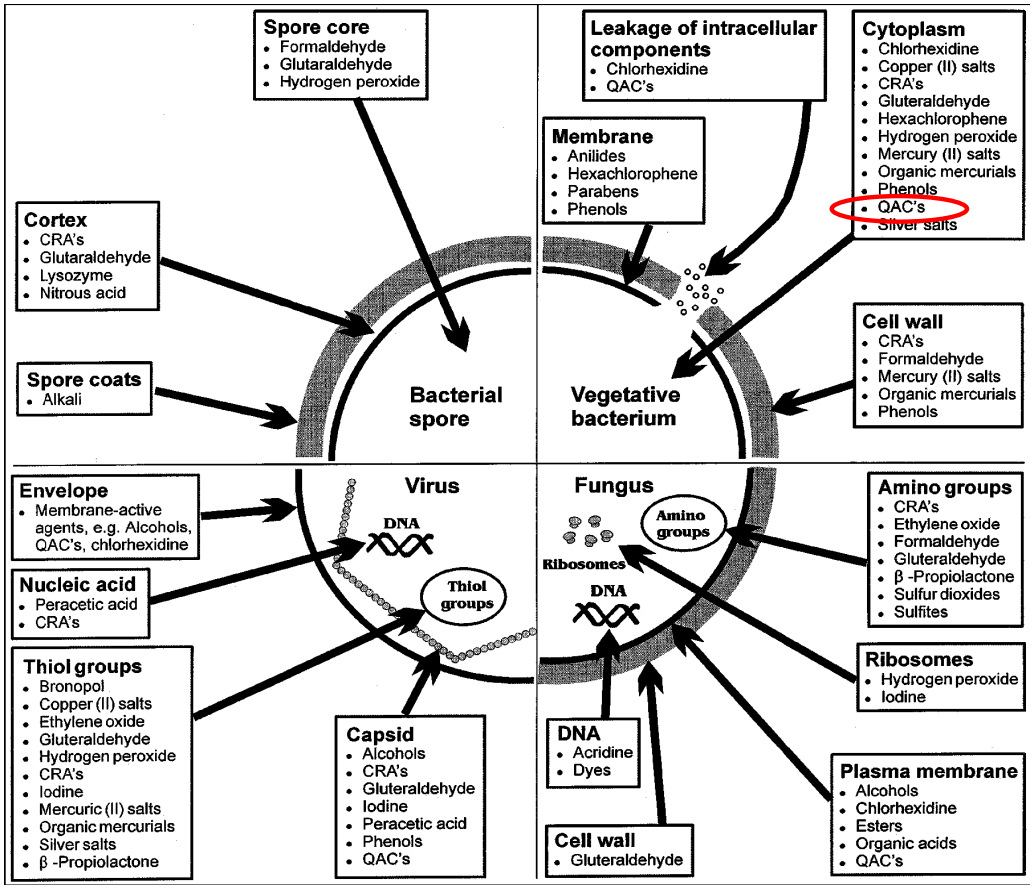


Figure 4: Mechanisms of microorganism inactivation by biocides (according Cloete *et al.*, 1998).

The details of their mechanism of action are not well identified. Several observations indicate the following sequence for a cationic surfactant (McDonnell and Russell, 1999):

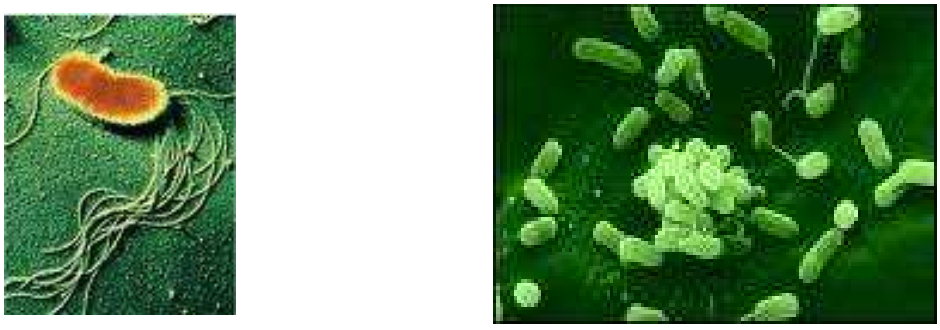
- Adsorption and penetration of the agent into the cell wall;
- Reaction with cytoplasmatic membrane (lipid or protein) followed by membrane disorganization;
- Leakage of intracellular low-molecular-weight material;
- Degradation of proteins and nucleic acids;
- Wall lyses caused by autolytic enzymes.

The QACs can be applied under neutral/alkaline conditions and can be in contact with any type of surface material including the ones used in food.

2.3 Microorganism

The microorganism used in this work was a strain of *Pseudomonas fluorescens*.

P. fluorescens are rod shape Gram-negative, motile, aerobic, with a very versatile metabolism and can be found in water and soil (Prescott et al, 1996) (figure 5). One of the most important characteristic of *P. fluorescens* is the ability of this species to form a biofilm in almost any conditions. The characteristics and the amount of a biofilm formed by this bacterium is directly related to factors such as temperature, composition of the media, osmotic pressure, pH, iron and dissolved oxygen (O'Toole *et al.*, 2000).



(a) (b)

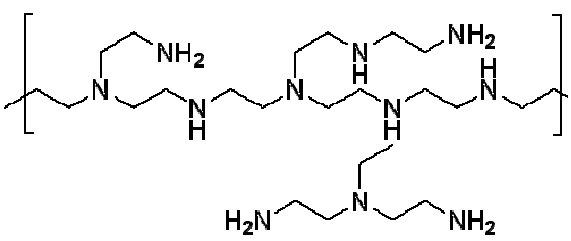
Figure 5: Scanning electron micrograph of *Pseudomonas fluorescens*: (a) aerobic soil isolate (www.scienceclarified.com/As-Bi/Bacteria.html) (b) wheat root-colonizing isolate (www.tau.ac.il/.../virtau/5-Evgeniy_A/evgeniy.htm)

The use of *P. fluorescens*, as a model microorganism, is related to the fact that this bacterium is ubiquitous in biofilms formed in industrial systems and has potential to cause serious problems in terms of operations of the process and final product safety in food industry (Pereira *et al.*, 1998; Pereira and Vieira 2001; Simões *et al.*, 2005). The availability of information regarding the growth conditions and biofilm formation properties and behaviour (Simões, 2005) was also a decisive factor behind that choice.

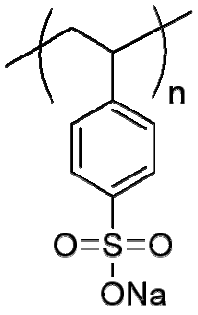
3 Materials and Methods

3.1 Particle's Production Process

Polyethyleneimine (PEI – M_w 750 000) 50% (w/v) in water, Poly(sodium 4-styrenesulfonate) (PSS – M_w 70 000) (figure 6) and boric acid (SigmaUltra minimum 99.5%) were obtained from Sigma-Aldrich. Benzyldimethyldodecylammonium chloride (BDMDAC – M_w 339.9) (figure 3) was obtained from Fluka. All chemicals were used without further purification.



(a)



(b)

Figure 6: Chemical structure of (a) polyethyleneimine (PEI) and (b) Poly(sodium 4-styrenesulfonate) (PSS).

Poly(styrene) (PS)-research particles 4.37 μm ± 0.07 μm 10% w/v aqueous solution were obtained from Microparticles GmbH (figure 7). These PS microparticles, also called Latex-microspheres, have the followed physical and chemical properties:

- Particle Density: 1.05 g/cm^3
- Refractive Index: 1.59
- High monodispersity and uniform spherical shape
- Hydrophobic surface
- Non-specific adsorption of proteins
- Low temperature resistance up to 100 ºC
- Soluble in organic solvents (dependent on the degree of cross-linking)

- Coefficient of Variation (C.V. value) of < 3% for research grade particles and for particle size standards

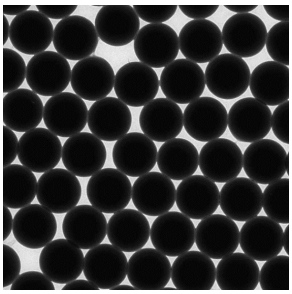


Figure 7: Transmission Electron Microscopy (TEM) images of 470 nm PS particles (h ttp:// www.microparticles.de/properties.html).

The particles were prepared using the layer-by-layer self-assembly (LbL) technique as state above. The oppositely charged polyelectrolytes (PEI and PSS) and BDMDAC, were assembled on polystyrene cores, in a process that comprises the following 3 steps according figure 8. Polystyrene particles were allowed to interact with the PEI solution (1 mg/mL in borate buffer solution) for 20 minutes, and then washed in borate buffer solution 0.1 M pH 9 to remove the excess polymer. After this procedure the core was positively charged and was used for the deposition of the polyanion PSS, followed by the cation 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 minutes, centrifuging at 4000 rpm for 4 minutes and resuspending them in borate buffer pH 9. This step was repeated twice.

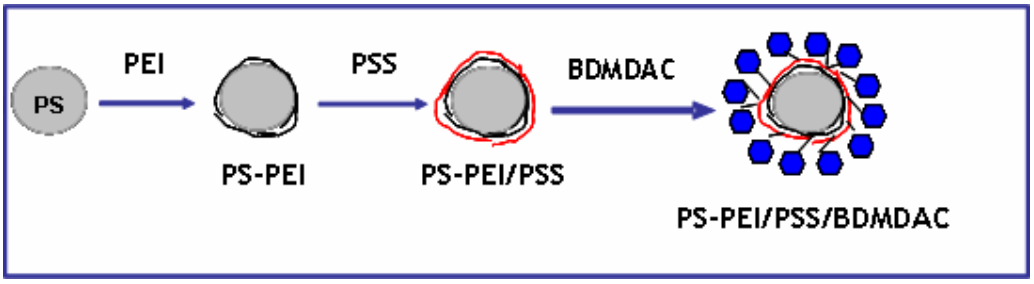


Figure 8: Schematic sequence of particle functionalization formation.

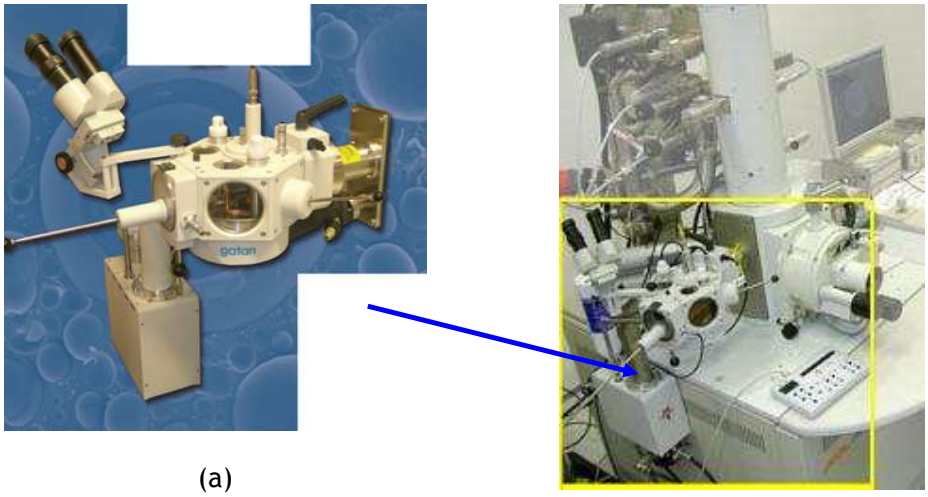
The solvent used in the whole process was borate buffer solution at pH 9. It was selected since the ionic strength of the solution as well as the pH value allows a better layer-by-layer process by promoting the right superficial charge for the different molecules intervening in the process.

3.2 Characterization of the Coated Particles

3.2.1 CryoSEM and X-ray Microanalysis

The coated particle integrity was analysed by CryoSEM (model Gatan ALTO 2500) (figure 9), at CEMUP (Centro de Materiais da Universidade do Porto).

Cryo preparation techniques for SEM have become essential for the observation of wet or "beam sensitive" sample. Using these techniques, the need for conventional preparation methods, such as critical point drying or freeze drying is removed. Besides, it allows observation of the sample in its natural hydrated state. CryoSEM is also a very rapid process, typically only a few minutes are needed.



(a) (b)

Figure 9: CryoSEM equipament, Cryo preparation chamber (a) (http://www. gatan.com /sem/alto_ 2500_.php) that attaches to a SEM (b).

The sample is fixed on a holder with a layer of carbon-rich conductive glue (conductive to allow discharge of electrons). It is rapidly frozen with a nitrogen "slush" that transmits cold well (figure 10). The holder with frozen sample is held under liquid nitrogen to be couple to a rod and pulled back into a small cylindrical container. This is done to transfer the sample to the high vacuum cryo unit and prevent too much contamination with gas particles while sliding in the sample into the cryo-chamber. The cryo-chamber is equipped with a knife that can be handled from outside by means of a level to fracture the sample to expose internal structure. Finally a thin conductive coating is usually applied to allow high resolution imaging or microanalysis in the SEM. The entire or fractioned sample is further placed into the observation chamber with a rod.

(a)

(b)

Figure 10: (a) sample in the support and (b) rapid freezing station that contains nitrogen 'slush'.

The X-ray microanalysis is an analytical technique for determination of the chemical composition of solid samples, thin layers or particles in electron microscopes like CryoSEM.

3.2.2 Size Distribution in Number and Volume

The size distribution of the particles was determined in a Coulter Particle Size Analyzer (model LS 230 – small volume module plus) by Laser Diffraction (figure 11). The analysis of the particle size was considered as volume distribution and number distribution.

Figure 11: Coulter Particle Size Analyser.

A sample placed in the fluid module is circulated through a sample cell at a constant speed. A beam of laser light shone through the cell is diffracted by particles within the sample, and the forward scattered (or diffracted) light is collected by series of detectors. The

distribution of light falling on the sensors enables the size distribution of the sample to be calculated.

3.2.3 Zeta Potential

The zeta potential of the particles was determined using a Nano Zetasizer (Malvern instruments, UK) (figure 12).

Figure 12: Nano zetasizer (Malvern instruments).

Most particles dispersed in an aqueous system acquire a surface charge. These surface charges modify the distribution of surrounding ions, resulting in a layer around the particles that is different from the bulk solution. If the particle moves, this layer moves as part of the particle. The zeta potential is the potential at the point in this layer where it moves past the bulk solution. Zeta potential is a measure of one of the main forces that mediate interparticle interactions.

Zeta potential is measured by applying an electric field across the dispersion. Particles within the dispersion with non-zero zeta potential will migrate toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential.

3.3 Biocidal Effect of BDMDAC Coated Particles – Determination of the Minimum Bactericidal Concentration (MBC)

The bactericidal effect of the BDMDAC coated particles (PS-PEI/PSS/BDMDAC) was tested by comparison with the effect of the non-coated particles. The *P. fluorescens* strain was cryopreserved in a refrigerated chamber at -80ºC, in a mixture of nutrient broth and 15% glycerol. Bacteria propagation was obtained by removing a inoculum of cryoval with a sterile inoculating loop. The bacteria were then distributed evenly over the surface of solid medium of Plate Count Agar (PCA) using the streak plate technique and incubated for 24 h at 30 ºC.

The biomass obtained was used to prepare a suspension in sterile saline solution (0.85% NaCl) with an optical density (O.D.) of approximately 0.22 at 610 nm was obtained. The suspension was serially diluted (1 mL of sample was transferred to a tube containing 9 mL sterile saline solution) until 1:100000. An aliquot of 1.0 mL was colleted and was used to test biocide effect of the coated particles (figure 13).

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 biocidal effect of the different systems was evaluated at different incubation times (0, 30 and 60 minutes). The experiments were performed as shown in the figure 13. After each incubation time, 100 µL of sample were spread on Plate Count Agar (PCA) with a sterilized glass rod and incubated for 24 h at 30 °C. The viable cells were counted to assess the biocidal effect.

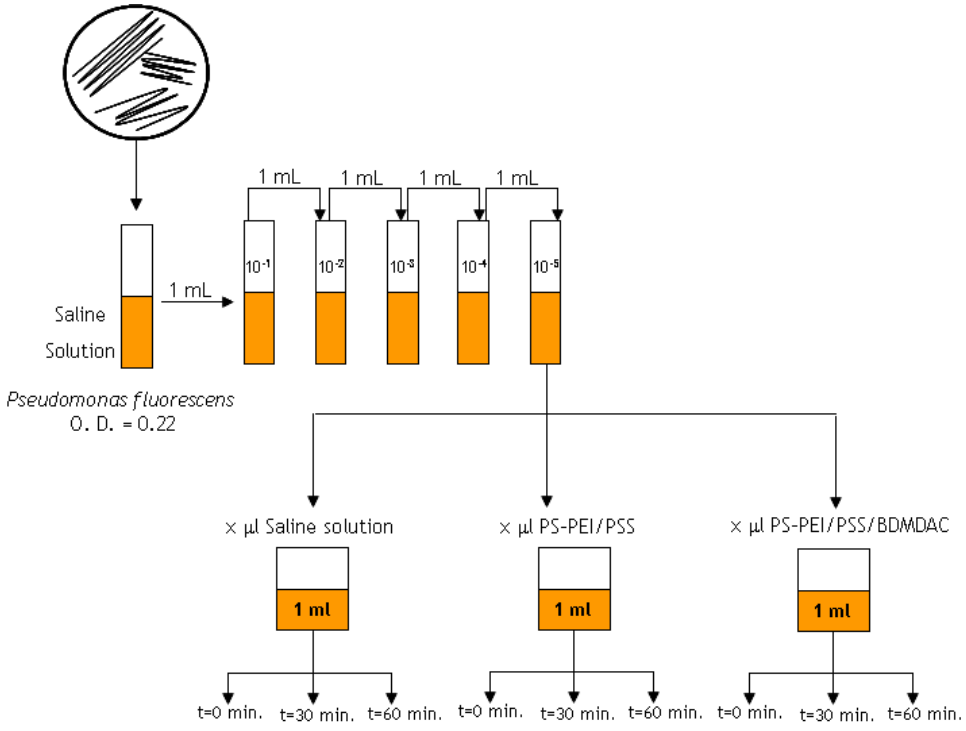


Figure 13: Schematic representation of the experimental procedure. x is the volume tested.

The evaluation of the minimum amount of surfactant/biocide needed for effective microbial reduction was carried out through the determination of the survival ratio (ratio between the CFU's in test/ CFU's in saline solution) of the microbial population after different periods of exposure to BDMDAC coated particles.

3.4 Evaluation of the Possibility of Reutilization of Biocide Coated Particles

The goal of this experiment was to evaluate the likelihood of reutilization of the BDMDAC coated particles. The experiment was similar to the one described in section 3.3, but in this case the procedure was repeated twice. In the second test, the BDMDAC coated particles were the same that were used in the first test. The experiments were performed as shown in the following scheme (figure 14).

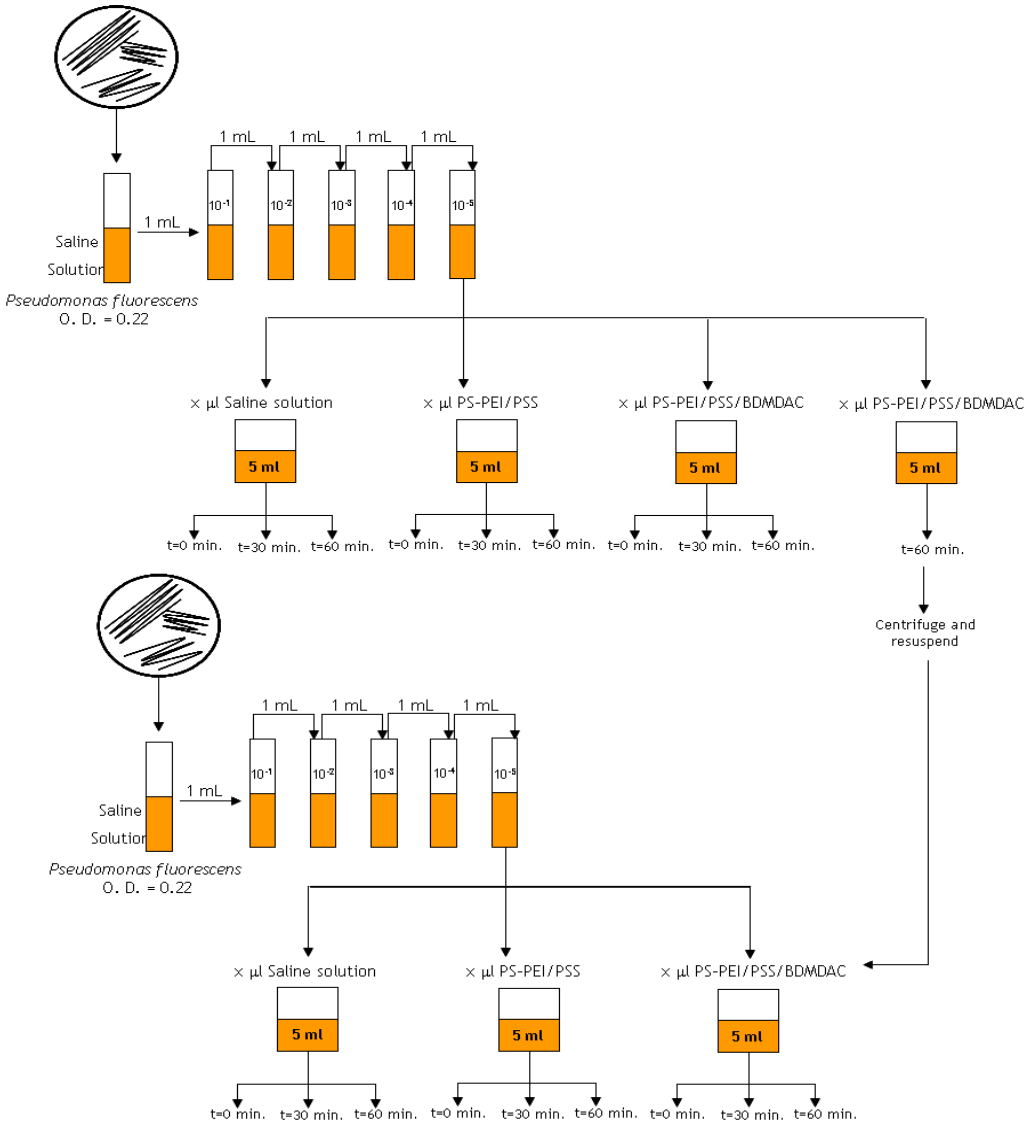


Figure 14: Schematic representation of reutilization experiment of BDMDAC coated particles.

The x value was the volume of the BDMDAC coated particles used. This experiment was performed at the minimum concentration of BDMDAC coated particles needed for biocidal effect.

4 Results and Discussion

Figure 15 show optical fluorescence microscopy images of the PS-PEI/PSS/BDMDAC particles stained with rhodamine. The excitation wavelength used was 566 nm. This analysis allows to evaluate the particle preparation and the degree of the aggregation.

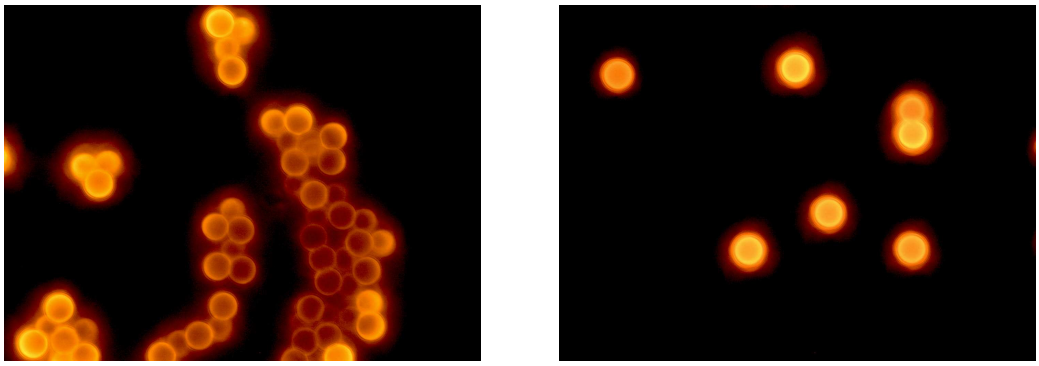


Figure 15: Micrographs of optical fluorescence microscope (×2000) PS-PEI/PSS/BDMDAC particles stained with Rhodamine.

4.1 CryoSEM and X-ray Microanalysis

CryoSEM was used to visualize the morphology of the particles as well as the presence of the external layer (BDMDAC and/or PSS). The selection of this technique was mainly related to the fact that it allows the analysis of particles in the hydrated state. X-ray microanalysis coupled with CryoSEM was used to confirm the elemental constitution of the particles surface.

Cryo-SEM images of PS-PEI/PSS particles show that they are spherical and have a rough surface (see example in figure 16).

The particles with an additional layer after contact with BDMDAC solution can be seen in Figures 17 and 18. X-ray microanalysis indicates that the external layer is mainly composed by C (carbon) and its content is considerably higher in PS-PEI/PSS/BDMDAC particles when compared with PS-PEI/PSS particles (Figures 16 and 18). This would be expected because BDMDAC has a very long carbon chain (Figure 3).

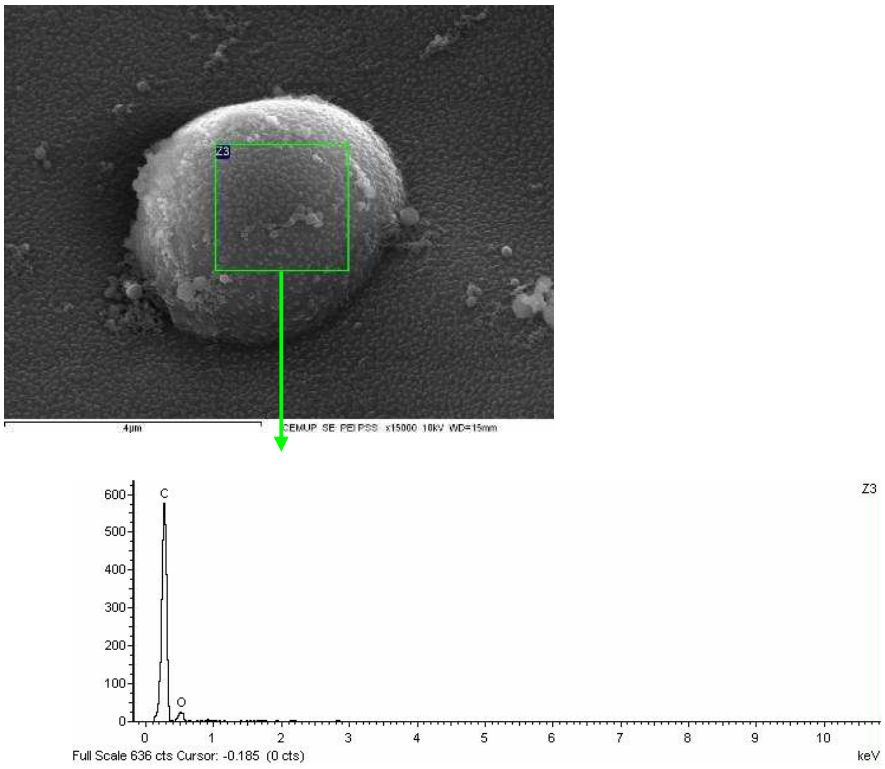


Figure 16: CryoSEM image (×15000, 10 kV) and X-ray microanalysis of PS-PEI/PSS particles.

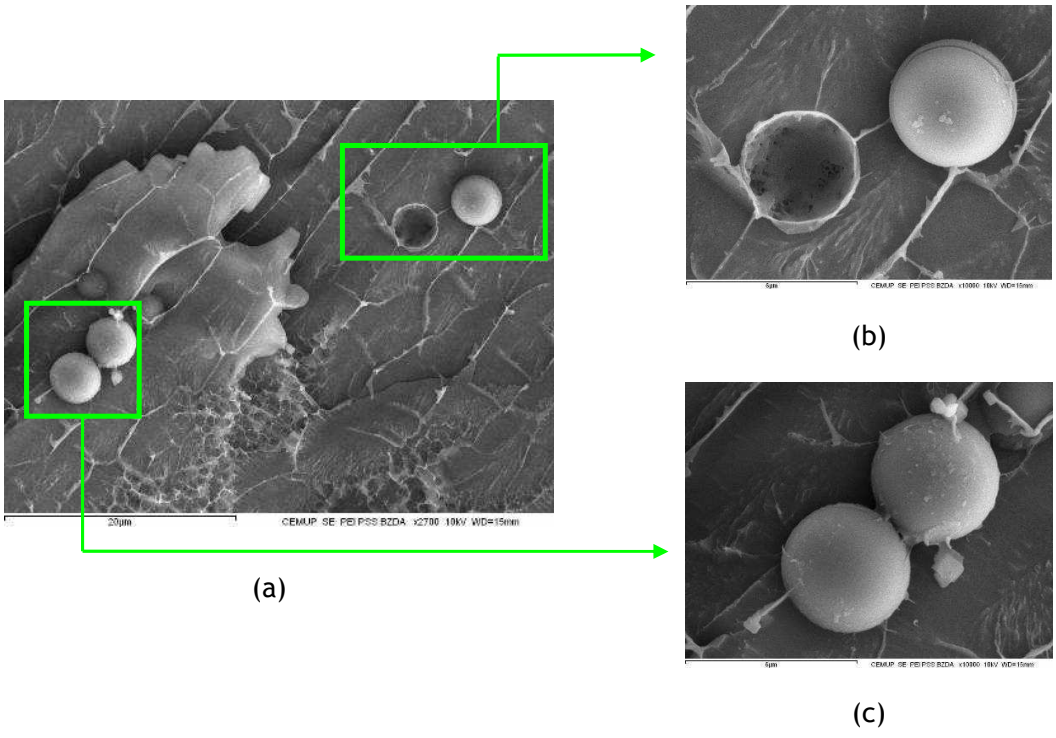


Figure 17: CryoSEM images (a) (×2700, 10 kV) of PS-PEI/PSS/BDMDAC particles; (b) and (c) are higher magnification view of the particles (×10000, 10 kV).

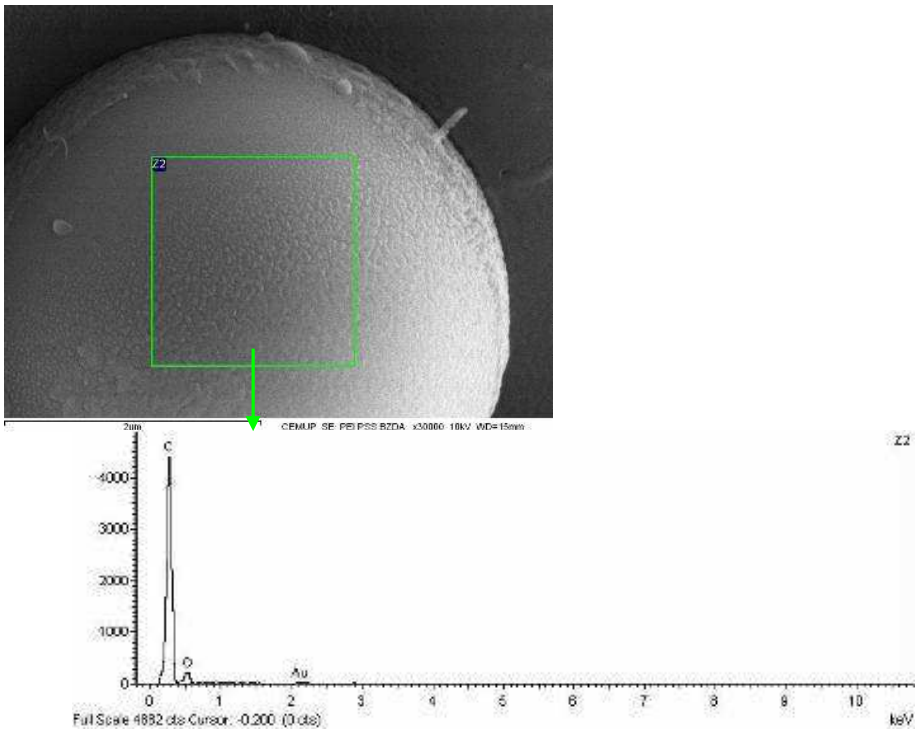


Figure 18: CryoSEM image (×30000, 10 kV) and X-ray microanalysis of PS-PEI/PSS/BDMDAC particles.

4.2 Size Distribution in Number and Volume

The size distribution of the particles is depicted in Figure 19 and Figure 20. Three populations could be found for PS-PEI/PSS particles with an average size 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), whereas a wider distribution could be seen between 6 µm and 20 µm (Figure 19). This can be related to particle aggregation due to the hydrophobic interactions between the carbon chains of BDMDAC. However, when consider the size distribution in number only the two populations of smaller diameters (3.0 and 4.4 µm) are observed, indicating that the number of particles of about 20 µm is not significant, for all type of particles (Figure 20).

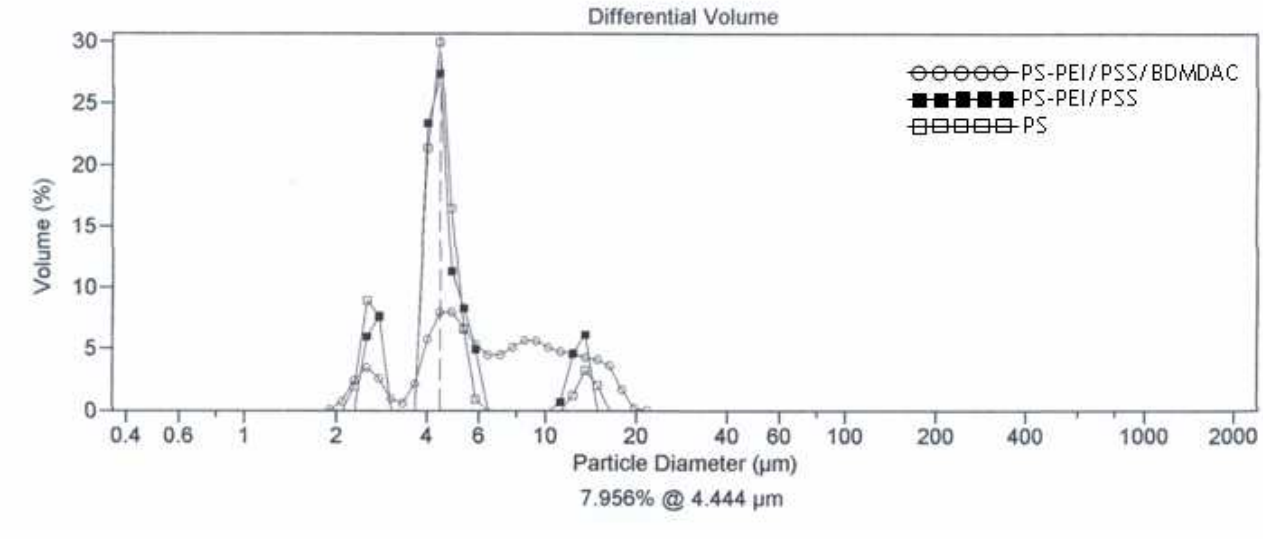


Figure 19: Volume distribution of the particles (PS, PS-PEI/PSS and PS-PEI/PSS/BDMDAC).

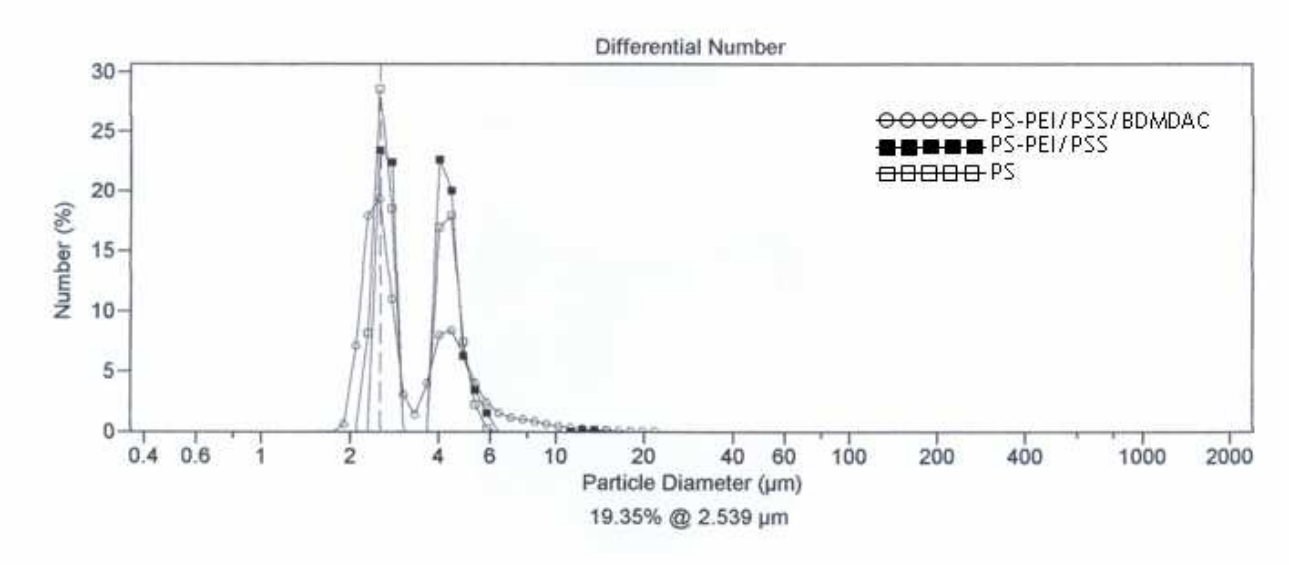


Figure 20: Size distribution of the particles in number (PS, PS-PEI/PSS and PS-PEI/PSS/BDMDAC).

4.3 Zeta Potential of the Coated Particles

The zeta potential of the particles PS-PEI/PSS at pH 9.0 in borate buffer solution is -33.9±4.03 mV.

The zeta potential of PS-PEI/PSS/BDMDAC particles was also determined in borate solution at different pH values, 3.6, 7.0 and 9.0, and was 0, -16.4±3.6 and -20.7±4.9 mV, respectively (Figure 21).

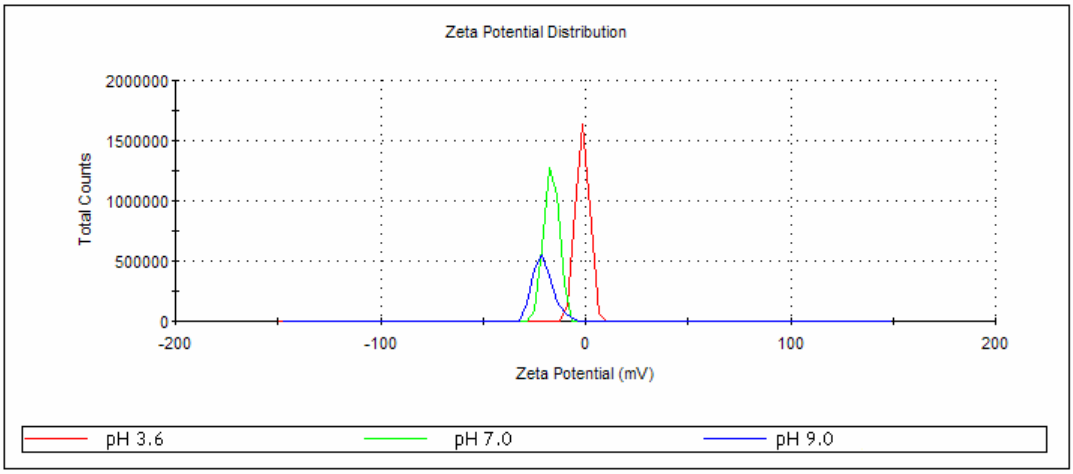


Figure 21: Zeta potential of PS-PEI/PSS/BDMDAC particles at different pH values in borate buffer solution.

The isoelectric point of the particles in borate buffer solution was found to be around 3.6 (zeta potential 0 mV). The fact that the particles have a higher in absolute number zeta potential at pH 9 (zeta potential -20.7±4.9 mV), and consequently are more stable in solution, explains the option of producing the particles at this pH value.

The shift in the zeta potential values of the PS-PEI/PSS and PS-PEI/PSS/BDMDAC particles to less negative values allows to confirm the presence of the BDMDAC layer.

The zeta potential of the biocidal particles was also measured in water at neutral pH to mimic the real conditions at which they will be used. When dispersed in water, the isoelectric point (zeta potential ~0 mV) of the particles seemed to have changed to around 7.

The different value of zeta potential found for a pH of around 7 for water and borate buffer solution also reinforces the importance of the dispersion medium on the superficial properties of the particles because the medium has to be appropriate for the adhesion of polyelectrolytes.

4.4 Minimum Concentration of Biocide for Bactericidal Effect (MBC)

The biocidal effect for the different systems was assessed by Heterotrophic Plate Count (HPC) and determination of Colony Forming Units (CFU) after incubation for 24 h at 30 ºC in PCA medium. The effect of the biocide was determined as a survival ratio defined as CFU's / CFU's in saline solution instead of CFU only, to allow normalization between the results from the different experiments.

The test was performed at different particle concentrations. The stock particles concentration was estimated considering the PS particle mass existent in 5.0 mL of the particle suspension divided by the mass of a single particle (see annex 1).

The correspondent mass of the total particles was 5.04×10^{-2}g. Thus, the number of particles in the 5.0 mL volume was 3.45×10^{9}.

Different volumes of the stock suspension were tested on 1.0 mL of the microorganism suspension. The number of particles of each volume used is given in Table 1.

Table 1: Volume taken from stock solution and the corresponding number of coated particles.

Volume taken from batch solution of coated particles (µl)	Nº of particles in test (coated particles)
10	6.90×10^{6}
100	6.90×10^{7}
150	1.04×10^{8}
175	1.21×10^{8}
200	1.38×10^{8}
250	1.73×10^{8}
300	2.07×10^{8}
450	3.11×10^{8}
500	3.45×10^{8}

The results obtained for the misture of PS-PEI/PSS particles with the suspension of the *P. fluorescens* showed that they have no bactericidal effect (survival ratio around 1) (Figure 22). The number of microorganisms in the presence of these particles was approximately the same as the control suspension (in average 1.10×10^{3} CFU's/mL, annex 2).

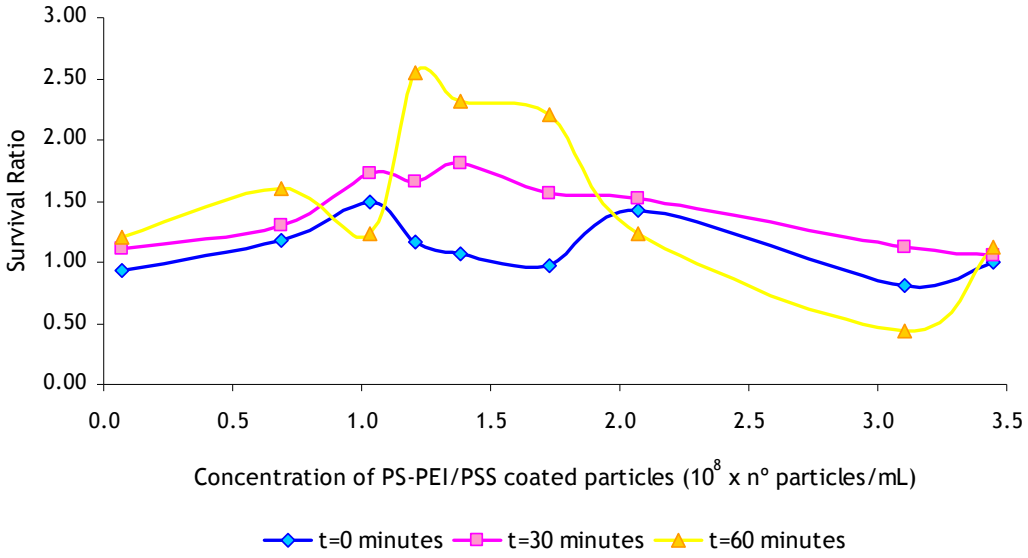


Figure 22: Representation of the survival ratio (CFU/CFU in saline solution) versus concentration of the PS-PEI/PSS particles.

On the other hand, for the particles coated with BDMDAC, (PS-PEI/PSS/BDMDAC), an efficient biocidal effect (survival ratio = 0) was registered for a concentration of 1.73×10^8 particles/mL after 30 minutes in contact with the microorganism and 1.21×10^8 particles/mL after 60 minutes. For time zero, no biocidal effect was registered independently of the concentration of the coated particles used, indicating most probably the need of an induction period for the biocidal effect to start (figure 23).

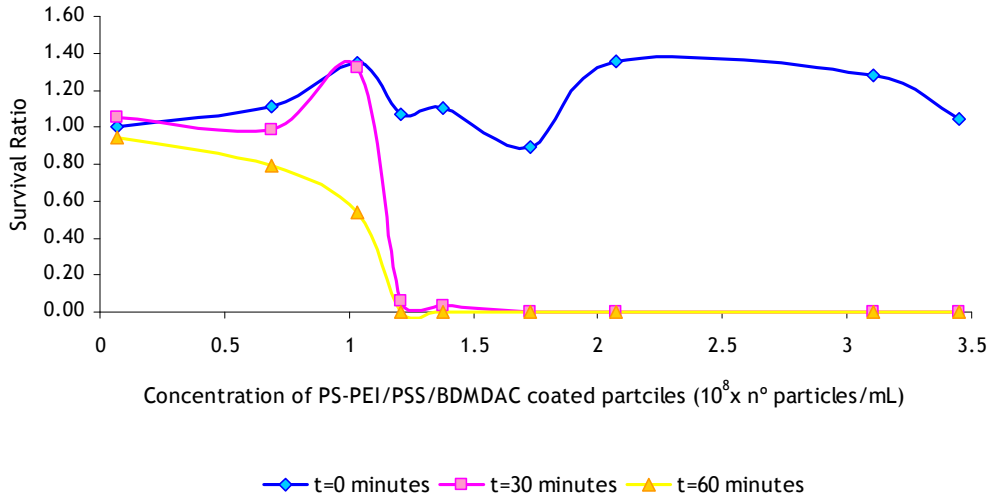


Figure 23: Representation of the survival ratio (CFU/CFU in saline solution) versus concentration of the PS-PEI/PSS/BDMDAC particles.

The concentration of PSS and BDMDAC adhered to the coated particles was estimated as described in the annex 3 and is presented in table 2 for the different particle volumes tested.

Table 2: Concentration of PSS and the BDMDAC in coated particles.

Volume taken from batch solution of coated particles (µL)*	Concentration of PSS in coated particles (mg/mL)	Concentration of BDMDAC in coated particles (mg/mL)
10	5.50×10^{-4}	9.06×10^{-4}
100	5.05×10^{-3}	8.23×10^{-3}
150	7.25×10^{-3}	1.19×10^{-2}
175	8.28×10^{-3}	1.36×10^{-2}
200	9.26×10^{-3}	1.53×10^{-2}
250	1.11×10^{-2}	1.83×10^{-2}
300	1.28×10^{-2}	2.11×10^{-2}
450	1.72×10^{-2}	2.84×10^{-2}
500	1.85×10^{-2}	3.05×10^{-2}

(*) this volume is added to 1 mL of suspension of microorganism.

The survival ratio versus PSS and BDMDAC concentration in coated particles is shown in Figure 24 and Figure 25, respectively.

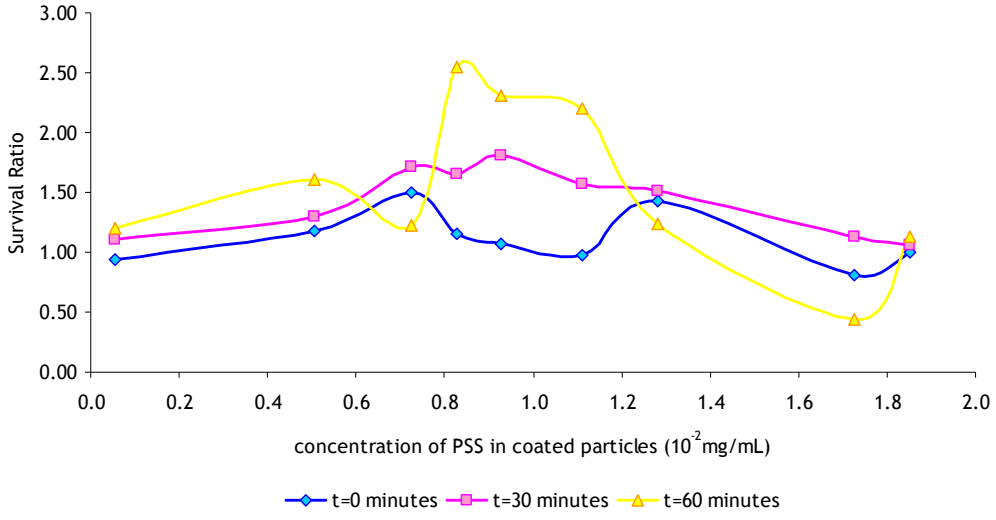


Figure 24: Representation of the survival ratio (CFU/CFU in saline solution) versus theoretical concentration of PSS in the coated PS-PEI/PSS particles.

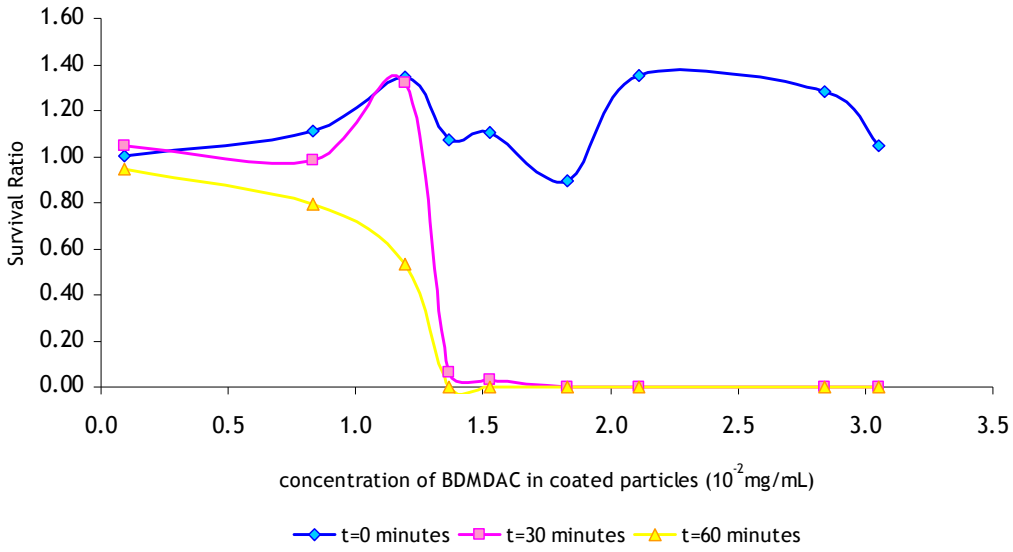


Figure 25: Representation of the survival ratio (CFU/CFU in saline solution) versus theoretical concentration of BDMDAC in the coated PS-PEI/PSS/BDMDAC particles.

As stated above, coated PS-PEI/PSS particles have no effect on the microorganism since the survival ratio is always different from 0. The particles coated with PS-PEI/PSS/BDMDAC have an efficient biocidal effect (survival ratio=0) at a concentration of 1.83×10^{-2} mg/mL after 30 minutes in contact with microorganism and 1.36×10^{-2} mg/mL after 60 minutes.

However, the biocide concentration at the particle surface needs to be experimentally quantified and for that an HPLC technique is being optimized. The methodology is already selected and tested but it still needs to be improved before presenting the final results.

4.5 Evaluation of the Likelihood of Reutilization of the Biocide Coated Particles

The reutilization experiment was performed with 2.07×10^{8} particles/mL that corresponds to 2.11×10^{-2} mg/mL of BDMDAC on coated particles for a volume of 300 µL. The minimum number of coated particles needed to have biocidal effect is 1.73×10^{8} particles/mL that corresponds to 1.36×10^{-2} mg/mL of BDMDAC. However, a slightly higher concentration of particles was used to compensate for possible losses during the experiments.

This experiment allowed concluding that the coated particles can be effectively reused. At this biocidal concentration, the particles have biocidal effect after 30 minutes in contact with the microorganisms when the fresh solution is used and after 60 minutes when reused (figure 26).

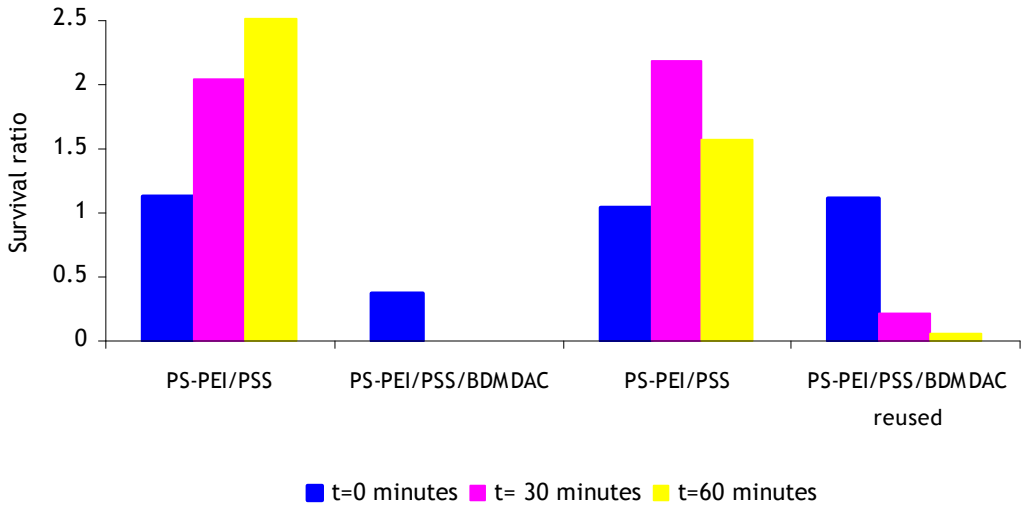


Figure 26: Survival ratio of microorganism in the presence of coated particles freshly prepared and after being used once (reused).

The need of longer incubation time for killing the bacteria can be related either to the fact that some particles may be lost during the reutilization process or some particles may have lost their activity. The particle loss may be due to the centrifugation step needed for reutilization of the particles after the first test (section Material and Methods). After the first test, the particles are collected from the test tube and washed by centrifugation before being reutilized.

The fact that the particles can be reused suggests an interpretation of the mechanism of action of the biocide. One explanation for the interaction mechanism of the BDMDAC coated particles with the microorganisms is the adsorption and penetration of the hydrocarbon chain of the biocide in the cell wall and reaction with cytoplasmatic membrane causing lyses in cell. It is here proposed that the carbonate chain of the biocide is the external layer of the particle that penetrates in the membrane cell (Figure 27).

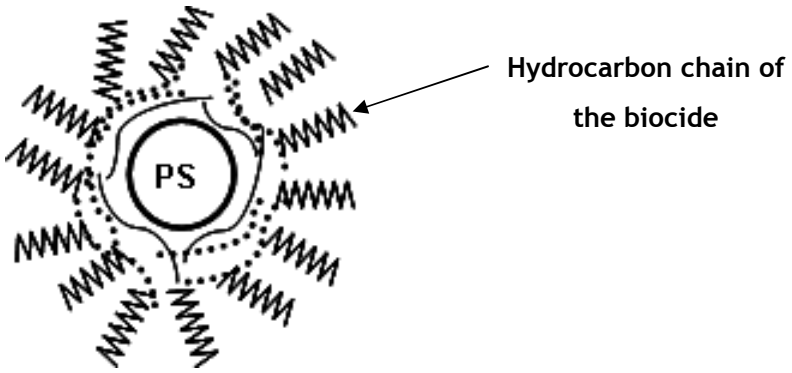


Figure 27: Scheme of the PS-PEI/PSS/BDMDAC particle.

According to this possible interpretation, the BDMDAC is linked to the particle by ion-ion interactions (strong forces), allowing the hydrophobic part of the biocidal molecule, hydrocarbon chain, to be free to interact with the cell membrane of the microorganism. After cell damage, the hydrophobic part of BDMDAC particles remains again free to affect other cells. Future experimental work is planned to verify this biocide action mechanism.

5 Conclusions

The goal of the present project was to develop and characterize microparticles with functionalized surfaces that act as carriers of biocide molecules. The biocide used was benzyldimethyldodecylammonium chloride (BDMDAC) and the particle cores were polystyrene (PS, 4 μm). Functionalized particles were prepared by the layer-by-layer technique using oppositely charged polyelectrolytes, polyethyleneimine (PEI) and poly(sodium 4-stryrenesulfonate) (PSS) and the biocide as the last layer.

The particles PS-PEI/PSS were not active against *Pseudomonas fluorescens* but the particles PS-PEI/PSS/BDMDAC were shown to be active against the microorganism. For the particles coated with BDMDAC, an efficient biocidal effect (survival ratio = 0) was found for a concentration of particles of 1.73×10^{8} particles/mL after 30 minutes of incubation with microorganism and 1.21×10^{8} particles/mL after 60 minutes of incubation. It has been as well demonstrated that the particles can be reutilized.

The present work is the beginning of a study that will have, in the future, a potential impact in reducing environmental costs, a better control of the health risks associated with the use of biocides, a reduction in energy consumption and a decrease in biocide costs in a large number of industrial plants.

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Annexes 1 – Estimative of the Number of the Particles in Stock Solution

The total number of PS particles is given by the mass of the total volume divided by the mass of a single particle, which is calculated using:

$$V = \frac{4}{3} \times \pi \times r^3 \tag{A1.1}$$

$$\rho = \frac{m}{V} \tag{A1.2}$$

The mass of a single PS particle is 1.46×10^{-11}g, knowing that the density is 1.05 g/cm^3. The mass of the particles in stock suspension is 5.04×10^{-2}g. From here it is easy to calculate the number of particles in stock suspension is 3.45×10^9particles.

$$n^o\ particles = \frac{mass\ of\ \ particles\ in\ stock\ solution(g)}{mass\ of\ one\ particle(g)} \tag{A1.3}$$

The stock suspension has 5 mL, so we can determine a particle concentration of 6.90×10^8particles/mL.

Different test volumes were taken from the stock suspension. The tests were carried out on 1 mL of the suspension of micro-organism. A given concentration of particles corresponds to each test volume (equation A1.4).

$$n^o\ of\ coated\ partciles\ in\ test = 6.90 \times 10^8 \times \frac{volume\ taken\ from\ batch\ solution(\mu l)}{1000} \tag{A1.4}$$

Annexes 2 – Experimental Results

Table A2.1: Number of the CFU's in test and corresponding survival ratio (CFU/CFU in saline solution) for PS-PEI/PSS and PS-PEI/PSS/BDMDAC particles.

	Number of CFU's in test		
Volume	**t=0 minutes**	**t=30 minutes**	**t=60 minutes**
10 µl saline solution	3242	3101	2262
10 µl coated particles PS-PEI/PSS	3030	3444	2732
10 µl of coated particles PS-PEI/PSS/BDMDAC	3257	3252	2141
Survival ratio* PS-PEI/PSS	0.93	1.11	1.21
Survival ratio* PS-PEI/PSS/BDMDAC	1.00	1.05	0.95
100 µl saline solution	2668	2052	1826
100 µl coated particles PS-PEI/PSS	3130	2662	2932
100 µl of coated particles PS-PEI/PSS/BDMDAC	2959	2019	1447
Survival ratio* PS-PEI/PSS	1.17	1.30	1.61
Survival ratio* PS-PEI/PSS/BDMDAC	1.11	0.98	0.79
150 µl saline solution	1127	1254	1277
150 µl coated particles PS-PEI/PSS	1685	2156	1570
150 µl of coated particles PS-PEI/PSS/BDMDAC	1518	1656	684
Survival ratio* PS-PEI/PSS	1.49	1.72	1.23
Survival ratio* PS-PEI/PSS/BDMDAC	1.35	1.32	0.54
175 µl saline solution	1392	670	505
175 µl coated particles PS-PEI/PSS	1616	1110	1287
175 µl of coated particles PS-PEI/PSS/BDMDAC	1492	41	0
Survival ratio* PS-PEI/PSS	1.16	1.66	2.55
Survival ratio* PS-PEI/PSS/BDMDAC	1.07	0.06	0
200 µl saline solution	1110	546	420
200 µl coated particles PS-PEI/PSS	1188	990	972
200 µl of coated particles PS-PEI/PSS/BDMDAC	1224	18	0
Survival ratio* PS-PEI/PSS	1.07	1.81	2.31
Survival ratio* PS-PEI/PSS/BDMDAC	1.10	0.03	0

Table A2.2: Number of the CFU's in test and corresponding survival ratio (CFU/CFU in saline solution) for PS-PEI/PSS and PS-PEI/PSS/BDMDAC particles. (Continuation)

	Number of CFU's in test		
Volume	**t=0 minutes**	**t=30 minutes**	**t=60 minutes**
250 µl saline solution	725	463	394
250 µl coated particles PS-PEI/PSS	706	725	869
250 µl of coated particles PS-PEI/PSS/BDMDAC	650	0	0
Survival ratio* PS-PEI/PSS	0.97	1.57	2.21
Survival ratio* PS-PEI/PSS/BDMDAC	0.90	0	0
300 µl saline solution	975	1105	1274
300 µl coated particles PS-PEI/PSS	1391	1677	1573
300 µl of coated particles PS-PEI/PSS/BDMDAC	1320	0	0
Survival ratio* PS-PEI/PSS	1.43	1.52	1.23
Survival ratio* PS-PEI/PSS/BDMDAC	1.35	0	0
450 µl saline solution	899	1124	1363
450 µl coated particles PS-PEI/PSS	725	1269	595
450 µl of coated particles PS-PEI/PSS/BDMDAC	1153	0	0
Survival ratio* PS-PEI/PSS	0.81	1.13	0.44
Survival ratio* PS-PEI/PSS/BDMDAC	1.28	0	0
500 µl saline solution	2415	2348	2010
500 µl coated particles PS-PEI/PSS	2408	2483	2265
500 µl of coated particles PS-PEI/PSS/BDMDAC	2528	0	0
Survival ratio* PS-PEI/PSS	1.00	1.06	1.13
Survival ratio* PS-PEI/PSS/BDMDAC	1.05	0	0

$$(*)\ survival\ ratio = \frac{CFU's}{CFU's\ in\ saline\ solution}$$

Annexes 3 – Estimative Quantification of the Concentration of PSS and BDMDAC Adhered to the Coated Particles

- ✓ **Quantification of the amount of PSS adhered to the particles:**

Firstly, the number of monomers in the polyelectrolyte (value n) was calculated. The molecular mass of one monomer ($C_8H_7NaO_3S$) of PSS is given by:

$$M(C_8H_7NaO_3S)=8\times M(C)+7\times M(H)+M(Na)+3\times M(O)+M(S) \quad \text{(A3.1)}$$

The values of the molar mass of the compounds are in the table A3.1.

Table A3.1: molar mass of different atoms.

Compound	Molecular mass (M) (g/mol)
Carbon (C)	12.01
Hydrogen (H)	1.008
Sodium (Na)	22.99
Oxygen (O)	16.00
Sulphur (S)	32.07
Chlorine (Cl)	35.45
Nitrogen (N)	14.01

The molecular mass of one monomer of PSS is 206 g/mol. The molecular mass of PSS is 70000 g/mol (information given by producer).

$$N^o\, monomers=\frac{M(molecule\, of\ PSS)}{M(one\, monomer)} \quad \text{(A3.2)}$$

The number of monomers is 340 for each PSS macromolecule. Then the surface area of one monomer of PSS was estimated, by calculating first the value of x and y represented in figure A3.1.

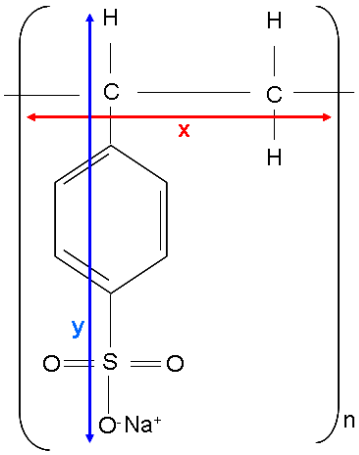

Figure A3.1: Schematic representation of one monomer of the polyelectrolyte PSS.

The bond lengths (pm) were taken from the literature (Atkins and Jones, 2004). In the case of PSS monomer, four type of bonds are present (table A3.2).

Table A3.2: Bond lengths (pm) (Atkins and Jones).

Bond	Average bond length (pm)
C-C	154
C-H	109
C⋯C in benzene	139
C-O	143

The x value (Figure A3.1) includes two C-C bonds and the y value includes one C-C bond, one C-S (~C-O) bond, one C-H bond, one S-O (~C-O) bond and two C⋯C benzene bonds. The value of x was calculated to be 308 pm and y 827 pm. As the polyelectrolyte has 340 monomers, the value of the horizontal axis (X) is 104720 pm:

$$horizontal\ value = 340 \times x \tag{A3.3}$$

The surface area (the area where the molecule is a rectangle) is 8.66×10^{-5} μm^2, which was calculated using:

$$A_{surface\ of\ PSS} = X \times y \tag{A3.4}$$

The area of the particle, that is, one sphere is given by:

$$A_{sphere} = 4 \times \pi \times \left(\frac{d}{2}\right)^2 \tag{A3.5}$$

The area of the particle is 60.00 μm^2. The number of the molecules of PSS on each particle is given by dividing the molecule area by the particle area. The number of PSS

molecules on each particle is 6.93×10^{5}. Considering the number of particles in stock solution, the total number of PSS molecules is 2.39×10^{15}.

- ✓ **Quantification of the amount of BDMDAC adhered to the particles:**

At this time, the number of PSS molecules that is needed to coat the particle is known. Now, the number of molecules of BDMDAC that is needed to coat the same particles has to be calculated. As BDMDAC has only one positive charge and PSS has 340 negative charges, theoretically 340 molecules of BDMDAC are needed to compensate for all the PSS charges.

The number of BDMDAC molecules needed to coat the particles is 8.22×10^{17} in stock suspension. Using the Avogadro constant (6.022×10^{23} mol^{-1}) the concentrations of PSS and BDMDAC in stock solution of particles are 3.97×10^{-9} mol and 1.36×10^{-6} mol respectively.

The molecular mass of BDMDAC is 339.99 g/mol. Therefore, the theoretical concentrations of PSS and BDMDAC in the particles used in each experience can be calculated. For example, for 10 µL of batch solution there are 6.90×10^{6} particles and 4.77×10^{12} molecules of PSS/coated particles. Using equation 10, the amount of PSS and the amount of BDMDAC that are in the particles can be calculated.

$$n(BDMDAC\ or\ PSS)(mol) = \frac{m(BDMDAC\ or\ PSS)(g)}{M(BDMDAC\ or\ PSS)(g/mol)} \qquad \text{(A3.6)}$$