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Development of Functionalized Microparticles with Immobilized Biocide

Dissertation for Master in Bioengineering

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“Tenho em mim todos os sonhos do mundo.”

Fernando Pessoa

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Abstract

Biofilms, known as attached microorganisms aggregated along their extracellular polymeric network, represent part of the strategy that microorganisms use in order to survive in changing and challenging environments. They are also a source of planktonic pathogenic microorganisms in drinking water distribution systems, which will remain viable and distributed to the consumer's tap. The control of undesirable biofilms often includes the use of chemical products with antimicrobial properties, such as chlorine, which are associated with the formation of harmful disinfection by-products. This study suggests the use of a new technological approach to minimize the use of antimicrobial agents and their deleterious effects, based on the principle of drug-delivery systems whereby the biocidal chemicals are transported on micro-nanoparticles. The microparticles were prepared using a layer-by-layer (LBL) self-assembly technique, through the subsequent assemble of oppositely charged molecules of polyethyleneimine (PEI), sodium polystyrene sulfonate (PSS), and benzyldimethyldodecyl ammonium chloride (BDMDAC) on $CaCO_3$, kaolin, silica, sea sand and cork cores. BDMDAC-coated particles were observed by scanning electron microscopy (SEM). Zeta potential measurements indicated that the particles surface was functionalized due to changes in surface charge after the deposition of each layer. The efficacy of the microparticles was assessed against *Pseudomonas fluorescen*s in both planktonic and the biofilm states by determining the cellular viability after different exposure periods to BDMDAC-coated particles. Tests were performed in the same conditions using free BDMDAC. The antimicrobial effects against planktonic cells was very pronounced using 100 mg/L of BDMDAC adsorbed on $CaCO_3$, kaolin and silica particles for an exposure period of 1 hour. The effects obtained with the application of free BDMDAC were similar to those promoted by the application of BDMDAC-coated particles. $CaCO_3$ microparticles were found to be very effective against biofilms, mainly after 2 hours of exposure to the cells. In contrast, kaolin microparticles showed insignificant effects on biofilms under the same conditions, regardless of the exposure period. The overall results indicate that this biocide immobilization method is a promising strategy for the control of microbial growth of planktonic cells and biofilms. Additionally, there are evidences that it may be possible to reuse the microparticles, which would reduce the environmental risks associated with excessive use of chemical agents, thereby providing real environmental and public health benefits.

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Resumo

Os biofilmes, agregados de microrganismos aderidos a superfícies em conjunto com as substâncias polimerias extracelulares que produzem, representam parte da estratégia que os microrganismos usam para sobreviver em ambientes desfavoráveis. Eles constituem também uma fonte de microrganismos patogénicos planctónicos em sistemas de distribuição de água potável, que vão permanecer viáveis e consequentemente distribuídos pelos consumidores. O controlo de biofilmes indesejáveis inclui frequentemente o uso de produtos químicos com propriedades antimicrobianas, tal como o cloro, que estão associados à formação de subprodutos nocivos. Este estudo sugere a utilização de uma nova abordagem tecnológica que visa minimizar o uso de agentes antimicrobianos e os seus efeitos prejudiciais, com base no princípio dos sistemas de entrega de fármacos em que os compostos químicos antimicrobianos são transportados em micro ou nanopartículas. As micropartículas foram produzidas usando a técnica "camada por camada" ou LBL (do inglês layer-by-layer), através da subsequente deposição das moléculas opostamente carregadas, de polietilenoimina (PEI), poliestireno sulfonato de sódio (PSS) e o biocida cloreto benzildimetildodecil de amónia (BDMDAC) em núcleos de $CaCO_3$, caulino, sílica, areia do mar e cortiça. As partículas revestidas com BDMDAC foram observadas através de microscopia eletrónica de varrimento (em inglês, SEM). As medições do potencial zeta indicaram que a superfície das partículas foi funcionalizada devido às mudanças na carga da superfície após a deposição de cada camada. A eficácia das micropartículas foi testada contra *Pseudomonas fluorescens* no estado planctónico e em biofilmes através da determinação da viabilidade celular após diferentes tempos de contacto com as partículas revestidas. Realizaram-se também testes nas mesmas condições usando BDMDAC na forma livre. O efeito antimicrobiano observado contra células planctónicas foi muito pronunciado usando uma concentração de 100 mg/L de BDMDAC imobilizado em partículas de $CaCO_3$, caulino e sílica para um período de contacto de 1 hora. Os efeitos obtidos através da aplicação de BDMDAC na forma livre foram semelhantes aos resultados obtidos com a aplicação de partículas revestidas. As micropartículas de $CaCO_3$ demonstraram ser muito eficientes contra biofilmes, principalmente após duas horas de exposição às células. Em contraste, as micropartículas de caulino mostraram efeito insignificante contra biofilmes nas mesmas condições, independentemente do tempo de exposição. Os resultados gerais indicam que este método de imobilização de biocida é uma estratégia promissora para o controlo

do crescimento microbiano de células planctónicas e biofilmes. Adicionalmente, há evidências de que poderá ser possível reutilizar estas micropartículas, o que levaria a uma redução dos riscos ambientais associados com o uso excessivo de agentes químicos no tratamento de água, proporcionando assim benefícios ao nível ambiental e ao nível da saúde pública.

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Nomenclature

Abbreviations

BDMDAC – Benzyldimethyldodecylammonium chloride

$CaCO_3$ – Calcium carbonate

DBPs – Disinfection by-products

DW – Drinking water

DWDS – Drinking water distribution systems

EPS – Extracellular polymeric substances

HPLC – High-performance liquid chromatography

N_{cells} – Number of cells

PEI – Polyethyleneimine

PEM – Polyelectrolyte multilayers

PSS – Poly(sodium 4-styrenesulfonate)

SD – Standard deviation

SEM – Scanning electron microscopy

SS – Stainless steel

QAC – Quaternary ammonium compound

Chapter 1

1. Work outline

1.1. Background and project presentation

Currently, the presence of biofilms in drinking water distribution systems represents a well-recognized hazard that directly affects the microbiological quality of the water that reaches the consumer's tap (Sharma et al., 2003). Additionally, they constitute a source of planktonic bacteria, including pathogens, leading to the dissemination of many waterborne diseases (Wingender & Flemming, 2011). The conventional disinfection methods used currently in drinking water treatment, mainly chemical disinfection, are commonly associated with a high efficiency in microbial pathogens control. However, they are often inefficient in biofilm control (removal and inactivation). Additionally, the presence of the chemicals is directly responsible for the formation of harmful disinfection by-products (DBPs) that often present carcinogenic characteristics (Sadiq & Rodriguez, 2004). Therefore, and considering also the increased resistance to common disinfection chemicals presented by some pathogens, it is clear the need of new cost-effective, environmental- and health-friendly strategies that allow an appropriate disinfection while avoiding the formation of harmful components (Li et al., 2008).

In this context, and considering the enormous potential already demonstrated by the use of micro and nanotechnology in diverse research fields (medicine, pharmaceutics, environmental), the development of microparticles with functionalized surfaces carrying a biocide is here proposed. The basis of this project is a previous study performed by investigators of the Biofilm Group of the Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE). Previous studies demonstrated that this novel antimicrobial strategy has potential for the control of microbial growth of planktonic cells and biofilms (Ferreira et al. 2010; 2013). Additionally, this biocide application strategy showed to allow the reuse of biocidal molecules, resulting in the reduction of environmental and public health risks associated with excessive use of those agents.

1.2. Objectives

The main purpose of this project was to develop cost-effective microparticles with functionalized surfaces that present antimicrobial properties. With this approach, it is expected the development of a new technology allowing the saving of considerable amounts of biocides in water treatment, reduction in environmental costs, a better control of the health risks associated with the use of biocides (e.g. the formation of organochlorinated compounds, potentially carcinogenic) and an overall reduction in the costs of drinking water disinfection.

In this project it was also supposed to explore the use of the layer-by-layer technique in the development of the mentioned microstructures, explore different materials as support/core of the entire structure considering low prices and high availability, characterize the developed structures (structure and morphology, size and agglomeration tendency, charge, biocide intake at the surface) and test the antimicrobial efficacy of the developed functionalized microparticles against suspended cells and biofilms.

1.3. Thesis organization

This thesis is divided in six chapters. The present one, Chapter 1, presents the motivations and the objectives of the overall project.

In Chapter 2 a short literature review about the current state of the problems associated with the presence of biofilms and pathogenic microorganism in drinking water distribution systems is presented. This chapter comprises also an overview about the current strategies for biofilm control, with special attention for micro- and nanotechnology methods for this purpose. The layer-by-layer technique, a crucial method for the development of the microparticles produced in this project, is described in detail in this chapter.

Chapter 3 comprises the characterization of the different types of microparticles developed using LBL technique. This characterization consists of scanning electron microscopy for structures and morphology analysis, Zeta potential for charge determination and Coulter counter for the determination of particles size.

Chapter 4 describes the study of the efficacy of the developed microparticles in a cellular suspension of *Pseudomonas fluorescens*. The variation of cellular viability with the use of different concentrations of biocide is presented, as well as the biomass decay

constant (K_d). A discussion of the results is presented by comparing the efficacy of the microparticles with the freely suspended biocide.

Chapter 5 describes the study on the efficacy of the developed microparticles against biofilms of *Pseudomonas fluorescens*. Two different types of biofilms were analysed: biofilms formed during 24 hours in a microtiter plate and biofilms formed during 5 days in a chemostat reator. The variation of cellular viability with the use of different concentrations of biocide is presented, as well as the as the biomass decay (K_d). A discussion of the results is presented by comparing the efficacy of the particles with the biocide in the free form.

Finally, Chapter 6 comprises the main conclusions of the study and suggestions for future work based on the needs and difficulties encountered.

Chapter 2

2. Literature Review

2.1. Drinking water quality and public health

Water is a chemical compound essential for all socio-economic development and for maintaining healthy ecosystems. Water affects food production, energy, environment quality and consequently the economics of both developing and industrialized countries (Shannon et al., 2008). Drinking water, also known as potable water, is water with quality enough to guarantee that its consumption does not represents immediate or long-term risk. The availability of safe drinking water represents an important priority for all civilized societies. However, today nearly 1 billion people worldwide lack access to sufficient safe drinking water and a total of 2.6 billion people currently lack adequate sanitation facilities, which causes significant problems for human health - millions of people die annually due to diseases transmitted by unsafe water or human excreta (Montgomery & Elimelech, 2007). Furthermore, millions of people have malnutrition problems as a result of intestinal parasitic infections and diarrheal diseases caused by waterborne bacteria and enteric viruses (Behrman et al., 2004). It is estimated that 50% of the population of developing countries suffers the impact of the different health problems that occur due to the lack of access to safe drinking water and adequate sanitation, and that 45% of all deaths in those countries are due to contaminated drinking water. In developing countries, mainly sub-Saharan Africa and Southeast Asia countries (Pitman, 2002), the concern about the presence of pathogens in drinking water is significantly more important than chemical contamination, which is nowadays a relevant point to consider in developed countries. Waterborne diseases (e.g. gastroenteritis, cholera, typhoid fever, meningitis, encephalitis, dysentery, hepatitis, legionellosis, poliomyelitis, leptospirosis, giardiasis and salmonellosis), one of the most important water-associated health problems, refer to any illness caused by the use of drinking water contaminated by pathogenic microorganisms or by chemical products. The waterborne microorganisms potentially causing illness include bacteria, fungi, viruses, prions, protozoa, helminthes and rickettsiae that are transmitted to people when they consume untreated or inadequately-treated water (World Health Organization, 2003). Most waterborne pathogens are

introduced into drinking-water supplies in human or animal faeces. Although most of these pathogens are not able to grow in water, a restrict group of microorganisms can effectively do it, such as *Legionella* spp., atypical mycobacteria, *Burkholderia pseudomallei*, *Acanthamoeba* spp. and *Naegleria fowleri* (World Health Organization, 2011). Besides ingestion, other routes of transmission include inhalation of water droplets (aerosols) in which the causative organisms have multiplied because of warm waters and the presence of nutrients, leading to infections of the respiratory tract (e.g. *Legionella* spp.). In the group of pathogens transmitted through drinking-water, bacteria are generally the most sensitive microorganisms to disinfection. Examples of pathogenic bacteria are *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Escherichia coli* – pathogenic and enterohaemorrhagic, *Francisella tularensis, Helicobacter pylori* (World Health Organization, 2011). Viruses and prions represent almost half of the total emerging pathogens in the last three decades and some are more difficult to detect than bacteria and protozoan parasites. In this category it is possible to include Adenoviruses, Astroviruses, Enteroviruses, Hepatitis A virus and Hepatitis E virus (World Health Organization, 2011). Protozoa are also of particular concern due to its high resistance to some disinfectants, such as chlorine, and include *Acanthamoeba* spp., *Cryptosporidium hominis/ parvum*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia intestinalis*, *Naegleria fowleri* (World Health Organization, 2011). Breakdown in water supply safety (source, treatment and distribution) may lead to large-scale contamination and potentially to detectable disease outbreaks.

The outbreaks of waterborne diseases are not exclusive of developing countries. Recent important outbreaks of waterborne illness in developed countries (gastroenteritis in Walkerton, Ontario, Canada, induced by *E. coli* in 2000; norovirus gastroenteritis outbreak in Sweden in 2009) suggested the need of a more restricted control of waterborne pathogens and of a constant re-evaluation of disinfection techniques through time to guarantee an effective process of disinfection respecting this two conditions: absence of stress for the environment and safety of the treatment itself for human health (Richardson, 2003).

2.2. Biofilms in drinking water distribution systems

There are now different evidences that contribute to the conviction that most bacteria normally colonise surfaces in organized biofilm communities rather than

growing in suspension as individuals in natural, industrial and medical habitats (Stickler, 1999). Biofilms are aggregates of microorganisms attached to a surface, growing on it (Costerton & Stewart, 2001). In fact, microorganisms have a natural tendency to attach to wet surfaces, to multiply and to produce extracellular polymeric substances (EPS) that compose a slimy matrix, forming a biofilm (Simões et al., 2010).

The adoption of biofilm mode of life by microorganisms allows them to experiment diverse advantages comparing with planktonic cells. In biofilms, microorganisms are protected from stress conditions (chlorine, shear stress, temperature), which allows them to remain viable. In drinking water distribution systems (DWDS), as well as in diverse industries and other facilities, biofilms represent a problem of considerable hygienic, operational and economical relevance. Nowadays, biofilms are often recognized as a source of infections. In the USA, it is estimated that around 80% of persistent bacterial infections are associated with biofilms (Janssens et al., 2008).

Many problems in DWDS occur due to the presence of microbes, including biofilm growth, nitrification, pipes corrosion and pathogens persistence (Simões & Simões, 2013). It is estimated that about 95% of the total biomass that is fed in these systems along with treated water attaches to pipe walls, while only 5% is in the water phase (Flemming et al., 2002). If cell dispersion occurs, biofilms become the primary source of microorganisms in this systems, affecting cell density in the bulk phase. Thus, the quality of the potable water is affected, increasing the possibility of diseases outbreak. It is known that microbial growth in DWDS is a very complex process. In particular, biofilms formed on these systems are composed by complex organized microbial communities embedded in a gelatinous matrix of EPS. Since normally DWDS pipes are colonized by biofilms, it is of special concern the interaction of pathogens with these biofilms, such as bacteria and protozoa, which may culminate in pathogens dissemination.

The quality of drinking water depends of efficient disinfection strategies to control microorganisms both in bulk and biofilm states. Although biofilm elimination is almost impossible, several aspects can be considered in order to prevent and control their growth.

2.2.1. Biofilm formation

The biofilm formation, represented in Figure 1, comprises a sequence of dynamic steps that include: 1) pre-conditioning of the adhesion surface by macromolecules present in the bulk liquid, 2) transport of planktonic cells to the surface, 3) adsorption of

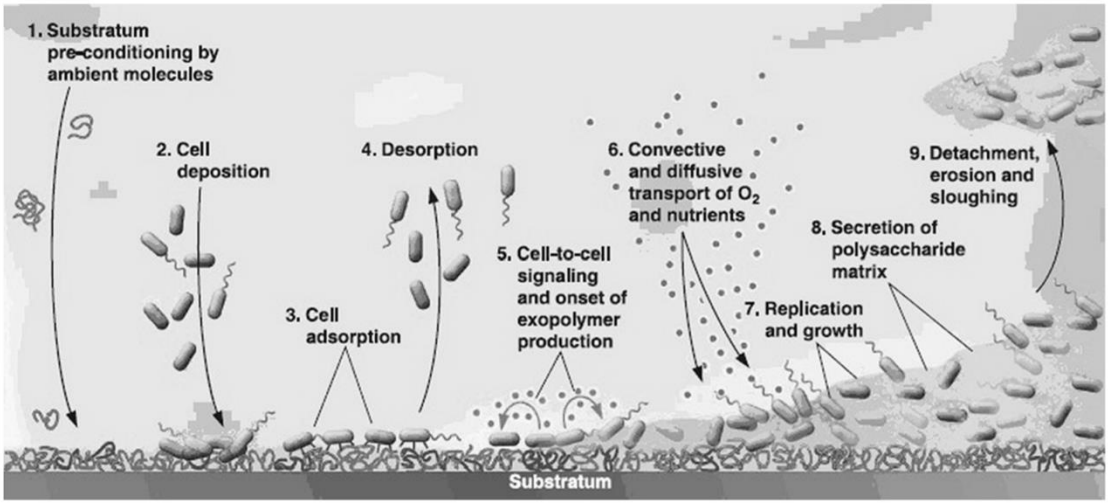

Figure 1 - Biofilm formation steps. Adapted from (Breyers & Ratner, 2004).

cells at the surface, 4) desorption of reversible adsorbed cells, 5) irreversible adsorption of bacterial cells at the surface, 6) production of cell-cell signalling molecules, 7) transport of substances to and within the biofilm, 8) Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm (Adapted from Breyers & Ratner 2004).

Both physicochemical properties of the bacterial cell surface and of the adhesion surface can influence the initial cell attachment. Generally, any surface can be the basis for biofilm development, including plastic, glass, metal, wood and food products. Furthermore, different physicochemical properties of the microorganism, adhesion surface and the bulk medium play a role in bacterial adhesion, such as the texture, roughness, surface charge, hydrophobicity, pH, temperature and nutrient composition of the preconditioning solution (Srey et al., 2013). The formation of a preconditioning film, composed of macromolecules such as natural organic substances is known to enhance the attachment of bacterial cells (Tang et al., 2009). In addition to the adhesion surface properties, also the physical and chemical characteristics of the microorganisms are crucial for initial attachment. It is known that the presence of extracellular appendages, cell surface hydrophobicity, cell-cell communication processes and the production of EPS contribute for biofilm formation and development (Allison, 2003; Simões et al., 2010). The extracellular filamentous appendages produced by some cells

interact with the surface through a smaller radius than the cell itself, contributing for the initial attachment. The hydrophobic interactions between the cell and the surface increase with the non-polar nature, which directly contributes for the cell-cell and cell-substratum attachment (Donlan, 2002). In the initial stage of attachment, the adherent cells do not possess a significant amount of EPS and some can move themselves independently by pilus-mediated twitching or gliding motility (Srey et al., 2013). Thus, in this stage the adhesion is reversible: the attached microorganisms are not yet committed to the differentiation process (morphological changes) that leads to biofilm formation and part of the cells can suffer desorption.

Irreversible attachment is characterized for a permanent bonding between the microorganism and the adhesion surface with the presence of EPS (Stoodley et al., 2002b). The accumulation and growth of microorganisms leads to the formation of the microcolonies and is directly related to the production of EPS (Donlan, 2002). The EPS comprises mainly polysaccharides, proteins, nucleic acids, lipids, phospholipids and humic substances (Sutherland, 2001). This matrix forms a barrier that protects microorganisms against adverse conditions, such as the presence of biocides (Simões et al., 2010). The presence of the EPS matrix makes difficult the penetration of biocides to reach the target by diffusion limitation and/or chemical interaction with the extracellular components (Heinzel, 1998). Cell-cell communication plays also a role in cell attachment and detachment from biofilms (Donlan, 2002). In fact, bacteria are colonial microorganisms that naturally search for systems of intercellular interactions and communications to facilitate their adaptation to changing environment, which depends on their ability to respond and modulate gene expression according to those conditions changes (Daniels et al., 2004). Quorum sensing is a process used by both Gram-negative and Gram-positive bacteria that enable them to communicate using secreted signalling molecules (autoinducers) in order to control and regulate a variety of physiological functions (Bassler, 1999).

In the final steps, biofilm formation goes through a maturation process where it develops into an organized structure which depends on the nutrient source (Chmielewski & Frank, 2003). Finally, external perturbations lead to bacterial cells dispersion, which constitutes the last step of biofilm formation cycle and where the cells return into their planktonic form, allowing the colonization of new niches (Stoodley et al., 2002b). The external perturbations may be increased by fluid shear, internal biofilm

processes (e.g. endogenous enzymatic degradation) and the release of EPS or surface-binding proteins (Stoodley et al., 2002a).

2.2.2. Strategies for biofilm prevention and control in drinking water distribution systems

Biofilm formation can be prevented and/or controlled through different strategies, including: a pre-treatment of the water (e.g. minimization of the concentration of organic matter entering the distribution system), pipe material selection, the use of chemical disinfection methods and alternative strategies (Simões & Simões, 2013).

2.2.2.1. Pre-treatment

The removal of organic and inorganic matter prior to disinfection, using either conventional treatments (coagulation, flocculation, sedimentation and filtration) or more efficient processes such as granular activated carbon (GAC) filtration, enhanced coagulation and membrane filtration (Sadiq & Rodriguez, 2004), can be effective in the reduction of the organic matter content in water, which represents an advantage for the maintenance of sufficient residual chlorine in the system (Simões & Simões, 2013). Additionally, the control of microbial growth can be obtained by the limitation of the essential nutrients for growth using another treatments, such as sedimentation, filtration and UV disinfection (Chandy & Angles, 2001). Since carbon is in general the growth-limiting element for microorganisms, the restriction of carbon concentration represents a way for microbial growth limitation.

2.2.2.2. Selection of surface materials

The characteristics of the materials used in drinking water systems pipes can influence biofilm proliferation and the development rate of microbes. In fact, different materials induce different biofilm growth propensities. Although currently there does not exist any material that totally prevents biofilm formation, some exhibit an inhibitory effect of biofilm growth, such as copper, when compared with other materials (Mueller et al., 1992). Furthermore, the type of material can also affect the disinfectant efficiency of biofilms. For example, iron pipes are more reactive with disinfectants, leading to the quenching of their antimicrobial effects (Kerr et al., 2003).

2.2.2.3. Chemical disinfection

The main strategy to control biofilm accumulation in DWDS is chemical disinfection (Simões & Simões, 2013). The water disinfection process emerged in the beginning of the 20th century and became one of the most important advances for

human health since it can eradicate pathogens from drinking-water (Sadiq & Rodriguez, 2004). Water disinfection is a process used to kill or irreversibly inactivate microorganisms present in water and to ensure microbiologically safe water through the system. Besides the effects in the inactivation of pathogens from drinking water, disinfectants can also be used for removing taste and colour, oxidizing iron and manganese, improving coagulation and filtration efficiency, preventing algal growth in sedimentation basins and filters and preventing biological regrowth in the water distribution system (EPA, 1999). Some characteristics to consider when choosing the right disinfectant for an efficient disinfection process must include: effectiveness of the disinfectant in the pH range used; stability of the disinfectant when diluted; toxicity of the disinfectant; microbial spectrum of the disinfectant; influence of temperature in disinfectant activity; stability of the disinfectant during the reaction with organic material; and possibility of surface corrosion by the disinfectant (Wirtanen et al., 2000). In water treatment, chlorine and its compounds are the most popular and commonly used disinfectants due to its potency, low cost and high oxidizing potential, which contributes for low levels of disinfectant residues in the treated water and protects against microbial recontamination (EPA, 1999). Currently, the residual concentration of free chlorine leaving the treatment system should be around 0.5 mg l^{-1} (World Health Organization, 2011). However, the levels of disinfectants normally employed in these systems are not sufficient to prevent the growth and development of microbial biofilms in pipe surfaces and after they are established their elimination is almost impossible (Simões & Simões, 2013). One strategy used to maintain the disinfectant level and guarantee microbial content control in water is the addition of supplementary chlorine in strategic points along the distribution system, commonly known as rechlorination stations. However, as mentioned above, some pathogens present high resistance to chlorine, such as *Mycobacterium* spp. and *L. pneumophila* (World Health Organization, 2011).

The efficiency of the disinfection process is influenced by physicochemical and biological factors, such as the process conditions (e.g. temperature, pH, contact time, dose) and the type of organisms. Regarding chlorine disinfectants, different studies demonstrated that generally microbial inactivation increases with the increase of the residual disinfectant and the contact time of chlorine in the water (Sadiq & Rodriguez, 2004). As mentioned previously, the use of chorine as a disinfectant for drinking water

treatments maintains adequate residual levels to avoid recontamination in the water distribution system. However, the residual concentration depletes rapidly at high temperatures (Sadiq & Rodriguez, 2004). Consequently, in order to maintain an adequate level of residual disinfectant in the distribution system, higher doses are applied in the summer to control microbial activity which is also higher in warm water than in cold water (Arora et al., 1997).

Chemical disinfectants are capable to kill pathogens in drinking water. However, they are also powerful oxidants, oxidizing the organic matter, anthropogenic contaminants and bromide/iodide naturally present in most source waters, forming disinfection by-products (DBPs) which provides an unintended health hazard (Sadiq & Rodriguez, 2004; Richardson, 2003). The concerns on the presence of DBPs in drinking water started only in the early 1970s. In 1974, Rook et al. identified the first DBPs in water treated with chlorine, more specifically chloroform and other trihalomethanes (THMs). This was recognized as a public health issue only after the publication of results by the U.S. Environmental Protection Agency showing that those DBPs were ubiquitous in chlorinated drinking water, and other results published by the National Cancer Institute showing that chloroform was carcinogenic in laboratory animals (Kopfler et al., 1976; National Cancer Institute, 1976). Since those events, more than 600 different types of DBPs have been reported in the literature and significant research efforts have been made to understand DBPs formation, occurrence and its effects in human health (Plewa et al., 2002; Krasner et al., 2006). The quantified DBPs in drinking water are generally present at sub-µg/L (ppb) or low- to mid-µg/L levels (Richardson et al., 2007).

As an alternative to the treatment with chlorine, many drinking water companies are changing its use to alternative disinfectants, such as ozone, chlorine dioxide and chloramines, in order to avoid the formation of THMs to meet new regulations. However, this can result in new issues. The use of ozone (ozonation), which has powerful oxidation properties, has been shown to efficiently remove microorganisms, taste and odour. Although it generates relatively few DBPs, it leads to the formation of bromate mainly in the presence of high levels of bromide in water (Ozekin et al., 1998). Bromate represents a harmful compound since it has been shown to be carcinogenic in animals (Kurokawa et al., 1986). Chloramines are less effective than free chlorine and produce similar DBPs to chlorine but in much lower concentrations. Disinfection with

chlorine dioxide leads to the formation of chlorite and chlorate which have human health risk implications (US EPA, 2001). More recently, ultraviolet (UV) light is being used for this purpose, either alone or in combination with chlorine (Wang et al., 2012).

Overall, the formation of chlorinated DBPs in drinking water like THMs has emphasized the need for exploring alternate disinfectants and new treatment technologies.

2.2.2.4. Alternative techniques

Disinfecting methods used in drinking water treatment and decontamination represent an effective approach in the control of microbial pathogens. However, as stated before, the use of chemical disinfectants such a chlorine can lead to the formation of DBPs, which are carcinogens, as a result of the reaction between those chemicals with different components present in natural water. Furthermore, the resistance of some pathogens to conventional disinfectants has brought the need to use higher dosages of these chemicals, aggravating DBPs formation (Li et al., 2008). Thus, it is clear the need of a revaluation of conventional disinfecting methods and search innovative methods to improve disinfection efficiency while avoiding DBPs formation. Alternative techniques for disinfection and microbial control in drinking water have already been described. For example, UV radiation is a non-chemical disinfecting method in which energy is applied to destroy the microorganisms by altering their genetic material and rendering them unable to reproduce. This method is a very effective against all bacteria, viruses and protozoa cysts found in clarified waters (Hijnen et al., 2006). However, UV leaves no residual disinfectant in the water, which can be overcome by applying a second disinfectant to generate a residual amount, such as chlorine. The combination of UV light with chemical treatments (chlorine and chlorine dioxide) was shown to be more effective in eradicating drinking water biofilms than the two treatments applied separately (Simões & Simões, 2013). Another promising approach for water disinfection is the combination of UV irradiation and direct electrolysis (Bergmann et al., 2002). Also, electron-beam radiation, characterized for the use of ionizing radiation produced by electron accelerators represents an environmentally-friendly technique with potential in the disinfection of drinking water and wastewaters (Sampa et al., 1995). More recently, the potential application of antimicrobial nanomaterials for water disinfection and microbial control started to be considered (Shannon et al., 2008).

2.3. Micro and nanotechnology for water treatment and biofilm control

The field of micro and nanotechnology has been gaining significant interest in environmental and biological applications in the last years. Nanotechnology concerns the control of structures in a scale between 1 and 100 nm. Within that range of values, materials have been showing unique physicochemical properties, such as large surface to mass ratio, high reactivity and unique interactions with biological systems (Zhang et al., 2008). The large specific surface area and high reactivity of nanomaterials makes them excellent adsorbents, catalysts and sensors. Furthermore, since biofilms can serve to promote pathogens persistence due to their relative impermeability, considering the unique properties of nanomaterials already stated, nanotechnology may provide the answer to penetrate such biofilms, leading to a decrease in biofilm formation (Taylor & Webster, 2009). In recent years, both natural and engineered nanomaterials have shown antimicrobial properties. In contrast with conventional disinfectants, antimicrobial nanomaterials are not reactive in water due to lack of oxidant properties. In this way, when used correctly, they represent a good alternative to chemical disinfectants, considering the absence of DBPs production. Examples of antimicrobial nanomaterials reported in the literature are chitosan, silver nanoparticles, photocatalystic TiO_2, to name a few (Li et al., 2008).

Chitosan is obtained from chitin in arthropod shells that presents antibacterial activity, especially at nano-scale, a characteristic that makes it a potential compound to use in drinking water disinfection, in addition to its normal use as a coagulant/flocculant in water treatment systems (Zeng et al., 2008). Although its effectiveness occurs only in the presence of organics, when compared to other disinfectants, chitosan has higher antibacterial activity and presents lower toxicity to animals. According to the literature, some antimicrobial nanomaterials present similar or superior antimicrobial activities comparing to conventional chemical disinfectants (Li et al., 2008). They can also be used in combination with another existing technologies for water treatment, such as UV disinfection or membrane filtration. In the last case, nanotechnology can make the membranes “reactive” instead of a simple physical barrier, which contributes for a more effective treatment and a reduction in the required stages of treatment due to fouling minimization. An example of this approach with successful results is the use of filters composed of low-cost materials (e.g. cation resin substrate) coated with silver nanoparticles (Ag) for disinfection of groundwater, through a complete elimination of

the pathogens tested (Mpenyana-Monyatsi et al., 2012). TiO_2 in nano scale is another material with antimicrobial properties. Due to its stability, low-cost and non-toxicity, is one of the most studied nanomaterials that is suitable for applications in water treatment. Under UV-A irradiation, TiO_2 presents photocatalystic properties that can be used for microbial inactivation (Cho et al., 2005). The photoactivity in the UV-A range and the potential visible light activity when doped with metals makes TiO_2 photocatalystic disinfection especially useful in developing countries where electricity is not available. Another promising approach is the use of carbon nanotubes for inhibiting microbial attachment and biofouling formation on surfaces. Carbon nanotubes are grapheme sheets rolled into a tube which present antimicrobial activity against Gram-positive and Gram-negative bacteria and viruses (Kang et al., 2007). A drawback related to the use of carbon nanotubes is related with the difficulty of their dispersion in water. To overcome this problem surfactants or polymers such as sodium dodecyl benzenesulfate (SDBS) or polyvinylpyrolidone (PVP) can be used (Li et al., 2008). Alternatively, carbon nanotubes can be used to coat reactors surface in contact with the pathogens. Kang et al. (2007) immobilized single-walled nanotubes (SWNTs), a single pipe with diameter from 1 to 5 nm, on a membrane filter surface and observed 87% killing of *Escherichia coli* in a period of two hours. Another approach using nanomaterials is based in the application of nanoscale zero-valent iron to decentralized drinking water systems in order to improve the performance of point-of-use devices by the effective elimination of viruses, bacteria, chlorine, DBPs and other chemicals (Kharisov et al., 2012). However, this technique is not yet widely used.

The large specific surface area and high reactivity of nanomaterials gave rise to another possible approach regarding nanoparticles – the loading of drugs into nanoparticles through physical encapsulation, adsorption, or chemical conjugation. By loading drugs into nanoparticles through this strategies, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts (Zhang et al., 2010). Encapsulation of various substances into different micro and nanoparticles such as capsules, polymer spheres or liposomes, has received considerable attention due to increased interest in biotechnology, medicine, catalysis, ecology, nutrition, and others (Volodkin et al., 2004a). Polyelectrolyte capsules development from layer-by-layer (LBL) self-assembly technique have shown diverse applications in storing, protection, release and delivery of different functional agents

(Johnston et al., 2006). Besides the use of nanoparticles as functional agents for drugs immobilization through encapsulation, the desired molecules may also adsorb at the particles surface (Cakmak et al., 2004; Gomes et al., 2012). Sequential LBL polyelectrolyte adsorption has been used to fabricate multilayer films utilizing electrostatic interaction between oppositely charged molecules at each adsorption step, as discussed in the next section.

2.3.1. Layer-by-layer self-assembly technique

Assembling of nanoscale objects through electrostatic attraction in a LBL manner was firstly proposed by Iler in 1966, although the realization of the idea was only performed few decades later by Decher (Iler, 1966; Decher & Hong, 1991). The simplicity of the proposed procedure led to the rapid spread of the technique within various research communities for the preparation of assemblies of a diversity of materials, including polymers, biomaterials and inorganic substances (Ariga et al., 2011). This technique uses electrostatic attraction between polyanions and polycations to form supramolecular multilayer assemblies of polyelectrolytes, whose size and shape can be controlled, as well as the wall thickness and composition (Caruso et al., 1998). The fabrication of this complex involves step-wise deposition of polyelectrolytes from aqueous solutions and it is formed by the alternate adsorption of oppositely charged layers (Figure 2).

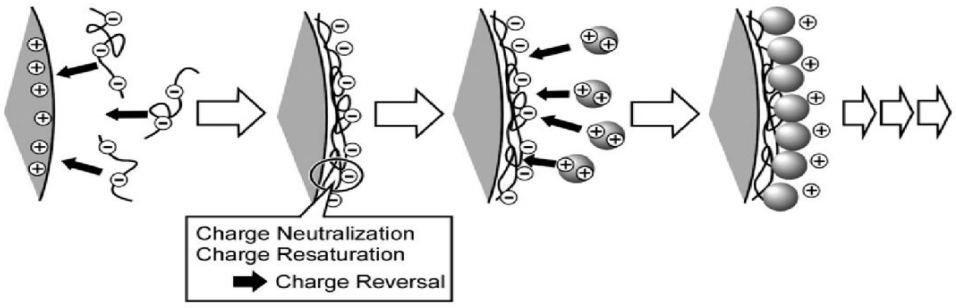

Figure 2 - Schematic representation of LBL technique**.**
The addition of a polyanion to a positively charged substrate changes the overall charge to a negative charge. The subsequent addition of a polycation will cause a new charge reversion. Adapted from Ariga et al. 2011.

Considering that each adsorption step leads to charge inversion of the surface, the subsequent deposition finally results in a layered complex, stabilised by strong electrostatic forces. For the construction of such self-assembled polyelectrolyte multilayers (PEMs) different building blocks have been used, including inorganic nanoparticles, functional polymers, proteins, chromophores, and biopolymers such as DNA (Hammond, 2000; Lvov et al., 1993). This method can be applied in the

construction of planar layers, involving the use of different materials as a matrix for functional or biological molecular entities, e.g. for sensor applications, as separation membranes, or as tailored surface modification. For example, Trybała et al. (2009) investigated the adsorption of polyelectrolytes at surgical stainless steel, at titanium plates and silicon plates in order to explore the LBL application area to real surfaces.

The LBL assembly method presents various attractive advantages, including its simplicity and low cost and also the variability of the applicable materials. In addition to conventional polyelectrolytes, such as poly(allylamine hydrochloride) (PAH), poly(diallyldimethylammonium chloride) (PDDA), and poly(ethyleneimine) (PEI), poly(sodium styrenesulfonate) (PSS), various biomaterials have demonstrated to assemble by electrostatic LBL. This phenomenon occurs due to the presence of charged sites on their surface, leading to the formation of assembled films of proteins (Lvov et al., 1995), DNA (Lvov et al., 1993) and charged polysaccharides. Charged inorganic substances, such as colloidal nanoparticles, clay, nanosheets, modified zeolite crystals, two-dimensional perovskite, and polyoxometalates have also been used for LBL assembly (Ariga et al., 2011).

A pioneer innovation in the LBL assembly reported in 1998 involves the application of the technique onto a colloidal particle core (Caruso et al., 1998). In other words, the LBL films were assembled sequentially, similarly to the conventional assemblies, on a colloidal core. In a further step, dissolution of the central particle core upon exposure of the particles to appropriate solvents results in hollow capsules (Sukhorukov et al., 1998). Micro and nanometer-sized capsules found application in diverse areas, including medicine, biotechnology and synthetic chemistry. Since their introduction, capsules prepared using LBL technique have attracted particular interest, especially due to the easiness to adapt their properties (e.g. size, composition, porosity, stability, surface functionality, colloidal stability). They have been showing potential in diverse applications, including catalysis, drug delivery, chromatography separation, chemical reactors, controlled release of various substances, protection of environmentally sensitive biological molecules, and lightweight filler materials (Caruso, 2001; Jiang et al., 2001). Until the present, numerous efforts have been performed to generate inorganic hollow structures such as polystyrene latex spheres, spherical silica, carbon spheres, emulsion droplets, micelles, vesicles, and gas bubbles (Jiang et al., 2012). Although LBL hollow shells have been widely used in many applications in

fundamental science, a special attention has been given to their application in the medical field as drug delivers. Shu and coworkers (2010) reported the entrapment and controlled release of proteins using biodegradable LBL microshells of water-soluble chitosan and dextran sulphate, using bovine serum albumin as model protein for entrapment in LBL shells. Kumara *et al*. (2009) tested the encapsulation and release of the antituberculosis drug, rifampicin, using hydrogen-bonded LBL microshells of poly(vinyl pyrrolidone) and poly(methacrylic acid). Rifampicin release from the LBL microshells had the same efficacy as the free drug, indicating that encapsulation process did not affect drug properties. More recently, researchers proposed a method for the fabrication of hydroxyapatite hollow microspheres with potential application in water treatment due to their unique selective adsorption activity for the heavy metal Pb^{2+} (Jiang et al., 2012).

Despite the simplicity associated to the procedures of the LBL strategy, the assembling mechanism stills not completely understood. According to experienced LBL researchers film structures and qualities depend significantly on experimental conditions (salt concentration, secondary interactions, temperature, etc.) (Schonhoff, 2003). Furthermore, deposition of materials in the LBL assembly is not always uniform. Although the LBL technology remains under development, the method already assures fabrication of nanostructures with positioning of target materials in desired geometries. The step-wise formation of these structures allows the introduction of multiple functionalities, providing opportunities to engineer a new class of materials with unprecedented structure and function (Schonhoff, 2003). Furthermore, the surfaces can be modified to alter the functionality and improve the colloidal stability of the structure (Hammond, 2000). The easiness associated to this technique has been stimulating researchers in various fundamental fields including chemistry, physics, and biology, attracting them to work in practical applications including nanotechnology and materials technology (Ariga et al., 2007).

2.3.2. Limitations of micro and nanotechnology for water treatment

The main challenges associated with the use of nanomaterials with antimicrobial properties are related to their dispersion and retention in water, as well as the sustainability of antimicrobial activity (Li et al., 2008). In fact, the aggregation of this type of nanoparticles can influence their high reactivity potential which is beneficial for

their functions. Oppositely, if the nanoparticles are well-dispersed and suspended in the solution, an efficient downstream separation method is needed (e.g. membrane filtration) to retain the particles and recycle them, which can be advantageous due to the cost associated with the particles. Besides the cost benefits associated, the retention of the nanoparticles is mandatory because of their potential nanotoxicity with subsequent impacts on human health and ecosystems (Wiesner et al., 2006). Nowadays, the immobilization of antimicrobial nanoparticles on reactor surfaces constitutes the main alternative to avoid the need of methods for nanomaterials separation from the suspension. However, despite the benefits of nanoparticles immobilization, the available surface area in the reactor limits the effective dosage for the specific treatment, which decreases disinfection efficiency comparing with the reactor with suspended nanoparticles. Furthermore, because of the lack of residual disinfectant due to their retention along with nanoparticles in the treatment system, a secondary disinfectant must be used for that purpose (Li et al., 2008). Thus, nanoparticles with antimicrobial properties may not be a complete approach for the total replacement of conventional chemicals.

Currently, there is a scarcity in information about nanoparticles toxicity against humans. Thus, it is difficult to evaluate the risk of its presence in drinking water and, more specifically, their allowable concentration. Therefore, for a large-scale use of antimicrobial nanoparticles in water treatment systems more studies need to be performed about this issue and new methods for nanoparticles retention must be developed.

The use of nanotechnology for water treatment is also limited by the costs associated, which are still high comparing with conventional disinfectants (Li et al., 2008). Therefore, low-cost nanomaterials should be explored so nanotechnology can compete with conventional treatments. Cost-benefits and economic analysis are needed to evaluate a large-scale application of nanotechnology in water treatment, and they must have into account the benefits associated with this emerging technology, such as lower DBPs formation and reduction in the environmental impacts resulting from the release of chemical compounds.

Chapter 3

3. Microparticles development and characterization

3.1. Introduction

The rapid growth of micro and nanotechnology in the past years sparked considerable interest in their use in a diversity of areas. Currently, there are several studies and practical applications of emerging approaches using materials at micro and nano scale, especially in medical and pharmaceutical fields (Zhang et al., 2010). However, few studies reveal the development of similar approaches for environmental applications. It is believed that when properly developed, the unique characteristics presented by some materials at micro and nano scale may be incorporated in water treatment approaches in order to replace or enhance conventional disinfection methods (Li et al., 2008). In this project different types of materials at micro scale were used as the basis (core) of a functionalized structure constructed through the use of the LBL technique, as stated in Chapter 2. Two polyelectrolytes, oppositely charged, were assembled sequentially in distinct cores (calcium carbonate, kaolin, silica, sea sand and cork), with a final layer of a Quaternary ammonium compound (QAC).

3.1.1. Core materials

Calcium carbonate ($CaCO_3$) is one of the most abundant minerals in the nature and is widely used in the industrial and pharmaceutical fields. Vaterite, one of the six different polymorphisms of $CaCO_3$, is the most interesting form for $CaCO_3$ microparticles production by chemical synthesis (Islan et al., 2015). This polymorph has spherical shape (range of 0.05-5 μm), low density and porous inner. Vaterite microparticles can be produced in different many ways, but most of them imply special equipment and conditions. The simplest, fastest and cost efficient method is based in the mixing of saturated aqueous stock solutions containing calcium and carbonate ions, which culminates in a crystallization process. The quality of the resultant microparticles depend on the rate of the nucleation process, which is affected by the experimental conditions such as the type of the salts used, their concentration, pH, temperature, solutions mixing rate and agitation intensity of the reaction mixture (Volodkin et al., 2004b). These type of $CaCO_3$ microparticles have been widely used for proteins, drugs

and other molecules encapsulation by the combination of molecules adsorption and LBL technique, for further delivery to the target site (Volodkin et al., 2004b; Wang et al., 2006). The porosity of these particles provide special and important properties such as enhanced drug absorption and release kinetics for medical and pharmaceutical applications (Preisig et al., 2014). An important characteristic to consider regarding calcium carbonate polymorphs is their rapid dissolution at acidic pH (Trushina et al., 2014).

Kaolin is an abundant industrial clay mainly composed of a hydrated aluminium silicate mineral named as kaolinite ($Al_2Si_2O_5(OH)_4$), between other minerals present in less amounts such as dickite, nacrite, and halloysite (Štengl et al., 2014). This type of clay can be found in all continents on Earth, with the exception of Antarctica. Due to its unique mineralogical, morphological, chemical and physical characteristics, kaolin is an important raw material appropriate for many different applications, such as paper coating and fillers, oil-based exterior industrial primer, medicines, pharmaceuticals, cosmetics, fertilizers, detergents, pesticides, white cement, ink, catalysts, to name a few (Hosseini & Ahmadi, 2015). Kaolinite consists of a regular stack of $(Si_2O_5)^{2-}$ tetrahedral sheets linked through oxygen atoms to parallel octahedral sheets of alumina octahedral $[Al_2(OH)_4]^{2+}$. While silica sheets are pH-independent and permanently negatively charged, the presence of surface hydroxyls (-OH) on the alumina sheets causes them to display charge heterogeneity that is pH and ionic strength dependant of surrounding medium. In order to comprise aluminol (Al-OH) and silanol (Si-OH) groups, at the edges of the kaolin particles the bonds between alumina and silica sheets are broken. Therefore, due to this functional groups, kaolin particles edges present charge heterogeneity (Avadiara et al., 2015).

Silicon dioxide (SiO_2), commonly named silica, is one of the most abundant oxide materials on Earth that is present in nature as sandstone, silica sand or quartzite. Silica is widely used worldwide as a material on its own and as a precursor to the fabrication of ceramic products. Because of their high chemical and thermal stability and high surface area for functionalization, in recent years silica microspheres have been receiving special attention for potential applications in distinct fields, including the physicochemistry, medicine, biochemistry, colloidal chemistry, drug delivery, and others (Waldron et al., 2014). Thus, many experimental methods have been tested to produce SiO_2 microparticles with a spherical shape, such as spray gelling, spray-drying,

sol-gel, reaction with supercritical ethanol, precipitation by supercritical antisolvent process and hydrolysis of silicon esters in an emulsion system (Montes et al., 2013; Yu et al., 2006; Waldron et al., 2014).

A conventional and low-cost method for treating water supplies is based in water filtration with a stratified bed of graded gravel and sand (Albers et al., 2015). Sand water filtration is achieved by two distinct mechanisms: mechanical filtration of bacteria through the grains of the sand and further adsorption of bacteria to the biofilm layer formed over time. Since bacteria are often larger than the spaces between sand grains, many of these microorganisms will stay entrapped in the sand filter, leading to the formation of a biofilm layer that will be a point of adhesion for new microorganisms (Clark et al., 2012). Furthermore, this process permits the elimination of another incoming particles through electrostatic interactions with the sand. In the past years, several studies have explored the possibility of modifying filtration sand in order to enhance their ability to remove microorganisms and dissolved impurities. Filtration sand showed to be able to successfully remove heavy metals, microorganisms, sulphate, manganese and others, when coated with iron, aluminium and manganese oxides (Hu et al., 2004; Ngwenya et al., 2015).

Cork is a biological material prevenient from the external covering of cork oak stem and branches. Nowadays, Portugal is the worldwide leader in the market of cork by producing three-quarters of the world production (Barbosa et al., 2012). This raw material is composed of a homogeneous tissue of thin-walled closed cells, regularly arranged without empty spaces between contiguous cells. In this cellular material the solid volume percentage is around 15%, a value typically lower than the one presented by other cellular materials. Chemically, cork cell walls are mainly composed of suberin, a lipidic biopolymer, which confers impermeable properties to the material. The low superficial tension presented by the cork (around 32 mJ/m^2) when compared to metallic or ceramic materials imply high contact angles and, consequently, low wettability. When water contacts with cork, initially the contact angle tends to be relatively high (around 84°) (Fortes et al., 2004). However, this value tends to decrease with time since the material starts to absorb the water, resulting in the change of the superficial properties.

3.1.2. Antimicrobial agent

The surface of the microparticles produced in this study was functionalized with a layer of a benzyldimethyldodecylammonium chloride (BDMDAC), a QAC. QACs are compounds with a nitrogen atom with covalent bonds to four residues, making the nitrogen positively charged. BDMDAC is included in alkyldimethylbenzylammonium salt benzalkonium chloride (BAC) group, a subgroup of QACs. Because the alkyl groups of these compounds derive from natural sources, such as coconut and soya bean oil, BACs alkyl chains differ in its length. These molecules are surfactants, which are amphiphilic due to the presence of hydrophobic groups (carbon chain) and hydrophilic groups (cationic, anionic, non-ionic and ampholytic). BAC is commonly used as a disinfection agent in hospitals, homes and public places (Ferk et al., 2007) and shampoos. Regarding BDMDAC, it is a cationic BAC with a 12 carbons chain mostly applied as a disinfectant in swimming polls (concentrations of 2 mg/L in day-to-day water treatment) and in cooling water systems (Block, 1983).

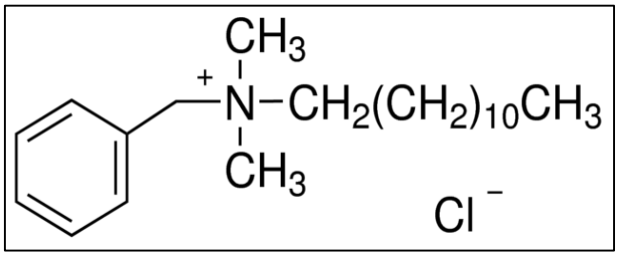

Figure 3 - BDMDAC chemical structure.

Despite the broad spectrum of action attributed to QACs, it has been reviewed for many years that QACs target site is predominantly the cytoplasmic membrane (McDonnell & Russel, 1999). As a cationic surfactant, BDMDAC can be either bactericidal or bacteriostatic. Generally, QACs are bactericidal towards vegetative cells of both Gram-negative and Gram-positive organisms and yeasts, and they can inhibit filamentous fungi, mycobacteria and bacterial spores outgrowth. To damage the cell, the long alkyl chains of QACs permeate the membrane and disrupt its physical and chemical properties, while the charged nitrogen remains at the surface leading to the disruption of the charge distribution. This process culminates in the leakage of intracellular low-molecular-weight material, degradation of proteins and nucleic acids and cell lyses (Wessels & Ingmer, 2013).

The purpose of the study presented in this chapter was to analyse the microparticles produced in terms of structure, morphology, surface charge and size distribution. The

analysis was performed in the microparticles without any treatment and after the coating process with the polyelectrolytes and BDMDAC.

3.2. Materials and methods

3.2.1. Particles manufacture process

The five types of microparticles used in this project were prepared using LBL technique trough the successive assembly of polyelectrolytes in different basic structures, as described by Ferreira et al. (2010).

3.2.1.1. Particles cores

The oppositely charged electrolytes Polyethyleneimine (PEI; molecular weight 750,000) 50% (w/v) in water (Sigma-Aldrich, Portugal), poly (sodium 4-styrenesulfonate) (PSS; molecular weight 70,000; Sigma-Aldrich, Portugal), and BDMDAC (molecular weight 339,9; Fluka, Portugal) were assembled on five distinct cores (Figure 4): calcium carbonate (prepared manually), kaolin (natural source), silicon dioxide microparticles (Sigma-Aldrich, Germany), sea sand (Merk, USA) and cork (natural source).

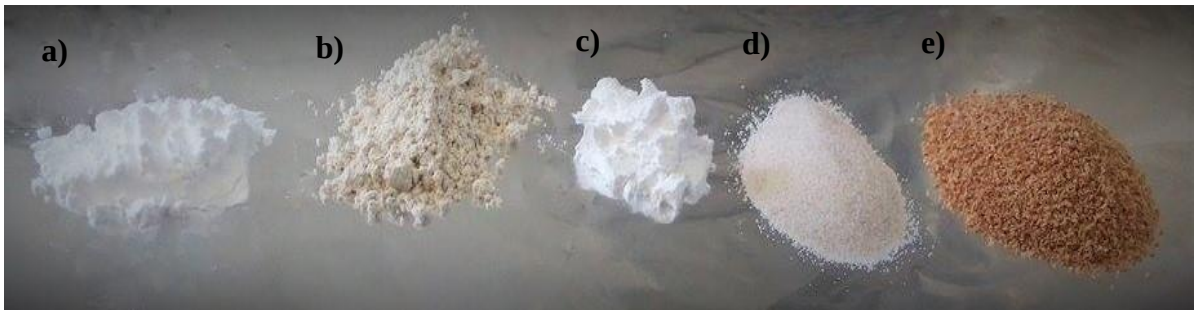

Figure 4 - Microparticles cores studied: a) calcium carbonate, b) kaolin, c) silica, d) sea sand and e) cork.

3.2.1.1.1. Calcium carbonate

Calcium carbonate microparticles were prepared by putting in contact a solution of 0.33 M $CaCl_2$ with a solution of 0.33 M $NaHCO_3$ during 30 seconds with agitation (1000 rpm) at room temperature (Figure 5). Then, the particles stayed at room temperature without agitation for 5 minutes. The obtained solutions were centrifuged for 10 minutes at 3220 *g*. The resultant pellet was resuspended and washed three times in ultra-pure water. A final washing step was performed using 99% ethanol. At last, the particles were dried in an incubator at 60 °C and stored at room temperature.

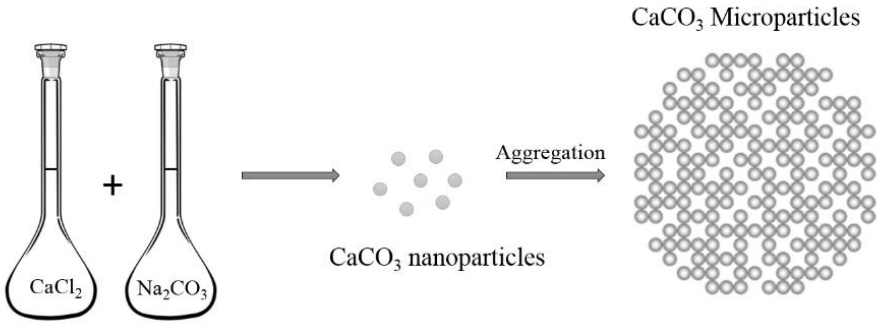

Figure 5 - Schematic representation of the $CaCO_3$ microparticles manufacture.

3.2.1.1.2. Kaolin

The kaolin used in this studies was the one described and characterized in (Martins, 2014). This clay, extracted from a natural source, was identified as kaolin through chemical and mineralogical analysis. For this project, kaolin characteristics determined for the referred author were considered, more specifically the BET surface area of 13.8 $m^2\ g^{-1}$ resulted from the analyses on textural properties of the clay, and the particle size average of 15 μm obtained with a Coulter Counter LS 230. The mineralogical composition was provided by LNEG (Laboratório Nacional de Energia e Geologia) and is presented in Table 1.

Table 1 - Mineralogical composition of the clay (Martins, 2014)**.**

Elements	Kaolinite	Mica	Quartz	Feld K	Hematite
Sample composition (%)	77	16	3	4	Traces

3.2.1.1.3. Silica

The silicon dioxide (SiO_2) beads were purchased from Sigma-Aldrich (Portugal). These microparticles present a diameter of 3 μm and a specific gravity of 1.8-2.0 g/cm^3.

3.2.1.1.4. Sea sand

The purified sea sand cores (SiO_2; molecular mass of 60.09 g/mol)) were purchased from Merk (Germany), with a particle size in the range of 0.1-0.315 mm and a bulk density of 200 - 1430 kg/m^3.

3.2.1.1.5. Cork

The cork used in this studies was obtained from a natural source. The sample was reduced to microparticles with an electric mill (IKA®, Portugal). Further, the resulting

particles were separated in different range of sizes using sieves with distinct grading (Retsch, Germany). Particles with a size range of 63-500 μm were selected. The chemical composition of the sample was not analysed in this project.

3.2.1.2. Microparticles coating process

This process was used for the five types of particles studied. In order to have a final stock solution with the same amount of BDMDAC at particles surface, the equivalence between the amounts of each type of particles to consider for the coating process was done regarding the total superficial area (see Appendix A.1). First, the particles were allowed to interact with a PEI solution (1 mg/mL in 0.1 M borate buffer solution) for 20 min and were then washed twice in 0.1 M borate buffer solution (pH 9), in order to remove the polymer excess. The resulting cores, positively charged, were then allowed to interact with a PSS solution (1 mg/mL in 0.1 M borate buffer solution) for 20 minutes and washed twice in 0.1 M borate buffer solution. Finally, a BDMDAC solution (1 mg/mL in borate buffer solution) was added to interact with the cores during 24 hours. Then, two washing steps using borate buffer were performed and the final pellet was resuspended in borate buffer. The washing steps were carried out by centrifuging at 3220 *g* for 5 minutes. The borate buffer (0.1 M) that was used in the whole process was selected due to its ionic strength.

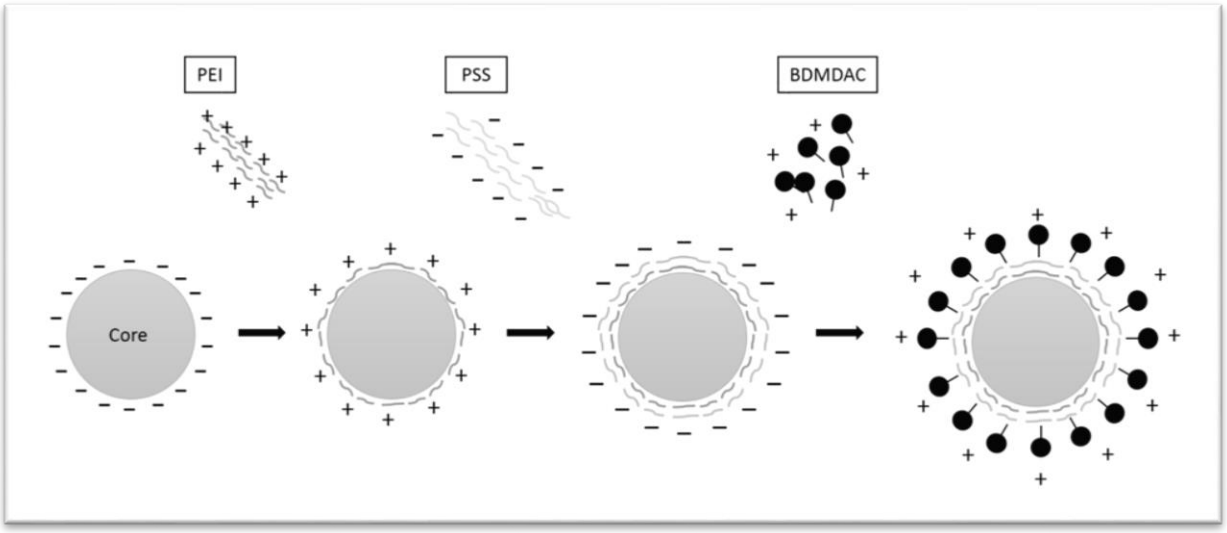

Figure 6 - Schematic representation of microparticles functionalization through LBL technique.

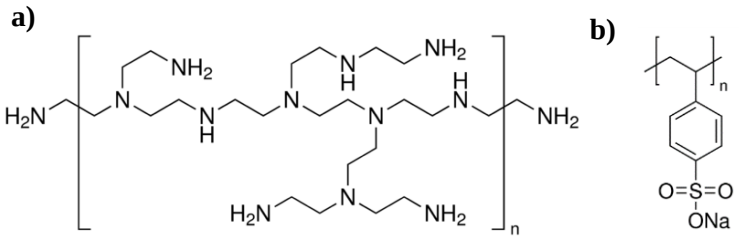

Figure 7 - Chemical structure of a) PEI and b) PSS.

3.2.2. Particles characterization methods

3.2.2.1. Zeta potential

The zeta potential of the particles was determined using a Nano Zetasizer (Malvern Instruments, UK). Through the application of an electric field to the dispersion of the particles, they move toward the electrode of opposite charge with a velocity (electrophoretic mobility) related to their zeta potential. Therefore, the calculation of the electrophoretic mobility allows the calculation of the zeta potential and zeta potential distribution. The zeta potential of the particles was measured in the different stages of the preparation of the particles ($CaCO_3$, Kaolin, Silica, sea sand and cork cores; core-PEI; core-PEI/PSS; core/PEI/PSS/BDMDAC). The measurements were repeated twice.

3.2.2.2. Number and volume size distribution

The size distribution of the particles was determined in a Coulter Particle Size Analyser (model LS 230 – small volume module plus) by laser diffraction. The analysis of particle size was considered in terms of volume and size distribution. Particles before and after the coating process were analysed.

3.2.2.3. Scanning electron microscopy

SEM was performed to analyse the integrity/morphological characteristics of the non-coated and coated particles. The samples were analysed at the Centre for Materials Characterization at the University of Porto (CEMUP), using a Quanta 400 FEG ESEM/EDAX Genesis X4M, a high resolution (Schottky) environmental SEM with X-ray microanalysis and electron backscattered diffraction analysis. Samples were coated with a Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment.

3.3. Results and discussion

The analysis of the particles surface with and without the coating was performed using SEM. The resulting images are presented in Figure 8.

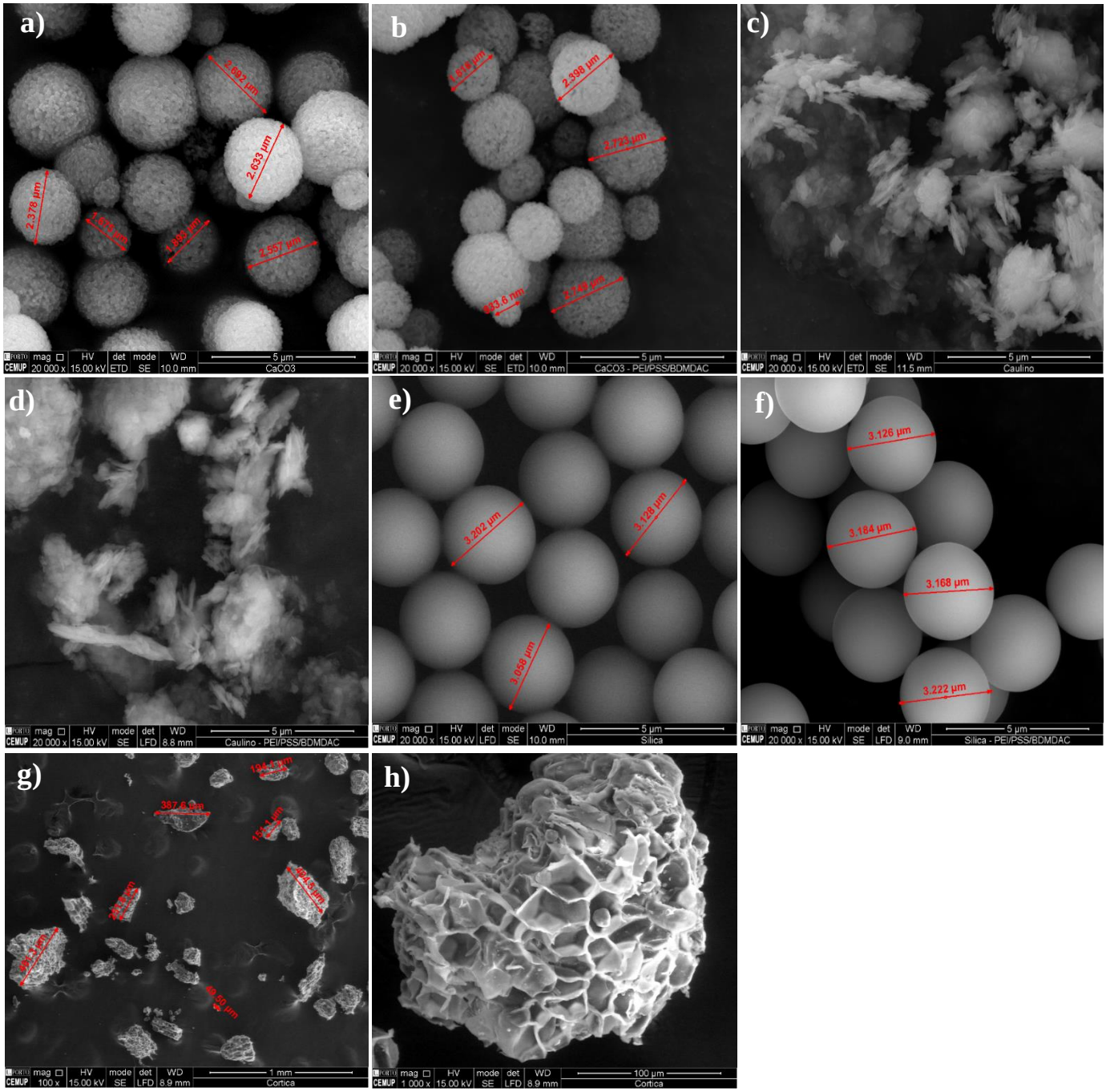

Figure 8 - SEM images of a) $CaCO_3$ particles; b) $CaCO_3$-PEI/PSS/BDMDAC particles; c) kaolin particles; d) kaolin-PEI/PSS/BDMDAC particles; e) silica particles; f) silica-PEI/PSS/BDMDAC particles and g/h) non-coated cork particles.

SEM images of $CaCO_3$ and $CaCO_3$-PEI/PSS/BDMDAC particles (Figures 8a and 8b) show that they are spherical (diameter range 1-3 µm, approximately) and have a spongy surface, regardless of the presence and absence of BDMDAC. However, by comparing coated and non-coated particles with more detail, it is possible to observe

that the particle surfaces with biocide are slightly smoother than the surfaces of particles without biocide. Probably, this phenomenon occurs due to the addition of layers that stay strongly attached to the particles surfaces and will cover up those referred surfaces. This suggests also that the biocide is bounded to the microparticles by strong interactions (ionic bonding), using the LBL technique, and therefore it will not be released from the particles when interacting with the biological structures. Higher magnifications of $CaCO_3$ microparticles allow the identification of nanoparticles that are aggregated in order to form a sphere, as represented in Figure 5 (see Appendix A.2).

Regarding kaolin SEM images (Figures 8c and 8d), it is possible to observe several aggregates of particles with undefined shapes. Higher amplifications of SEM images of kaolin particles allow the observation of particles with lamellar shape sequentially positioned (see Appendix A.2). For kaolin particles, both the shape and the surface characteristics seem to remain equal after the BDMDAC coating procedure. Thus, the overall coating process does not seems to modify the structural characteristics of kaolin microparticles.

Through the analysis of the SEM analyses of the coated and non-coated commercial silica microparticles (Figures 8e and 8f) a perfect spherical shape is observed for both cases, with a very similar diameter (approximately 3 μm). Again, it is not possible to observe any visible differences between coated and non-coated particles, which suggests that the coating process does not seems to promote structural changes.

The structure observation of cork microparticles was only performed without coating, for a preliminary use of this material as a possible support for the functionalized particles (Figures 8g and 8h). The size distribution of the particles is in agreement to what was determined through the separation process. Furthermore, as expected, cork microparticles revealed a homogeneous tissue of thin-walled closed cells, regularly arranged without empty spaces between contiguous cells. This can represent an advantage for the process of coating, since there is a huge superficial area for the functionalization with the biocide.

The zeta potential values of the different types of microparticles before and after the addition of each layer is presented in Table 2. Note that the preparation of the samples and the measurements were performed under pH 9, since previous studies demonstrated that it allows a better LBL process by promoting the right superficial charge for the different molecules intervening in the process for the $CaCO_3$ microparticles coating

process (Ferreira et al., 2013). Although in this project other types of materials were used for the functionalized microparticles production, the same procedures and conditions (pH, temperature, solution concentrations, etc.) were used, as a preliminary assay.

Table 2 - Zeta potential (mV) of the particles in the different stages of preparation using LBL self-assembly technique. These values are means ± SDs of three independent measurements.

Zeta potential (mV)	Non-coated particles	Core-PEI	Core-PEI/PSS	Core-PEI/PSS/BDMDAC
$CaCO_3$	-21.8 ± 1.1	6.1 ± 4.5	-23.1 ± 3.1	-16.9 ± 3.6
Kaolin	-29.7 ± -2.0	-20.7 ± 4.3	-34.9 ± 5.0	-21.2 ± 1.7
Silica	-77.6 ± 8.4	-15.3 ± 2.1	-61.8 ± 3.0	-43.9 ± 0.7
Sea sand	-40.0 ± 2.3	-11.7 ± 2.9	-24.9 ± 13.1	-7.5 ± 3.2
Cork	-21.1 ± 1.4	-16.0 ± 5.9	-25.9 ± 2.2	-13.1 ± 1.5

By analysing the zeta potential values presented in Table 2 it is possible to identify a change in the charge of the particles after each layer assembly. This observation corroborates the idea of the LBL self-assembly technique, in which the build-up of multilayer assemblies is achieved by consecutively alternating adsorption of anionic and cationic polyelectrolytes, causing charge reverse (Decher & Hong, 1991). At pH 9, all cores showed negative charge, which is favourable for the electrostatic interactions with the cationic polyelectrolyte PEI. Because the first layer acts as a precursor for the multilayer films, the type of polyelectrolyte used plays an important role in structure stability. PEI has been used in several studies as a precursor layer to stabilize the multilayer films (Boulmedais et al., 2004; Kolasińska & Warszyński, 2005). In the case of the present study, the addition of PEI was not sufficient to revert the charge of the particles to positive (except for $CaCO_3$ particles), although all of them suffered a decrease (less negative charge). After PSS layer addition, the charge of the particles became more negative again due to ionic attraction between opposite charges (ion-ion bounding). Finally, after BDMDAC layer addition, the superficial charges of all particles became more positive (although still negative), suggesting that the biocide was able to adhere to the particles surface, through ionic bounding with PSS. The unpronounced shift from negative to positive charge after BDMDAC addition may be explained by the fact that PSS is a polyanion that induces high negative zeta potential

values (Volodkin et al., 2004b), while each BDMDAC molecule only provides one positive charge to link to the particle. Furthermore, the hydrophobic interactions between the carbon chains of BDMDAC and the water block the access of more BDMDAC molecules that bind to the free negative charges of PSS.

Although in this project the quantification of the amount of biocide in particles though the use of HPLC was not possible to perform due to lack of resources and schedule issues, the samples in order to asses it were stored for a complete analysis in the future.

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 non-coated particles was assessed (see Appendix A.3). The medium diameter obtained for each type of particle is presented in Table 3. In the case of $CaCO_3$ microparticles, a wide size distribution was seen until 20 μm, with a medium value of approximately 8.74 μm, which is not in accordance with the sizes observed in the SEM inspections, probably due to aggregation phenomenon. After the coating process with BDMDAC, two distinct populations were observed: a population with a similar size range as the non-coated particles and a population with an average size of 80 μm. This can be related with particle aggregation due to hydrophobic interactions between the BDMDAC carbon chains.

Table 3 – Average diameter of coated and non-coated particles (μm) obtained with Coulter Counter.

Microparticles	**Average diameter (μm)**
$CaCO_3$	8.74 ± 2.04
$CaCO_3$-PEI/PSS/BDMDAC	13.15 ± 3.52
Kaolin	6.74 ± 4.62
Kaolin-PEI/PSS/BDMDAC	8.97 ± 1.58
Silica	2.66 ± 0.12
Silica-PEI/PSS/BDMDAC	5.19 ± 0.61
Sea Sand	208.2 ± 65.2
Sea Sand-PEI/PSS/BDMDAC	218.3 ± 60.0
Cork	475.4 ± 274.3
Cork-PEI/PSS/BDMDAC	487.5 ± 291.3

Regarding kaolin microparticles results, for both coated and non-coated particles there is none individualized populations and a high disparity of sizes until 50 μm can be observed. These results corroborate the observations assessed through kaolin SEM images, where aggregates of different sizes were observed. This event may occur due to the instability of kaolin particles that is a consequence of the charge heterogeneity of these specific particles (Avadiara et al., 2015). Note that, as mentioned previously, kaolin particles edges present charge heterogeneity. Therefore, the coating process of these particles needs optimization. It would be of interest to study the variation of the charge with the pH in order to determine the most suitable pH value for and efficient coating process.

In the case of silica particles, it was possible to clearly observe an individualized population with an average size of 2.66 μm regarding non-coated particles, as also suggested by the SEM analysis. However, since a wide range of sizes from 1 to 15 μm was observed for silica particles after coating, it is suggested that these particles suffer also aggregation, possibly due to the hydrophobic interactions between the BDMDAC carbon chains.

Finally, in the case of sea sand and cork particles, there is no difference in the sizes of both coated and non-coated particles. Thus, the aggregation scenario is not applied here, mainly due to the bigger size and weight when compared to the other three types of particles. The average size obtained through this method for sea sand and cork samples is in agreement with the range of values mentioned in the materials section.

3.4. Conclusions

The surface of $CaCO_3$, kaolin, silica, sea sand and cork microparticles was successfully functionalized with a QAC using LBL self-assembly technique. Overall, the different analysis performed suggested that there was a sequential deposition of the layers. Through SEM, it was possible to observe that for the five types of microparticles developed, no significant structural and morphological changes were noticed after the coating process. Although they seem similar before and after the coating, zeta potential analysis after the assembly of each polyelectrolyte layer indicates that there was indeed a deposition of polyelectrolytes at the surface of the particles, accompanied with a change in the superficial charge. The size distribution analysis revealed that BDMDAC-coated $CaCO_3$, kaolin and silica microparticles tend to aggregate, possibly due to

hydrophobic interactions between the carbon chains of the QAC. Since the physical and structural properties of the developed microparticles may be directly related with their efficacy against microbes, aggregation phenomenon must be minimized in order to allow the total availability of the biocide present at the surface of the referred particles.

Chapter 4

4. Microparticles efficacy against planktonic cells

4.1. Introduction

The supply of microbiologically and chemically safe water is a factor of crucial importance to ensure the health of the populations and to sustain viable industry and the agriculture. The consumption of contaminated drinking water by humans is the cause of several diseases and health-related problems (World Health Organization, 2011). Although the use of chemical disinfectants is commonly very efficient against microbes, they often react with organic matter, leading to the formation of harmful DBPs, as described in Chapter 2. Accordingly, professionals belonging to different areas, such as physical, biological and engineering sciences, currently challenge themselves in order to explore new technologies to overcome this concern. One of the main requirements in order to develop innovative and effective strategies to obtain safe water is based on profound understanding of the microbial diversity of DWDS (Berry et al., 2006). However, since most of the bacterial cells in these systems are either non-cultivable by current cultivation methods or are present in a viable but non-cultivable state, there is a current underestimation of the real composition of bacterial populations (Oliver, 2010).

The microbial composition of DWDS depends of the microflora characteristics of the raw water source. In general, in this system the predominant bacterial genera found have been *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*/*Corynebacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Methylobacterium*, *Moraxella*, *Xanthomonas*, *Serratia*, *Staphylococcus*, *Mycobacterium*, *Sphingomonas* and *Pseudomonas*, being this last one the most abundant organism in different water sources (Berry et al., 2006). Although most of these microorganisms growing on DWDS are harmless, their presence may also be considered due to their role in biofilm formation (Wingender & Flemming, 2011). Nevertheless, several studies detected the presence of different pathogens in DWDS, including *Mycobacterium* spp., infectious enteroviruses and adenoviruses, *Legionella* spp., *Pseudomonas aeruginosa*, *Giardia* spp., filamentous fungi, to name a few (Berry et al., 2006).

Considering the abundance of *Pseudomonas* spp. in natural and industrial environments, *Pseudomonas fluorescens* was the microorganism used in this project to evaluate the efficacy of the functionalized microparticles produced. The bacteria in the *P. fluorescens* species complex are Gram-negative with motile rods (Figure 9). They are primarily aerobic, unable to ferment glucose, and chemoorganotrophic and grow at a pH between 4 and 8 (Scales et al., 2014). Because of its versatile metabolic capabilities, this bacterium have the ability to persist in a wide range of environments, including soil, surfaces of plants, non-sterile pharmaceuticals, indoor surfaces and also in mammalian hosts (Silby et al., 2009). Generally, environmental isolates, which have optimal temperatures of growth of 25–30 °C, are not virulent to human cells. However, certain strains of *P. fluorescens* isolated from clinical samples have a higher permissive growth range (up to 37 °C) and show increased virulence against human cells, especially in patients with compromised immune systems (Donnarumma et al., 2010).

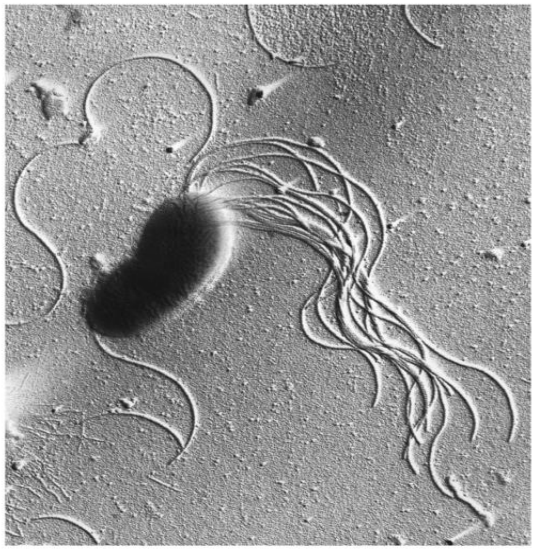

Figure 9 - Scanning electron micrograph of *P. fluorescens* (from Scales et al. 2014).

As presented in Chapter 2, biocides are commonly used both in DWDS and industrial systems to prevent and control microbial growth and biofouling. Accordingly, for the better design and develop effective antimicrobial strategies, the mechanisms of action of biocides and the mechanisms of bacterial resistance are two important points to consider when choosing the appropriate disinfectant. A study of Ferreira et al. (2011) demonstrates that the QAC used in this study, BDMDAC, is an effective biocide against *P. fluorescens* that binds by ionic and hydrophobic interactions to the cell membrane, causing changes in membrane properties and function. These events cause cellular

disruption and loss of membrane integrity with consequent leakage of essential intracellular constituent. Nevertheless, *P. fluorescens* has already demonstrated the potential to form disinfectant-resistant biofilms and the ability to express resistant proteins when exposed to a QAC (cetyltrimethyl ammonium bromide) (Simões et al., 2006b).

The purpose of the present study was to evaluate the efficacy of the BDMDAC-coated microparticles against planktonic *P. fluorescens* at different concentrations and during distinct time periods. The efficacy of the functionalized microparticles was compared with the action of BDMDAC in the free state (without immobilization at microparticles surface) using the same concentrations and conditions.

4.2. Materials and methods

4.2.1. Microorganism and culture conditions

P. fluorescens was isolated from a drinking water distribution system and identified by 16S ribosomal DNA sequence analysis as described by Simões et al. (2007). The optimal growth conditions were 27±3 °C, pH 7 with glucose as the main carbon source. The *P. fluorescens* strain was cryopreserved at -80 °C, in a mixture of nutrient broth and 15% (v/v) of glycerol. Bacterial propagation was obtained by removing an inoculum from the cryovial. The bacterial cells were then distributed evenly over the surface of Plate Count Agar (PCA) and incubated for 24 h at 27±3 °C. For each experiment, bacterial cells were grown overnight at 30 °C under agitation (120 rpm) in an orbital incubator (New Brunswick Scientific, I26, USA), using a broth consisting of 5.5 g/L glucose, 2.5 g/L peptone, and 1.25 g/L yeast extract, in 0.2 M phosphate buffer at pH 7.

4.2.2. Microparticles efficacy tests against planktonic cells

The antimicrobial effect of the BDMDAC-coated particles was compared with the effect of free biocide. Planktonic cells obtained from liquid medium were centrifuged and resuspended in a sterile saline solution (0.85% NaCl) to an $OD_{610nm} = 0.2 \pm 0.02$ (bacterial cell density of approximately 1.41×10^8 CFU/mL). An aliquot of 1.0 ml was collected and used to test the antimicrobial effects of the coated particles and the biocide in the free form. BDMDAC coated particles and free BDMDAC effect was tested at different concentrations (10, 25, 50 and 100 mg/L) during 30 and 60 minutes of incubation at room temperature under agitation (Figure 10). After each incubation time,

the suspension was serially diluted to 10^{-5} and the droplet method was used to spread the samples on PCA (10 µL of sample for each drop). After an incubation period of 24 h at 30 °C, the number of CFUs was counted. Control experiments were performed with bacteria in 0.85% saline solution.

Figure 10 - Schematic representation of the antimicrobial tests with planktonic cells.

4.2.3. Statistical analysis

The data were analysed using the statistical program SPSS 22.0 (Statistical Package for the Social Sciences). The mean and SD within samples were calculated for all treatments. To compare the different conditions tested the Student's *t*-test was used. Statistical calculations were based on confidence levels ≥95% ($p < 0.05$ was considered statistically significant).

4.3. Results and discussion

The antimicrobial effect of BDMDAC was investigated against planktonic cells in both free and microparticles immobilized forms. For both cases, the biocidal agent was in contact with the cell suspension for 30 and 60 minutes, and the results are presented in Figure 11. The curves presented were approximated to a pseudo first order reaction and an inactivation constant (K_d) was determined (Table 4).

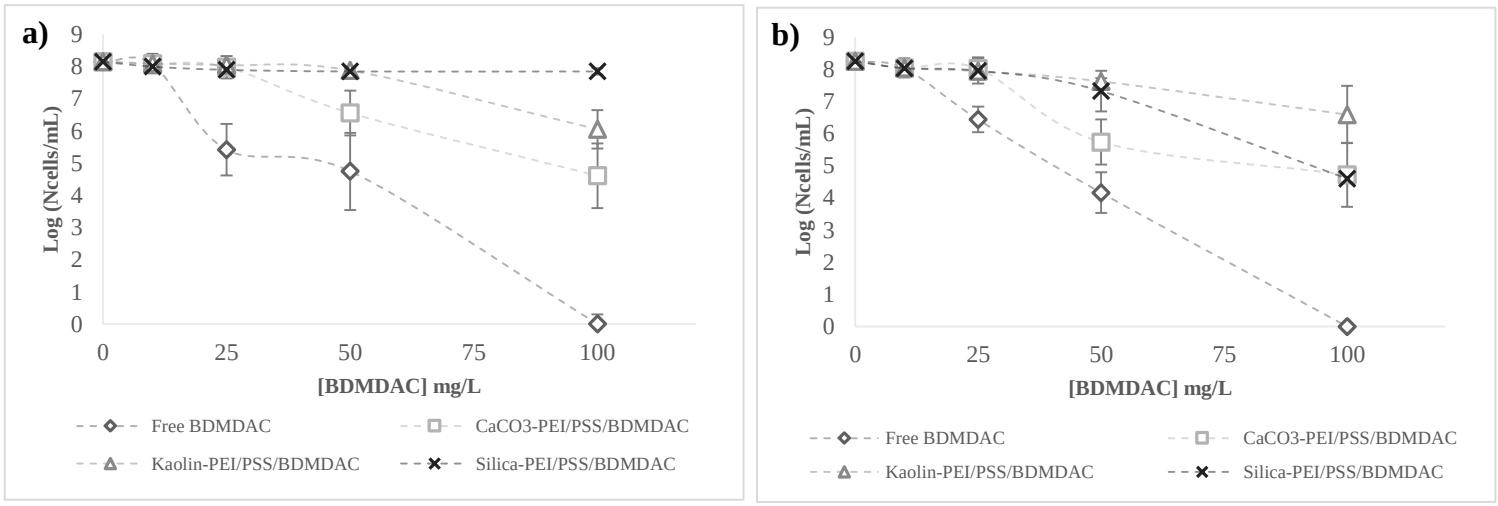

Figure 11 - Logarithm of the number of cells *per* mL of planktonic *P. fluorescens* exposed to different concentrations of the BDMDAC adsorbed on particles during a) 30 minutes and b) 60 minutes. Each symbol indicates the mean ± the SD of three independent experiments.

First, it is important to mention that the results obtained through the use of non-coated $CaCO_3$, kaolin and silica microparticles showed that they have no antimicrobial effects, since the numbers of bacterial cells in the presence of these particles was approximately the same as for the saline solution ($p > 0.05$). In contrast, when the particles were functionalized with BDMDAC, they showed clear antimicrobial effects after 30 and 60 minutes of contact with the cellular suspension (except for silica microparticles contacting for 30 minutes).

Regarding coated $CaCO_3$ microparticles, they showed to be the microparticles with the highest biocidal activity for both incubation times (reduction of 3 in the logarithmic scale). Also, the inactivation constant (K_d) was not significantly different comparing 30 and 60 minutes of contact (0.038 and 0.039 $[mg/L]^{-1}$, respectively), suggesting that the particles rapidly interacted with the cells and exercise their antimicrobial effects. In the case of coated kaolin microparticles, the antimicrobial effect was less pronounced for a higher contact time. This occurrence may be due to the aggregation potential of kaolin particles, as suggested by the characterization of the particles. Finally, coated silica microparticles demonstrated null antimicrobial effect after a contact time of 30 minutes, since the number of bacterial cells in the presence of these particles was approximately the same as for the saline solution ($p > 0.05$). However, by increasing the contact time to 60 minutes, the biocidal effects were so pronounced as for the other type of particles, suggesting that for silica microparticles the contact time favours the antimicrobial effects.

Table 4 – Inactivation constants $[mg/L]^{-1}$ and correlation coefficients (R^2) obtained from the linear approximation of the curves presented in Figure 11. The presented values are means ± the SD of three independent experiments.

	Sample	Inactivation constant (K_d; $[mg/L]^{-1}$)	Correlation coefficient (R^2)
30 min	Free BDMDAC	0.076 ± 0.001	0.93 ± 0.01
	$CaCO_3$-PEI/PSS/BDMDAC	0.038 ± 0.003	0.98 ± 0.03
	Kaolin-PEI/PSS/BDMDAC	0.021 ± 0.008	0.93 ± 0.01
	Silica-PEI/PSS/BDMDAC	0.003 ± 0.007	0.77 ± 0.06
60 min	Free BDMDAC	0.088 ± 0.003	0.99 ± 0.06
	$CaCO_3$-PEI/PSS/BDMDAC	0.039 ± 0.004	0.96 ± 0.09
	Kaolin-PEI/PSS/BDMDAC	0.016 ± 0.013	0.99 ± 0.06
	Silica-PEI/PSS/BDMDAC	0.037 ± 0.010	0.96 ± 0.08

Despite the clear antimicrobial effect exercised by the three types of coated microparticles in the planktonic tests, it was less pronounced when compared with the application of equivalent concentrations and exposure periods of free BDMDAC. This event may occur due to the aggregation suffered by the microparticles after the coating process, which contributes for the decrease of the availability of the biocide when compared with its free similar. Nevertheless, the differences were not statistically significant ($p > 0.05$).

In the final stage of this study, sea sand and cork were used as the core for the BDMDAC functionalized study considering their low cost and high availability. For a preliminary test, both microparticles types were functionalized using the procedure of coating of $CaCO_3$, kaolin and silica particles. However, since the quantification of the biocide uptake at particles surface was not performed and an approximation to the other types of particles would encompass a significant error, for this preliminary test two different aliquots were collected from the stock solution to putt in contact with the cellular suspension during 60 minutes (see Appendix A.1). The quantities collected were selected taking into account the best results obtained for $CaCO_3$ microparticles and were named “lower concentration” (aliquot of 250 μL) and “higher concentration” (aliquot of 500 μL). The effect was determined as a survival ratio between the CFU for the antimicrobial test and the CFU in saline solution (Figure 12).

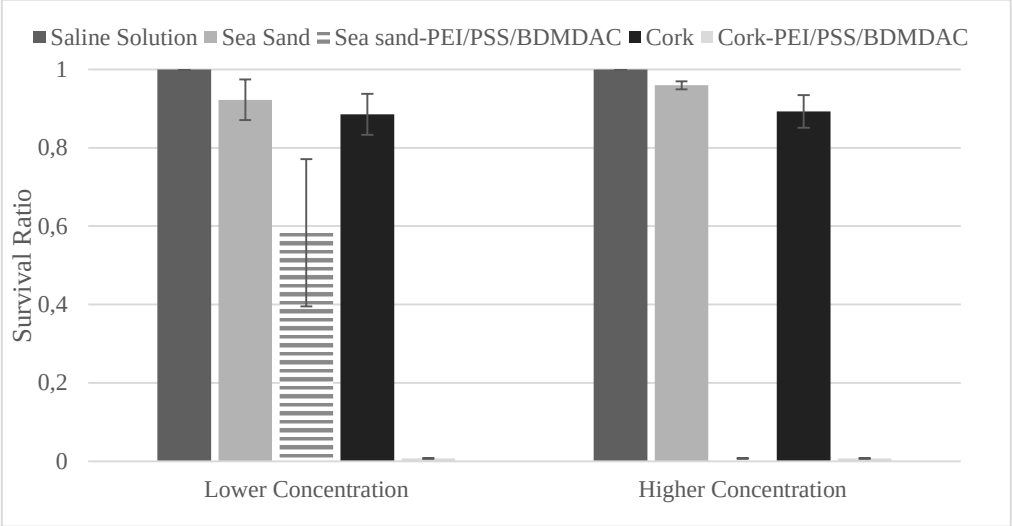

Figure 12 - Survival ratio of planktonic *P. fluorescens* exposed to control conditions (saline solution, sea sand and cork particles) and to BDMDAC-coated particles and free BDMDAC at two different concentrations, for a 60 min exposure period. Each symbol indicates the mean ± the SD of two independent experiments.

According to these results, non-coated sea sand and cork have no antimicrobial effects, since the number of bacterial cells in the presence of these particles was not statistically different from the number of cells in saline solution ($p > 0.05$). In contrast, both types of particles coated with BDMDAC demonstrated significant antimicrobial effects, especially the coated cork microparticles. No CFU were detected after exposure to both biocide concentrations experimented. Probably, the higher porosity of cork microparticles led to high adsorption rate of the biocide into its surface. A profound analysis of the coated nanoparticles of cork need to be performed in the future to better understand the coating process and to explore the amount of biocide that they can incorporate at the surface. Regarding sea sand microparticles, the higher concentration tested appeared to exercise a higher antimicrobial effect than the lower one (survival ratio of 58% and 2%, respectively). Comparing sea sand with silica microparticles, which have the same chemical composition (SiO_2) but different sizes, the antimicrobial effect appears to be more pronounced for the first ones (note that the aliquot of 500 µL collected is an approximation of the concentration 100 mg/L presented in Figure 11). Possibly, the absence of aggregation in BDMDAC-coated sea sand microparticles leads to a higher availability of the biocide, resulting in a lower survival ratio of the bacterial cells.

Overall, the planktonic tests indicated a pronounced antimicrobial effect of BDMDAC when coating the particles. In general, when compared to other QACs, BDMDAC presents significant antimicrobial effect (Guerin-Mechin et al., 2000; Simões

et al., 2006a). As mentioned previously, BDMDAC binds by ionic and hydrophobic interactions to the cell membrane, causing changes in membrane properties and function, as manifested by phenomena such as cellular disruption and loss of membrane integrity with consequent leakage of essential intracellular constituents (Ferreira et al., 2011). In other hand, previous preliminary studies with BDMDAC-coated microparticles developed on the reuse of the particles showed promising results on antibacterial tests, even after three times reprocessing (Ferreira et al., 2013). In this context, there are evidences that suggest that the biocidal agent is not consumed during the contact with the cells. In other words, apparently the QAC immobilized on the particle surface can damage the bacterial cells membrane with its long carbonated chain and remains linked to the particle by ionic linkage. Furthermore, Ferreira et al. (2013) found also that after 18 months in borate buffer, pH 9 (kept at 4 °C), the particles of polystyrene coated with BDMDAC released only 15% of the QAC. Therefore, there is an opportunity associated with particles reuse, if they are properly removed from the suspension. In practice, this approach would save significant amounts of biocide in drinking water treatment systems, reducing the costs associated with the process. Furthermore, this method may allow a better control of the residual amount of biocide in DWDS, avoiding the formation of undesired compounds through the reaction of the chemicals with organic matter.

4.4. Conclusions

The functionalized microparticles developed in this study were found to be effective against planktonic *P. fluorescens*. The BDMDAC-coated $CaCO_3$ were the type of particles with the highest antibacterial activity. Kaolin coated microparticles, selected for this project due to the high availability of the clay and low cost, were moderately effective in the decrease of cellular viability. However, more studies need to be performed in order to optimize the coating process of kaolin to obtain better results. Silica microparticles demonstrated also high antimicrobial efficiency, mainly after one hour of exposure to the planktonic cells. Sea sand and cork microparticles were found to be a promising material to consider in future experiments using this coating technique, with the advantages of being highly available and cheap.

Chapter 5

5. Microparticles efficacy against biofilms

5.1. Introduction

Microbes are well known for their capacity to attach to available surfaces and readily form biofilms. A biofilm is a community of microorganisms attached to a surface that produce EPS and exhibiting an altered phenotype compared with corresponding planktonic cells, especially regarding growth, gene transcription, protein production and intercellular interaction (Mah & O'Toole, 2001). In DWDS, the inner-surfaces of the pipes are inevitably colonized by biofilms, even in the presence of residual disinfectants. It is estimated that 95% of the overall biomass is attached to pipe walls, while only 5% is in the water phase (Flemming et al., 2002). Therefore, there is an increased possibility of pipes corrosion, taste and odour problems, and most importantly, pathogens dissemination into the water bulk phase (Percival & Walker, 1999; Wingender & Flemming, 2011). In fact, the interaction of pathogenic microorganisms with biofilms, especially in DW systems, has been of particular concern. Pathogenic bacteria such as *L. pneumophila* and intestinal coliforms have already been associated with biofilms in potable water systems (World Health Organization, 2011).

Biofilms constitute a protected mode of growth, allowing microorganisms to enjoy many advantages when compared with their planktonic counterparts, including reduced susceptibility to dehydration, phagocytosis, metal toxicity, physical breakdown and to biocides and antibiotics exposure (Lindsay & von Holy, 2006). Biofilms have been reported as being 100-1000 times less susceptible towards biocides and antibiotics that are their equivalent populations of planktonic bacteria (Gilbert et al., 2002). The reduced susceptibility to antimicrobial agents in biofilms is commonly attributed to different factors, including the EPS production (which implies reduced penetration of the antimicrobial), slow growth (and subsequent lower metabolic rates) and changes in a range of other metabolic processes, such as quorum-sensing (Mah & O'Toole, 2001). Several studies demonstrated that generally bacteria in planktonic culture are more susceptible to biocides than attached cells depending on the maturation state of the biofilm, which ranges from cells attached to surfaces for hours to samples extracted

from continuously fed biofilm reactors that are weeks old (LeChevallier et al., 1988). For example, in DWDS, disinfection with chlorine conduce to a reduction in the concentration of planktonic bacteria, but its effect on the concentration of biofilm bacteria is barely null (Gagnon et al., 2005).

The purpose of the present study was to evaluate the efficacy of the BDMDAC-coated microparticles in contact with a *P. fluorescens* biofilms established in 24 hours in a microtiter plate, and the same biofilm produced in stainless steel slides in a well-stirred continuous reactor during 5 days. The efficacy of the functionalized microparticles was compared with the action of BDMDAC in the free state (without immobilization at microparticles surface) in the same concentration.

5.2. Materials and methods

5.2.1. Biofilm setup in a microtiter plate

Biofilms were developed in 96-well microtiter plate (Orange scientific) at 27 ±3 °C for a period of 24h under agitation as described by Simões et al. (2007). *P. fluorescens* was grown overnight in a nutrient solution consisting of 5.5 g/L glucose, 2.5 g/L peptone, and 1.25 g/L yeast extract, in 0.2 M phosphate buffer at pH 7. The bacterial suspension was diluted to achieve an OD_{610nm}= 0.02 and the wells were filled with 200 µL of the diluted bacterial suspension.

5.2.2. Antimicrobial test with a 24 h microtiter plate biofilm

After 24 h of incubation, the medium was discarded with a pipet and the wells were washed twice with 0.85% saline water. Afterwards, the bactericidal solution was added at the desired concentration (20 µL of stock solution + 180 µL of saline water). To perform this assays the different cores coated with BDMDAC were experimented at different concentrations. For the two positive controls, only saline water and non-coated particles were added to the wells. The plate was incubated in independent experiments during different periods (1 and 2 h) at 27 ± 3 °C under agitation. The biocide was then discarded with the pipet and the wells were washed twice with saline water. Using 250 µL of saline water, the biofilm was scraped from each well and reserved in an *eppendorf*. This step was repeated once and 500 µL were added to a final volume of 1 mL. The biofilm was then serially diluted to 10^{-5} and the droplet method was used to

spread the samples on PCA (10 µL of sample for each drop). After an incubation period of 24 h at 30 °C, the number of CFUs was counted.

5.2.3. Biofilme setup in a continuous reactor

Biofilms were developed in a well-stirred continuous reactor at 27 ± 3 °C as described by Ferreira et al. (2013). *P. fluorescens* was grown in a 2 L polymethyl methacrylate (Perspex) reactor, suitably aerated and magnetically agitated (Figure 13). The reactor was continuously fed with 0.20 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 bacterium was grown in the reator by adding 500 mL of bacterial suspension ($OD_{610\ nm}$ = 1.0) to 1.5 L of a 0.85% saline solution for approximately 2 h before the beginning of the continuous feeding process. Twenty slides (2.0 cm × 2.0 cm × 0.1 cm) of stainless steel (SS) AISI 316 were placed vertically in contact with the bacterial suspension for 5 days for biofilm growth.

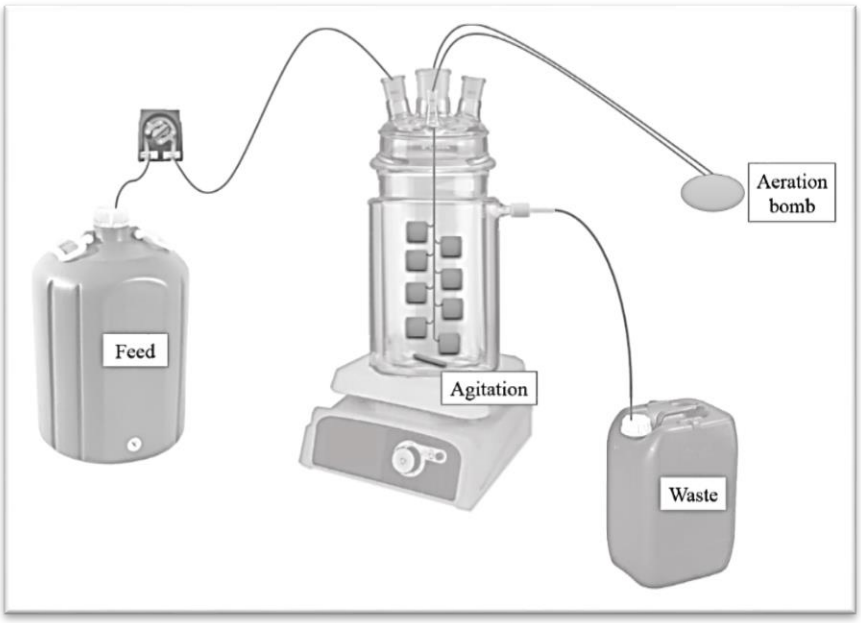

Figure 13 - Schematic representation of the biofilm reator setup.

5.2.4. Antimicrobial test with a 5 days reactor biofilm

After biofilm development, the biofilm-covered slides were carefully transferred to a closed flask that contained the BDMDAC coated particles solution. The flask was placed in an orbital shaker throughout the chemical treatment, to ensure the same temperature and agitation conditions as in the reactor (Figure 14). Biofilm-covered slides were exposed to BDMDAC calcium carbonate and kaolin coated particles at a

100 mg/L concentration during 2 h. As control assays, biofilm-covered slides were also placed in saline solution and in a solution of non-coated particles at the same concentration. For comparison, free BDMDAC at the same concentration as in the coated particles was also tested in the same conditions. Afterwards, the stainless steel slides with accumulated biofilm were carefully removed from the solution of BDMDAC-coated particles. Biofilm control action was measured as the variation in the CFU. Each slide was then placed in a flask containing 10 mL of saline solution, biofilm was scraped off and vortexed for 2 min, and the necessary dilutions were performed to determine the variation in the number of CFU. The bacterial samples were diluted to the adequate cellular concentration in sterile saline solution and the droplet method was used to spread the samples on PCA (10 µL of sample for each drop). Colony enumeration was carried out after 24 h at 27 ± 3 °C. The evaluation of the microbial reduction was carried out through the determination of the survival ratio (ratio between CFU in the antimicrobial test and CFU in the control with saline).

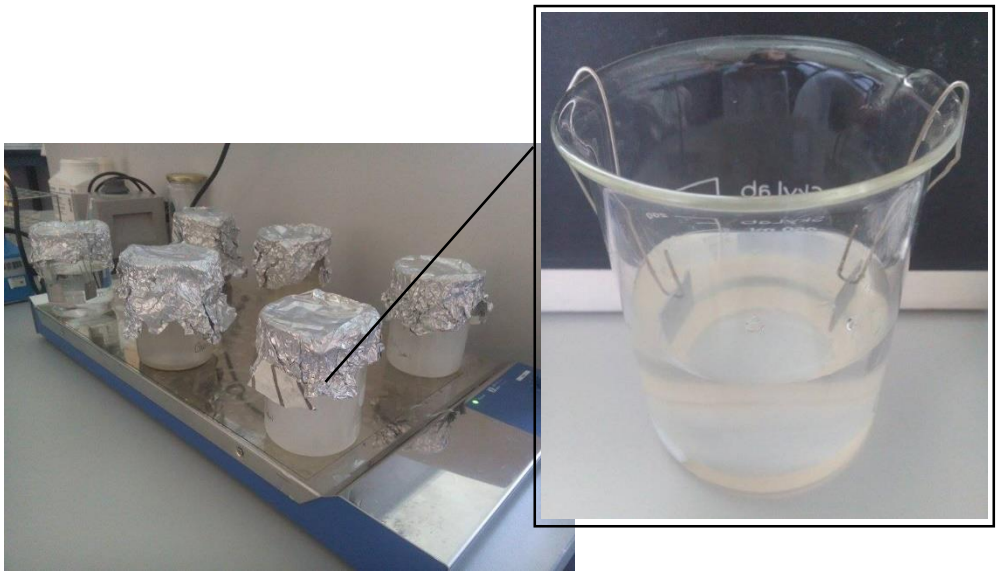

Figure 14 - Images of the antimicrobial test setup.

5.2.5. Statistical analysis

The data were analysed using the statistical program SPSS 22.0 (Statistical Package for the Social Sciences). The mean and SD within samples were calculated for all treatments. To compare the different conditions tested the Student's *t*-test was used. Statistical calculations were based on confidence levels ≥95% ($p < 0.05$ was considered statistically significant).

5.3. Results and discussion

The effects of the application of $CaCO_3$, kaolin and silica coated microparticles against biofilms was first performed in a *P. fluorescens* biofilm developed in a microtiter plate for 24 hours. Sea sand and kaolin microparticles were not tested in these conditions because of size concerns. Different concentrations of BDMDAC were tested during exposure periods of 1 and 2 hours. In accordance with a study of Ferreira et al. (2011), the minimum bactericidal concentration (MBC) associated with the contact of free BDMDAC with planktonic *P. fluorescens* was 10 mg/L. Therefore, and considering that biofilms have been reported as being 100-1000 times less susceptible towards biocides that are they equivalent populations of planktonic bacteria, higher concentrations were selected in this test than the ones used in Chapter 4. For each condition, the number of cells *per* mL was determined and the results are presented in Figure 15.

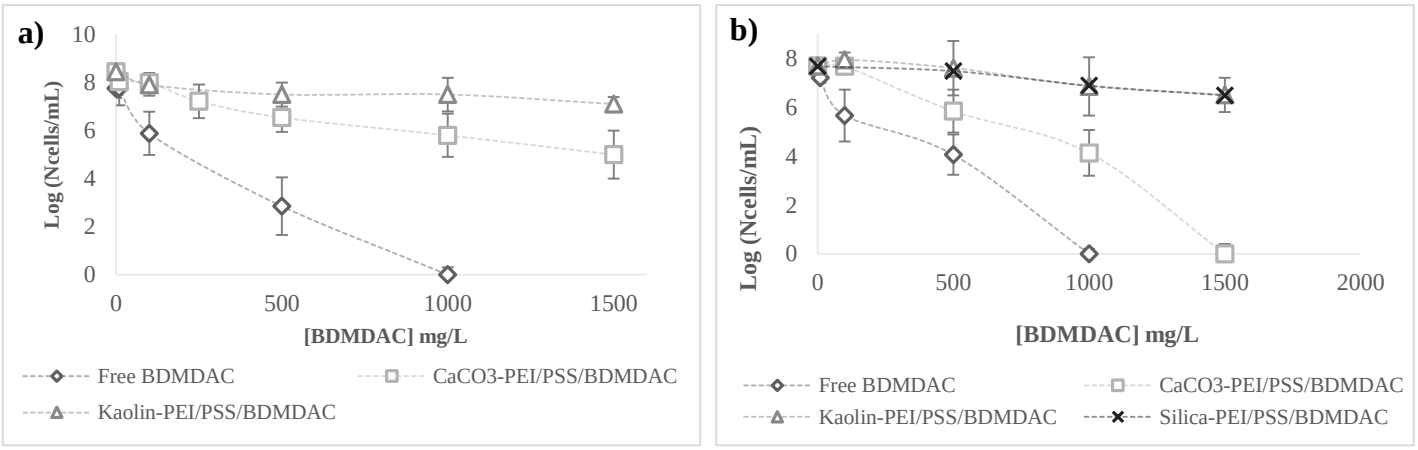

Figure 15 - Logarithm of the number of cells *per* mL of *P. fluorescens* biofilm exposed to different concentrations of BDMDAC adsorbed on particles during a) 1 hour and b) 2 hours. Each symbol indicates the mean ± the SD of three independent experiments.

Additionally, the curves presented in Figures 15a and 15b were approximated to a pseudo first order reaction and the inactivation constant (K_d) was determined (Table 5).

Table 5 - Inactivation constants $[mg/L]^{-1}$ and correlation coefficients (R^2) obtained from the linear approximation of the curves presented in Figure 15. The presented values are means ± the SD of three independent experiments.

	Sample	**Inactivation constant (K_d; $[mg/L]^{-1}$)**	**Correlation Coefficient (R^2)**
1 h	Free BDMDAC	0.010 ± 0.001	0.98 ± 0.01
	$CaCO_3$-PEI/PSS/BDMDAC	0.002 ± 0.003	0.97 ± 0.03
	Kaolin-PEI/PSS/BDMDAC	0.001 ± 0.008	0.89 ± 0.01
2 h	Free BDMDAC	0.007 ± 0.003	0.93 ± 0.06
	$CaCO_3$-PEI/PSS/BDMDAC	0.004 ± 0.004	0.99 ± 0.09
	Kaolin-PEI/PSS/BDMDAC	0.001 ± 0.013	0.96 ± 0.06
	Silica-PEI/PSS/BDMDAC	0.001 ± 0.010	0.98 ± 0.08

It is important to mention that the results obtained through the use of non-coated $CaCO_3$, kaolin and silica microparticles showed that they have no antimicrobial effects against *P. fluorescens* biofilms under the test conditions. The number of bacterial cells in the presence of these particles was approximately the same as for the saline solution (p>0.05).

The particles functionalized with BDMDAC demonstrated antimicrobial effects after both 1 and 2 hours of contact with the biofilms (note that silica functionalized microparticles were only tested for 2 h of contact). Coated $CaCO_3$ microparticles had a pronounced antimicrobial effect at 1000 mg/L, especially after an exposure period of 2 hours (reduction of 3 N_{cells}/mL in the logarithmic scale). Also, for this period of incubation, the inactivation constant (K_d) was the double when compared with an incubation period of 1 hour (0.004 $[mg/L]^{-1}$ and 0.002 $[mg/L]^{-1}$, respectively). By comparing the application of equivalent concentrations and exposure times of free BDMDAC, it was verified that the antimicrobial effect of this QAC was more significant without immobilization at microparticles surface. However, the difference is not statistically significant (p>0.05). Nevertheless, this occurrence may be explained for the easiness of microparticles deposition on the microtiter plate bottom, which may not

be overcome by the agitation provided, leading to a decrease in the contact of the biocide with the biofilm in the walls of the well.

Regarding the results obtained for coated kaolin microparticles, for all the concentrations tested it was not possible to verify a significant antimicrobial effect for both exposure times of contact. The reduction of the number of cells *per* mL was similar regardless the time, as well as the inactivation constant (0.001 $[mg/L]^{-1}$). Again, the high aggregation of kaolin microparticles, mainly after the surface functionalization, may be the cause of the insignificant antimicrobial effect associated with these particles.

Finally, the antimicrobial effect of coated-silica microparticles was also insignificant, since the number of bacterial cells in the presence of these particles was approximately the same as for the saline solution ($p > 0.05$). The deposition of the particles at the bottom of the microtiter plate may explain these results. Thus, more studies need to be performed under optimized process conditions.

The effects of the application of free BDMDAC and BDMDAC coated particles were then tested against biofilms formed on SS slides for a period of 5 days in a well-stirred continuous reactor, by determining the survival ratio of the biofilm population. The results are represented in Figure 16. In this study only $CaCO_3$ and kaolin microparticles were tested with a time of contact of 2 hours, since best results were obtained in the microtiter plate tests with this condition. Furthermore, silica microparticles were not available to perform a test with this magnitude, where a high amount of particles was necessary. In other hand, sea sand and cork microparticles were only introduced in the project in the final period. Thus, due schedule issues, they were not tested against biofilms.

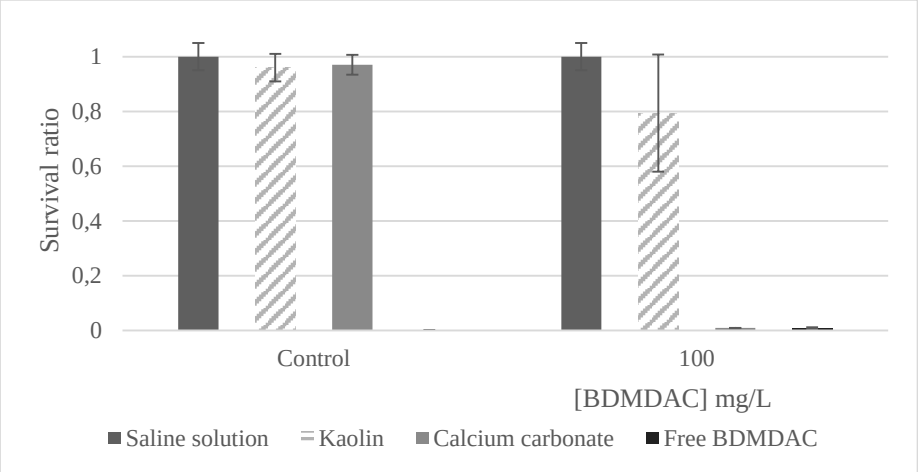

Figure 16 - Survival ratio of *P. fluorescens* after 2 hours of exposure to free biocide, and $CaCO_3$ and kaolin coated and non-coated particles.

In the case of $CaCO_3$ microparticles, the treatment of the biofilm with particles without coating did not promote a significant decrease in biofilm viability (4%) when compared with the control with saline solution (p>0.05). Unlike non-coated particles, the biofilm exposure to the biocidal $CaCO_3$ particles with a concentration of BDMDAC of 100 mg/L resulted in a viability decrease of 99.1% of the total biofilm population. Similarly, the application of an equivalent concentration and exposure period of free BDMDAC led to a viability decrease of 99.2%. The differences observed were not statistically significant (p>0.05).

In contrast, and in accordance with the results obtained in the antimicrobial test with biofilms in microtiter plates, BDMDAC-coated kaolin particles promoted only a moderate decrease in the viability of the cells (approximately 21%). However, when compared with the results obtained for the application of an equivalent concentration and exposure period of free BDMDAC and coated $CaCO_3$ microparticles, the differences were statistically significant (p<0.05). In fact, the interaction of $CaCO_3$ with the biofilms in the slides was visually more pronounced than the interaction of coated kaolin microparticles (see Figure 17).

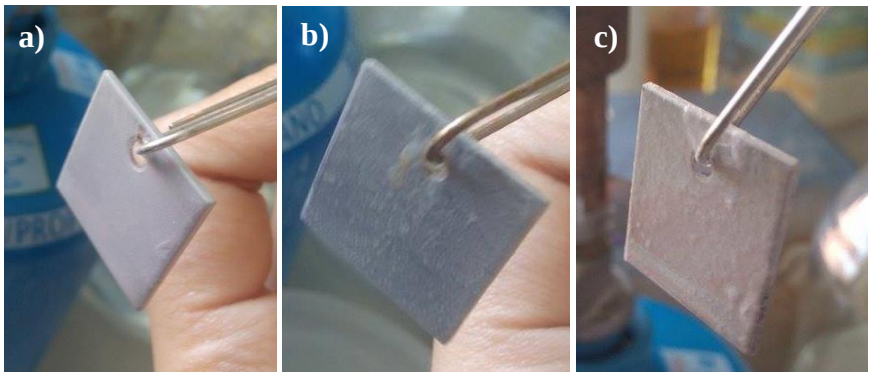

Figure 17 – Biofilm formed in the SS slides after treatment with a) saline water; b) coated kaolin microparticles and c) coated $CaCO_3$ microparticles.

The higher antimicrobial effects associated with the QAC in the free state when compared to the results associated with immobilized biocide may be related with diffusion limitations associated with particles size and shape (Peulen & Wilkinson, 2011; Pal et al., 2007). In natural biofilms, the diffusion may be impaired by: the porous structure of the biofilm, the type of interaction of the particles with the cells, non-diffusing macromolecules or the EPS network, and the adsorption of the solute to freely diffusing species, abiotic particles, or gas bubbles (Stewart, 2003). For example, Peulen and Wilkinson (2011) found that the diffusion of the microspheres used in contact with a biofilm of *P. fluorescens* was highly dependent upon the size of the particles and the

porosity of the biofilm. It is conceivable that reducing the particle size would increase the antimicrobial effects. Also, other authors concluded that silver nanoparticles undergo shape-dependent interaction with the Gram-negative bacterium *E. coli* (Pal et al., 2007). Accordingly, it might be that the undefined shape and the big size of BDMDAC-coated kaolin microparticles is a possible reason for the low antimicrobial activity against biofilms. Oppositely, the lower size and spherical shape of $CaCO_3$ microparticles (3 μm) may be favourable for biofilm penetration.

Nowadays, the use of chemical antimicrobial agents in water treatment is of concern mainly because of two important points: the possibility of the spread of disinfectant residues in the food-chain if they reach water consumers and the development of resistance to the chemicals by some microorganisms. Residual QACs that are released into sewage treatment plants and natural environment are concern due to their toxicity to a lot of organisms (Zhang et al., 2015). For example, QACs could inhibit the nutrients uptake by algae, leading to a lower nutrient removal in wastewater treatment plants (Liang et al., 2013). On the other hand, it is well known that the prolonged exposure of microorganisms to sub-lethal concentrations of chemicals promotes antimicrobial resistance and cross-resistance events (McDonnell & Russel, 1999). Long-term exposure of microbial populations to QACs not only increases the selection of QAC-resistant bacteria but also the selection of antibiotic-resistance bacteria (Gaze et al., 2005). A previous study reported the ability of *P. aeruginosa* to adapt to increasing concentrations of benzalkonium chloride and verified the co-resistance to other membrane-active biocides (Loughlin et al., 2002). In this context, since it allows the reuse of antimicrobial agents, the strategy of biocide immobilization used in this study may have potential public health, environmental, and economic benefits by effectively limiting the levels of biocides used in cleaning and disinfection practices.

Possible difficulties in the application of this strategy may come from the current cost associated with the entire process when compared with the price of the disinfectants commonly used in DWDS. Thus, it is of extreme importance the use of relatively cheap cores, as the ones proposed in this study. In order to avoid the excessive use of polyelectrolytes, which also have costs associated, in the future it would be of advantage the exploration of different processes. For example, several authors report the use of cationic polyelectrolytes with antimicrobial activity (Cakmak et al., 2004; Lichter

& Rubner, 2009; Sridhar et al., 2014). This approach would save in the use of QACs providing real benefits associated with environmental and economic issues.

5.4. Conclusions

The BDMDAC-coated $CaCO_3$ microparticles developed in this study were highly effective against biofilms of *P. fluorescens*. Furthermore, the efficiency of those particles was not significantly different from the action of the biocide in the free state. However, coated kaolin and silica microparticles were not efficient under the same conditions. This might be due to aggregation problems of the particles, probably because of the hydrophobic interactions between the carbon chains of the QAC used. Furthermore, it might be that the coating process is not the most suitable for this particles (note that an estimative of the total amount of biocide adhered to the particles was done, taking into account the amount adhered on $CaCO_3$ microparticles already studied in detail by other researchers). Thus, the analysis of kaolin and silica coating process needs to be assessed in more detail in the future.

It was also clear that in the conditions of the tests performed with biofilms in the microtiter plate the concentration of BDMDAC-coated particles needs to be at least ten times higher than the ones used in the planktonic tests. Probably, this occurs due to the presence of EPS along with the biofilm, constituting a barrier for microparticles action. Furthermore, there are evidences that the diffusion processes into the biofilms are dependent on the size and shape of the particles. This might explain the low antimicrobial activity of coated kaolin microparticles, since they present undefined shapes and big size due to the aggregation phenomenon.

Chapter 6

6. Concluding remarks and future work

Although several studies reported the use of micro and nanotechnology and, more specifically, the use of LBL self-assembly technique at micro and nanoscale in medical and pharmaceutical fields, there is a scarcity of information about the use of those techniques for water treatment processes. In this context, the goals of this work were to develop and characterize innovative microparticles with functionalized surfaces that act as carriers of antimicrobial molecules using LBL technique, and to test them against both planktonic *P. fluorescens* and biofilms formed by the same bacteria.

The microparticles coated with a QAC (BDMDAC) used in this study revealed considerable antimicrobial effects against planktonic *P. fluorescens*, especially $CaCO_3$ microparticles. Kaolin microparticles seemed to suffer the phenomenon of aggregation, leading to the decrease of the availability of the biocide contacting with cells. Preliminary tests using coated sea sand and cork showed that they are a promising approach to consider in the future since they demonstrated to significantly decrease cell viability. The particular structure of cork must be investigated in detail in the future in order to take advantage of it for the immobilization of high biocide doses.

In addition, the $CaCO_3$ microparticles coated with BDMDAC revealed also to be effective against biofilms formed by *P. fluorescens*. However, the same was not observed by using silica and kaolin microparticles, probably due to the potential of the aggregation associated with this particles in the conditions tested. Furthermore, studies reported in the literature reveal that the shape and the size of particles may influence their diffusion into the biofilm. Thus, kaolin seemed to be unappropriated for biofilm treatment under the tested process conditions. Additionally, considering the particular structure and composition of biofilms, it would be of interest to investigate how the microparticles interact with EPS and diffuse through biofilms.

Overall, it is clear the need of a better evaluation of the characteristics of the particles produced. In particular, it is essential to quantify the amount of biocide adhered on the particles in order to adjust the results obtained in this study. Moreover, different types of biocides should be tested.

Concerning the prices associated with the production of these particles, future studies will therefore focus on the use of new particle cores, in addition to the ones already proposed. It is suggested also the use of magnetic materials, such as magnetite, associated with the advantage of being easier to recover from the water by magnetic separation. This improvement would favour the large scale application of this process against both planktonic and biofilm cells.

In the future, it would also be of interest to test the efficacy of the developed particles in a larger scale and with different types of microorganisms, in multispecies biofilms, in order to mimic the ecologic diversity found in DWDS and to test this strategy against natural contaminants. For example, the microparticles could be tested in a fluidized bed where the particles were allowed to interact with the bacterial cells.

In conclusion, the results obtained in the present study clearly demonstrate that this novel and promising biofilm control strategy may have potential public health, environmental, and economical benefits.

References

Albers, C.N., Ellegaard-Jensen, L., Harder, C.B., Rosendahl, S., Knudsen, B.E., Ekelund, F. & Aamand, J. (2015). Groundwater Chemistry Determines the Prokaryotic Community Structure of Waterworks Sand Filters. *Environmental Science & Technology*. 49. p.pp. 839–846.

Allison, D. (2003). The biofilm matrix. *Biofouling*. 19. p.pp. 139–150.

Ariga, K., Hill, J.P. & Ji, Q. (2007). Layer-by-layer assembly as a versatile bottom-up nanofabrication technique for exploratory research and realistic application. *Physical chemistry chemical physics : PCCP*. 9. p.pp. 2319–2340.

Ariga, K., Lvov, Y., Kawakami, K., Ji, Q. & Hill, J.P. (2011). Layer-by-layer self-assembled shells for drug delivery. *Advanced Drug Delivery Reviews*. 63. p.pp. 762–771.

Arora, H., LeChevallier, M. & Dixon, K. (1997). DBP occurrence survey. *American Water Works Association*. 89 (6). p.pp. 60–68.

Avadiara, L., Leonga, Y. & Fourieb, A. (2015). Physicochemical behaviors of kaolin slurries with and without cations—Contributions of alumina and silica sheets. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 468. p.pp. 103–113.

Barbosa, A., Silva, L., Ochsner, A., Abenojar, J. & Real, J. (2012). Influence of the Size and Amount of Cork Particles on the Impact Toughness of a Structural Adhesive. *The Journal of Adhesion*. 88. p.pp. 452–470.

Bassler, B. (1999). How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current Opinion in Microbiology*. 2. p.pp. 582–587.

Behrman, J., Alderman, H. & Hoddinott, J. (2004). Hunger and malnutrition in Copenhagen Consensus—Challenges and Opportunities. *London School of Hygiene and Tropical Medicine*.

Bellar, T., Lichtenberg, J. & Kroner, R. (1974). The occurence of organohalides in chlorinated drinking waters. *American Water Works Association*. 66 (12). p.pp. 703–706.

Bergmann, H., Iourtchouk, T., Schöps, K. & Bouzek, K. (2002). New UV irradiation and direct electrolysis—promising methods for water disinfection. *Chemical Engineering Journal*. 85. p.pp. 111–17.

Berry, D., Xi, C. & Raskin, L. (2006). Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*. 17. p.pp. 297–302.

Block, S.R. (1983). *Disinfection, sterilization and preservation*. In: Philadelphia: Leg & Feblger, pp. 309–329.

Boulmedais, F., Frisch, B., Etienne, O., Lavalle, P., Picart, C., Ogier, J., Voegel, J.C., Schaaf, P. & Egles, C. (2004). Polyelectrolyte multilayer films with pegylated polypeptides as a new type of anti-microbial protection for biomaterials. *Biomaterials*. 25 (2004). p.pp. 2003–2011.

Breyers, J. & Ratner, J. (2004). Bioinspired implant materials befuddle bacteria. *ASM News*. 70. p.pp. 232–237.

Cakmak, I., Ulukanli, Z., Tuzcu, M., Karabuga, S. & Genctav, K. (2004). Synthesis and characterization of novel antimicrobial cationic polyelectrolytes. *European Polymer Journal*. 40. p.pp. 2373–2379.

Caruso, F. (2001). Nanoengineering of Particle Surfaces. *Advanced Materials*. 13 (1). p.pp. 11–22.

Caruso, F., Caruso, R. & Möhwald, H. (1998). Nanoengineering of inorganic and hybrid hollow spheres by colloidal templating. *Science*. 282 (5391). p.pp. 1111–1114.

Chandy, J.P. & Angles, M.L. (2001). Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. *Water Research*. 35 (11). p.pp. 2677–2682.

Chmielewski, R. & Frank, J. (2003). Biofilm formation and control in food processing facilities. *Comprehensive Reviews in Food Science and Food Safety*. 2 (1). p.pp. 22–32.

Cho, M., Chung, H., Choi, W. & Yoon, J. (2005). Different Inactivation Behaviors of MS-2 Phage and Escherichia coli in TiO 2 Photocatalytic Disinfection Different Inactivation Behaviors of MS-2 Phage and Escherichia coli in TiO 2 Photocatalytic Disinfection. *Applied and Environmental Microbiology*. 71 (1). p.pp. 270–275.

Clark, P., Pinedo, C., Fadus, M. & Capuzzi, S. (2012). Slow-sand water filter: Design, implementation, accessibility and sustainability in developing countries. *Med Sci Monit*. 18 (7). p.pp. 105–117.

Costerton, J. & Stewart, P. (2001). Battling biofilms. *Scientific American*. 285 (1). p.pp. 74–81.

Daniels, R., Vanderleyden, J. & Michiels, J. (2004). Quorung sensing and swarming migration in bacteria. *FEMS Microbiology Reviews*. 28. p.pp. 261–289.

Decher, G. & Hong, J. (1991). Buildup of ultrafin multilayer films by self-assembly process 1. Consecutive adsorption of anionic and cationic bipolar amphiphiles on charged surfaces. *Macromol. Chem.* 46. p.pp. 321–327.

Donlan, R. (2002). Biofilms: microbial life on surfaces. *Emmerging Infectious Diseases*. 8. p.pp. 881–890.

Donnarumma, G., Buommino, E., Fusco, A., Paoletti, I., Auricchio, L. & Tufano, M. (2010). Effect of temperature on the shift of Pseudomonas fluorescens from an environmental microorganism to a potential human pathogen. *Int J Immunopathol Pharmacol*. 23 (1). p.pp. 227–234.

EPA, U. (1999). Microbial and disinfection by-product rules—simultaneous compliance guidance manual. *United States Environmental Protection Agency EPA 815-R-99-015*.

Ferk, F., Mišík, M., Hoelzl, C., Uhl, M., Fuerhacker, M., Grillitsch, B., Parzefall, W., Nersesyan, a., Mičieta, K., Grummt, T., Ehrlich, V. & Knasmüller, S. (2007). Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB), two common quaternary ammonium compounds, cause genotoxic effects in mammalian

and plant cells at environmentally relevant concentrations. *Mutagenesis*. 22 (6). p.pp. 363–370.

Ferreira, C., Pereira, a. M., Pereira, M.C., Melo, L.F. & Simões, M. (2011). Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens. *Journal of Antimicrobial Chemotherapy*. 66. p.pp. 1036–1043.

Ferreira, C., Pereira, A., Pereira, M., Simões, M. & Melo, L. (2013). Biofilm Control With New Microparticles With Immobilized Biocide. *Heat Transfer Engineering*. 34 (8-9). p.pp. 712–718.

Ferreira, C., Rosmaninho, R., Simões, M., Pereira, M., Bastos, M., Nunes, O., Coelho, M. & Melo, L. (2010). Biofouling control using microparticles carrying a biocide. *Biofouling*. 26 (2). p.pp. 205–212.

Flemming, H.-C., Percival, S.L. & Walker, J.T. (2002). Contamination potential of biofilms in water distribution systems. *Wat. Sci. Tech. Wat. Supp.* 47 (5). p.pp. 271–280.

Fortes, M., Rosa, M. & Pereira, H. (2004). *The Cork*. 1st Ed. Lisbon: IST Press.

Gagnon, G. a., Rand, J.L., O'Leary, K.C., Rygel, a. C., Chauret, C. & Andrews, R.C. (2005). Disinfectant efficacy of chlorite and chlorine dioxide in drinking water biofilms. *Water Research*. 39. p.pp. 1809–1817.

Gaze, W.H., Abdouslam, N., Hawkey, P.M. & Wellington, E.M.H. (2005). *Incidence of Class 1 Integrons in a Quaternary Ammonium Compound-Polluted Environment Incidence of Class 1 Integrons in a Quaternary Ammonium Compound-Polluted Environment*. 49 (5). p.pp. 1802–1807.

Gilbert, P., Allison, D.G. & McBain, a J. (2002). Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance? *Symposium series (Society for Applied Microbiology)*. p.p. 98S–110S.

Gomes, A.P., Mano, J.F., Queiroz, J. a. & Gouveia, I.C. (2012). Layer-by-Layer Deposition of Antibacterial Polyelectrolytes on Cotton Fibres. *Journal of Polymers and the Environment*. 20. p.pp. 1084–1094.

Guerin-Mechin, L., Dubois-Brissonnet, F., Heyd, B. & Leveau, J.Y. (2000). Quaternary ammonium compound stresses induce specific variations in fatty acid composition of Pseudomonas aeruginosa. *International Journal of Food Microbiology*. 55. p.pp. 157–159.

Hammond, P.T. (2000). *Recent explorations in electrostatic multilayer thin film assembly*. 4. p.pp. 430–442.

Heinzel, M. (1998). Phenomena of biocide resistance in microorganisms. *International Biodeterioration & Biodegradation*. 41. p.pp. 225–234.

Hijnen, W.A.M., Beerendonk, E.F. & Medema, G.J. (2006). Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Research*. 40. p.pp. 3–22.

Hosseini, M. & Ahmadi, A. (2015). Biological beneficiation of kaolin: A review on iron removal. *Applied Clay Science*. 107. p.pp. 238–245.

Hu, P., Hsieh, Y., Chen, J. & Chang, C. (2004). Characteristics of manganese-coated sand using SEM and EDAX analysis. *Journal Colloid Interface Science*. 272. p.pp. 308–313.

Iler, R.K. (1966). Multilayers of colloidal particles. *Journal Colloid Interface Sci.* 21. p.pp. 569–594.

Islan, G., Cacicedo, V., Bosio, V. & Castro, G. (2015). Development and characterization of new enzymatic modified hybrid calcium carbonate microparticles to obtain nano-architectured surfaces for enhanced drug loading. *Journal Colloid Interface Science*. 439. p.pp. 76–87.

Janssens, J., Steenackers, H., Robijns, S., Gellens, E., Levin, J. & Zhao, H. (2008). Brominated furanoes inhibit biofilm formation by Salmonella enterica serovar typhimurium. *Applied and Environmental Microbiology*. 74 (21). p.pp. 6639–6648.

Jiang, P., Bertone, J.F. & Colvin, V.L. (2001). A Lost-Wax Approach to Monodisperse Colloids and Their Crystals. *Science*. 291. p.pp. 453–457.

Jiang, S.D., Yao, Q.Z., Zhou, G.T. & Fu, S.Q. (2012). Fabrication of hydroxyapatite hierarchical hollow microspheres and potential application in water treatment. *Journal of Physical Chemistry C*. 116. p.pp. 4484–4492.

Johnston, A., Cortez, C., Angelatos, A.S. & Caruso, F. (2006). Layer-by-layer engineered capsules and their applications. *Current Opinion in Colloid and Interface Science*. 11. p.pp. 203–209.

Kang, S., Pinault, M., Pfefferle, L.D. & Elimelech, M. (2007). Single-walled carbon nanotubes exhibit strong antimicrobial activity. *Langmuir*. 23. p.pp. 8670–8673.

Kerr, C.J., Osborn, K.S., Rickard, A.H., Robson, G.D. & Handley, P.S. (2003). *Biofilms in water distribution systems In: The handbook of water and wastewater microbiology*. In: D. Mara & N. Horan (eds.). London: Academic Press, pp. 757–775.

Kharisov, B.I., Rasika Dias, H. V., Kharissova, O. V., Jiménez-Pérez, V.M. & Péreza, Betsabee Olvera Floresa, B.M. (2012). Iron-containing nanomaterials: synthesis, properties, and environmental applications. *RSC Advances*. 2 (25). p.pp. 9325–9358.

Kolasińska, M. & Warszyński, P. (2005). The effect of nature of polyions and treatment after deposition on wetting characteristics of polyelectrolyte multilayers. *Applied Surface Science*. 252. p.pp. 759–765.

Kopfler, F., Melton, R., Lingg, R. & Coleman, W. (1976). GC/MS determination of volatiles for the national organics reconnaissance survey (NORS) of drinking water. *Identification and Analysis of Organic Pollutants in Water , Ann Arbor Science*. p.pp. 87–104.

Krasner, S.W., Weinberg, H.S., Richardson, S.D., Pastor, S.J., Chinn, R., Sclimenti, M.J., Onstad, G.D. & Thruston, A.D. (2006). Occurence of a new generation of disinfection byproducts. *Environ. Sci. Technol*. 40. p.pp. 7175–7185.

Kurokawa, Y., Aoki, S., Matsushima, Y., Takamura, N., Imazawa, T. & Hayashi, Y. (1986). Dose-response studies on the carcinogenicity of potassium bromate in F344 rats after long-term oral administration. *Journal of the National Cancer Institute*. 77 (4). p.pp. 977–982.

LeChevallier, M.W., Cawthon, C.D. & Lee, R.G. (1988). Inactivation of biofilm bacteria. *Applied and Environmental Microbiology*. 54 (10). p.pp. 2492–2499.

Li, Q., Mahendra, S., Lyon, D.Y., Brunet, L., Liga, M. V, Li, D. & Alvarez, P.J.J. (2008). Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. *Water research*. 42 (18). p.pp. 4591–602.

Liang, Z., Ge, F., Zeng, H., Xu, Y., Peng, F. & Wong, M. (2013). Influence of cetyltrimethyl ammonium bromide on nutrient uptake and cell responses of Chlorella vulgaris. *Aquatic Toxicology*. 138-139. p.pp. 81–87.

Lichter, J. a. & Rubner, M.F. (2009). Polyelectrolyte multilayers with intrinsic antimicrobial functionality: The importance of mobile polycations. *Langmuir*. 25 (29). p.pp. 7686–7694.

Lindsay, D. & von Holy, a. (2006). Bacterial biofilms within the clinical setting: what healthcare professionals should know. *Journal of Hospital Infection*. 64. p.pp. 313–325.

Loughlin, M.F., Jones, M. V & Lambert, P. a (2002). Pseudomonas aeruginosa cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. *The Journal of antimicrobial chemotherapy*. 49. p.pp. 631–639.

Lvov, Y., Ariga, K., Ichinose, I. & Kunitake, T. (1995). Layer-by-layer Architectures of Concanavalian A by means of Electrostatic and Biospecific Interactions. *J. Chem. Soc.* (22). p.pp. 2313–2314.

Lvov, Y., Decher, G. & Sukhorukov, G. (1993). Assembly of thin films by means of successive deposition of alternate layers of DNA and poly(allylamine). *Macromolecules*. 26 (20). p.pp. 5396–5399.

Mah, T.F.C. & O'Toole, G. a. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology*. 9 (1). p.pp. 34–39.

Martins, D. (2014). *Interaction of Chemicals Contaminants with Suspended Particles and Attached Inorganic Deposits in Drinking Water Systems*. Faculty of Engineering of the University of Porto.

McDonnell, G. & Russel, A. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews*. 12 (1). p.pp. 147–179.

Montes, A., Gordillo, M.D., Pereyra, C., De La Rosa-Fox, N. & Martínez De La Ossa, E.J. (2013). Silica microparticles precipitation by two processes using supercritical fluids. *Journal of Supercritical Fluids*. 75. p.pp. 88–93.

Montgomery, M.A. & Elimelech, M. (2007). Water and sanitation in developing countries: including health in the equation. *Environ. Sci. Technol*. 41 (1). p.pp. 17–24.

Mpenyana-Monyatsi, L., Mthombeni, N.H., Onyango, M.S. & Momba, M.N.B. (2012). Cost-Effective Filter Materials Coated with Silver Nanoparticles for the Removal of Pathogenic Bacteria in Groundwater. *Int J Environ Res Public Health*. 9 (1). p.pp. 244–271.

Mueller, R.F., Characklis, W.G., Jones, W.L. & Sears, J.T. (1992). Characterization of initial events in bacterial surface colonization by two Pseudomonas species using image analysis. *Biotechnol. Bioeng.* 39. p.pp. 1161–1170.

National Cancer Institute (1976). *Report on the carcinogenesis bioassay of chloroform (CAS no. 67-66-3)*. Bethesda, MD.

Ngwenya, B., Curry, P. & Kapetas, L. (2015). Transport and viability of Escherichia coli in clean and iron oxide coated sand following coating with silver nanoparticles. *Journal of Contaminant Hydrology*. 179. p.pp. 35–46.

Oliver, J.D. (2010). Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiology Reviews*. 34. p.pp. 415–425.

Ozekin, K., Westerhoff, P., Amy, G.L. & Siddiqui, M. (1998). Molecular Ozone and Radical Pathways of Bromate Formation during Ozonation. *Journal of Environmental Engineering*. 124 (8). p.pp. 456–462.

Pal, S., Tak, Y.K. & Song, J.M. (2007). Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium Escherichia coli. *Applied and Environmental Microbiology*. 73 (6). p.pp. 1712–1720.

Percival, S.L. & Walker, J.T. (1999). Potable water and biofilms: A review of the public health implications. *Biofouling*. 14 (June 2015). p.pp. 99–115.

Peulen, T.O. & Wilkinson, K.J. (2011). Diffusion of nanoparticles in a biofilm. *Environmental Science and Technology*. 45. p.pp. 3367–3373.

Pitman, G. (2002). Bridging Troubled Waters—Assessing The World Bank Water Resources Strategy. *World Bank Publications*.

Plewa, M., Kargalioglu, Y., Vankerk, D., Minear, R. & Wagner, E. (2002). Mammalian cell cytotoxicity and genotoxicity of drinking water disinfection by-products. *Environ. Mol. Mutagen*. 40 (2). p.pp. 134–142.

Preisig, D., David, H., Varum, F., Bravo, R., Alles, R. & Huwyler, J. (2014). Drug loading into porous calcium carbonate microparticles by solvent evaporation. *European Journal of Pharmaceutical and Biopharmaceutics*. 87. p.pp. 548–558.

Richardson, D., Plewa, M. & Wagner, E. (2007). Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research. *Mutation Research - Reviews in Mutation Research*. 636 (1-3). p.pp. 178–242.

Richardson, S. (2003). Disinfection by-products and other emerging contaminants in drinking water. *Trends in Analytical Chemistry*. 22 (10). p.pp. 666–684.

Rook, J. (1974). Formation of haloforms during chlorination of natural waters. *Water Treat. Exam*. 23. p.pp. 234–243.

Sadiq, R. & Rodriguez, J. (2004). Disinfection by-products (DBPs) in drinking water and predictive models for their occurrence: a review. *Science of The Total Environment*. 321 (1-3). p.pp. 21–46.

Sampa, M.H.O., Borrely, S.I., Silva, B.L., Vieira, J.M., Rela, P.R., Calvo, W.A.P., Nieto, R.C., Duarte, C.L., Perez, H.E.B., Somessari, E.S. & Lugão, A.B. (1995). The use of electron beam accelerator for the treatment of drinking water and wastewater in Brazil. *Radiation Physics and Chemistry*. 46 (4-6). p.pp. 1143–1146.

Scales, B.S., Dickson, R.P., LiPuma, J.J. & Huffnagle, G.B. (2014). Microbiology, Genomics, and Clinical Significance of the Pseudomonas fluorescens Species Complex, an Unappreciated Colonizer of Humans. *Clinical Microbiology Reviews*. 27. p.pp. 927–948.

Schonhoff, M. (2003). Self-assembled polyelectrolyte multilayers. *Current Opinion in Colloid and Interface Science*. 8. p.pp. 86–95.

Shannon, M., Bohn, P., Elimelech, M. & Georgiadis, J. (2008). Science and technology for water purification in the coming decades. *Nature Publishing Group*. 452 (7185). p.pp. 301–310.

Sharma, S., Sachdeva, P. & Virdi, J.S. (2003). Emerging water-borne pathogens. *Applied microbiology and biotechnology*. 61. p.pp. 424–428.

Silby, M.W., Cerdeño-Tárraga, A.M., Vernikos, G.S., Giddens, S.R., Jackson, R.W., Preston, G.M., Zhang, X.-X., Moon, C.D., Gehrig, S.M., Godfrey, S. a C., Knight, C.G., Malone, J.G., Robinson, Z., Spiers, A.J., Harris, S., Challis, G.L., Yaxley, A.M., Harris, D., Seeger, K., Murphy, L., Rutter, S., Squares, R., Quail, M. a, Saunders, E., Mavromatis, K., Brettin, T.S., Bentley, S.D., Hothersall, J., Stephens, E., Thomas, C.M., Parkhill, J., Levy, S.B., Rainey, P.B. & Thomson, N.R. (2009). Genomic and genetic analyses of diversity and plant interactions of Pseudomonas fluorescens. *Genome biology*. 10 (5). p.p. R51.

Simões, L. & Simões, M. (2013). Biofilms in drinking water: problems and solutions. *RSC Advances*. 3. p.pp. 2520–2533.

Simões, M., Pereira, M.O., Machado, I., Simões, L.C. & Vieira, M.J. (2006a). Comparative antibacterial potential of selected aldehyde-based biocides and surfactants against planktonic Pseudomonas fluorescens. *Journal of Industrial Microbiology and Biotechnology*. 33. p.pp. 741–749.

Simões, M., Simões, L. & Vieira, M. (2010). A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*. 43. p.pp. 573–583.

Simões, M., Simões, L.C., Machado, I., Pereira, M.O. & Vieira, M.J. (2006b). Control of Flow-Generated Biofilms with Surfactants. *Food and Bioproducts Processing*. 84 (December). p.pp. 338–345.

Srey, S., Jahid, I. & Ha, S. (2013). Biofilm formation in food industry: A food safety concern. *Food Control*. 31. p.pp. 572–585.

Sridhar, R., Sundarrajan, S., Vanangamudi, A., Singh, G., Matsuura, T. & Ramakrishna, S. (2014). Green processing mediated novel polyelectrolyte nanofibers and their antimicrobial evaluation. *Macromolecular Materials and Engineering*. 299. p.pp. 283–289.

Štengl, V., Popelková, D. & Grygar, T. (2014). Composite pigments based on surface coated kaolin and metakaolin. *Applied Clay Science*. 101. p.pp. 149–158.

Stewart, P.S. (2003). Diffusion in Biofilms. *Society*. 185 (5). p.pp. 1485–1491.

Stickler, D. (1999). Biofilms. *Current Opinion in Microbiology*. 2. p.pp. 270–275.

Stoodley, P., Cargo, R., Rupp, C., Wilson, S. & Klapper, I. (2002a). Biofilm material properties as related to shear-induced deformation and detachment phenomena. *Journal of Industrial Microbiology & Biotechnology*. 29 (6). p.pp. 361–367.

Stoodley, P., Sauer, K., Davies, D. & Costerton, J. (2002b). Biofilms as complex differentiated communities. *Annual Review of Microbiology*. 56. p.pp. 187–209.

Sukhorukov, G.B., Donath, E., Davis, S., Lichtenfeld, H., Caruso, F., Popov, V.I. & Möhwald, H. (1998). Stepwise polyelectrolyte assembly on particle surfaces: a novel approach to colloid design. *Polymers for Advanced Technologies*. 767. p.pp. 759–767.

Sutherland, I. (2001). The biofilm matrix - an immobilized but dynamic microbial environment. *Trends in Microbiology*. 9. p.pp. 222–227.

Tang, X., Flint, S., Bennett, R., Brooks, J. & Morton, R. (2009). Biofilm growth of individual and dual strains of Klebsiella oxytoca from the dairy industry on ultrafiltration membranes. *Journal of Industrial Microbiology & Biotechnology*. 36 (12). p.pp. 1491–1497.

Taylor, E.N. & Webster, T.J. (2009). The use of superparamagnetic nanoparticles for prosthetic biofilm prevention. *International Journal of Nanomedicine*. 4. p.pp. 145–152.

Trushina, D.B., Bukreeva, T. V., Kovalchuk, M. V. & Antipina, M.N. (2014). CaCO3 vaterite microparticles for biomedical and personal care applications. *Materials Science and Engineering C*. [Online]. 45. p.pp. 644–658. Available from: http://dx.doi.org/10.1016/j.msec.2014.04.050.

Trybała, A., Szyk-Warszyńska, L. & Warszyński, P. (2009). The effect of anchoring PEI layer on the build-up of polyelectrolyte multilayer films at homogeneous and heterogeneous surfaces. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 343. p.pp. 127–132.

US EPA (2001). Controlling disinfection by-products and microbial contaminants in drinking water. *United States Environmental Protection Agency, EPA*. 600-R-01-1.

Volodkin, D., Petrov, A.I., Prevot, M. & Sukhorukov, G.B. (2004a). Matrix polyelectrolyte microcapsules: new system for macromolecule encapsulation. *Langmuir : the ACS journal of surfaces and colloids*. 20 (7). p.pp. 3398–3406.

Volodkin, D. V., Larionova, N.I. & Sukhorukov, G.B. (2004b). Protein encapsulation via porous CaCO3 microparticles templating. *Biomacromolecules*. 5. p.pp. 1962–1972.

Waldron, K., Wu, W.D., Wu, Z., Liu, W., Selomulya, C., Zhao, D. & Chen, X.D. (2014). Formation of monodisperse mesoporous silica microparticles via spray-drying. *Journal of Colloid and Interface Science*. 418. p.pp. 225–233.

Wang, C., He, C., Tong, Z., Liu, X., Ren, B. & Zeng, F. (2006). Combination of adsorption by porous CaCO3 microparticles and encapsulation by polyelectrolyte multilayer films for sustained drug delivery. *International Journal of Pharmaceutics*. 308. p.pp. 160–167.

Wang, X., Hu, X., Wang, H. & Hu, C. (2012). Synergistic effect of the sequential use of UV irradiation and chlorine to disinfect reclaimed water. *Water Research*. 46 (4). p.pp. 1225–1232.

Wessels, S. & Ingmer, H. (2013). Modes of action of three disinfectant active substances: A review. *Regulatory Toxicology and Pharmacology*. 67 (3). p.pp. 456–467.

Wiesner, M.R., Lowry, G. V., Alvarez, P., Dionysiou, D. & Biswas, P. (2006). Assessing the risks of manufactured nanomaterials. *Environ. Sci. Technol*. 40. p.pp. 4336–4337.

Wingender, J. & Flemming, H.C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *International Journal of Hygiene and Environmental Health*. 214 (6). p.pp. 417–423.

Wirtanen, G., Saarela, M. & Mattila-Sandholm, T. (2000). Biofilms - Impact on hygiene in food industries. In: *Biofilms II: Process analysis and applications*. pp. 327–372.

World Health Organization (2003). *Emerging Issues in Water and Infectious Disease*.

World Health Organization (2011). *Guidelines for Drinking-water Quality*. Fourth. WHO (ed.).

Yu, J., Zhao, L. & Cheng, B. (2006). Preparation of monodispersed microporous SiO2 microspheres with high specific surface area using dodecylamine as a hydrolysis catalyst. *Journal of Solid State Chemistry*. 179. p.pp. 226–232.

Zeng, D., Wu, J. & Kennedy, J.F. (2008). Application of chitosan flocculation for water treatment. *Carbohydrate Res*. 71 (1). p.pp. 135–139.

Zhang, C., Cui, F., Zeng, G., Jiang, M., Yang, Z., Yu, Z., Zhu, M. & Shen, L. (2015). Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. *Science of The Total Environment*. 518-519. p.pp. 352–362.

Zhang, L., Gu, F.X., Chan, J.M., Wang, A.Z., Langer, R.S. & Farokhzad, O.C. (2008). Nanoparticles in medicine: therapeutic applications and developments. *Clin. Pharmacol. Ther*. 83. p.pp. 761–769.

Zhang, L., Pornpattananangkul, D., Hu, M. & Huang, C. (2010). Development of Nanoparticles for Antimicrobial Drug Delivery. *Current Medicinal Chemistry*. 17. p.pp. 585–594.

Appendix

A.1. Estimative of the number of particles in the stock solution

After the coating process with PEI, PSS and BDMDAC, the particles were resuspended in 10 mL of borate buffer, as described in Chapter 2. This solution of 10 mL was considered the stock solution for all the experiments. Each stock solution contained a specific number of microparticles, depending on the type of microparticles base material. Considering a previous study of Ferreira et al. (2013) where $CaCO_3$ microparticles were characterized in detail regarding the same coating method, the characteristics of the particles presented in the study were considered in this project.

A.1.1. $CaCO_3$ microparticles

Considering a medium diameter of 3 μm, the volume of the microparticles was determined through the Equation 1:

$$V = \frac{4}{3} \times \pi \times r^3 \qquad \text{Eq. 1}$$

Knowing that the density of the particles was 2.83 g/cm^3, the mass of a single particle was determined as being 4.001 x 10^{-11} g, using Equation 2.

$$\rho = \frac{m}{V} \qquad \text{Eq. 2}$$

For each stock solution preparation, **0.09g** of $CaCO_3$ non-coated microparticles were used. Thus, considering the mass of a single particles determined previously, a total of 2.25x10^9 particles was calculated as the number of particles presented in the stock solution.

In accordance with the study of Ferreira et al. (2013), the analysis using HPLC for the determination of the amount of biocide demonstrated that each particle of $CaCO_3$ has **1.33x10^{-9}** mg of BDMDAC at the surface. Thus, considering the number of particles present in the stock solution, it was possible to state that there are 3 mg of BDMDAC in 10 mL of solution, or **0.3 mg/mL**.

A.1.2. Kaolin microparticles

The determination of the mass of kaolin microparticles to weight for the preparation of a stock solution of coated particles was based in the equivalence of the superficial

area between kaolin and $CaCO_3$ microparticles. First, the superficial area of each $CaCO_3$ particles was determined using Equation 3, and a value of $2.83x10^{-7}$ cm^2 was obtained.

$$Sup_{Area} = 4\pi r^2 \quad \text{Eq. 3}$$

Therefore, the total superficial area available for surface functionalization in a stock solution was $2.83 \times 10^{-7} cm^2 \times 2.25 \times 10^9 = 636.17 \frac{cm^2}{0.09g} = 7068.56 \frac{cm^2}{g} = 0.7069 \frac{m^2}{g}$. In other hand, according to Martins (2014), the superficial area of the kaolin used is 13.8 m^2/g. Accordingly, an amount of $\frac{0.7069}{13.8} = \mathbf{0.05\ g}$ of the clay was weighted in the preparation of the stock solution. Since the total superficial area available for surface functionalization was equivalent, **the amount of BDMDAC adsorbed on the total amount of kaolin particles was assumed to be the same as for $CaCO_3$ microparticles (0.3 mg/L in the stock solution).**

A.1.3. Silica microparticles

Since silica microparticles have the same diameter as $CaCO_3$ microparticles (approximately 3 μm), the total superficial area was considered the same. Therefore, for the preparation of a stock solution of silica microparticles also 2.25×10^9 particles of silica were used. Considering that the solid content of the purchased silica microparticles flask was 5% WT and the density of the particles equal to 1.8 g/cm^3, it was determined that the flask had 9.84×10^9 particles of silica. Thus, **1.14 mL** was collected from the flask for the further application of the coating process. Again, **the amount of BDMDAC immobilized on the total amount of silica particles was assumed to be the same as for $CaCO_3$ microparticles (0.3 mg/L in the stock solution).**

A.1.4. Sea sand microparticles

Using the medium values of the diameter and density of sea sand provided, the mass of one single particles and the superficial area were calculated using Equations 2 and 3, respectively. The superficial are obtained for one single particle was 0.0013 cm^2. Accordingly, in order to have a total of 636.17 cm^2 (equivalence to $CaCO_3$ microparticles), a total of $\frac{636.17}{0.0013} = 500000$ sea sand particles was necessary. Since one

particle weights 3.35×10^{-6} g, the amount of sea sand particles weighted for the preparation of a stock solution was **1.7 g**. Again, **the amount of BDMDAC immobilized on the total amount of sea sand particles was assumed to be the same as for $CaCO_3$ microparticles (0.3 mg/L in the stock solution).**

A.1.5. Cork microparticles

Since no data was available about the characteristics of cork microparticles, the reasonable value of 1.5 g was weighted for the preparation of the stock solution.

A.2. SEM analysis

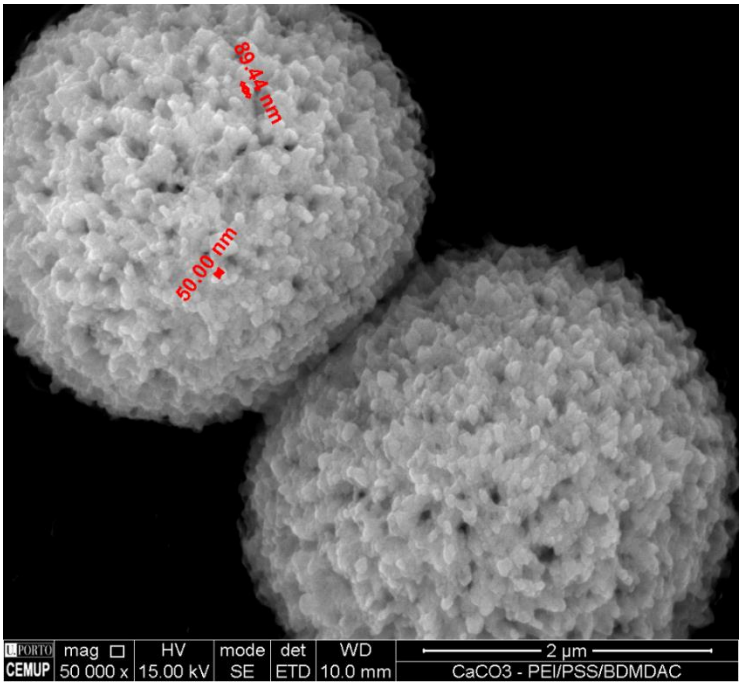

Figure A.2.1 - SEM images of $CaCO_3$-PEI/PSS/BDMDAC particles.

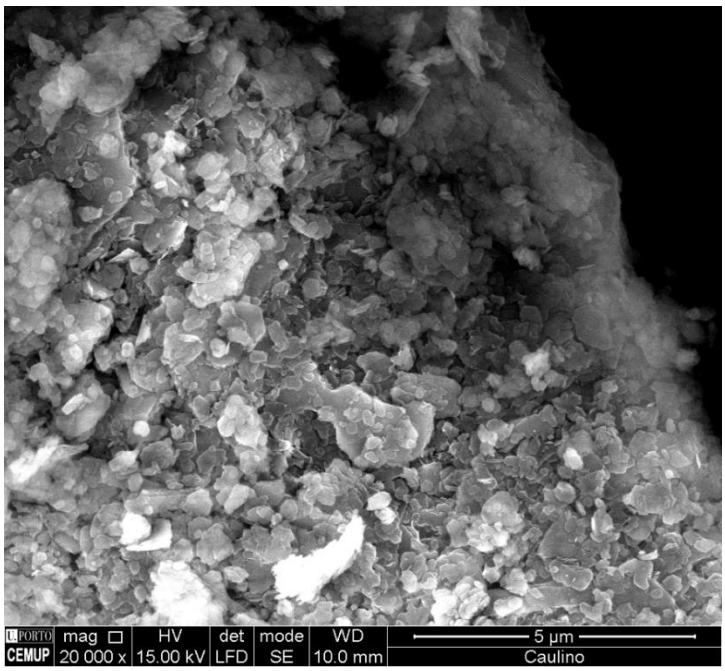

Figure A.2.2 - SEM images of kaolin particles.

A.3. Size distribution analysis

The size distribution in volume of coated and non-coated $CaCO_3$, kaolin, silica, sea sand and cork is represented in Figures A.3.1 - A.3.5.

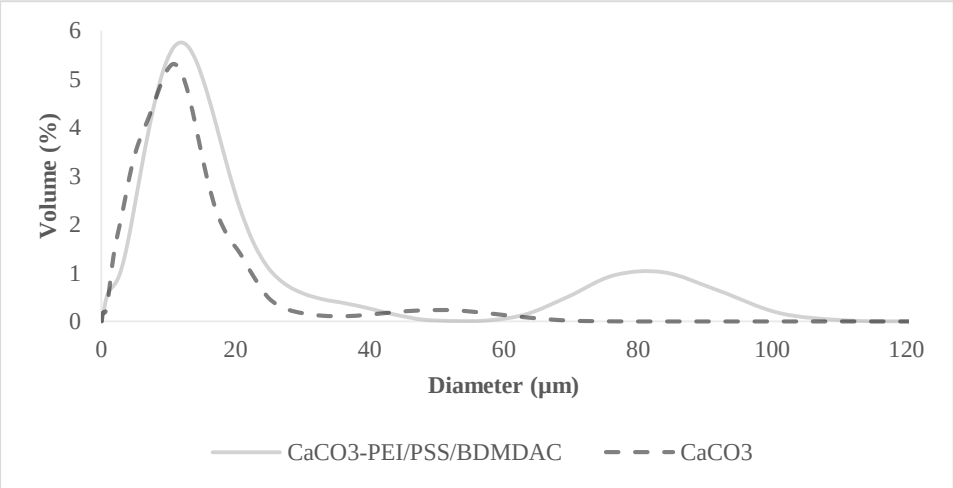

Figure A.3.1 – Size distribution (µm) in volume (%) for coated and non-coated $CaCO_3$ microparticles.

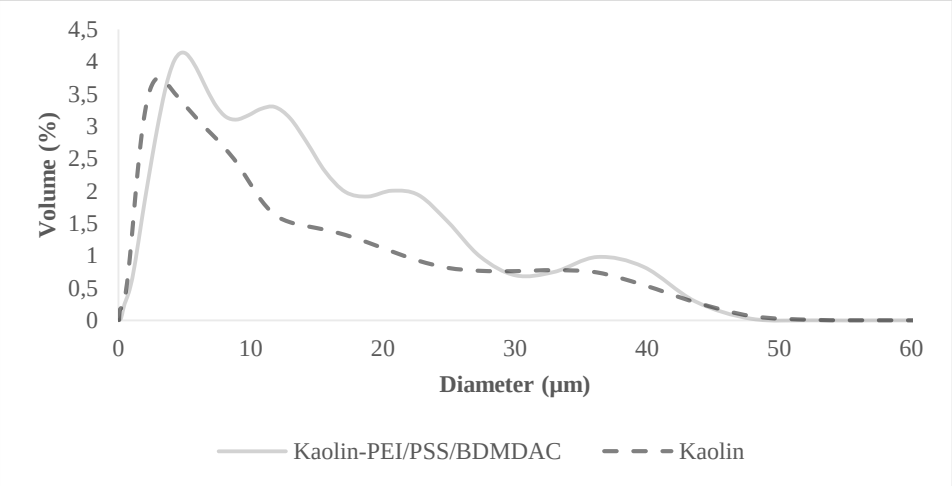

Figure A.3.2 – Size distribution (µm) in volume (%) for coated and non-coated kaolin microparticles.

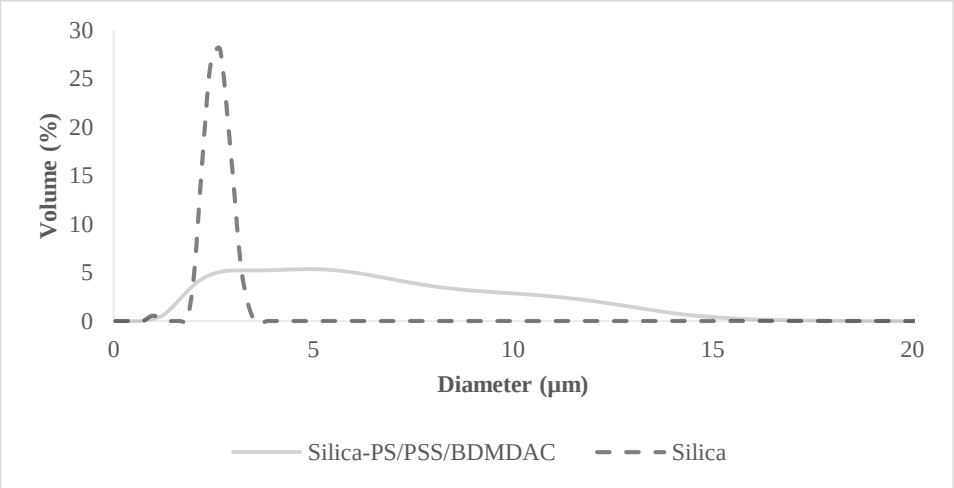

Figure A.3.3 – Size distribution (µm) in volume (%) for coated and non-coated silica microparticles.

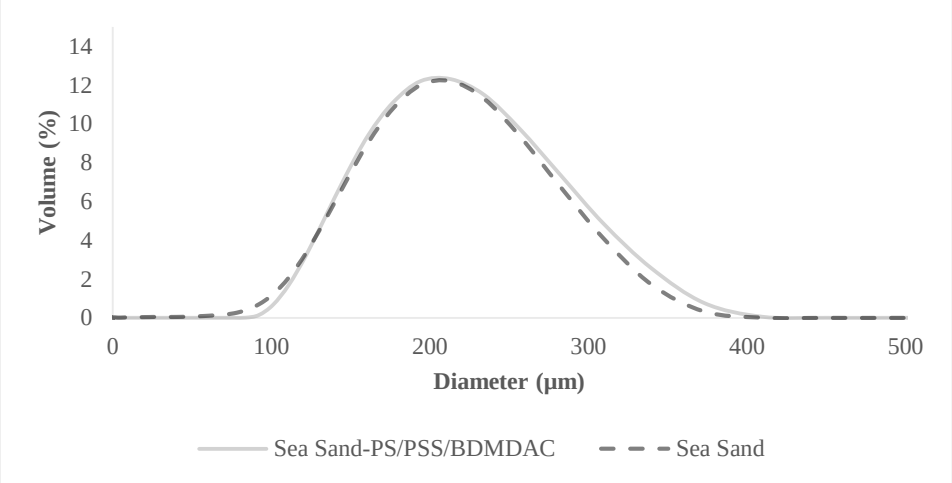

Figure A.3.4 – Size distribution (µm) in volume (%) for coated and non-coated sea sand microparticles.

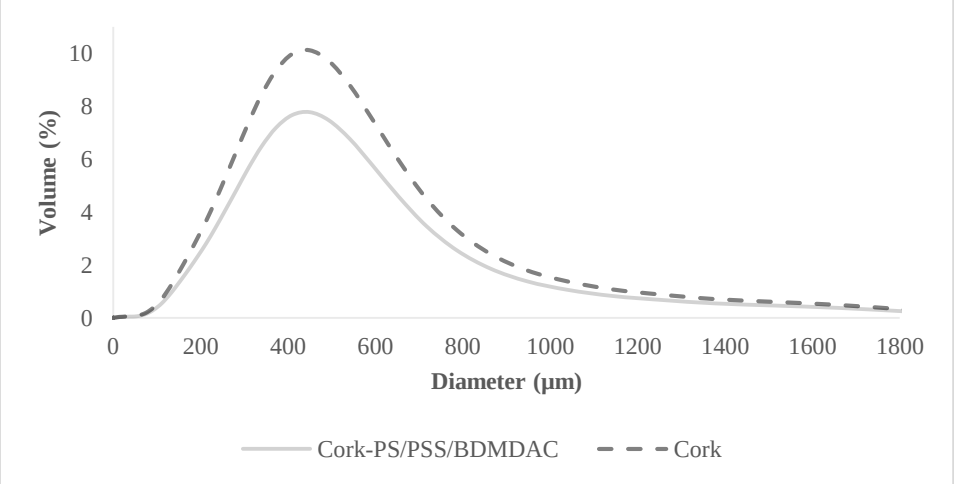

Figure A.3.5 – Size distribution (µm) in volume (%) for coated and non-coated cork microparticles.