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Functionalization of Hydroxyapatite Microparticles with a Biocidal Agent: an alternative solution for Biofilm Prevention

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Mestrado Integrado em Bioengenharia

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Abstract

Biofilms are ubiquitous, developing on most surfaces immersed in natural and anthropogenic aqueous environments. Being one of the most successful modes of life on Earth, these structures allow protection to the colonizing species, not only from competing microorganisms and environmental factors, but also from antimicrobial compounds.

Henceforth, the mitigation of undesired biofilms reveals itself as a challenging task, even more so, since the most commonly applied disinfection procedures carry with them harmful consequences. Amongst the latter, highlight should be given to the formation of disinfection by-products, that are often toxic and may end up in the discharged waters or even in the final products intended for consumption, thus compromising ecosystems and public welfare. In view of this reality, the value of *prevention* approaches, instead of control ones, should not be overlooked.

This thesis offers an alternative to the conventional strategies in use, by the development of benzyldimethyldodecyl ammonium chloride coated microparticles, through micro-nanotechnology. With this end in mind, the Layer-by-Layer (LbL) self-assembly technique was employed through the subsequent assemble of oppositely charged molecules of polyethyleneimine (PEI), sodium polystyrene sulfonate (PSS), and benzyldimethyldodecyl ammonium chloride (BDMDAC) on hydroxyapatite (HAp) and calcium carbonate cores ($CaCO_3$). Both types of microparticles were characterized, before and after functionalization, by BET (Brunner-Emmett-Teller) analysis. Furthermore, release assays by the dialysis method (DM), and the quantification of the amount of BDMDAC in the particles by high perfomance liquid chromatography (HPLC) were carried out. These tests proved the process's efficacy, since the specific surface area decreased upon coating and no release of biocide was detected.

The free biocide's antimicrobial activity was tested against planktonic *Pseudomonas fluorescens*, leading to the conclusion that its efficiency was dose dependent and that the minimum bactericidal concentration (MBC) was 100 mg/L, for 1 hour contact. When attempting to test the microparticles' antimicrobial activity, total bacteria reduction was obtained at 200 mg/L, at a 2 hour contact. This decrease was attributed to an alternative mode of action and to the tendency particles have for agglomerating. Lastly, the capability of the particles, at 200 mg/L, to prevent biofilm formation was assessed. A deeper understanding of the results was acquired with resource to OCT (Optical Coherence Tomography) images. It was clearly perceived that the use of this microtechnology solution allowed to hinder biofilm formation to a great extent. Overall, the HAp microparticles displayed better results in every assay, which was in part attributed to their higher specific surface area and porosity.

All things considered, the results indicate that both the method and materials applied are a promising strategy for the prevention of biofilm formation, although further research is necessary to fulfil their full potential.

Keywords: biofilm, prevention, layer-by-layer (LbL), BDMDAC, hydroxyapatite (HAp);

Resumo

Os biofilmes são considerados ubíquos, tendo a capacidade de se formar na maioria das superfícies imersas em ambientes aquáticos, tanto naturais, como antropogénicos. Sendo um dos modos de vida mais bem sucedidos no nosso planeta, estas estruturas conferem às espécies colonizadores proteção não só de microorganismos que com elas competem, mas também de factores ambientais.

Por este motivo, a mitigação de biofilmes indesejados apresenta-se como uma tarefa trabalhosa, com a agravante de que os processos de desinfeção mais comuns carregam consigo consequências prejudiciais. Entre estas, é de realçar a formação de subprodutos, frequentemente tóxicos, que poderão acabar presentes nas águas de descarga ou até em produtos finais com destino ao consumidor, compromentendo o ambiente e o bem-estar da população. Tendo presente esta realidade, o valor de estratégias de prevenção, ao invés de estratégias de control, não deve ser preterido. Nesta tese é proposta uma alternativa aos métodos convencionais em uso, através do desenvolvimento de partículas cobertas de cloreto benzildimetildodecil de amónia (BDMDAC), com recurso à micro-nanotecnologia. Para este fim, a técnica camada-por-camada, ou LBL (do inglês, layer-by-layer), foi empregue através da subsequente deposição das moléculas opostamente carregadas, de polietilenoimina (PEI), poliestireno sulfonato de sódio (PSS) e do biocida BDMDAC em núcleos de hidroxiapatite (HAp) e carbonato de cálcio ($CaCO_3$). Ambos os tipos de partículas foram caraterizados, antes e depois da sua funcionalização, através de análise BET (Brunner-Emmett-Teller). Para além disto, foram realizados ensaios de libertação com recurso ao método de diálise, ou DM (do inglês, Dialysis Method). Foi também quantificada a quantidade de biocida aderido às partículas através de cromatrografia líquida de alta eficiência, ou HPLC (do inglês, High Performance Liquid Chromatography). Os ensaios realizados vieram provar a eficácia do processo aplicado, já que a área superficial específica diminuiu após funcionalização e não foi detetada libertação de biocida.

Foi averiguada a atividade antimicrobiana do biocida contra *Pseudomonas fluorescens* no estado plantónico, concluindo-se que a sua eficácia era dependente da dose aplicada e que a concentração bactericida mínima, ou MBC (do inglês, Minimum Bactericidal Concentration) foi 100 mg/L, para uma hora de contacto. Testada posteriormente a atividade antimicrobiana das micropartículas, a redução total de bactéria foi apenas obtida a uma concentração de 200 mg/L, para 2 horas de contacto. Esta diminuição de eficácia foi atribuída a diferentes modos de ação e à tendência de aglomeração das partículas. Por fim, a capacidade das partículas para prevenir a formação de biofilmes, a uma concentração de 200 mg/L, foi investigada. Foi claramente constatado que o uso desta solução da microtecnologia permitiu afetar, em grande extensão, a formação de biofilme. No cômputo geral, as partículas de HAp demonstraram melhores resultados em todos os ensaios realizados, resultados atribuídos em parte a uma maior área superficial específica, bem como maior porosidade. Em suma, os resultados obtidos indicam que tanto o método como os materiais aplicados se apresentam como estratégias promissoras na prevenção da formação de

biofilmes, apesar de uma investigação mais profunda ser crucial para que estas atinjam o seu máximo potencial.

Keywords: biofilme, prevenção, camada-por-camada (do inglês, Layer-by-Layer), hidroxiapatite (HAp)

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"All you've got to do is own up to your ignorance honestly,
and you'll find people who are eager to fill your head with information."

Walt Disney

Contents

List of Figures

List of Tables

Abbreviations

AMR	Antimicrobial Resistance
BDMDAC	Benzyldimethyldodecylammonium chloride
BET	Brunner-Emmett-Teller
COPD	Chronic Obstructive Pulmonary Disease
DLVO	Derjaguin-Landau-Verwey-Overbeak
DM	Dialysis Method
EDL	Double Layer Forces
EPS	Extracellular Polymeric Substances
HA	Hyaluronic Acid
HAp	Hydroxyapatite
HPLC	High Performance Liquid Chromatography
LbL	Layer-by-Layer
MF	Microfiltration
MP	Microparticle
NF	Nanofiltration
NP	Nanoparticle
PAH	Poly(allylamine hydrochloride)
PEI	Polyethyleneimine
PS	Poly-styrene
PSS	Sodium Polystyrene Sulfonate
PM	Particulate Matter
QAC	Quaternary Ammonium Compound
RO	Reverse Osmosis
OCT	Optical Coherence Chromatography
UV	Ultra-Violet
UF	Ultrafiltration
vdW	Van der Waals
3D	Three-Dimensional

Chapter 1

Work Outline

1.1 Background and Thesis Presentation

Bacteria often exist in compact, surface-associated communities, named biofilms. Not only are these structures found in natural, but also in anthropogenic environments, where they often represent a major issue. In industrial and health-care facilities biofilms carry the weight of increased costs of production and maintenance, as well as public health and environmental welfare. In fact, economic impacts come hand in hand with energy losses due to increased fluid resistance and heat transfer resistance, preventive dimensioning and design of operation units, premature replacement of equipment and cleaning costs, amongst other technical repercussions propelled by the existence of biofilms (Flemming and Wingender, 2010; Paulus, 2005; Melo and Flemming, 2010).

The prevention of biofilm formation is just as important as their posterior control, and both these strategies can often be separated into physical or chemical techniques (Abe et al., 2012). Chemical techniques generally employ the use of biocides, disinfectants that may represent a hazard for the environment. These compounds, besides amplifying antimicrobial resistance (AMR), may not react in totally during the process, leading to discharge into the environment or wastewater plants and the formation of toxic by-products (DBPs) by reaction with other water components (McDonnell and Russell, 1999; Ferreira, 2016; Li et al., 2008). A more effective and controlled use of these products would therefore be a solution of added value since it would allow to resolve multiple problems at once.

In this sense, the use of micro-nanotechnology is regarded as a promising approach to tackle the issues mentioned. Its broad applications have been well described in literature, and the list continues to grow while the scientific community becomes ever more aware of their potential.

In fact, at such scale, materials present unique properties and can be functionalized in order to carry biocides, allowing to decrease the dosage of the later. Besides this, it also circumvents the formation of DBPs and the costs associated with wastewater treatment to remove the remaining biocide. Furthermore, many of the materials available for micro-nanotechnology applications, such as hydroxyapatite (HAp), are attained through waste recovery of other industries, which adds up to their sustainability.

The foundations for this thesis lay in previous work performed by researchers in LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy). In this connection, highlight should be given to the work developed in (Ferreira, 2016), the starting point for this project. By studying biocide coated microparticles as a strategy for biofilm control and prevention, the positive contributions of this project are:

i) Increase of profit due to the reduction of capital costs associated to several industries where biofilms are ubiquitous.

ii) The reduction of detrimental consequences for the environment as a result of lowering biocide release into water systems.

iii) Public health protection, by diminishing the consequences inherent to the use of biocides.

iv) Further scientific insight into the behavior, characterization, and advantages of functionalized microstructures, particularly regarding HAp microparticles.

1.2 Objectives

The main goal of this thesis was to develop microparticles with functionalized surfaces that present antimicrobial activities and are effective in the mitigation of biofilm development, with a focus on preventing initial formation of such structures, an approach that is often disregarded. The particles herein developed aimed to be cost-effective, to reduce the health issues associated with the use of biocides, which are known for forming DBPs, for their carcinogenic potential and for their influence in the increase of antimicrobial resistance, and to represent a sustainable and environmental-friendly solution. Furthermore, the strategy developed hopes to reduce the overall costs of disinfection in the many industries affected by this problem, which are further described in Chapter 2.

With this end in mind, this project analyses the layer-by-layer technique (LbL) comparing the coating of two different microparticle cores, one of which is hydroxyapatite (HAp), a material that lacked study in previous research carried out within this framework. Moreover, a characterization of the microparticles is supplied, including release assays, that provide a deeper insight into their mode of action. Antimicrobial tests were carried out using free and immobilized biocide, benzyldimethyldodecylammonium chloride (BDMDAC), in the context of planktonic cells and in conditions propitious to the formation of *P. fluorescens* biofilms.

1.3 Thesis Organization

This thesis is divided in 7 Chapters. The current chapter, Work Outline, intends to describe the scope and the objectives of the work developed.

In Chapter 2, the reader will find a Literature Review that aims to provide a summary of relevant information related to biofilm formation, its consequences, strategies for its control and prevention and the advantages of going micro-nanoscale to rise to the biofilm challenge. In Chapter 3, the action of the free biocide benzyldimethyldodecylammonium chloride (BDMDAC) against a strain of *P. fluorescens* is presented. Chapter 4 describes the process of development of functionalized hydroxyapatite (HAp) and calcium carbonate ($CaCO_3$) microparticles by the Layer-by-Layer method as well as their characterization in terms of the quantification of the amount of biocide using high performance liquid chromatography (HPLC), their morphology by BET (Brunner-Emmett-Teller) analysis, and release assays by the dialysis method (DM). In Chapter 5, the action of such particles against planktonic cells of *P. fluorescens* and a comparison between both cores is assessed. Chapter 6 studies the efficacy of the coated particles in preventing biofilm formation. Last but not least, Chapter 7 summarizes the main conclusion of this work and points at the future direction.

Chapter 2

Literature Review

2.1 Biofilms: An overview

2.1.1 A Microbial Home

A microbial biofilm can be defined as a sessile microbial consortia established in a three-dimensional structure and consists of multicellular communities composed of prokaryotic and/or eukaryotic cells embedded in a matrix composed, at least partially, of material synthesized by the microbial community (Azeredo et al., 2017). Over the years, the study of biofilms has witnessed a major advance, providing a continuously growing knowledge on their structure and functioning. Several new methodologies have been recently developed for, or adapted to, biofilm studies that have contributed to deeper knowledge on biofilm physiology, structure and composition. In short, the observation of aggregated microorganisms can be dated to the primordia of microbiology itself, although the concept of biofilm in its fullest is relatively recent (Høiby, 2014).

Bacterial biofilms may, at sight, seem like a paradigm to Darwin's theory regarding evolution through natural selection. In fact, biofilms generally comprise many species leading to the formation of stable micro consortia, development of physiochemical gradients and strong cell–cell communication, which amounts to highly competitive environments (Flemming and Wingender, 2010). In order to understand this, one must contemplate biofilm communities from an ecological point of view and regard a concept of evolution that substitutes competition for association, in order to achieve proliferation instead of the survival of the fittest/individual selection (Caldwell and Costerton, 1996). Biofilms are one of the most successful modes of life on Earth benefiting from emergent properties such as social interaction and others-which will be explained in further

detail-that are not present in planktonic cells. These associations result in elaborated systems with a cell density between 10^8 to 10^{11} cell/g wet weight (Flemming et al., 2016).

Biofilm development can usually be divided in three crucial steps: attachment, growth/maturation and detachment/dispersal (Stoodley et al., 1999). The first step refers to the attachment of planktonic cells to a surface, establishing the bridge between the two forms of life. This stage comprises two distinct checkpoints. The process begins when the cells attach reversibly to the surface, through the intervention of *flagella* and *pili*. From this point forward, the bacteria can either return to its initial arrangement or proceed to develop a structured biofilm, by the intervention of surface proteins that promote its adhesion. Nonspecific physicochemical forces of interaction act between the molecules and microorganisms and the surface, such as Van der Waals forces, hydrophobic, electrostatic and London dispersion forces (Nikolaev and Plakunov, 2007). Bacteria then undergo growth by cell proliferation and maturation process, alongside with the formation of the EPS (Extracellular Polymeric Substances). Lastly, there's biofilm dispersion: the final stage in the biofilm lifecycle and, concomitantly, the beginning of a new one. During the maturation process, some cells transfer to planktonic growth and disperse to find a new surface to attach. Once again, this step can be divided it two: active and passive dispersion. While the first is triggered by changes in environmental chemical conditions and depends on call motility or degradation of EPS, the second depends on physical factors such as shearing force under liquid flow conditions (Stoodley et al., 1999).

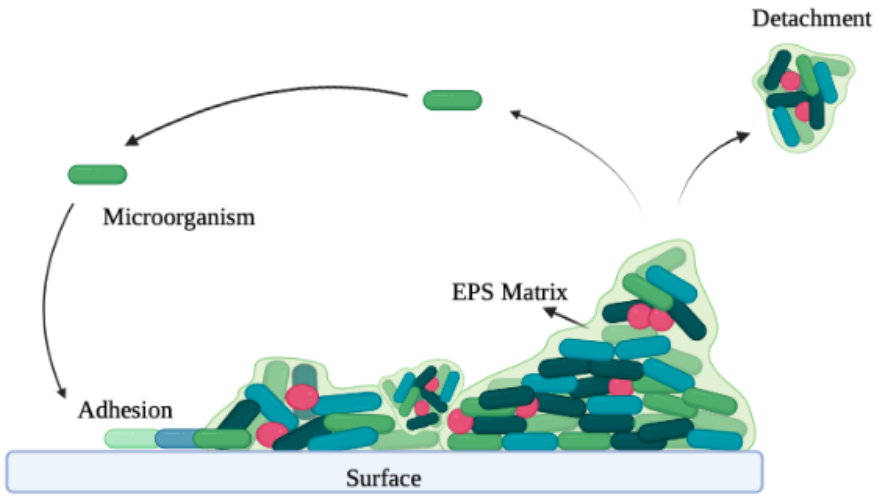

Figure 2.1: Biofilm Lifecycle and Structure. (adapted from Melo (2003))

There are some factors that affect the formation and properties of biofilms, such as: characteristics of the microbial species and strains; composition and roughness of the surface material where the microorganisms are attached; nutrient composition and concentration; fluid pH, temperature, ionic strength and the hydrodynamics (velocity and turbulence) (Melo, 2003). The EPS matrix defines the biofilm's architecture, stability, and the existence of pores and channels. Matrix formation relies on the availability of nutrients, synthesis and secretion of extracellular material, shear stress and social competition, which consequently translates in a high energetic cost to the cells. Nevertheless, it comes as an advantageous expense, since it allows the emergence of beneficial properties to the biofilm (Flemming et al., 2016). These are not predictable when studying free-living bacterial cells and include social cooperation (i.e synergistic microconsortia), habitat formation (through the generation of localized gradients that provide diversity), enzyme retention and resource capture. In fact, the generation of the EPS matrix originates a physical shelter, promoting the accumulation of nutrients, encountered in the water phase of the biofilm or associated with the substratum, and altering the physicochemical environment of the biofilm itself (Stoodley et al., 2002). Besides this, the EPS matrix grants enhanced survival of exposure to antimicrobials, which constitutes a major obstacle in the mitigation of biofilms (Konopka, 2009). For this reason, and given the context of the work developed, it is particularly important to understand this property, in order to justify the alternative means proposed.

Regarding human health, the ability of biofilms to survive exposure to disinfectants, toxic metals and small-molecule antibiotics makes it harder to prevent contagion. Tolerance to multiple compounds that target metabolic processes during growth comes up as a consequence of the slow rate at which this growth occurs. Besides this, tolerance also derives from the quenching of the antimicrobial's activity, carried out by molecules present in the EPS matrix by diffusion-reaction inhibition. These reactions can involve chelation by complex formation, enzymatic degradation of antimicrobials or sacrificial reaction of EPS (for example, with oxidizing disinfectants) (Daddi Oubekka et al., 2012). This process results in a decrease of antimicrobials to sublethal concentrations, allowing exposed cells to survive and therefore develop resistance. Furthermore, resistance can also arise from the gene transfer of resistance genes, made easy by the proximity of cells within the biofilm (Flemming et al., 2016).

More particularly and in the context of this thesis, genus Pseudomonas covers a wide variety of species that can be found in several technological processes, easily forming biofilms on different types of surfaces. *P. fluorescens* forms biofilms with extremely complex, three-dimensional (3-D)

structures (Iltanen et al., 2006; Scales et al., 2014). Although it is mostly studied regarding its part in the soil and the rhizosphere, this bacterium possesses numerous traits, giving it the ability to grow and thrive in mammalian hosts. Amongst other processes, quorum sensing and biofilm formation are integral to the various environmental niches occupied by *P. fluorescens* and allow it to colonize surfaces such as hospital equipment and food-grade stainless steel surfaces, as well as plant surfaces, showerheads, and even indoor wall surfaces (Scales et al., 2014; Feazel et al., 2009; Tuttlebee et al., 2002; Ude et al., 2006). Besides this, more often than not, it is shown to be disinfectant-resistant (Simões et al., 2005).

2.1.2 The Detrimental Impacts of Biofilms

Pathogenic and spoilage bacterial species capable of forming biofilms pose a significant problem for the healthcare and food industries, as their biofilm forming ability protects them from common cleaning processes and allows them to remain in the environment post-sanitation (Coughlan et al., 2016). Besides the possibility of leading to the contamination of drinking water, which is in the origin of several waterborne diseases, biofouling also compromises energy and food production, as well as the biomedical, environmental, and other industrial and economic fields (Shannon et al., 2010). In fact, biofilms of various species amount to a major water contaminant as most of the microorganisms are comprised within these structures (Manuel et al., 2009).

There are three main properties of biofilms that affect the environment and normal functioning of industrial technological process: physical (structural), chemical (metabolic) and biological (living) (Paulus, 2005). Biofilms from spoilage and pathogenic bacteria usually accumulate in the inside of mixing tanks and vats, compromising safety and quality of the process and resulting product (Paniagua et al., 2020). Economic impact caused by biofilms in cooling water systems is due to the energy losses that come as a consequence of not only increased fluid frictional resistance, but also increased heat transfer resistance at power plant condensers and process heat exchangers. Since thermal conductivity of biofilms is significantly less than that of metal heat transfer surface materials, their accumulation on the surfaces of heat exchange tubes drastically diminishes the heat transfer rate (Bajpai, 2015). Consequently, these units are frequently designed with excess capacity to match the decrease of efficacy of heat transfer caused by biofouling (Flemming, 2011). This preventive dimensioning of equipments increases the capital costs of manufacturing plants, and these implications occur in all industries using heat exchangers, including food, paper manufacturing and oil production. Moreover, fouling leads to increased capital costs for premature

replacement of equipment, unscheduled turnarounds or downtime to clean equipment that fouled (Paulus, 2005). Overall, biofilms can cause reduction of heat transfer, increased pressure drop, increased corrosion of metallic surfaces, contamination of the fluids flowing through the tubes or channels and of potable water produced in membrane systems. Furthermore, it reflects in increased environmental costs associated to the need to treat wastewater containing the chemicals not consumed by the interaction with the microorganisms and increased costs of equipment and reagents for cleaning and disinfection (Melo and Flemming, 2010).

Once again in a closer view, while far less virulent than other strains such as *P. aeruginosa*, *P. fluorescens* can cause serious infections in humans and has been found in clinical samples from the mouth, stomach, and lungs, although the most common site of *P. fluorescens* infection is the bloodstream. For instance, in a survey developed at the University of Michigan Hospital, it was shown that *P. fluorescens* was regularly cultured from respiratory specimens, more specifically, from over 240 respiratory specimens over 11 years. It was also shown that from all patients with positive cultures, 38.8% had a pulmonary condition called cystic fibrosis, followed by other chronic airway diseases such as chronic obstructive pulmonary disease (COPD), asthma and non-cystic-fibrosis bronchiectasis (Scales et al., 2014). Studies have demonstrated that the presence of *P. fluorescens* promotes the persistence of other detrimental bacteria such as *L. pneumophila*, responsible for large outbreaks of Legionnaires' disease and Pontiac fever (McDade, 2008). In fact, increased resistance to sanitizers was observed in multispecies biofilms in which one of the strains was an EPS producer. EPS-producing strains of one species conferred protection to non EPS-producing strains of another, ultimately protecting both from sanitation (Coughlan et al., 2016).

In order to maximize the yield of action of biocides and to design efficient antimicrobials there is a growing need to comprehend not only the mechanisms of action of biocides, but also the mechanisms of resistance of bacterial cells (Russell, 2003).

2.1.3 Control Strategies

As it is possible to convey, in almost every industry there is a set of characteristics that propel the growing of biofilms. In reality, there are detrimental consequences caused by biofouling in virtually all the industries using non-sterile water. For this reason, biofilm control is crucial to circumvent the issues described above. The most employed methods are the chemical and physical.

In the context of this thesis, the first one will be described in further detail, exploring the concept of biocide.

The physical methods, as the name implies, are related to the mechanical cleaning of the surfaces where biofilms are present. In this category, some of the most widely used techniques are hydrodynamic stress, ultrasonication, pigging and air or gas injection. Hydrodynamic stress is based on the manipulation of the shear stress conditions by increasing the fluid velocity until the hydrodynamic forces at the surface surpass the limit of the internal cohesive forces in the biofilm, thus leading to detachment (Abe et al., 2012). Ultrasonication consists of the application of ultrasound waves with frequencies that range from 20kHz to 10MHz with the objective of promoting breakage and detachment of the biofilm from the surface by vibration. In this technique, the most important factors are the intensity of application and the frequency itself and it is advised the use of many treatments of single pulses of high ultrasound amplitudes, rather than less treatments of multiple pulses (Chemat et al., 2011). The main disadvantages inherent to this method are its inefficiency in bacterial inactivation and the cost associated to the devices and their installation (Bott, 2000; de Lemos, 2016). In its turn, pigging is attractive for the possibility of performing maintenance operations in pipelines, without the need of interrupting the flow or dismantling the system. This method consists in the insertion of temporary pigs of different configurations that can be adapted to the cleaning purpose, such as the brush and cage system and ice pigging (Bott, 1998; Ainslie et al., 2009; Bott, 2011). Lastly, the injection of air or other adequate gas is configured for the erosion and dislodgement of biofilms, through the high turbulence applied by the air bubbles (de Lemos, 2016). Yet another physical technology to remove undesired compounds is membrane filtration, which can be categorized into microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), although microfiltration in particular is designed for the removal of suspended bacteria and protozoa. However, the membranes themselves are susceptible to fouling, benefiting from a pre-treatment to remove bacteria and avoid this issue. As such, mechanical methods are usually used conjointly with chemical methods of disinfection (da Costa, 2016).

Cleaning and disinfection are both essential operations in diverse areas and, when applied to industrial processes, fundamental for the quality of the final product and safety (Carpentier and Cerf, 1993). Although effective cleaning procedures break up or dissolve the EPS matrix removing 90% or more of the biofilm mass attached, they may not be sufficient to kill the microorganism that will then be able to recolonize other surfaces (Gibson et al., 1999; Srinivasan et al., 1995). This makes disinfection a fundamental part of biofilm control. Disinfection is generally defined

as the use of antimicrobial chemicals to destroy microorganisms and its aim is to decrease the population of viable cells and prevent microbial growth on surfaces (Maukonen et al., 2003). The process of choosing a disinfectant must consider its effectiveness in the pH range used, stability when diluted, toxicity and safety, microbial spectrum, corrosiveness at the surface, stability when reacting with organic material and surface activity (Troller, 2012; Wirtanen, 1995; Wirtanen et al., 2000).

In this context, biocides are chemical compounds with antimicrobial activity that will eradicate the microorganisms or prevent their growth in the water. The EU Biocidal Products Directive 98/8/EC defines biocides as *'active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means'* (Ferreira et al., 2011). The most common classification distinguishes between oxidising and non-oxidising biocides. Oxidising biocides are based in radical-mediated reaction oxidizing organic material, and include the halogenated agents, ozone and peroxygens. Non-oxidising agents can include a rather large range of biocides like electrophilic agents, surface active agents and weak acids. Unlike the antibiotics usually used to treat clinical infections, biocides do not act on a specific cellular target, but cause damages varying from sub-lethal damage to cell death (Denyer and Stewart, 1998). Due to the biocides large range of antimicrobial activity, other concepts may be more precise, including 'bacteriostatic' as agents which inhibit growth and 'bactericidal' for the group of agents that kill the target microbes in suspension or on surfaces (McDonnell and Russell, 1999). The mechanism of action of these agents can be described in three levels of interaction with microorganisms: through outer cellular components, over the cytoplasmic membrane and by interaction with cytoplasmic constituents (Morente et al., 2013).

Quaternary ammonium compounds (QAC), such as benzyldimethyldodecylammonium chloride (BDMDAC) used in this thesis, have a broad spectrum of action and their antimicrobial activity is closely connected with their long chain with an alkyl group (Ferreira et al., 2011). These cationic surfactants can act as both a bactericidal or bacteriostatic. They act by the formation of an electrostatic bond with the negatively charged sites on cell walls, as a consequence of their positive charge (McDonnell and Russell, 1999; Simões et al., 2005). The first step is the adsorption to and diffusion over the cell wall, followed by interaction and disruption of the cytoplasmic membrane causing the release of cellular components and, at last, precipitation of cell content

and death (Block). Ferreira *et al*. (2011) shows that the BDMDAC effectively acts against *P. fluorescens*, binding by ionic and hydrophobic interactions to the cell membrane, which changes the properties and function of later. This ultimately leads to cellular disruption as well as loss of membrane integrity, with subsequent leakage of essential intracellular constituent. Today, QAC's are applied in diverse consumer products, and food and health care industries. Despite their low toxicity and versatility of applications and targets, several recent studies have focused on their part in the development of resistance among bacterial microbes (Gerba, 2015; Russell, 2004).

Besides the use of disinfectants, such as biocides, other chemical strategies are at use, such as UV radiation and the use of metal ions. UV radiation has the advantage of being a simple technique and not adding chemicals to the water, however it comes as a high expense, it is not optimized, and it is classified as a point-of-contact treatment, exposing the rest of the system (Zhou and Smith, 2001). In short, UV radiation operates mainly in small wastewater treatment plants by neutralizing or killing viruses by producing hydroxyl radicals (Parrotta and Bekdash, 1998; Lehtola et al., 2003). Metal ions are usually applied on recirculating hot-water systems, and are believed to interact with enzymes implicated in cellular respiration and bind to specific sites in the DNA. The most commonly used metal ions are copper, usually combined with silver, due to synergy of this combination (Kim et al., 2002).

2.1.4 Better Safe than Sorry: A Matter of Prevention

When assessing the consequences brought upon different processes by the presence of microorganisms, one must have in account that in many cases they don't constitute a major concern when in the planktonic state. This comes to show that avoiding the initial attachment would not only solve some of the mentioned problems, while in other cases it would, at the minimum, facilitate disinfection and reduce capital costs (Meyer, 2003). Although the general term "biofilm control" is used, this concept must therefore include the prevention of initial colonization, having into account the industrial equipment design and choice of surface materials, the aforementioned development of removal strategies against the already formed biofilm and quantitative microbial risk assessment, thus minimizing human exposure (Science et al., 2019).

Prevention starts with the correct choice of materials and the design of operation units that are less vulnerable to biofilm accumulation. In dead ends, corners, cracks, crevices, gaskets, valves and joints, the probability of such phenomenon to occur is higher (Xu et al., 2017). However,

previous literature has shown, in an attempt to identify materials capable of suppressing or preventing to some extent biofilm formation in drinking water distribution systems, that such objects are scarce (Rogers et al., 1994). Another logical possibility would be to limit the microorganism's carbon source. Nevertheless, it has been reported that ultra-pure water systems also supported the development of biofilms (Griebe and Flemming, 1998). Yet again in a perspective of altering the environmental conditions, it has been suggested that by supplying the microorganisms with growth factors, the first step of biofilm formation could be avoided, since this would no longer represent a beneficial survival strategy. Avoiding biofilm formation can also pass by the incorporation of antimicrobial agents into surface materials or the coating of surfaces with antimicrobial agents (Meyer, 2003; Thouvenin et al., 2003). These solutions have been used extensively in the biomedical field, in applications such as coatings for implants (Simões, 2005). Besides this, prevention of biofilm formation can also involve the regular disinfection of the process units (Meyer, 2003). Since this is a process that involves the repeated use of biocides, enhancing the issue of microorganism resistance, it is important to develop strategies that allow their application more efficiently and to decrease the dosage of such products.

2.2 Micro-Nanotechnology Approaches

2.2.1 Plenty of Room at the Bottom [1] : Thinking Big by Working Small

The prime role of nanotechnology is the search for an understanding and control of materials in the range of 1-100 nm. At this scale, incomparable physicochemical properties such as ultra-small size, large surface to mass ratio, high reactivity and singular interactions with biological systems emerge (Zhang et al., 2010). Nanomaterials are also good adsorbents, catalysts, and most importantly, able to react to external signals changing their own structure and properties. Given this particularity they are often called "smart" materials, manipulated to fulfil specific functions (Obi and Chibueze, 2018). As such, it is of little wonder that the field of micro and nanotechnology has been gaining significant interest in environmental and biological applications throughout the years. More specifically, nanobiotechnology holds the key to understand and transfigure biosystems by applying this technology and structures, namely to biofouling control (Ferreira et al., 2010; Roco,

[1] (Feynman, 1959). "There's Plenty of Room at the Bottom: An Invitation to Enter a New Field of Physics" was a lecture presented by the physicist Richard Feynman at Caltech, on December 29, 1959. The physicist was a pioneer in nanotechnology, sharing the vision that the manipulation of individual atoms was a possibility that could take us further than any form of other synthetic chemistry. His work went unnoticed at the time, and it wasn't until the beginning of the 1980s that he started gaining more recognition as the nanotechnology advocates attempted to establish the scientific credibility of this field. Today, his contribution to several areas of science are undeniable (Forstner, 2020).

2003). The existent antimicrobial nanomaterials, being relatively inert in water, do not form prejudicial DBPs which constitutes an advantage when compared to the use of free biocides (Obi and Chibueze, 2018). The formation of DBPs, which can be toxic or even carcinogenic, comes as a consequence of the interaction of chemicals such as free chlorine, chloramines and ozone with some constituents of natural water (Li et al., 2008). Over 600 DBPs have been identified in literature (Krasner et al., 2007). Furthermore, nanotechnology is a major asset in the fight against antimicrobial resistance (AMR), which over the years has been pointed out as one of the major threats to human health (Bush et al., 2011). In truth, these concepts are correlated due to the fact that an increased AMR implies a higher disinfectant dosage, which ultimately worsens the formation of DBPs. A shift from the development of new antibiotics to the search of novel nanotechnology methods for the prevention of biofilm formation has been verified in the scientific community, since several of these techniques have been proven capable of overcoming the limitations inherent to the current strategies (Elbourne et al., 2019). Some of the strategies in use to tackle industrial biofilm control include the use of inorganic nanoparticles (*e.g.* silver, metal oxide, gold and multi-metallic nanoparticles), organic nanoparticles (*e.g.* liposomes, polymeric nanoparticles), dendrimers and nanoenzymes (Obi and Chibueze, 2018). Nanoparticles can be used to control biofilm in water systems, especially in decentralized or in specific points of use along the water systems (Dankovich and Gray, 2011).

Nowadays, antibacterial and antibiofilm materials that aim to affect single and multi-species biofilms can be generally divided into three major categories: engineered nano- and micro-topography surfaces that inhibit bacterial adhesion, surfaces with covalently immobilized antimicrobials that kill bacteria upon contact, such as cationic macromolecules containing quaternary ammonium and phosphonium moieties and biocide releasing surfaces (Ivanova, 2017). The research around nanoparticles started by approaching them as antimicrobial coatings. When interacting with microorganisms, different mechanisms can follow: interruption of electrons transport through the membrane, oxidation of the cell's components or production of secondary products, harmful to the organism. Many nanomaterials, both engineered and natural, present antimicrobial properties. Some of the most regularly mentioned in literature are titanium dioxide, various metal nanoparticles, chitosan and photocatalytic TiO_2 (Li et al., 2008). Chitosan is a derivative from a natural polysaccharide called chitin, which can be found on arthropod shells. By the interaction of the chitosan's positively charged molecules with the negative charges present in the cell membranes, it leads to an increase of membrane permeability and, consequently, membrane rupture (Li et al.,

2008). In its turn, metal nanoparticles are advantageous for their unique physical and chemical properties, which can be used to construct biosensors by the immobilization of biomolecules (Du et al., 2016). When it comes to TiO2, the advantages lay on its stability, low-cost and non-toxicity, making it suitable for water treatment (Hammond, 1999). As the knowledge around micro and nanotechnology unveils, these particles are also analyzed from a different prism: as cores for coated particles, instead of simply coatings. In this sense, these particles are reactive and because their ratio between area and volume is high, they are capable of carrying a high dose of biocide (Ferreira et al., 2013). To this end, (Ferreira et al., 2010) applied poly-(styrene) (PS) core particles, treated with polyethyleneimine (PEI) and sodium polystyrene sulfonate (PSS), carrying benzyldimethyldodecylammonium chloride (BDMDAC) to the control of *P. fluorescens* biofilms, obtaining promising results. On the other hand, (Ferreira et al., 2013) compared two different core particles (PS and calcium carbonate ($CaCO_3$)) prepared with the same layer-by-layer (LbL) technique and biocide, parallelly with the work developed in this thesis in which the comparison of calcium carbonate and hydroxyapatite cores is studied.

According to a new market report published by Lucintel, "the future of the global microsphere market looks promising with opportunities in the composites, medical technology, life science and biotechnology, and painting coating industries". The global microsphere market is expected to recover in 2021 from the decline due to global economic recession led by COVID-19 and is predicted to reach an estimated 4.2 billion by 2025 with a CAGR (Compound Annual Growth Rate) of 4% to 6% from 2020 to 2025 (Mic).

2.2.2 Functionalized Microparticles and the Particular Case of Hydroxyapatite

The concept of microparticle (MP) is the designation used for structures with usually spherical configuration and diameters in the micrometer range, normally from 1-1000 μm. Antimicrobial polymeric microparticles are generally formed by a polymer matrix in which a small amount of an active compound can be immobilized (Campos et al., 2013). For the purpose of this thesis, two particle cores were studied: $CaCO_3$, as mentioned in Ferreira *et al.* 2013, and HAp. Since the action of BDMDAC coated $CaCO_3$ particles against *P.fluorescens* is already deeply described in previous work, such as Ferreira *et al.* 2016, this thesis shines a new light on the promising alternative that HAp particles may represent. For this reason, it is imperative to understand the main characteristics and advantages inherent to this material.

In many fields, such as medicine, food industry and the plastic industry, calcium carbonate is an object of study (Kitamura, 2001). In its essence, calcium carbonate has three different types of anhydrous crystalline polymorphs: vaterite (polycrystalline spheres), aragonite (needles), and calcite, with rhombohedral, orthorhombic and hexagonal structures (Volodkin et al., 2004; Cölfen, 2003). The type of salts, as well as their concentration, pH, temperature, mixing rate of the solutions and intensity of agitation are considered to be key factors in its crystallization (Sukhorukov et al., 2004). Calcium carbonate is considered to be an economic alternative to other existents materials, such as poly-(styrene) (PS), but it is increasingly soluble in water as the pH decreases (Ferreira, 2016).

Hydroxyapatite (HAp) is a substance with two facets, being both a mineral and a biological material (Rial et al., 2021). Deriving from the mineral apatite, the most abundant naturally occurring phosphate on Earth, hydroxyapatite is the main constituent of the inorganic part of human and animal hard tissues such as bone and teeth (George et al., 2020). For this reason, its value for medical purposes arouses a great deal of interest. As a mineral, HAp as a density of 3.16 g/cm^3, a hardness of 5Mohs and a melting point above 1500ºC and a much lower solubility in water than calcium carbonate. Its stoichiometric chemical formula is $Ca_{10}(PO_4)_6(OH)_2$, with a ratio Ca/P of 1.67, from which characteristics such as bioactivity, osteointegration and osteoinduction emerge (Rial et al., 2021).

Thereby, HAp is the go-to choice of material in areas such as alveolar ridge, dental and bone implants. In fact, HAp is well known for its great biocompatibility and chemical stability, promoting the development of nano and microparticles for several applications mainly in the biomedical field (Silva-Holguín and Reyes-López, 2020). Amongst them are the delivery of growth factors, antibiotics, anticancer drugs, enzymes and antigens for slow-release vaccination (Motskin et al., 2009). Moreover, HAp is attractive for other physical and chemical properties, such as high adsorption capacity, low water solubility, availability and high stability under oxidizing and reducing conditions (Ghahremani et al., 2017). These characteristics lead to its application in areas outside medical development, such as water and wastewater treatment. In this context HAp has the added advantage of allowing the treatment of drinking water without constituting an element of toxicity and resulting in calcium rich water (George et al., 2020). This material goes even further in its versatility, as it can also be applied to ion exchange, catalysis, development of batteries and sensors, and filtration of microbes, virus, phosphorus, particulate matter (PM) and soil pollution (Liu et al., 2002; Rial et al., 2021; Tsuchida et al., 2008).

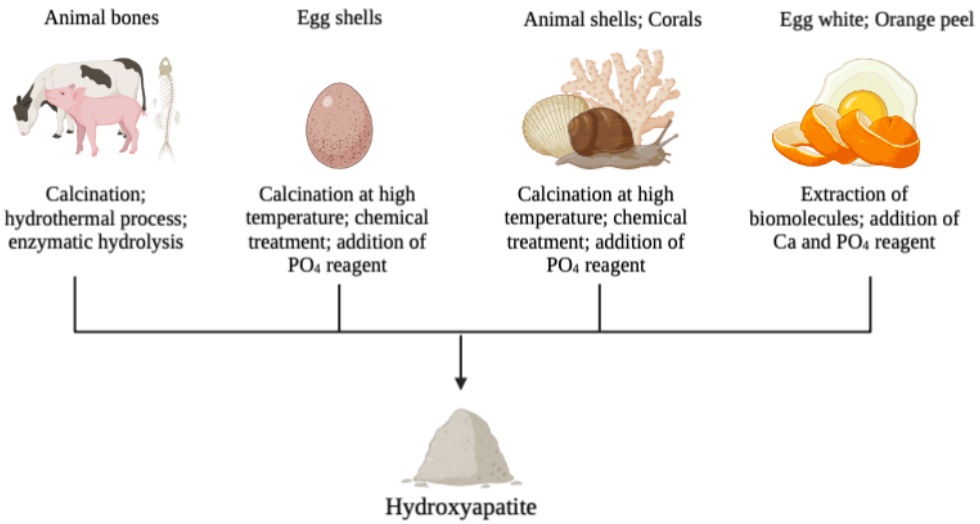

Figure 2.2: Raw materials and means of production of HAp from biogenic sources. (adapted from George *et al* 2020)

As pointed out in subsection 2.1.2, biofilms are a major concern in almost every industry leading to increased cost of production and maintenance, besides raising public health and environmental issues (Vishwakarma, 2020). It seems clear that there is a growing sense of responsibility in resolving these problems and finding sustainable and greener solutions. Action is being taken in the sense of using low-cost natural materials that besides being cheap raw material, would be considered waste (mainly from the food industry) if no other application was given to them. In this context, HAp is a particularly captivating solution for the possibility of using natural (biogenic) sources that can be attained through waste recovery, adding up to an economic and environmental sustainability (George et al., 2020). Figure 2.2 sums up some of the biogenic sources that have been in use as well as the methodology they are processed by.

The characteristics of the particles are deeply dependent of the method of synthesis by which they are produced, starting with the raw material. For instance, HAp particles of sizes between 30-150 nm were obtained through the use of eggshells, but HAp produced from bovine bone sources was found to be in the range of 50-250 μm (George et al., 2020). Not only the size, but also shape is affected. Using fish bones as the raw material, irregularly shaped HAp between 200-720 nm was obtained (Ozawa and Suzuki, 2002). However, particles from eggshells by Gergely *et al* (2010) were described as needle-like and flower-shaped particles from the combination of eggshell membrane and bamboo membrane were reported in (Zhang and Darvell, 2011). In addition, these features will dictate and may be adjusted for the adequate applications (George et al., 2020). In

fact, for almost each specific design there seems to be a particular route, which means it is possible to adjust the methodology to the objective, from obtaining HAP in powder, granular, macro- or microporous form, to manipulating the degrees of crystallinity, amongst other variables. The several methods to produce HAp particles are well summarized in (George et al., 2020), as well as the morphology, phase purity and size of the products obtained by such procedures. The processing time and temperature required for the synthesis of Hap from various biogenic sources were 3–4 h and 500–1300°C. In order to obtain Hap with high purity using biogenic sources, temperature in the range of 650–800°C with the reaction time of 3–5 h is favorable (George et al., 2020). The main routes for HAp production are through dry methods, wet methods and high temperature methods. However, other routes have been developed such as 3D processes, nanofabrication, ion doping and synthesis from natural sources (Rial et al., 2021).

Dry methods include solid state and mechanochemical methods. The first one relies on the calcification of chemical precursors, while the second induces the chemical reaction of precursors by mechanical processes such as milling or compression, where rotation speed and milling time are the main factors that affect the final product (Rhee, 2002; Cox et al., 2015; Yeong et al., 2001). The main disadvantages are the power and time required as well as the heterogeneity of the particles (Rhee, 2002; Yeong et al., 2001). In its turn, wet processes include low-temperature chemical precipitation, hydrolysis reactions and the hydrothermal method. These processes consist of mixing aqueous solutions of compounds possessing CA^{2+} and PO_4^{3-} resulting in the precipitation of HAp (Rial et al., 2021). The main variables are the reactant addition rate, concentration, drying/heat treatment conditions and, above all, pH and temperature. Chemical precipitation leads to the formation of heterogenous chemical composition and higher porosity. As opposed to this method, hydrothermal techniques produce well-crystallized powders of homogenous composition (Rodríguez-Lugo et al., 2018). Wet processes possess the advantages of being more economic and undemanding. Moreover, they are less susceptible to contamination during synthesis and the by-product is in great majority water (Rial et al., 2021).

To sum up, HAp is a promising nanomaterial for its unique characteristics, innumerous raw materials and synthesis routes and versatility of applications, not only in but out of the medical field. Furthermore, it represents an environmentally friendly alternative to other conventional options. In truth, the nanotechnology advances around this material, whether as a core particle or coating, are in constant development, proving its full potential is yet to be achieved.

2.2.3 LbL Technique

Multiple techniques, physical and chemical, have been developed with the purpose of modifying numerous surfaces, as well as medical devices. These strategies include self-assembled monolayers, graft polymerization, Layer-by-Layer (LbL) assembly and covalent attachment. However, LbL stands out for the myriad of design possibilities by the deposition of active agents.

LbL was first developed in its original form by Decher and co-workers with the goal of obtaining pure polymer multilayers on planar supports, back in 1991 (Decher, 1997). This strategy allows, for example, the creation of multifunctional materials and coating either with anti-adhesive or antimicrobial properties, circumventing the need for chemical modification of surfaces and use of crosslinking agents (Ivanova, 2017). The main advantages of this method for the assembly of polyelectrolytes lay on how easy, versatile and cost-effective it is. Besides this, the size, porosity, shape, surface functionality, stability, wall thickness and wall composition can be finely tuned (Cordeiro et al., 2004).

To put it simply, this technique works by sequentially depositing layers with opposite charges on to the original particle. The process begins with the step-wise deposition of polyelectrolytes from aqueous solutions, which will subsequently lead to the formation of the polyelectrolyte multilayer film. To remove the non-adsorbed polyelectrolyte in solution, cyclic centrifugation or filtration and washing are applied (Donath et al., 1998). As to the building blocks tested for creating these self-assembled polyelectrolyte multilayers (PEMs), they include inorganic nanoparticles, functional polymers, proteins, chromophores, and biopolymers, (*e.g.*: DNA) (Hammond, 1999; Lvov et al., 1993).

Amongst the existing techniques for creating tailor-made polyelectrolyte containing nanocoatings, such as immersive, spin, spray, electromagnetic, and fluid, the immersive method is the most extensively used. This process, propelled by the electrostatic interactions between its components, allows a better control of thickness by dipping a charged substrate into an oppositely charged aqueous solution of the active agent (Richardson et al., 2015). Other present non-electrostatic interactions worthy of mention are hydrophobicity, hydrogen bonds, Van der Waals forces and charge transfer halogen interactions. Multi layering can be either linear or exponential, depending on the previously mentioned interactions, their charge density, pH or strength of the solution. The first one occurs on the presence of polyelectrolyte pairs that have a strong interaction, as it is the case of poly(styrenesulfonate)/poly(allylamine hydrochloride) (PSS/PAH) allowing the thickness to increase linearly. As such, the layers formed will not be susceptible to diffusion of the polyelec-

trolytes within the layers themselves. On the other hand, weak interactions, as perceived in the case of hyaluronic acid (HA) and poly(L-lysine) (HA/PLL), result in an exponential growth. Consequently, the multilayers allow the diffusion of at least one of the two polyelectrolytes, resulting in highly hydrated coatings that can be used as a platform for immobilising proteins or enzymes onto materials and surfaces with no notable loss of biological activity (Ivanova, 2017). Other commonly used polyelectrolytes are poly(diallyldimethylammonium chloride) (PDDA), poly(ethyleneimine) (PEI) and poly(sodium styrenesulfonate) (PSS) (Ferreira, 2016). Other less conventional materials display the capability of assembly through LbL, such as colloidal nanoparticles, clay, nanosheets, modified zeolite crystals, two-dimensional perovskite and polyoxometalates (Ariga et al., 2007).

As it is possible to comprehend, the development of materials with the capacity of repelling and killing pathogens is tightly linked to changes in surface, roughness and charge. As aforementioned in previous sections, prevention is of the utmost importance when it comes to tackling biofilm related issues. For this purpose, several bacterial-repelling materials have been developed with resource to the LbL technique, such as hydrophilic polymers (i.e: PEG, poly(l-glutamic acid) (PGA), heparin and hyaluronic acid, that increase surface wettability by forming a hydrated layer that prevents bacterial adherence (Ivanova, 2017). However, although materials such as anti-adhesives act primarily upon the bacterial attachment, they may not be impeditive to pathogenic bacterial growth in solution. Consequently, cells may live to further colonize other surfaces. To overcome the point at issue, several functional multilayer assemblies have been developed. The majority of them involve cationic compounds that by interacting with the anionic cell wall result in its damage and bacterial death (Fernandes et al., 2014). One of the main obstacles in this procedure comes from the complexity in affecting bacterial cell membrane, which might be hindered by layer rigidity and strong interactions of the active compound with the anionic compound. A solution for this problem is varying the pH during and/or after the LbL construction which has been proven to be highly effective for increasing surface charge and mobility (Lichter and Rubner, 2009). As such, it is important to underline that both the solutions pH and ionic strength are crucial for the development of stable materials (Séon et al., 2015).

It is possible to perceive that despite the many advances and simplicity of such procedures, the mechanisms behind LbL are still not fully understood, as the quality and structures themselves depend also on experimental conditions such as salt concentration, secondary interactions, temperature, and others (Ferreira, 2016). Nowadays, the use of antibiofilm coatings capable of affecting bacterial cell-to-cell communication and consequently biofilm integrity present themselves

as a good strategy for addressing the hazard of bacteria resistance. This comes as a response to the emergence of resistant strains that result from the continuous use of biocides, triggering the need for new strategies that allow a sustained and controlled release of the previous. In this context, LbL leads to the possibility of integrating diverse agents, be it classic antibiotics and/or novel antimicrobials, producing hybrid multilayer coatings with synergetic modes of action. The manipulation of distinct levels in coating where the active compounds are embedded, allows to achieve the synchronous or successive delivery of each active principle at the desired stage of the bacterial lifecycle (Ivanova, 2017). This comes has a major advantage since it ultimately reduces toxic side effects, as a consequence of lower drug dosage while improving the prophylactic and therapeutic efficacy (Fayaz et al., 2010). Besides this, lower drug usage represents a decrease in the costs as opposed to the continuous application of antimicrobial chemicals. These advantages, in addition to the use of biogenic waste to produce HAp, lead to an economy friendly solution.

Chapter 3

Antimicrobial Activity

3.1 Introduction

The success or failure of antimicrobial treatments can be evaluated through the performance of antimicrobial susceptibility tests (Cerf et al., 2010). As antimicrobial susceptible tests, MICs and MBCs are used to explore the differences in bacteria susceptibility or resistance to the antimicrobial agent tested. Despite this, scholars have argued that the MIC values present some drawbacks. In fact, small variations in methodology can result in large variations of the MIC (*ie*: extended incubation tends to increase MIC and lower inoculum concentrations tend to diminish the MIC). Besides this, MIC is related to the inhibition of visible growth. In other words, it may not be clear if the microorganisms were in fact killed (Lambert and Pearson, 2000; Pagès et al., 2010).

Due to these limitations, the determination of MBCs was considered a more appropriate approach. In short, this value defines the necessary concentration to kill most cells ($> 99.9\%$) after 24h incubation under a given set of conditions (Cerf et al., 2010; Elbourne et al., 2019; French, 2006). Taking this into consideration, the minimum bactericidal concentration was determined to evaluate the susceptibility of *P. fluorescens* to BDMDAC. More specifically, the lowest concentration of BDMDAC where no colony forming units (CFU) were detected on solid medium was taken as the MBC (Smith et al., 2008).

The activity of biocides is widely affected by a myriad of factors, including the period of contact with microorganisms, presence of interfering materials, formulation effects, temperature of contact and the intrinsic characteristics of the microorganism itself. In the work developed by Russell and McDonnell (2000) special attention was given to the effect of concentration. It was argued that this is a parameter of invaluable importance in the study of the true antimicrobial

activity and modes of resistance of biocidal-type agents. In fact, since most biocides are developed for optimal, broad-spectrum antimicrobial activity at high concentrations, this factor has both practical and clinical consequences (Russell and Mcdonnell, 2000). Despite the complexity of the relation between antimicrobial activity and concentration, mathematical models that allow the calculation of the concentration exponent (η) were developed. Previous literature also speculates on the information the value n can supply about the cellular targets of the biocide. In this work, the concentration exponent was calculated with resource to the Chick-Watson mathematical model of disinfection (Grobe et al., 2002) and its meaning further discussed.

3.2 Materials and Methods

3.2.1 Microorganism and Culture Conditions

The *P. fluorescens* in use, isolated from a drinking water distibution system, was previously identified by 16S ribossomal sequence analysis. For achieving optimal growth conditions the main carbon source was glucose. The optimal temperature was 27 ± 3ºC and the pH 7. The *P. fluorescens* was cryopreserved in a refrigerated chamber at –80ºC, in a mixture of nutrient broth and 30% (v/v) of glycerol. An inoculum was removed from the cryovial, and subsequently distributed evenly over the surface of Plate Count Agar (PCA – Merck, VWR) and incubated for 24 h at 27 ± 3ºC, to allow bacteria propagation (Ferreira et al., 2013).

3.2.2 Antimicrobial Product

The chosen biocide for the development of this work was benzyldimethyldodecyl ammonium chloride (BDMDAC - Sigma Aldrich), a cationic quaternary ammonium compound (QAC) with a long carbon chain composed of 12 carbons (Figure 3.1).

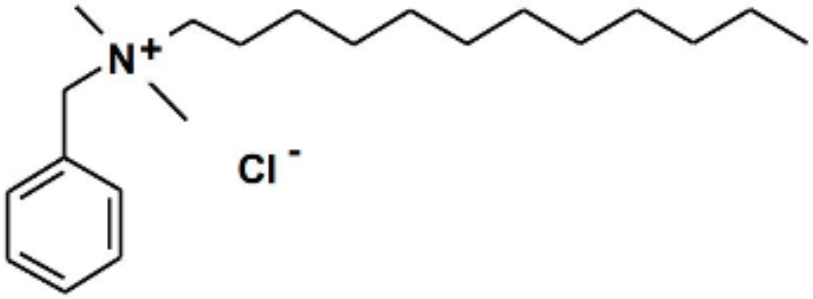

Figure 3.1: Chemical Structure of BDMDAC.
(Ferreira et al., 2011)

For the experiments regarding the minimum bactericidal concentration (MBC) with free biocide, BDMDAC solutions were prepared from a stock solution (1 g/L) with ultrapure water (UPW).

3.2.3 Determination of the Minimum Bactericidal Concentration (MBC)

Bacterial cultures were prepared in 100 mL sterile flasks containing 25 mL of sterile growth medium (5 g/L glucose, 2.5 g/L peptone, 1.25 g/L yeast extract, 2.60 g/L disodium phosphate and 1.88 g/L potassium dihydrogen phosphate) and an appropriate volume of bacterial inoculum. An overnight grown culture of *P. fluorescens* (30°C, 120 rpm) was centrifuged (4000 g, 10 min) and washed twice with saline solution (0.85% NaCl). In order to obtain an optical density at 610nm (OD_{610nm}) of approximately 0.2 ± 0.02, corresponding to roughly 1.5×10^8 cells/mL, bacteria were resuspended in saline solution.

Afterwards, an aliquot of 100 μL was sampled and increasing biocide concentrations (5, 10, 15, 20, 25, 30, 50 and 100 mg/L) were tested. The process applied consisted in a maximum 60 minute contact time (25°C, 160 rpm) after which BDMDAC was chemically neutralized (10 minute contact time), as in (Johnston et al., 2002). The solution used for this effect was composed as follows, for 100 mL of solution, in which the components were added by the correspondent order: L-histidin (0.1 g), lecithin (0.3 g), thiosulphate (5 H_2O) (0.79 g), saponin (3 g), dH_2O (96 mL), phosphate buffer (0.25 M, pH 7) (1 mL) and Tween 80 (3 mL). During contact time sampling was carried out at 5, 15, 30, 45 and 60 minutes. A volume of 100 μL of bacterial suspensions were diluted to an adequate cellular concentration (from $10^0 - 10^5$) in saline solution. Next, a volume

of 10 μL of each suspension was transferred onto PCA plates with resource to the drop method and later incubated at 37°C (Herigstad et al., 2001). Colony enumeration was carried out after 24 hours. The lowest concentration of BDMDAC where no colony forming units (CFU) were detected on solid medium was taken as the minimum bactericidal concentration (MBC) (Smith et al., 2008). Three independent experiments were performed for each condition tested.

3.2.4 Determination of the Dose Response

An analysis of concentration dependence of bacterial killing was also elaborated, with resource to the Chick-Watson mathematical model of disinfection (Grobe et al., 2002):

$$\frac{dX}{dt} = -k_{dis}C^{\eta}X$$

(3.1)

In the equation presented above (3.1), X is the viable density, t is time, k_{dis} is a disinfection rate coefficient and C is the antimicrobial agent concentration. The variable of interest is the exponent η, which reflects the concentration dependence of killing. By the assumption that the biocide concentration is constant, it is possible to reach the intended solution by integration:

$$ln(\frac{X}{X0}) = -k_{dis}C^{\eta}t$$

(3.2)

Another variable is yet needed to calculate η, which is named apparent rate of disinfection (r) when bacteria is exposed, in this case, for one hour to the biocide BDMDAC.

$$r = (\frac{-ln(R)}{t})$$

(3.3)

The reduction, R, is obtained by the subtraction of the log (CFU/mL) calculated for the control and the log (CFU/mL) calculated for each concentration after one hour of exposure. For each concentration tested, the results of 3 independent experiments were subjected to this process. By the combination of equations (3.2) and (3.3) it is therefore possible to obtain (3.4):

$$ln(r) = ln(k_{dis}) + \eta ln(C)$$

(3.4)

The software GraphPad Prism 9.2.0 for macOS (GraphPad Software, La Jolla California, USA) was used for applying linear regression with slope η and y-interception ln(kdis). Model quality was assessed through the coefficient determination (r^2).

3.3 Results and Discussion

3.3.1 Minimum Bactericidal Concentration (MBC)

Figure 3.2 summarizes the results obtained for the Log counts of *P. fluorescens* as a function of the dissolved BDMDAC concentration tested.

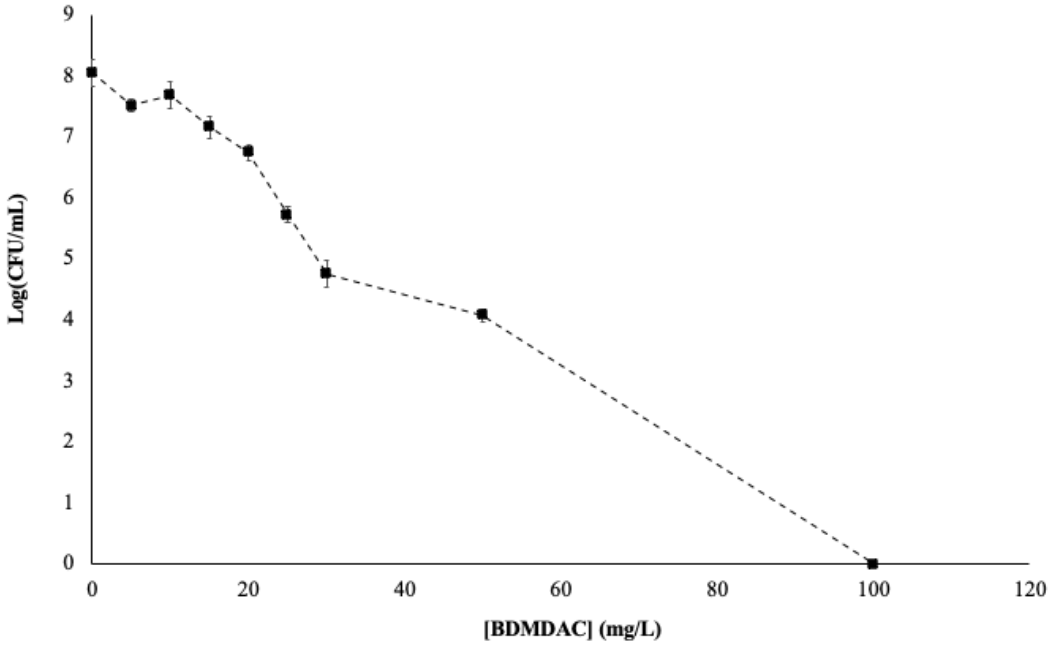

Figure 3.2: Log CFU counts of *P. fluorescens* as function of BDMDAC concentration (0, 5, 10, 15, 20, 25, 30, 50, 100 mg/L) after 1 h exposure period. The means ± SDs for three independent experiments are illustrated. The MBC value was determined as the lowest concentration where no CFU counts were detected.

Analyzing the graphic, it is possible to acknowledge that increasing the biocide concentration resulted in increasing antimicrobial activity until a maximum was reached, leading to total reduction of cell culturability. As such, the MBC obtained was 100 mg/L, when no CFU was detected after biocidal exposure.

The concentration of biocides most used in disinfection procedures is 50 mg/L, according to literature (Ferreira et al., 2011; Simões et al., 2007). As it should be expected however, this value varies with the purpose of disinfection, its target and the product applied. For example, in the food industry, QACs are used at concentrations ranging from 200 to 400 mg/L for different food-contact surfaces. In health centers, concentrations of QACs close to 2000 mg/L are used, applying these products as low-level disinfectants (Mazzola et al., 2009). According to the CDC, these are appropriate for noncritical items that contact with the skin, mainly as stethoscopes, blood pressure and tourniquet cuffs, EKG (Electrocardiogram) leads, bedside equipment, and environmental surfaces (Amodio et al., 2020). It seems safe to admit BDMDAC could serve as a good alternative to the biocides already in use.

To further validate the findings, previous work developed in LEPABE was studied. In the thesis by Antão (2015), the minimum bactericidal concentration for BDMDAC action against planktonic *P. fluorescens* had also been 100 mg/L, confirming the results. However, in the work developed in Ferreira *et al.* (2011), that laid the foundations for this thesis, the MBC found was much lower. In fact, an MBC of 10 mg/L was determined, although some limitations were pointed out to the CFU quantification method. In truth, total membrane damage was only obtained at 20 mg/L, when using the Live/Dead BacLight viability kit (Ferreira et al., 2011). Even so, since this value is significantly different from the one reported herein, the methodologies in use were thoroughly compared. It was noted that in the work previously mentioned the neutralizers differed in composition. Having this in consideration, the effect of the neutralizer in the procedure was questioned.

An experiment at a concentration of 20 mg/L was conducted in the same conditions, but using the neutralizer as described in Ferreira *et al.* (2011) ((w/v) 0.1% peptone (Sigma; Portugal), 0.5% Tween 80 (Sigma) and 0.07% lecithin (Sigma), dissolved in saline solution). No significant difference was noted between the results and the ones already presented in this thesis, since the count of log counts were of 6.97 ± 0.09. Additionally, and to exhaust all hypothesis, the quality conditions of the biocide were put in question. New biocide was ordered from (BDMDAC - Sigma Aldrich). An experiment at a concentration of 20 mg/L was once more conducted, using the new

biocide. Once again, the results obtained for this experiment were of 6.65 $\pm$ 0.09 log, which doesn't constitute a significant difference from the ones in Figure 3.2. The same experiments were then carried out at a concentration of 100 mg/L. In both cases, no CFU were detected after biocide exposure, meaning the MBC determined in the first place was most likely correct. Each of the hypothesis was tested by conducting three independent assays.

Several explanations can be held accountable for this gap. Maillard (2018) exposes several different resistance mechanisms intrinsic to and acquired by bacteria to biocides that can be responsible for possible emerging insusceptibility.

Furthermore, by carrying out sampling at different times, it was possible to verify that time has an effect on the antimicrobial activity of BDMDAC (Annex A). This opens the possibility of using lower biocide concentrations if a higher contact time is applied. However, the repercussions and cost of such strategy must be carefully balanced before implementation, since it may not be advantageous to the process units and application for which the disinfection is intended.

3.3.2 Dose response

For the same exposure time, if n=1 log CFU/mL reduction increases in direct linear proportion, and if n=2, log CFU/mL reduction is proportional to the square of the biocide concentration (Fernandes et al., 2014). The concentration exponent found in this was 0.84 $\pm$ 0.16, which is in accordance with previous findings and, once more, validates that the efficacy of BDMDAC is concentration dependent. Table 3.3 presents several examples of the concentration exponents (η) for different biocides (Lelieveld et al., 2005).

Biocide	Exponent
Phenolics	4–9.9
Alcohol	
Benzyl alcohol	2.6–4.6
Aliphatic alcohols	6.0–12.7
Cationic biocides	
Chlorhexidine	2
Polymeric biguanides	1.5–1.6
QACs	0.8–2.5
Crystal violet	0.9
Aldehydes	
Formaldehyde	1
Glutaraldehyde	1
Peroxygens	
Hydrogen peroxide	0.5
Metallic salts	
Silver nitrate	0.9–1.0
Mercurials	0.03–3.0
Organic acid	
Parabens	2.5
Sorbic acid	2.6–3.2
Potassium laurate	2.3

Figure 3.3: Examples of typical concentration exponents (η) for different compounds (Lelieveld et al., 2005).

The values found for QACs suggest that these cationic biocides will retain much of their lethal activity over a considerably wide concentration range. Furthermore, it is also stated that compounds with n<2 comprise, amongst others, quaternary ammonium compounds such as the biocide in use and are considered to interact strongly with their target by chemical or ionic bonding (Lelieveld et al., 2005). This is also coherent with the mode of action previously described for BDMDAC.

3.4 Conclusions

From this Chapter it is possible to take several conclusions regarding the antimicrobial activity of BDMDAC against *P. fluorescens*.

The MBC found for such action, despite dubious at first, was reasoned with resource to previous literature and further experiment, allowing the conclusion that its value was 100 mg/L. Having in account the concentrations typically used for disinfection procedures, and the fact that these vary according to the intended applications, the MBC found was admissible and regarded as favorable.

The concentration exponent found by application of the Chick-Watson mathematical model, 0.84 ± 0.16, was also in consistent with the typical values for QACs, showing that the antimicrobial action is, as expected, dose dependent.

Chapter 4

Particle Development

The primary source for the removal and killing of microorganisms from various surfaces and water has often been physical and chemical methods (Melo and Bott, 1997). Notwithstanding, the detrimental consequences of the chemical strategies currently employed have raised questions regarding their sustainability and safety for the human health (Li et al., 2008; Gerba, 2015). The scientific community now views micro-nanotechnology as a new generation of solutions and hopes to find in it the key to solve the issues regarding the increased antimicrobial resistance to biocides and the toxic effects of the latter. In this context, this Chapter is dedicated to the particle development by the LbL method as well as the characterization of the microparticles as a result of that process, regarding several parameters. LbL stands out for the myriad of design possibilities by the deposition of active agents.

The characteristics of the particles are deeply dependent of the method of synthesis by which they are produced, starting with the raw material. Two types of cores were used in this study: hydroxyapatite (HAp) and calcium carbonate ($CaCO_3$). HAp is well known for its great biocompatibility and chemical stability, promoting the development of nano and microparticles for several applications mainly in the biomedical field (Motskin et al., 2009; Rial et al., 2021). In its turn, $CaCO_3$ will serve as term of comparison for HAp. These microparticles are well-known for being cheap, porous and made of a mineral very abundant in nature (Ferreira, 2016).

For the quantification of BDMDAC on the surface of coated particles, a high performance liquid chromatography (HPLC) method was developed. This allowed to assess with more accuracy the concentration of biocide adhered do the particles, as opposed to a simple estimation of that value.

The BET (Brunner-Emmett-Teller) theory serves as a mean of assessing the size, specific surface area, surface irregularities, pore walls and pore size distribution of the particles, before and after functionalization, allowing a deeper study of their morphology (Akbari et al., 2011). This technique is based on the phenomena of adsorption and desorption, occurring in the surface of the particles (Dollimore et al., 1976). The amount of gas adsorbed depends not only on the exposed surface but also on the temperature, gas pressure and strength of interaction between the gas and solid. The data is then processed by plotting the correspondent isotherms of this process, from which the BET equation (Appendix A.2.1) uses the information to determine the surface area of the sample (Akbari et al., 2011).

In order to assess the biocide release from the particles, the dialysis method (DM) was proposed. This is argued to be one of the most common and versatile methods to assess drug release. Based on physical diffusion of the biocide through a dialysis bag, the set-up simplicity and ease to perform sampling, make it attractive to examine different nanosized materials such as nanospheres, liposomes, emulsions and nanosuspensions (D'Souza, 2014). As every strategy, this method also has it disadvantages. One of them lays on the fact that it is unsuitable for drugs that are susceptible to binding with the dialyzing membrane (Heng et al., 2008). Besides this, if not properly sealed, leakage of the suspension and release media are highly likely (D'Souza, 2014).

In short, this Chapter intends to provide wider comprehension of the microparticles before testing their efficacy against *P. fluorescens*, thus allowing a proper evaluation of their behavior and advantages.

4.1 Materials and Methods

4.1.1 Layer-by-Layer (LbL) technique

Hydroxyapatite and calcium carbonate functionalized spherical core particles were obtained through the LbL self-assembly procedure described by Ferreira *et al* (2013). The HAp microparticles (nanoXIM-HAp202; Fluidinova S.A) had, as described by the manufacturer's lot characterization, a particle size distribution of 5.0 ± 1.0 μm. In resemblance, the $CaCO_3$ microparticles in use also had an average size of 5.0 μm. These particles were provided by LEPABE and produced as described in Ferreira *et al* (2016).

Polyethyleneimine (PEI – molecular weight 750 000 g/mol) 50% in water, poly(sodium4-styrenesulfonate) (PSS – molecular weight 70 000 g/mol) and boric acid (H_3BO_3) were purchased from Sigma Aldrich. BDMDAC (molecular weight 339.9 g/mol) was purchased from Sigma Aldrich. Borate buffer (0.1 M) was used in the whole process, chosen by its ionic strength. The pH was kept at 9 since this was considered to be the value that guarantees the adequate superficial charge for the different molecules that are engaged in the process, which comprises three major steps. The concentration of particles in solution was 9 mg/mL.

In the first step, the negatively charged particles are put in contact with a PEI (i.e: polycation) solution (1 g/L in 0.1M borate buffer solution) for 20 minutes. As described in Donath *et al* (1998) the removal of the non-adsorbed polyelectrolyte in solution is done by centrifugation and washing. The solution is vortexed, centrifuged at (4000 rpm, 5 min), resuspended in an adequate volume of borate buffer (0.1M) and centrifuged once again (4000 rpm, 5min). The supernatant is discarded, leading to the next step.

In the second step, the now positively charged particles are allowed to interact with a PSS (polyanion) solution (1 g/L in 0.1M borate buffer solution) for another 20 minutes. Once again, a washing step similar to the previous one is applied. The particles are now negatively charged.

In the third step the negative charges will allow the deposition of BDMDAC. The particles are left in contact with a solution of BDMDAC (1 g/L in 0.1M borate buffer solution) overnight.

All the interaction steps were carried out in a roller mixer SRT6D, so that the contact polymers/biocide and the core was homogeneous. The process is depicted in Figure 4.1:

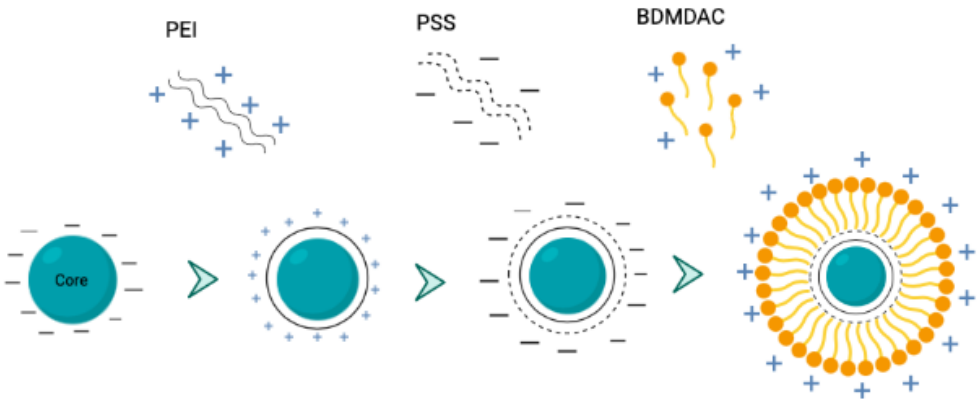

Figure 4.1: Schematic representation of the LbL method in use.

After contact with the biocide, two washing steps were implemented. Since the amount of BDMDAC adhered to the particles was assessed using HPLC (High-Performance Liquid Chromatography), the supernatants that resulted from these steps were collected for analysis.

4.1.2 Quantification of the amount of biocide

For the quantification of BDMDAC on the surface of coated particles a high performance liquid chromatography (HPLC) method was adapted and applied as described in Santos *et al* 2010. HPLC was carried out using a Shimadzu LC-2040 C 3D system, equipped with a PDA detector. The analytical column was a 100-3 ACE CN (150 mm x 4.6 mm). The gradient initiates with mobile phase A (0.025% trifluoroacetic acid in water:acetonitrile (90:10, (*v/v*)), posteriorly ramped to the mobile phase B (0.025% trifluoroacetic acid in water:acetonitrile (39:70, (*v/v*)). For sample dilution, acetonitrile is used (Santos et al., 2010).

Two calibration curves of BDMDAC were made, one with concentrations ranging from 1 to 50 mg/L and another with concentration ranging from 10 to 300 mg/L. The chromatograms were obtained at a wavelength of 214 nm (Santos et al., 2010). Before injection, all samples were filtered through syringe filters (0.2 μm).

The concentration of BDMDAC in the particles, was determined by the following equation:

$$C_p = C_{BST} - C_{S0} - C_{S1} - C_{S2} \tag{4.1}$$

Where C_p is the concentration of BDMDAC in the microsuspension (mg/L), C_{BST} is the concentration of BDMDAC in the biocide stock solution (1000 mg/L), and C_{S0}, C_{S1} and C_{S2} are the concentrations of BDMDAC in the sequential supernatants resulting from each centrifugation step after the overnight contact of the microparticles with the biocide. It should be noted that hereon, despite the concentration of biocide present in each particles' stock solution, the necessary volume of microparticle suspension for each assay according to the intended concentration was calculated, as it will be described further ahead.

4.1.3 BET (Brunner-Emmett-Teller) Analysis

The particle size plays a crucial role in nanoparticle properties and therefore an essential task in property characterization of nanoparticles is particle morphology. Most properties of nanoparticles are size-dependent. In fact, the novel properties of nanoparticles do not prevail until the size has been reduced to the micro-nanometer scale (Akbari et al., 2011). To study the morphology of the particles, in terms of specific surface area (S_{BET}; m^2/g), total pore volume, (Vp; cm^3/g) and pore size distribution, a BET analysis was carried out.

In this work, the equilibrium isotherms of nitrogen adsorption at -196ºC for both types of particles, before and after functionalization, were determined using a Quantachrome NOVA 4200e equipment. In a first stage, the samples were degassed at 120ºC for 3 hours. To texturally characterize the samples, the specific surface areas were determined by the BET method (S_{BET}), applying the BET equation (Annex B.1) in the linear area of the graphic, included in the P/P_0 range of 0.05 to 0.30. Regarding $CaCO_3$ and HAp microparticles, 3 and 7 points, respectively, were used in this range. The total volume of pores was determined for P/P_0. The pore size distribution was calculated using the nonlocal density functional theory (NLDFT), available on the software of the analysis equipment, assuming a cylindrical model.

4.1.4 Release Assays: Dialysis Method (DM)

To assess the biocide release from the particles, the dialysis method (DM) was used. The set-up simplicity and ease to perform sampling make it attractive to examine different nanosized materials such as nanospheres, liposomes, emulsions and nanosuspensions (D'Souza, 2014).

In short, it consists of the use of a dialysis membrane, designed in such a way that allows periodical sampling. Firstly, the particles suspension is introduced inside an inner compartment that is actually a dialysis bag. The inner compartment is sealed and introduced into a larger vessel, containing release media. This vessel possesses a stir bar to promote agitation, minimizing unstirred liquid layer effects and propelling the release. The volume of the inner media usually ranges from 1 to 10 mL, while the outer media can typically go until 90 mL. This volume is adjusted in function of the total release media required for the study (D'Souza, 2014). The process that occurs therein is the physical diffusion of the drug release from the particles through the dialysis membrane to the outer vessel, where sampling is conducted.

Figure 4.2 illustrates the method's set-up as well as the direction of diffusion (from the inner to the outer compartment).

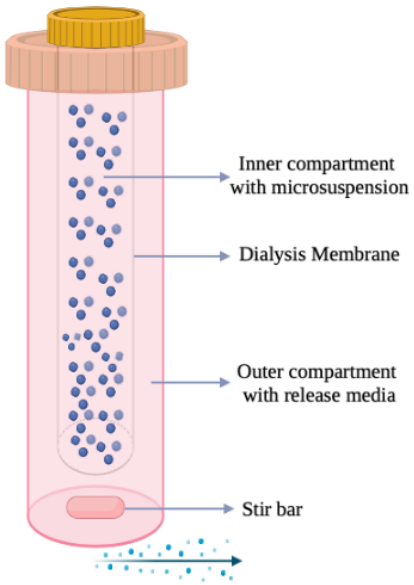

Figure 4.2: Dialysis Method (DM) set-up applied herein. (adapted from D'Souza (2014))

In the purpose of this thesis, DM was employed to evaluate the release of BDMDAC from the hydroxyapatite and calcium carbonate particles. The dialysis membrane used was Spectra-Por® Float-A-Lyzer® G2 (Sigma). In this work, two independent release assays were conducted by this

method with different BDMDAC concentrations in the dialysis bag: 300 mg/L and 1000 mg/L. In each assay, two replicates for each type of particle were used.

The necessary volume of particle suspension was centrifuged (4000 rpm; 5 min), resuspended in 10 mL (the volume of the dialysis bag) of borate buffer and carefully pipetted into the dialysis membrane. The outer compartment was filled with 27 mL of borate buffer (0.1 M; pH 9).

Sampling of 100 μL of the outer media was performed at 15 and 30 min, 1, 2, 4, 6, 24, 48, 72, and 96 hours and 1 and 2 weeks for HPLC analysis. The HPLC method used was as described in 4.1.2, with the slight difference that the vials used here possessed inserts for maximum content retrieval. Since the volume of the outer media is subjected to sampling and evaporation through time, it is important to add borate buffer accordingly as to keep it constant.

4.2 Results and Discussion

The results obtained by BET, regarding the textural properties of the particles are described in Table 4.1.

Sample	S_{BET} (m^2/g)	$V_{p\ P/P0=0.99}$ (cm^3/g)
$CaCO_3$_bf	8	0.030
$CaCO_3$_af	5	0.030
HAp_bf	89	0.429
HAp_af	70	0.360

Table 4.1: Results obtained by BET analysis; bf stands for *before functionalization* and af stands for *after functionalization*.

Regarding the analysis of the non-functionalized particles, it is possible to comprehend that the HAp ones have a superior specific surface area, of approximately 11 times that of the $CaCO_3$ particles. The specific surface areas of both $CaCO_3$ and HAp microparticles are not surprising, since they should be in the range of 5-24 m^2/g and approximately 100 m^2/g respectively, according to literature and the technical description of the product (Martin-Martinez, 2002). The total pore volume is also superior in the HAp microparticles. It has been stated that the specific surface area is inversely proportional to the size of the particles (McCoy, 1974; Baker and Christensen, 1988; Block, 2001). In this case, since the size of the two particles is the same, 5 μm, but the specific surface area is higher in the case of hydroxyapatite, it seems safe to admit that this increase is precisely due to the difference in the total pore volume. The pores sizes of HAp presented a more

heterogeneous distribution than the ones from $CaCO_3$ microparticles, although they are overall wider (Annex B.2).

The specific surface area of the coated HAp particles is 14 times the one obtained in the case of calcium carbonate. There is much proof that particle size and shape have a major impact in antibacterial activity (Fatin-Rouge et al., 2004; Behzadi et al., 2017). In this regard, it is expected that particles with a higher specific surface area will be more effective. This should be observed in the HAp particles' action against *P. fluorescens*.

Analyzing each type of particle separately it is possible to observe a decrease in S_{BET} after applying the LbL technique. This results from the sequential adhesion of layers which increases the size of the particle and consequently decreases the specific surface area. In the case of $CaCO_3$ particles the difference in this parameter doesn't seem to be significant, alongside with an unaltered total pore volume. This may suggest that, upon functionalization, the polyelectrolytes and biocide only adhere outside of the pores in the $CaCO_3$ particles. To have a further insight on the porous characteristics of the particles, one should analyze the pore size distribution where indeed, for these particles, no significant changes were observed (Annex B.2). Overall, the results indicate that the LbL technique was successful for biocide immobilization.

Adding up to this, the adhered biocide in the microparticles, varied with the type of core used. In the case of HAp, the minimum and maximum concentrations achieved were 242 and 555 mg/L. In the case of $CaCO_3$ these values were inferior, ranging from 133 to 494 mg/L. Once again, this seems to show that the higher specific surface encountered in the HAp particles is advantageous, allowing a greater adhesion of BDMDAC.

Upon these observations, it is now crucial to assess if the functionalized particles release BDMDAC from their surface. In the assay at a concentration of 300 mg/L the HPLC did not detect peaks that reflected significant release in any type of the particles, apart from punctual small ones that were considered to be close to the quantification limit (5 mg/L). For this reason, it was argued that increasing the concentration could contribute to more accurate results.

A second release assay was executed, where the immobilized concentration of BDMDAC in the dialysis bag was 1000 mg/L. Once again, no release was detected apart from sporadic peaks close to the quantification limit, which was not considered significant nor conclusive.

Suspicion raised as to if the method employed was appropriate and if the agitation provided by the stirring bar was enough to promote the release of biocide from the particles. To clear these doubts, a fast test at 1000 mg/L was proposed in which the assay would proceed in 25 mL

flasks, with agitation at 160 rpm, for 24 hours. By the end of the stipulated time, the microparticle suspension was centrifuged (4000 rpm, 5 min), and the supernatant was sampled as described in the previous section, for HPLC analysis. Despite the increase in agitation, no release was detected in any type of particles. This led to the conclusion that, indeed, the coated microparticles by LbL did not release the biocide to a relevant extent, indicating that the coating process was efficacious and that the biocide adhered successfully to both cores.

However, as it will be further explained in the next Chapter, microparticles may possess a tendency to agglomerate at certain concentrations, which would interfere with this process, raising the necessity of evaluating the accuracy of these results.

4.3 Conclusions

In conclusion, it is understood that the LbL technique was successful, allowing the biocide to be well incorporated to the particle's core in both cases. However, the concentrations obtained for the $CaCO_3$ were generally lower than for the HAp particles.

Regarding their morphology, the HAp microparticles presented a higher porosity and specific surface area, either before or after functionalization, which is expected to be reflected in a higher antimicrobial activity. In the case of $CaCO_3$ the differences in the specific surface area and porosity, before and after functionalization, were not considered significant.

Another important parameter tested was the release of BDMDAC from the particle's surface. It was concluded that they did not reveal any significant release.

Overall, the functionalization of both particle cores was considered a success and therefore assessing their true antimicrobial activity against *P. fluorescens* was the next stage.

Chapter 5

Particles' Efficacy Against Planktonic Cells

5.1 Introduction

Once the antimicrobial action of BDMDAC in its free form against *P. fluorescens* and the characteristics of the microparticles developed are studied, the next phase is to evaluate their performance. In fact, the eradication of planktonic cells is a crucial step in biofilm prevention, since it compromises their attachment to the surface and maturation into biofouling.

In this context, it is important to denote that the cell-microparticle interaction is highly dependent on a myriad of factors, that consequently affect the particles' performance. Amongst them is the effect of size and shape, surface charge, hydrophobicity, and surface functionality (Behzadi et al., 2017). Another important phenomena, often linked with the previous ones, to take into consideration might be particle agglomeration. This process plays an important part in determining reactivity, toxicity, fate, transport, and risk in the environment (Hotze et al., 2010). Furthermore, although there is extensive research around the possible modes of action of surface bound QACs, the scientific community as not yet reached a consensus on the matter, opening up space for several hypothesis (Barros et al., 2021; Kaur and Liu, 2016).

As such, this Chapter intends to determine the efficiency of both types of functionalized particles, and perform a comparative study of these results with the ones obtained in the Chapter 3, obtaining giving a new insight into their mode of action.

5.2 Materials and Methods

The *P. fluorescens* strain, as well as its culture conditions, chosen for this work was the same as described in 3.2.1. Planktonic cells obtained from liquid medium were centrifuged and resuspended in a sterile saline solution (0.85% NaCl) to an OD_{610nm} = 0.2 ± 0.02 (bacterial cell density of approximately 1.5×10^8 CFU/mL). An aliquot of 2.0 mL was collected and used to test the antimicrobial effects of the coated particles. BDMDAC coated particles were tested at different concentrations (100, 200 and 400 mg/L) during 15, 30, 60 and 120 minutes of incubation under agitation (160 rpm, 25ºC). These concentration values reflect the mass of particle-incorporated biocide divided by the whole volume of the liquid suspension. The necessary volume of coated particle suspension was calculated according to the following equation:

$$C_p \cdot v_p = C_t \cdot v_t \tag{5.3}$$

Where C_p is the concentration of BDMDAC (mg/L) in the initial microsuspension, v_p is the necessary volume of the latter, C_t (mg/L) is the tested concentration and v_t (mL) is the total volume of bacterial suspension in which the particles are immersed (2.0 mL). 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) (Herigstad et al., 2001). After an incubation period of 24 h at 37°C, the number of CFUs was counted. Control experiments were performed with bacteria in 0.85% (*v/v*) saline solution.

5.3 Statistical Analysis

Statistical analysis was performed using Graphpad Prism6 Software (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed through two-way ANOVA using Tukey's and Sidák's mutiple comparisons tests with a p-value <0.05 considered statistically significant. Three independent experiments were considered. Data are expressed as mean ± SD.

5.4 Results and Discussion

In this Chapter the efficacy of both types of particles against *P. fluorescens* planktonic cells were assessed, for a contact time of 2 hours, and at increasing concentrations of BDMDAC of 100, 200 and 400 mg/L. The triplicate results obtained are depicted in Figure 5.1.

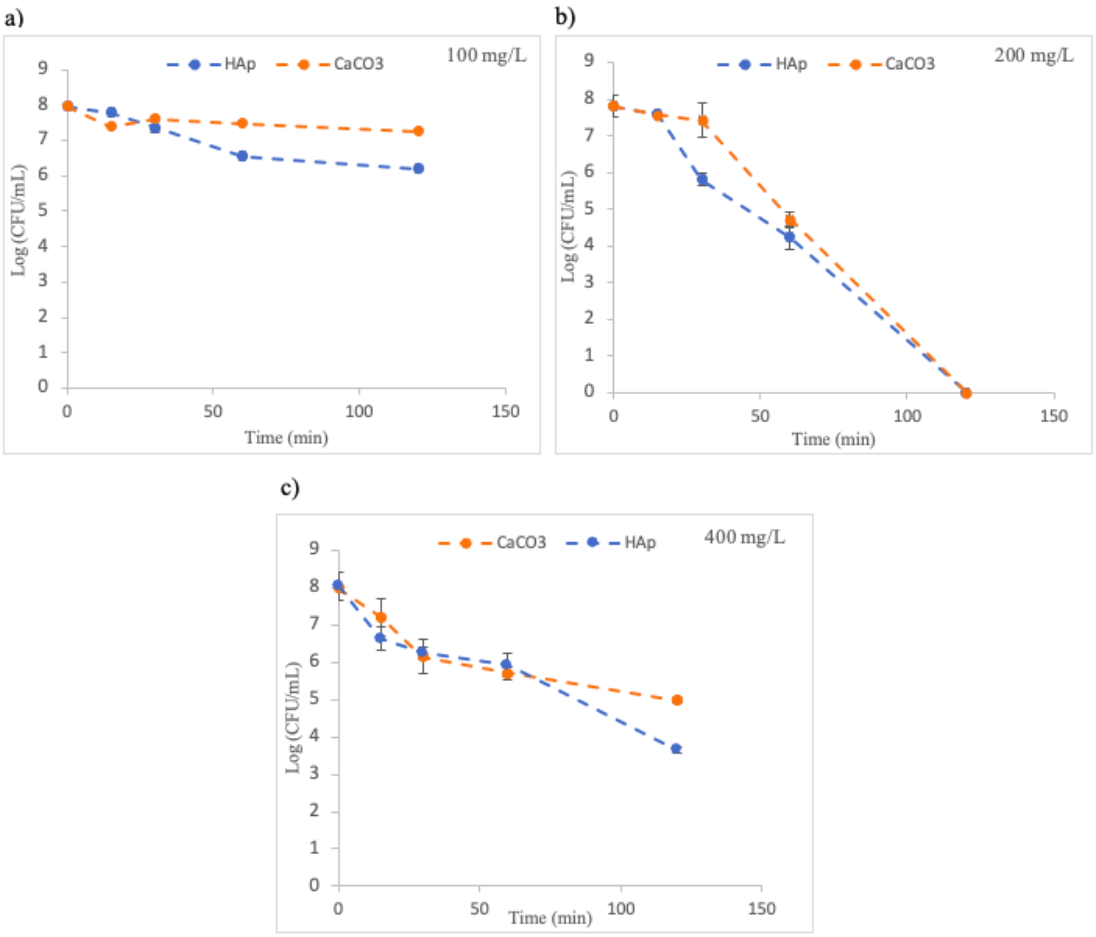

Figure 5.1: Log (CFU/mL) counts of *P. fluorescens* in contact with HAp and $CaCO_3$ coated microparticles as function of time (0, 15, 30, 60, 120 min), for BDMDAC concentrations of a) 100 mg/L, b) 200 mg/L, and c) 400 mg/L. The means ± SDs results of three independent experiments are illustrated.

When analyzing the results obtained at a concentration of 100 mg/L one can conclude that both functionalized particles have a clear antimicrobial effect, since the number of bacterial cells in their presence was significantly less than the value obtained for the control (i.e.: 0 min of contact time) ($p < 0.05$) Between the two cores used, it is safe to say that HAp had a better performance when compared to the $CaCO_3$ microparticles ($p < 0.05$), allowing almost a 2 log

reduction. This may be due to the latter's lower specific surface area and porosity when compared with HAp microparticles, decreasing the available BDMDAC. Despite this, their efficiency seems to be much reduced comparatively to when the free biocide is applied.

From Figure 5.1 b), it is concluded that despite the results obtained for the concentration of 100 mg/L, at 200 mg/L both coated cores allowed a total reduction of the CFU log counts, proving it is possible to kill all bacteria by their use and supporting their efficacy. However, evaluating the killing kinetics, HAp microparticles were seemingly, once again, more effective.

By observation of Figure 5.1 c), it is clear that, this time, the increase in concentration to 400 mg/L led to a decrease in the microparticles efficiency, as compared with the 200 mg/L. Nonetheless, at this concentration, it was noticeable that at the maximum contact time the HAp microparticles obtained better results than the $CaCO_3$ ones ($p < 0.05$), achieving approximately a 4.5 log reduction.

Overall, these results raise two main questions that will be addressed hereon.

i) **What resides behind the difference between the results obtained with the use of free biocide and the coated microparticles?**

As it was seen in Chapter 3 the value found for the MBC was, in fact, 100 mg/L for 1 hour contact with the biocide dissolved in the liquid (Figure 3.2). In view of this reality, one must wonder what could be responsible for this difference, more notably since even an extended time of contact did not allow for the total reduction of CFU log counts at this concentration (Figure 5.1 a)). In this concern, if the underlying mechanism by which the biocide acts upon bacteria is different in the free and immobilized forms, divergent results are foreseeable.

At this point, it should be stressed that assuming the biocide is not released from the particles, its consumption will be minimal when compared to the case where the same biocide is dissolved in water. This means that free and immobilized concentrations are not comparable *per se* when assessing the biocidal efficacy. In truth, it seems conceivable that the interaction between the biocide and bacteria will differ amongst the two forms, and therefore the use of the microparticles could be *apparently* more disadvantageous. Although many studies have been conducted on the efficiency of biocide activity, the mechanism of antimicrobial particles is far from being completely unveiled, leading to the formulation of different conjectures as to the mode of action behind immobilized biocides.

Delving deeper into this matter, many possible means by which the coated microparticles kill planktonic cells have been proposed (Kaur and Liu, 2016). However, this study will focus on three likely possibilities, based on previous work by Barros *et al* (2021): a) the slow release of free BDMDAC to the bulk solution; b) direct contact of the coated microparticles with the cells; c) a combination of the two first hypothesis. Since the tests performed in Section 4.1.4 seem to show no significant release, hypothesis a) seems unlikely by merely itself [1]. In its turn, option b) seems to stand in a stronger foundation.

Contact killing is based on the use of non-eluting surfaces that, by killing bacteria upon contact, as the name itself indicates, possess a long-term durability, reduced development of resistance and are environmental-friendly, therefore being considered a sustainable alternative to biocide leaching approaches (Li et al., 2006; Kaur and Liu, 2016). In this matter, the specific modes of action of surface bound QACs have been studied over the years, originating several theories although a definitive consensus is yet to be achieved (Kaur and Liu, 2016). Despite this, their action is mainly attributed to electrostatic interactions between the positively charged biocide and the negatively charged phospholipids of the cytoplasmatic membrane, besides punching of the membrane by the hydrophobic alkyl chain of QACs (Barros et al., 2021). Assuming contact killing is present, it is possible to convey that in the presence of pores not all the biocide will be promptly available (Ricardo, 2017), thus increasing the necessary contact time, which is in accordance with the results obtained.

The hypothesis of a combination of the two effects can neither be completely held true, nor discarded, caressing a more thorough assessment, especially as to the elements that sustain hypothesis a). To further test this possibility, reutilization assays could be carried out in order to understand if the BDMDAC remains immobilized on the particles after killing.

Additionally, if microparticle agglomerates are being formed, as it will be discussed further ahead, they may diminish the amount of available biocide when compared with its free form. Although it is dubious if the extent of this phenomena at a concentration of 100 mg/L would be enough to justify the observed depletion, previous work has sustained and presented this as a probable proposition (Antão, 2015). Therefore, it should not be dismissed.

On the grounds of all the above explanation, it is understandable that different results are expectable.

[1] Once again, it is important to underline the importance of evaluating the existence of particle agglomerates that could be interfering with the results obtained in the release assays. Please, see Chapter 4.

ii) **What is the relation between the biocide's concentration and the particles efficiency?**

Intriguingly, the killing assays conducted at 400 mg/L raised new questions. It was noticed that, against what could be intuitively expected, increasing the concentration of biocide does not always correspond to an enhanced antimicrobial activity. The reason behind this might be related with an increase of the agglomeration/aggregation [2] effect and/or a carbon chain entanglement effect.

The processes of nanoparticle agglomeration are major obstacles in the reproducibility of toxicity assays, as well as in the interpretation of such results. In fact, various NPs (nanoparticles) and MPs (microparticles) are propitious to agglomeration in aqueous biological matrices, such as cell culture media, given the high ionic strength and neutral pH usually used in biological experiences (Bihari et al., 2008; Chithrani et al., 2006). In biological media, agglomeration can occur because of the screening of the surface charge due to the present electrolytes. By the shielding of electric field, the particles come together, and may, ultimately sediment (Li et al., 2012). A myriad of precursory literature provides a clear understanding that this effect is expected to influence particles' toxicity, by affecting cellular uptake, decreasing the effective specific surface area (Liu and Hurt, 2010), increasing the sedimentation rate (Kato et al., 2010) and changing the shape into a fractal (Li et al., 2012; Zook et al., 2011). Furthermore, it has been stated that various nanoparticles will agglomerate to some extent in biological or environmental solutions before they are able to reach their biological target (Sharma, 2009).

The most widely accepted theory of aggregation goes by the name of DLVO (Derjaguin-Landau-Verwey-Overbeak). In short, this theory defends that there are two main forces responsible for the interaction between particles: van der Waals (vdW) and electrostatic double layer forces (EDL). The first one reflects the attraction between particles, while the second possesses a charge that is an expression of the surface that it surrounds, as well as of the chemistry of the surrounding media. The sum of these forces ultimately predicts the attractive or repulsive nature of the particle's interaction. When the attractive forces surpass the repulsive ones, aggregation is likely to occur (Hotze et al., 2010).

This theory can be summarized by Figure 5.2.

[2] The two terms are often interchangeable. However, aggregation is related to strongly bonded or fused particles, while agglomeration relates to a weaker bond. The term agglomeration will be used when exploring the results throughout this thesis, as a wider term, since it is not known whether the energy involved in the chosen processes is enough to fuse the particles irreversibly.

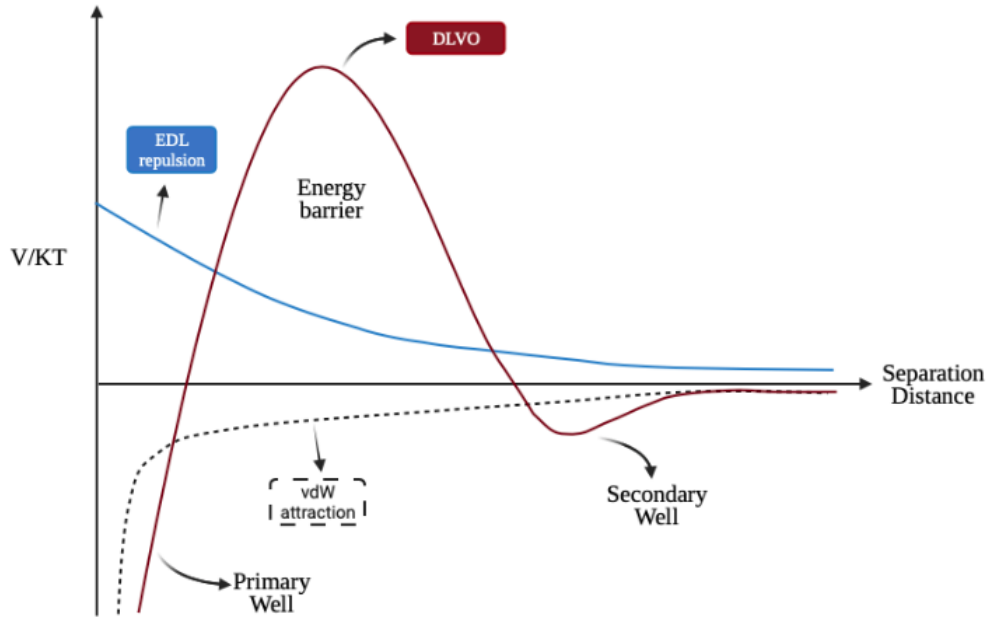

Figure 5.2: DLVO theory plot. (adapted from Hotze *et al* (2010))

From the represented plot, it is concluded that the relation between the separation distance and the likelihood of agglomeration is not a straightforward one. As such, it is possible to speculate the meaning of the results obtained from the following point of view.

On the starting point that a higher concentration of biocide corresponds to a higher volume of particles in solution, increasing concentration leads to more proximity between the microparticles. At 100 mg/L, although they are farther away from each other, thus minimizing agglomeration, the amount of biocide available per unit of volume seems to be insufficient to promptly kill the existent bacteria. By this logic, an intermediate concentration corresponds to an intermediate separation distance. In the assays at 200 mg/L, the amount of biocide is higher and, although the particles are closer together, this is not enough to promote their agglomeration. As opposed to this, at increased concentration of particles in solution, 400 mg/L, their proximity may favor the attraction by the effect of vdW forces, which ultimately promotes agglomeration.

Precursory studies have inferred the correlation between the aggregation state of nanoparticles and *in vitro* toxicity. In conclusion, it was postulated that the particle's behavior would be as depicted in Figure 5.3.

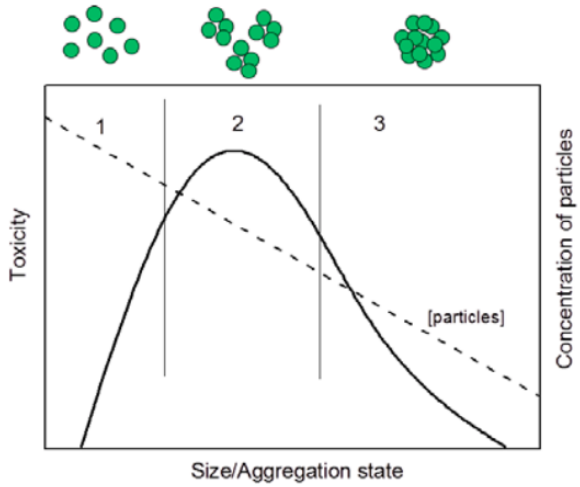

Figure 5.3: Relation between the aggregation and toxicity of nanoparticles: (1) Stable dispersion; (2) Small aggregates; (3) Big aggregates. (Ngoc, 2015)

By observation of this figure, it is possible to comprehend that until a certain point, agglomeration may be beneficial for the antimicrobial activity, but when surpassing a critical value it becomes so high that it interferes in a detrimental way with the killing processes. This seems to be in accordance with the pattern observed in the assays performed.

Parallelly to agglomeration, and as important, the effect of carbon chain entanglement must be considered. According to literature, in n-alkyl-QACs where the n-alkyl chain length is increased beyond 10, attraction between the adjacent hydrophobic chains may exceed the electrostatic repulsion of their charged nitrogen head groups (Gilbert and Moore, 2005). Consequently, when BDMDAC is associated with microparticles and above a certain concentration, the available biocide is reduced as a consequence of this effect, thus hindering the antimicrobial activity. This line of thinking can be applied to the assays at 400 mg/L, where the entanglement effect may indeed be propelled by a high concentration of biocide, leading to a reduced biocide availability and, consequently, efficacy.

5.5 Conclusions

By analysis of the killing kinetics and comparison between HAp and $CaCO_3$ particles, it is apprehended that the best results were obtained with HAp particles at a concentration of 200 mg/L and a contact time of two hours. These particles showed, for every concentration tested, a higher efficiency than the ones synthesised from $CaCO_3$, which is in accordance with their higher specific surface area. However, when increasing the concentration up to 400 mg/L, the likely agglomeration of the microparticles is held accountable for a significant decline in the antimicrobial efficiency, although further experiments would be necessary to understand this effect.

On the whole, the application of the microparticles should be judged weighing their antimicrobial effect alongside with other particularities, which are shown to be valuable advantages. In point of fact, although it is true that both higher concentration of biocide and higher contact time had to be employed in order to kill all bacteria, when compared with the use of free biocide, if it is confirmed they act mainly by contact killing this increase would not be a significant issue face to face with the advantageous impacts of this strategy. By not releasing BDMDAC to the bulk liquid, paving the way to the possibility of reutilization, the use of BDMDAC coated microparticles decreases the amount of DBPs formation, the release of toxic and carcinogenic compounds to the streams and increases the overall cost-benefit ratio of disinfection.

It is clear that the mechanisms of action of the BDMDAC coated particles need thorough study, particularly regarding the effects of agglomeration in antimicrobial activity.

Chapter 6

Microparticles Efficiency on the Prevention of Biofilm Formation

6.1 Introduction

As mentioned it Chapter 2, layers of microorganisms and their extracellular polymers lead to increased costs of production and maintenance, public health concerns and environmental issues. In the Section 2.1.4, the importance of applying preventive measures in order to circumvent these problems, instead of investing resources in fixing them, was also underlined. Although a wide number of such strategies are already commonly used, such as the optimization of process conditions and equipment design, research on new surfaces that minimize adhesion and the use of various cleaning and disinfection methodologies, these have often shown to be insufficient, to have a poor cost-benefit relation or to carry with them severe consequences. For instance, although liquid velocities of around 2 m/s are recommended in pipes to reduce biofilm growth by the increase of shear stress, one could argue that there are two sides to this coin, since high velocities result in compacter deposits, that are harder to remove/clean (Melo and Vieira, 1999; Simões, 2005). Regarding the research on new surfaces, the development of surfaces coated with antimicrobials are dependent on the relation between their cost and the costs that result from the presence of biofilms (Rosmaninho et al., 2007). Although these strategies often lead to the emergence of deposits that are more susceptible to cleaning and disinfection, they are faced with two major obstacles. The first one is related to the fact that biofilms do not attach uniformly to the surfaces and the second is that, to a great extent of these procedures, the disinfectants are carried as solutes by the bulk liquid and remain in the discharged waters, since in truth only a small percentage of the disinfec-

tants takes part in the disinfection reactions (Ludensky, 2003). Furthermore, the continuous use of such chemicals has implications in arising antimicrobial resistance (Tenover, 2006). Micro-nanotechnology represents an innovative, versatile, and promising alternative for creating more effective cleaning techniques that are environmentally friendly and minimize health risks.

Within this framework, this Chapter focuses on the use of coated antibacterial microparticles for the prevention of biofilm formation. For a better understanding and analysis of the results, it is important to understand the lifecycle of the bacterial strain in question, *P. fluorescens*. The study of this cycle in glucose medium, has been described by (Rasamiravaka et al., 2015), as depicted in Figure 6.1. This will serve as a point of reference to position ourselves in the stages of biofilm formation.

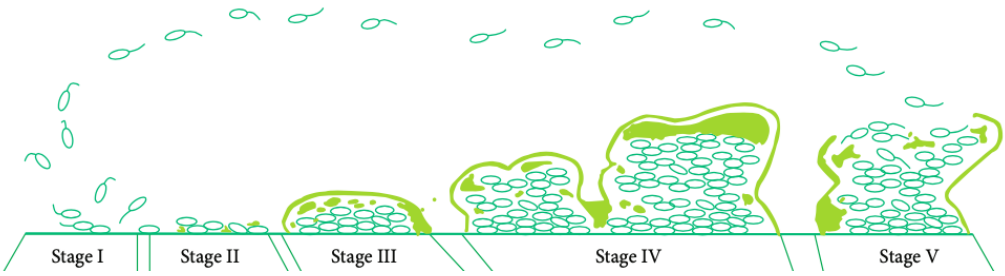

Figure 6.1: *P. fluorescens* biofilm lifecycle. In stage I (2 h), attachment to the surface is initiated by planktonic bacteria. This attachment becomes irreversible in stage II (8 h). In stage III micro-colony formation takes place (14 h). Stage IV depicts biofilm maturation and growth of the 3D community. In stage V, dispersion occurs - planktonic bacteria colonize other sites. The time spans described are according to the conditions in the referenced study (Rasamiravaka et al., 2015).

The biocide selected, benzyldimethyldodecylammonium chloride (BDMDAC), is usually applied in industrial systems as cooling water systems at a concentration of 50 mg/L of active product and, when cleaning is the goal, by recirculation for several hours (McCoy, 1974; Block, 2001). The strategy here proposed hopes to go a step forward in reducing the environmental load in the discharged waters, since the immobilization of the biocide on the particles should allow the reuse of the chemical that was not spent. In fact, regarding the $CaCO_3$ particles, reutilization assays have already been carried out in previous work, with positive outcomes (Ferreira et al., 2013).

This goal of this chapter is thus to assess the efficacy of both types of the coated particles, HAp and $CaCO_3$, in the prevention of biofilm formation.

6.2 Materials and Methods

6.2.1 Microorganism and Culture Conditions

The *P. fluorescens* strain chosen for this work was the same as described in 3.1.1. Bacterial cultures were prepared in sterile flasks containing sterile growth medium, Terrific Broth (TB), and an appropriate volume of bacterial inoculum. This medium is available as a nutritionally rich medium for the growth of bacteria, with increased concentrations of peptone, yeast extract and glycerol as a carbon source (Tartof, 1987). For the preparation of 1 L of this medium, 12 g of tryptone, 24 g of yeast extract and 4 mL of glycerol in 900 mL of H_2O are autoclaved and cooled off until 60ºC or less. Then, 100 mL of phosphate buffer (previously prepared, autoclaved and kept at 4ºC) are carefully added in the flow chamber. An overnight (16-18 h) grown culture of *P. fluorescens* (30ºC, 120 rpm) was centrifuged (4000 g, 10 min) and washed in TB. In order to obtain an optical density at 610 nm (OD_{610nm}) of approximately 0.2 ± 0.02 bacteria were resuspended in TB.

6.2.2 Microparticles Efficiency on the Prevention of Biofilm Formation

The assessment of the efficacy of the coated microparticles in the prevention of biofilm development was carried out in 12-well round-bottom polystyrene (PS) microtiter plates with a total well capacity of 3 mL. The surfaces chosen for testing biofilm formation were 9x9mm PVC coupons, previously sterilized and placed in the center of each well. With this purpose, coupons were first cleaned using humidified paper with 70% (v/v) alcohol. Double sided duct tape was put in each sampling well and placed in the flow chamber under UV for 30 minutes, alongside with the coupons in an open petri dish, with the bottom side turned up. The coupons were carefully placed on the duct tape, followed by another 30 minutes of UV to sterilize the upper face of the coupon.

The concentration of BDMDAC tested was based on the results presented in the previous chapter, having into consideration the one that showed to be the most effective in killing planktonic cells: 200 mg/L. It is important to underline, that although concentrations much higher than the MBC are used against previously established biofilms (since they can be 10 to 1000 times more resistant), the objective in these assays was to impede biofilm development in the first place, which would precisely have the added advantage of impeding such high dosages (Davies, 2003). The necessary volume of particles, calculated as described in Section 5.2, was centrifuged (4000 rpm, 5 min) and resuspended in 3 mL of bacteria. The wells were then filled according to Figure 6.2 in the flow chamber, and the plates were incubated for 48 hours (30º C, 160 rpm).

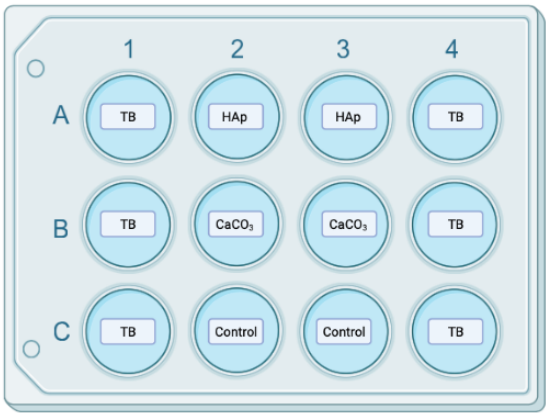

Figure 6.2: Microplate set-up as used in the experiments. TB-wells filled with only medium; HAp-bacteria in contact with HAp microparticles; $CaCO_3$-bacteria in contact with $CaCO_3$ microparticles; Control-bacterial suspension only.

Three independent assays were performed. In each, two plates were separately filled in the same conditions: one intended for CFU counts and another intended for OCT (Optical Coherence Tomography) analysis.

In a flow chamber, the coupons were carefully removed with a sterile tweezer and placed in another well previously sterilized and filled with saline solution, to remove the non-adhered cells. Each coupon was then placed in a falcon filled with 3 mL of saline solution, subjected to an ultrasound bath for 2 minutes and vortexed for another 2 minutes. Then, CFU counts were made as described in Section 5.2.

6.2.3 Optical Coherence Tomography (OCT)

The morphology of the developed biofilms was analyzed through optical coherence tomography (OCT) (Thorlabs Ganymede Spectral Domain Optical Coherence Tomography system, Thorlabs GmbH, Dachau, Germany). In the case of the coupons for OCT analysis, medium was removed by pipetting carefully not to disrupt any present biofilm and washed twice with saline solution (0.85%).

6.3 Statistical Analysis

Statistical analysis was performed using Graphpad Prism6 Software (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed through two-way ANOVA using Tukey's and Sidák's

mutiple comparisons tests with a p-value < 0.05 considered statistically significant. For each time span, three independent experiments with two replicates each were considered. Data are expressed as mean ± SD.

6.4 Results and Discussion

In a first approach, biofilms were allowed to develop for 48 hours in the Terrific Broth medium. However, as it will be further described, when analyzing the OCT images it was argued that by extending the time span of development to 72 hours, with medium replenishment at 24 hours (thus avoiding starvation of the cells and the accumulation of toxic compounds) the conditions of growth could be optimized and allow a better understanding of the effect of the functionalized microparticles in biofilm development. The results obtained in both experiments (each one involving three independent assays, with two replicates each), at a BDMDAC concentration of 200 mg/L are summarized in Figure 6.3.

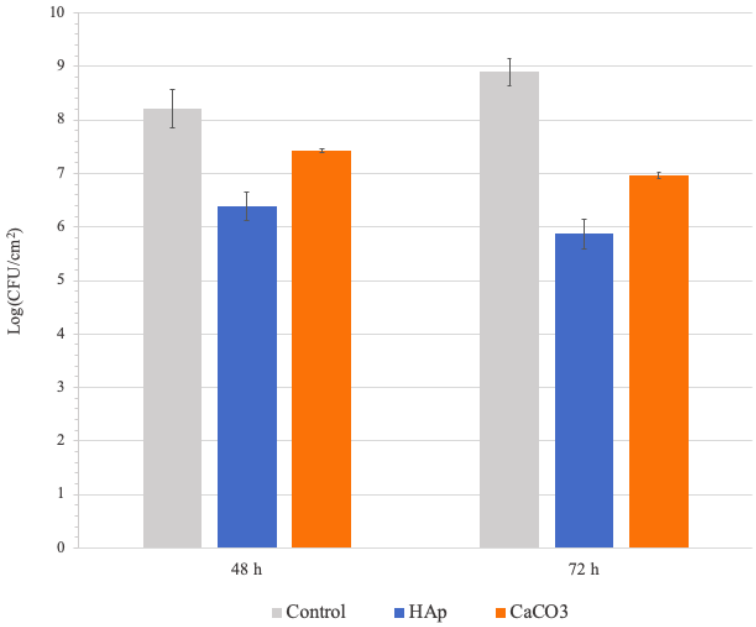

Figure 6.3: Log(CFU/cm^2) counts after 48 and 72 hours at BDMDAC concentrations of 200 mg/L (HAp and $CaCO_3$). In control experiments, the wells were filled solely with bacterial suspension. The means ± SDs for three independent experiments, each with two replicates, are illustrated.

It was verified that in the experiments carried out at 48 hours, there is a clear log CFU/cm^2 reduction when comparing the control wells and the wells where bacteria is in contact with HAp biocidal particles ($p < 0.05$) This once again shows their evident antimicrobial effect, accounting

for almost a 2-log reduction of bacteria. The log CFU/cm^2 counts from the control wells and the $CaCO_3$ particles were also statistically different ($p < 0.05$, although the decrease is less than 1 log and, therefore, not substantially significant. Between the two types of particles, as expected, the results reinforce the higher efficiency of HAp BDMDAC coated particles ($p < 0.05$)

Analyzing the results obtained in the 72 hours assays, the biofilms grown with no microparticle contact, showed to have higher bacterial density, than when compared with the ones developed for 48 hours in the same conditions ($p < 0.05$) Besides this, a difference between the control wells and the wells in which *P. fluorescens* was under antimicrobial action by $CaCO_3$ microparticles was now easily precepted ($p < 0.05$) This reflected in approximately a 2-log reduction. The same is easily conceivable analyzing the results obtained in the presence HAp microparticles, where a higher difference to the control was now perceived ($p < 0.05$) This translated to an approximately 3-log reduction. Comparing both types of particles, their efficacy was indeed, and once again, significantly different ($p < 0.05$)

The mere observation that at 72 hours a higher difference was obtained between the control and the microparticles, comes to show that biofilm development was hindered to a great extent. Moreover, even though for each tested particle the log counts at 48 and 72 hours were not statistically different ($p > 0.05$), their success in restraining biofilm formation is undeniable. In fact, as it can be acknowledged from Figure 6.1, no cell regrowth was noted in the extended experiments and, furthermore, a slight decrease can indeed be precepted. Overall, this brings a new perspective to the meaning of the outcomes, that will now be investigated deeper.

As mentioned before, the antimicrobial action of the biocide in its free form and in the coated particles is non-identical. In this matter, the absence of cell regrowth observed in the extended time experiments indicates that the particles are expected to not only initially kill the planktonic cells, affecting the first steps of biofilm formation, but also to compromise its entire development through the next phases. Adding up to this, it is known that throughout biofilm formation, bacteria sensitivity to drugs presents a dynamic change (Fu et al., 2021). In stage I, bacteria passes from a free-swimming phase to a surface-attached phase, in which the interactions between the cell and the surface may trigger mechanically sensitive channels on cell wall opening. From this effect, bacteria will be more susceptible to the biocide (Girish et al., 2019). Concomitantly, the cells that were able to attach start to interact with each other, by getting more proximate. This will allow cells to divide and grow, but also to become increasingly sensitive to the BDMDAC that may be still acting upon them at this phase. In fact, surface-attached cells can be more sensitive to

antibiotics than planktonic cells during early stage, and the 3D structures of early-stage biofilms have not been formed and are characterized by fragile structures (Fu et al., 2021). Furthermore, although microparticles could be removed to some extent upon medium replenishment, some may have remained in solution and contributed with a continuous antimicrobial effect through time. In short, it may be argued that the biocide in the microparticles is not consumed during contact with the cells, as opposed to the free biocide, meaning it has a persistent effect on the biofilm cells. This also has the added advantage of enabling the reutilization of the microparticles, which would not only save a considerable amount of biocide, but also allow to circumvent the aforementioned environmental issues associated with its use.

Despite the success reflected by the results until now, these raise questions regarding which factors may be interfering negatively with the process and the outcome.

Having into account the results obtained in Chapter 5, if the same concentration in such experiments for a 2 hour contact managed to kill all bacteria, it could be expected that the same would happen here and, therefore, no cells would be available for biofilm formation. Nevertheless, it should be noted that the medium in use was different, and that TB is a nutritionally rich medium for the growth of bacteria (Tartof, 1987).

In this concern, VBNC (Viable But NonCulturable) cells which were not evaluated and are potentially present in the assays of the Chapter 5, find in it optimal conditions for growth and can now be accounted for. The ability of cells to resuscitate from the VBNC state and return to an actively metabolizing and culturable form once the conditions are more favorable is well described in literature (Oliver, 2005). On the other hand, Fagerlind *et al.* (2012) stated the higher substrate concentration also resulted in faster biofilm development, which is in accordance with the characteristics of the Terrific Broth. Besides this, the influence of growth media on the sensitivity of different bacteria strains to biocides is well described in previous literature (Brill et al., 2006; Brown, 1977; Gilbert et al., 1987). Furthermore, it has been shown that cellular culture media are in dynamic change in composition and properties, which ultimately affects micro-nanoparticles' agglomeration and influence their cytotoxic effect. This comes to show that the colloidal stability of microparticles as a function of serum/extracellular environment/cellular growth media cannot be overlooked, since it can result in divergent conclusions in nanotoxicology, as observed (Alkilany et al., 2016).

In this scenario, agitation also plays its part since it may be sufficient to aid cell growth, but insufficient to avoid microparticles' agglomeration and sedimentation. These two factors combined,

act in favor of biofilm development.

All things considered, it is comprehensible that the results obtained are not directly comparable with the ones obtained in Chapter 5 and that what that seemed contradictory at first sight, is now reasoned and expected - although not fully understood to all their extent. In truth, the antimicrobial coated particles, and particularly HAp, show promise and may lay the foundation for a new strategy in biofilm prevention.

In search for a deeper understanding of the observed results and the hypothesis proposed, the OCT images are useful to evaluate the biofilms' morphology. Alongside with these images, comes the understanding that the extended time span of the experiment allowed, indeed, a better comprehension of the results. Figure 6.4 depicts representative images of the biofilm grown in the control wells.

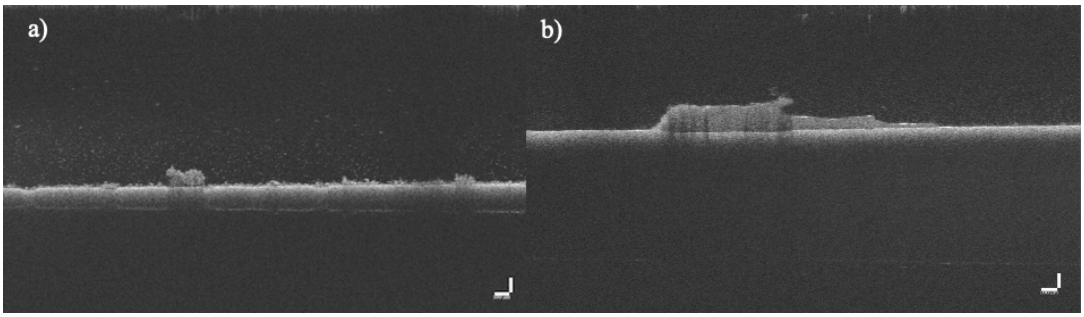

Figure 6.4: OCT images of *P. fluorescens* biofilms at a) 48 hours; and b) 72 hours; in TB medium.

It is possible to convey that the biofilms developed for 48 hours were distributed all over the surface, although thinner. At 72 hours biofilms were thicker and much more developed. These structures were also found to be well established all over the surface.

On the other hand, when cells were put in contact with the particles for 72 hours, the biofilms observed were much less developed, as depicted in Figure 6.5.

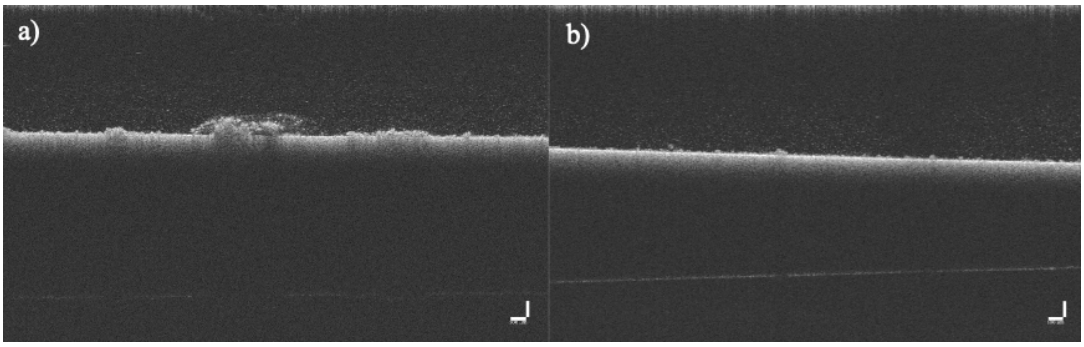

Figure 6.5: OCT images of *P. fluorescens* biofilms at 72 hours in contact with a) $CaCO_3$; and b) HAp microparticles.

Another interesting feature can be observed in the OCT images: the presence of what seem to be microparticle agglomerates. This comes to support the theory that particle agglomeration can be hindering the antimicrobial effect of BDMDAC, as depicted in Figures 6.6, regarding HAp microparticles.

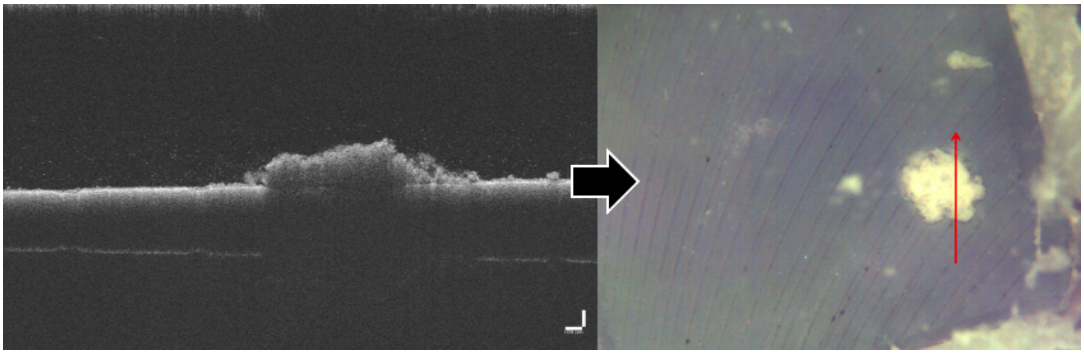

Figure 6.6: OCT images of possible HAp microparticles' agglomerate. On the right: Respective coupon's surface.

The same type of structure was identified for the cases where bacteria was in contact with $CaCO_3$ microparticles.

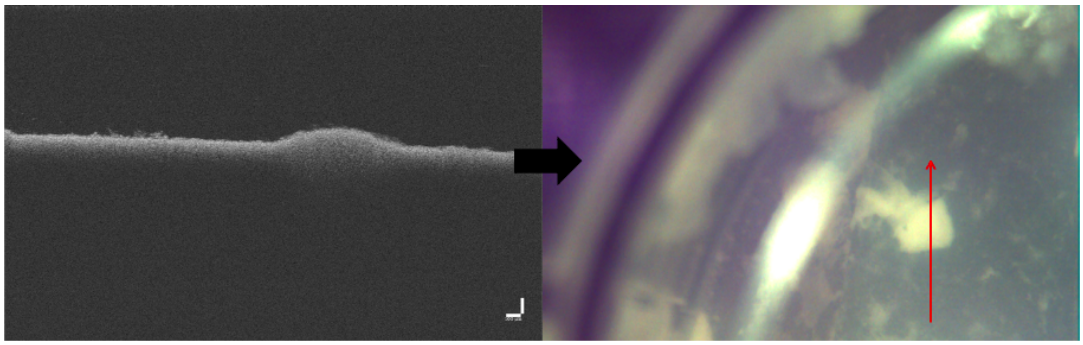

Figure 6.7: OCT images of possible $CaCO_3$ microparticles' agglomerate. On the right: Respective coupon's surface.

Overall, the results obtained were in accordance with the CFU/cm^2 log counts and provided more information about the possibility of microparticle aggregation.

6.5 Conclusions

From the presented results it can be concluded that none of the coated microparticles was able to completely prevent biofilm formation. This may be due to agglomeration effects, promoted by the rich medium, and the carbon chains entaglement effect.

Despite this, both particles presented a clear antimicrobial effect, and a promising performance against the development of *P. fluorescens* biofilms. The biocide is expected to act upon the first stages of biofilm formation, and hinder their entire development thereon, inhibiting cell regrowth. It was also stated that, in contrary to the free biocide, the BDMDAC in the particles is not consumed, which paves the way to their reutilization and to a sustainable strategy.

The hydroxyapatite coated particles are overall more effective than the $CaCO_3$ in achieving this, which is in accordance with the results obtained in the previous Chapters, regarding particles' characterization and effectiveness against planktonic cells. In fact, the higher efficiency of HAp is partly attributed to their higher porosity and specific surface area. All the above conclusions were reiterated by the visualization of OCT images.

To sum up, HAp BDMDAC coated microparticles once again proved their worth towards the development of new biofilm prevention strategies.

Chapter 7

General Conclusions and Future Work

7.1 Conclusion Remarks

The main goal of this work was to developed HAp functionalized microparticles, able to incorporate BDMDAC in such a way that it would allow a safe, economic, and environmental-friendly strategy for the prevention of biofilms in anthropogenic aqueous environments, such as industrial and healthcare facilities. As a term of comparison, $CaCO_3$ coated particles, already investigated in precursory studies, were also developed.

The antimicrobial activity of the biocide was first evaluated in terms of MBC and the concentration exponent. The results were considered to be in accordance with what was previously described in literature, regarding the typical dosage of QACs and their action.

The LbL technique was employed, using HAp and $CaCO_3$ cores, two different widely use polyelectrolytes (PEI and PSS) and the antimicrobial agent BDMDAC. The coating process was considered to be successful, as confirmed by HPLC and BET analysis. Moreover, the HAp microparticles were found to have a higher specific surface area and porosity, thus favouring a stronger antimicrobial activity.

When assessing the efficacy of the coated particles, the latter was shown to be affected by the concentration of immobilized biocide in solution and, consequently, the concentration of particles in solution. It was concluded that a higher concentration of BDMDAC does not always correspond to a higher efficacy. In fact, past a certain value, the outcome will actually be the opposite due to the possible microparticle agglomeration and carbon chain entanglement effects. In terms of results, amongst the concentrations tested, 200 mg/L was the only that allowed total bacteria reduction, doing so for both types of cores. In general, HAp microparticles exhibited a better performance.

Regarding the prevention it was shown that both functionalized microparticles bring undeniable consequences to biofilm formation, hindering their development into mature biofilms and impeding cell regrowth. Once again, the HAp microparticles showed to be more effective.

In conclusion, the work herein developed may pave the way for a new strategy in biofilm prevention, which shows many advantages. Firstly, and most importantly, it was indeed proven that the microparticles developed successfully affect and minimize biofilm formation. Besides this, the possibility of using waste from several industries as raw material makes this a sustainable alternative to other existent core options. Furthermore, by not leaching the biocide to the surrounding media, these particles circumvent the issues associated with DBP formation and release of toxic compounds in the water. By seemingly acting from a contact-killing mechanism, instead of leaching the biocide, the microparticles may even be reutilized, adding up to their sustainability and economic advantages.

7.2 Future Work

This work set out to be a step forward in the development of new strategies for biofilm prevention. In it is the hope of creating a more sustainable and safe generation of micro-nanotechnology solutions, minimizing health risks, environmental costs, and the economic repercussions for many industries. However, as it is possible to understand by the ongoing discussion throughout this thesis, some questions remain unanswered and would benefit from further research. Future work can be divided in 3 sections: MPs characterization, MPs antimicrobial activity and MPs sustainability.

MPs Characterization

i) Zeta Potential: By analysing the zeta potential values it is possible to identify charge changes of the particles after each layer assembly, allowing a deeper understanding of how the coating process affects particle charge. This also allows to to assess particle stability and their tendency to agglomerate.

ii) Number and Volume Size Distribution: To assess the effect of the BDMDAC coating process on the physical characteristics of the particles.

iii) SEM (Scanning Electron Microscopy): To analyse the integrity/morphological characteristics of the non-coated and coated particles, while giving further insight into their tendency for agglomeration.

MPs Antimicrobial Activity

i) Mode of Action: To study this matter, bacterial surface charge before and after exposure to BDMDAC can be assessed by zeta-potential analysis. Besides this, it is also important to further evaluate the existence of VBNC cells.

ii) Effect of particle concentration: It is clear that the relation between particle concentration and antimicrobial activity needs to be assessed more thoroughly. This parameter is closely related with the biocide's availability for killing bacteria in suspension due to agglomeration.

iii) Effects in Biofilm development: To better understand how the developed particles affect biofilm formation, EPS extraction could be carried out to understand the biofilm's composition.

MPs Sustainability

i) Reutilization Assays: The possibility of reusing the microparticles is one of the major assets of this strategy. As such, the particles performance upon reutilization must be evaluated.

By carrying out the tests proposed above, an appropriate choice of application could be carried out. As such, the efficacy of the developed particles could later on be tested in a larger scale to mimic the conditions found in industrial environments. Regarding this, the microparticles could, for example, 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 strategy shows promise and may be a step further in biofilm prevention, benefiting public health, the environment, and economy.

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Appendix A

Time Dependence

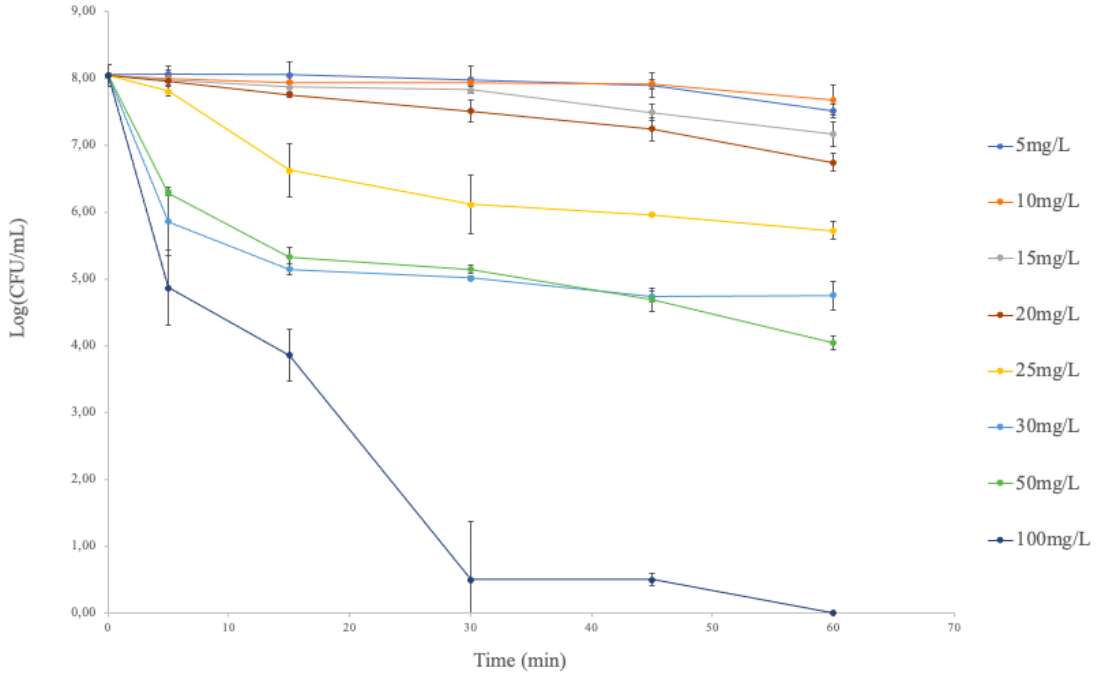

Figure A.1: Log (CFU/mL) counts of *P. fluorescens* for the different concentrations of free biocide tested (5, 10, 15, 20, 25, 30, 50, and 100 mg/L) as a function of time (5, 15, 30, 45, and 60 min). The means ± SDs results of three independent experiments are illustrated.

Appendix B

BET Analysis

B.1 BET Equation

In BET surface area analysis, nitrogen is usually used because of its availability in high purity and its strong interaction with most solids. The data is processed by plotting the correspondent isotherms of this process. The BET equation uses the information from the isotherm to determine the surface area of the sample, where X is the weight of nitrogen adsorbed at a given relative pressure (P/P_0, where P_0 is the saturation pressure), Xm is monolayer capacity, which is the volume of gas adsorbed at standard temperature and pressure (STP), and C is constant. STP is defined as 273 K and 1 atm.

Ideally five data points, with a minimum of three data points, in the P/P_0 range 0.025 to 0.30 should be used to successfully determine the surface area using the BET equation. At relative pressures higher than 0.5, there is the onset of capillary condensation, and at relative pressures that are too low, only monolayer formation is occurring (Hwang and Barron, 2011).

$$\frac{1}{X[(P_0/P)-1]} = \frac{1}{X_m C} + \frac{C-1}{X_m C}\left(\frac{P}{P_0}\right)$$

B.2 Pore Size Distribution

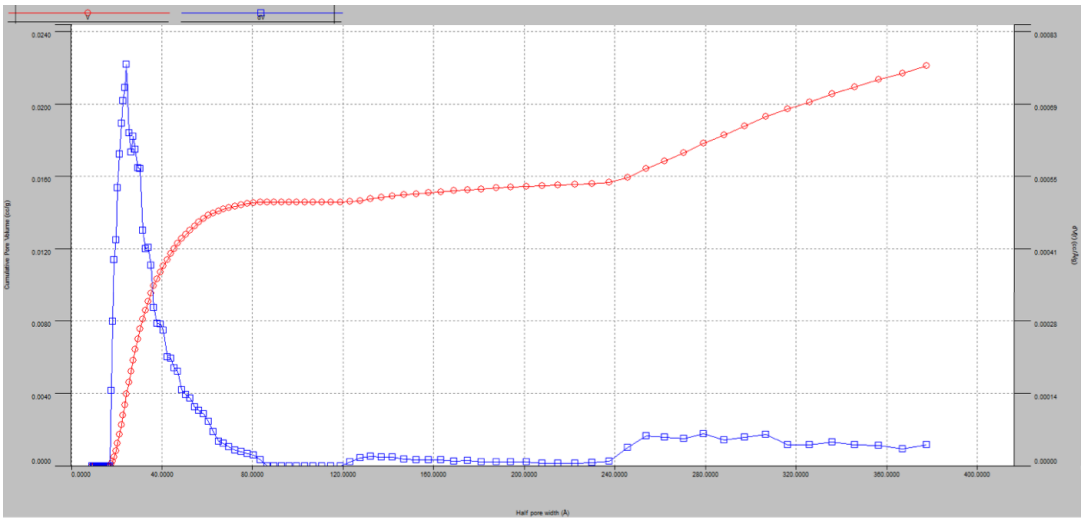

Figure B.1: Pore size distribution of $CaCO_3$ microparticles before functionalization.

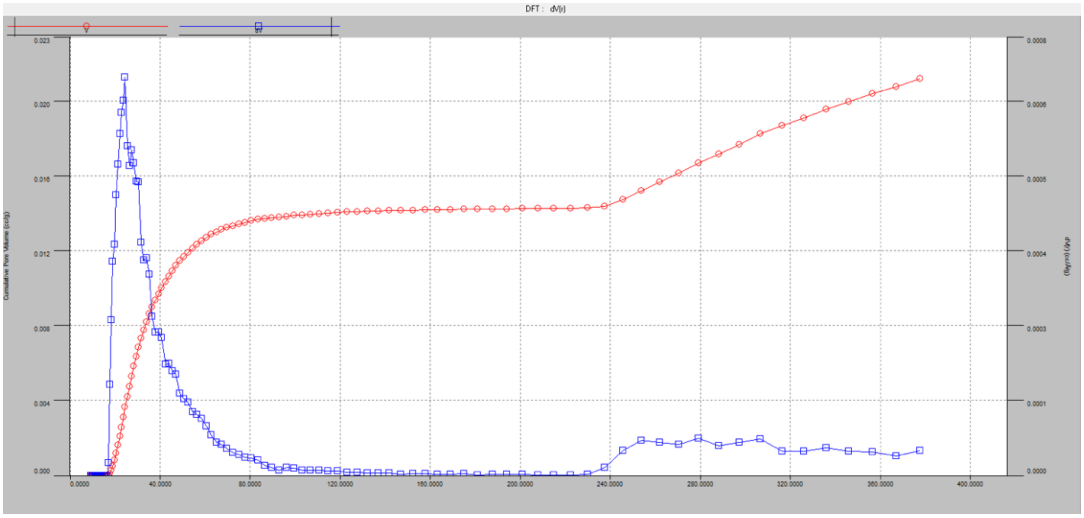

Figure B.2: Pore size distribution of $CaCO_3$ microparticles after functionalization.

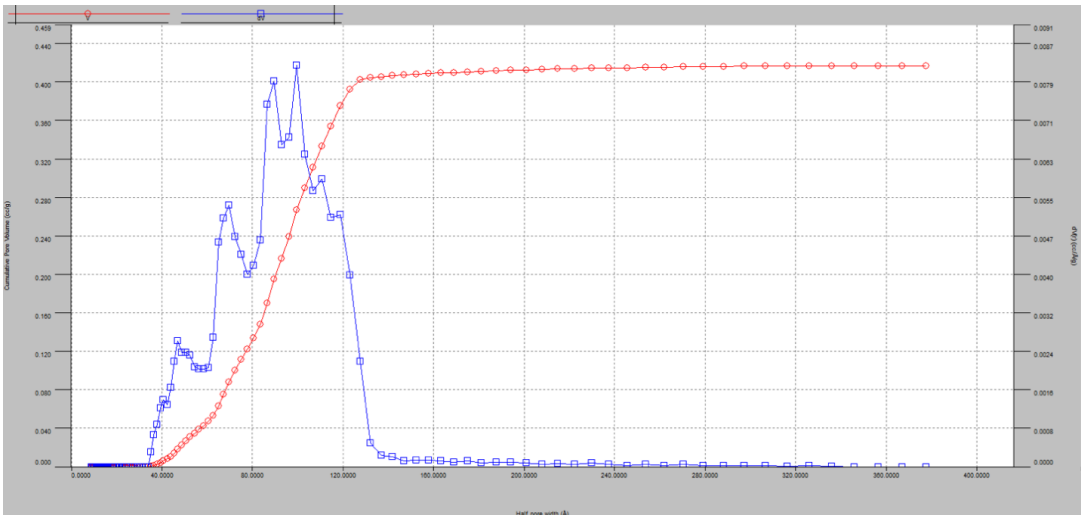

Figure B.3: Pore size distribution of HAp microparticles before functionalization.

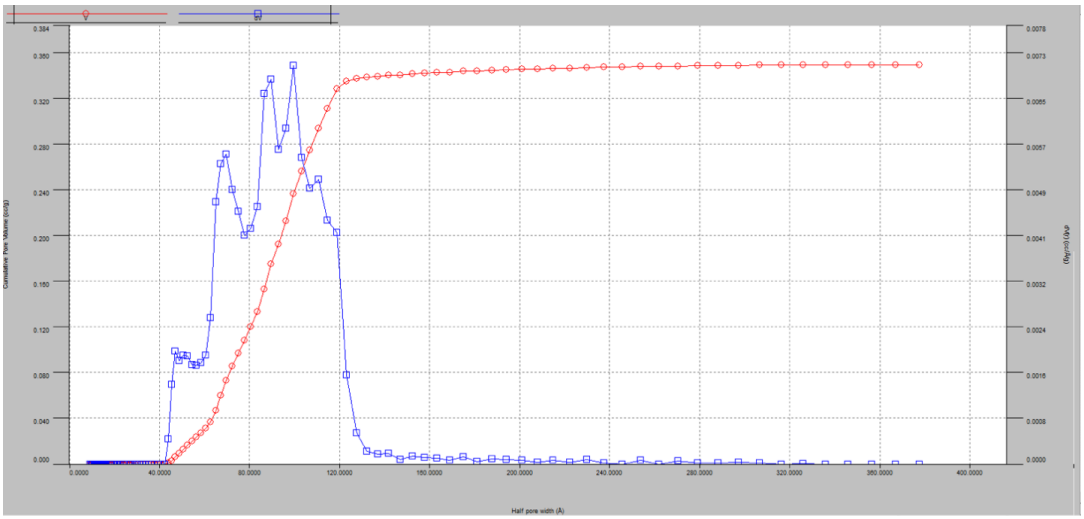

Figure B.4: Pore size distribution of HAp microparticles after functionalization.

The existence of pores with a higher diameter propels the incorporation of BDMDAC in the particles, which is therefore expected to happen at a greater extent in the case of HAp. HAp microparticles not only have a higher specific surface area, but also bigger pores, which should reflect in a higher antimicrobial activity.

Besides this, between non-functionalized and functionalized particles, in the HAp microparticles it is possible to observe that the wider pores decrease in diameter and pores of small size no longer appear in the size distribution after functionalization, which seemingly confirms the incorporation of BDMDAC in the pores. In the case of $CaCO_3$ pores, this difference comes more

unnoticed, supporting the theory that biocide incorporation occurs only at the surface and, therefore, pore sizing and total pore volume doesn't substantially change.