UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Engenharia Química

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Desenvolvimento de recobrimentos antibacterianos à base de quitosana por plasma-grafting

Development and characterization of chitosan coatings by plasma-grafting for antibacterial surfaces

Campinas, Agosto de 2017

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Tese em apresentada à Faculdade de Engenharia Química da Universidade Estadual de Campinas e à Faculdade de Engenharia de Materiais e Minas da Universidade Laval como parte dos requisitos exigidos para a obtenção do titulo de Doutora em Engenharia Química e em Engenharia de Materiais e da Metalurgia. Tese produzida no âmbito de um acordo de Cotutela, firmado entre a Unicamp (Brasil) e a Universidade Laval (Canada).

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RESUMO

O risco da colonização bacteriana em superfícies abióticas impõe desafios importantes para os diversos campos da ciência. Neste cenário, revestimentos antibacterianos têm sido desenvolvidos, usando um grande número de diferentes materiais. A modificação da superfície de polímeros permite melhorar as suas propriedades, com vistas ao desenvolvimento de materiais com respostas biológicas adaptadas ou adaptáveis ao ambiente onde serão implantados. A quitosana é um biopolímero com atividade antimicrobiana o qual pode ser utilizado numa ampla variedade de aplicações de cuidados de saúde e industriais, tornando-a particularmente interessante para o desenvolvimento e aplicação de novos materiais funcionalizados, ou seja, com propriedades antibacterianas. Neste estudo, diferentes tipos de quitosana foram caracterizadas de acordo com o seu grau de desacetilação (DDA) e massa molar (Mw), através de técnicas como ressonância magnética nuclear (^{13}C RMN) e cromatografia de exclusão de tamanho (SEC), entre outras. Os resultados obtidos através dessas análises revelaram a grande importância da caracterização de biopolímeros, uma vez que suas propriedades podem variar de acordo com os métodos de produção, o que pode influenciar no seu uso como aplicação. Em seguida, a metodologia aplicada para o tratamento e modificação de superfícies empregando as técnicas de plasma, para a funcionalização de superfícies e o *grafting* para a imobilização do recobrimento de quitosana foi validada. Inicialmente, filmes de PTFE (politetrafluoretileno) foram utilizados para verificar a eficácia da metodologia proposta para o tratamento e modificação de superfície. Três moléculas "ancoradoras" com diferentes características anidrido glutárico (GA), poli(etileno glicol) bis(carboximetil) (PEGb) e poli(anidrido etileno-alt-maleico) (PA), foram utilizadas visando ligar covalentemente o recobrimento de quitosana às superfícies de PTFE aminadas. Cada etapa do tratamento da superfície foi verificada por espectroscopia de fotoelétrons de raios-X (XPS), por medições de ângulo de contato e colorimetria sendo evidenciada as mudanças na composição química da superfície e sua molhabilidade. As alterações topográficas e de rugosidade após o *grafting* também foram observadas por microscopia eletrônica de varredura (MEV) e perfilometria. Esses resultados demonstraram que o tipo de molécula ancoradora tem uma influência primária no processo de produção dos recobrimentos seguido pela massa molecular dos diferentes tipos de quitosana. Para verificar a resposta antibacteriana dos diferentes tipos de recobrimentos obtidos, testes foram inicialmente realizados empregando a *Xylella fastidiosa* e revelaram a potencialidade dos substratos recobertos com quitosana. Assim, testes utilizando bactérias patogênicas como, *Escherichia coli, Pseudomonas aeruginosa* e *Staphylococcus aureus* foram realizados confirmando o comportamento antibacteriano das amostras PTFE-plasma-PA-CHIMW. Esses resultados encorajaram a aplicação desta metodologia em um substrato de PET (polietileno tereftalato), um polímero muito usado no ramo de têxteis convencionais como também na produção de têxteis hospitalares e biomateriais, demonstrando assim, que a metodologia de plasma-*grafting* aplicada neste estudo, para a produção de recobrimentos de quitosana, pode ser usada para a produção de superfícies onde a atividade antibacteriana é desejada, ou seja, esses revestimentos podem fornecer uma barreira adicional e complementar à transmissão de patógenos, enquanto podem atuar combinados com procedimentos normais de limpeza e desinfecção.

Palavras-chaves: quitosana, plasma, *grafting*, caracterização de superfícies, antibacteriano

ABSTRACT

The risk of bacterial colonization on abiotic surfaces poses important challenges in various fields of science. In this scenario, antibacterial coatings were developed, using a large number of materials. The surface modification of polymeric materials allows to improve surface properties, facilitating the development of optimized materials with biological responses adapted or adaptable to the environment in which they will be implanted. Chitosan is a biopolymer with inherent antimicrobial activity which can be used in a wide variety of health care and industrial applications, making it particularly interesting for the development and application of novel functionalized materials, *i.e.* antibacterial properties. In this study, different types of chitosan were characterized according to their degree of deacetylation (DDA) and molecular weight (Mw), using Nuclear Magnetic Resonance (^{13}C NMR) and Size Exclusion Chromatography (SEC), among others. The results obtained through these analyses revealed the great importance of the characterization of biopolymers since their properties can vary according to the production methods, which can influence its use as an application. Afterward, the methodology applied for the treatment and modification of surfaces using plasma, for the surface functionalization and grafting of molecules was validated. Initially, PTFE (poly(tetrafluoroethylene)) films were used to verify the efficiency of the proposed methodology for the treatment and surface modification. Three spacer molecules glutaric anhydride (GA), poly (ethylene glycol) bis (carboxymethyl) (PEGb) and poly (ethylene-alt-maleic anhydride) (PA), with different characteristics were used to covalently attach the chitosan coating to the aminated PTFE surfaces. Each step of the surface treatment was verified by X-ray Photoelectron Spectroscopy (XPS), through changes in chemical composition, by contact angle measurements and by colorimetry. The topographic and roughness changes after grafting were also observed by Scanning Electron Microscopy (SEM) and profilometry. These results demonstrated that the type of anchors has a greater influence on the coating process than the molecular weight of the different types of chitosan. To verify the antibacterial response of the different types of coatings obtained, tests were initially carried out using *Xylella fastidiosa* and revealed the potentiality of the substrates covered with chitosan. Tests using pathogenic bacteria such as *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* were performed confirming the antibacterial behavior of PTFE-plasma-PA-CHIMW samples. These results encouraged the application of this methodology in PET (polyethylene terephthalate) substrate, a polymer widely used in the field of conventional textiles as well as in the production of hospital textiles and biomaterials. Thus, the plasma-grafting methodology developed in this study, for the production of chitosan coatings, can be applied to the production of surfaces where antibacterial activity is desired.

Keywords: chitosan, plasma, *grafting*, surface characterization, antibacterian

RÉSUMÉ

Le risque de colonisation bactérienne sur des surfaces abiotiques pose des défis importants dans plusieurs domaines de la science. Dans cette optique, des revêtements anti-bactériens ont été développés à l'aide de différents matériaux. La modification de surface des polymères améliore ses propriétés, ce qui facilite le développement de matériaux ayant des réponses biologiques optimales adaptées ou adaptables à l'environnement dans lequel ils sont implantés. Le chitosane est un biopolymère avec activité anti-microbienne inhérente qui peut être utilisé dans une grande variété d'applications de soins de santé et de l'industrie, ce qui rend particulièrement intéressant pour le développement et l'application de nouveaux matériaux fonctionnalisés, ou avec des propriétés antibactériennes. Ce polymère est utilisé pour une grande variété d'applications dans les soins de santé et l'industrie, ce qui le rend particulièrement intéressant pour le développement et l'application de nouveaux matériaux fonctionnalisés. Dans cette étude, différents types de chitosane ont été caractérisés en fonction de leur degré de déacétylation (DDA) et de leur poids moléculaire (Mw) par des techniques telles que la Résonance Magnétique Nucléaire (^{13}C-RMN) et la Chromatographie d'exclusion par taille (SEC). Les résultats obtenus à partir de ces analyses révèlent de l'importance d'une caractérisation complète des biopolymères, puisque leurs propriétés peuvent varier en fonction des méthodes de production, ce qui peut influencer par la suite son utilisation et l'application. Ensuite, la méthodologie utilisée pour la modification de traitement et la surface en utilisant des techniques de plasma pour la fonctionnalisation de surfaces et le greffage de molécules a été validée. Dans un premier temps, les films en PTFE (polytétrafluoroéthylène) ont été utilisés pour vérifier l'efficacité de la méthodologie proposée pour le traitement et la modification des surfaces. Trois bras d'ancrages l'anhydride glutarique (GA), le poly (éthylène glycol) bis (carboxyméthyl) (PEGb) et le poly (anhydride éthylène-alt-maléique) (PA) ayant des caractéristiques différentes ont été utilisés dans le but de créer des liens covalents entre le recouvrement de chitosane et des surfaces aminées PTFE. Chaque étape du traitement de surface a été vérifiée par Spectrométrie Photoélectronique par Rayons-X (XPS), avec les changements de la composition chimique, ainsi que par des mesures d'angle de contact et par colorimétrie. Les changements topographiques et de rugosité après le greffage ont également été observés par la Microscopie Électronique à Balayage (MEB) et par la profilométrie. Ces résultats ont démontré que le type de bras d'ancrage a une plus grande influence sur le processus de production des revêtements que le poids moléculaire des différents types de chitosane. Pour vérifier la réponse antibactérienne des différents types de revêtements obtenus, les tests ont d'abord été réalisés avec *Xylella fastidiosa* et ont révélé le potentiel de ces substrats recouverts de chitosane. Des tests utilisant des bactéries pathogènes telles que *Escherichia coli, Pseudomonas aeruginosa* et *Staphylococcus aureus* ont été réalisés, confirment l'activité antibactérienne des échantillons de PTFE-plasma-PA-CHIMW. Ces résultats ont amené l'application de cette méthodologie sur une surface de PET (polytéréphtalate d'éthylène), un polymère largement utilisé dans le domaine des textiles conventionnels ainsi que dans la production de biomatériaux hospitaliers. Ainsi, les méthodologies par plasma et par greffage développées dans cette étude pour la production de revêtements de chitosane, peuvent être appliquées à la production de surfaces pour lesquelles l'activité antibactérienne est souhaitée.

Mots-clés : chitosane, plasma, *grafting*, caractérisation de la surface, antibactérien

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Abbreviations and symbols

Ads - Adsorption

ATR-FTIR - Attenuated Total Reflectance-Fourier Transform Infrared

CA - Contact Angle

CHI - chitosan

CHILW - chitosan low molecular weight

CHIMW - chitosan medium molecular weight

CHIOS - chitosan oligomeric

^{13}C CP/MAS NMR - Nuclear Magnetic Resonance spectroscopy

Da - Dalton

DBD plasma - dielectric barrier discharge plasma

DDA - degree of deacetylation

E. coli - *Escherichia coli*

EDAC - *N*-(3-dimethylaminopropyl)-*N'*-ethyl carbodiimide hydrochloride

EPS - extracellular polysaccharide

GA - glutaric anhydride

Grft - Grafting

HAI – Healthcare associated infection

MES - 2-(*N*-morpholino) ethane sulfonic acid hydrate

Mn - Number average molecular weight

Mw - Average molecular weight

Mw/Mn – polydispersity index

$-NH_2$ - amino groups

$-NH_3^+$ - protonated amino groups

NMR - Nuclear Magnetic Resonance

R_h - hydrodynamic radius

SEC - Size Exclusion Chromatography

SEM - Scanning Electron Microscopy

S. aureus - *Staphylococcus aureus*

PA - poly(ethylene-*alt*-maleic anhydride)

PEGb - poly(ethylene glycol)bis(carboxymethyl) ether

PET - polyethylene terephthalate

P. aeruginosa - *Pseudomonas aeruginosa*

PTFE - poly(tetrafluoroethylene)

XPS - X-ray Photoelectron Spectroscopy

X. fastidiosa - *Xylella fastidiosa*

Foreword

The constant threat of bacterial contamination, its social-economic burden, and the increasing number of antibiotic-resistant pathogens bacteria have stimulated the search of new alternatives to infection control, as the antibacterial coatings. These coatings can provide an additional and complementary barrier to pathogen transmission while can act combined with normal cleaning and disinfection procedures.

The work presented in this thesis was done through a co-supervision agreement between the University of Campinas - UNICAMP (Campinas, Brazil) and Université Laval (Québec, Canada). The study was focused on the use of chitosan to produce antibacterial coatings using a combination of two techniques: plasma and grafting of biomolecules on polymeric surfaces. Thus, the techniques and materials selected in order to produce the coating, the physicochemical and biological analyses were directly related to the respective expertise of the laboratories involved in this project: LBB (Laboratory for Biomaterials and Bioengineering) and LEQUIP (Laboratory of Engineering and Chemistry of Products).

The Laboratory for Biomaterials and Bioengineering (LBB) at Université Laval has been developing coatings for biomaterials by plasma treatment and grafting of functionalized molecules. In fact, these works have allowed the development of several research projects in the field of (nano)coatings. They are supported by a strong expertise related to several techniques of physicochemical characterizations. Among these techniques are the X-ray Photoelectron Spectroscopy (XPS), measurements of Contact Angle (CA), Scanning Electron Microscopy (SEM) and, Profilometry.

The Laboratory of Engineering and Chemistry of Products (LEQUIP) carry on an important part of its research on the development of materials based on natural polymers, polysaccharides such as alginate, fibroin silk, chitosan, etc. These materials are used in the fields of adsorption and layer-by-layer, etc. Thus, LEQUIP has been developing its expertise in polymer chemistry using also analytical techniques such as Nuclear Magnetic Resonance (NMR), Size Exclusion Chromatography (SEC), Infrared Spectroscopy (ATR-FTIR), UV-VIS colorimetry, among others and, recently, tests with bacteria.

The complementary expertise of the two laboratories enabled this multidisciplinary research work.

This thesis was organized in nine chapters, where **Chapter 1** presents a contextualization of the problematic related to bacterial contamination, the strategies employed and the general context that motivated this work. **Chapter 2** is focused on presenting in detail the research project in question, the objectives, and methodology to achieve each step of this study. **Chapters 3**, **4** and, **5** present the obtained results and discussion, which generated 3 scientific papers.

Chapter 3: Antibacterial coatings based on chitosan for pharmaceutical and biomedical applications.

Authors: Juliana Miguel Vaz, Daniele Pezzoli, Pascale Chevallier, Clayton Souza Campelo, Gabriele Candiani, and Diego Mantovani.

Article history:

Journal: Current Pharmaceutical Design

Submitted: 2017, February

This article had as objective to review the important developments in the field of antibacterial chitosan-based coatings related to applications in the biomedical and pharmaceutical field. It emphasized the biological aspects of bacterial contamination spread and its social-economic damages and the main strategies used to produce antibacterial coatings were critically evaluated, focusing on their advantages and limitations. This work covered also the physicochemical and biological characteristics of chitosan with a special focus on its immunogenicity, allergenicity, and genotoxicity, this is of particular interest because this issue is often overlooked or not clearly treated in the literature. The main techniques targeting to obtain chitosan antibacterial coatings, their advantages and limitations and the necessity to perform adequate surface preparation before coating deposition were presented and discussed. This was the great value because it is the first time that these subjects were summarized and presented in a comprehensive review.

Juliana, Pascale Chevallier, and Daniele Pezzoli jointly identified the scope of the review. I wrote the draft of this article. However, Daniele Pezzoli, Pascale Chevallier, Clayton Campelo and Gabriele Candiani complemented some issues. Clayton Campelo worked to produce Figures and Schemes. All the authors contributed to the corrections and final version.

Chapter 4: Covalent grafting of different types of chitosan on plasma treated PTFE surfaces.

Authors: Juliana Miguel Vaz, Éléonore C. Michel, Pascale Chevallier, Marisa Masumi Beppu and Diego Mantovani.

Article history:

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This article had as first objective to characterize different chitosan samples and the second one was to validate, by physicochemical characterizations, the methodology developed to produce the chitosan coatings by plasma-grafting using PTFE surfaces. The NMR, FTIR, potentiometric titration and SEM were performed in the Institute of Chemistry and in LRAC, both at the University of Campinas by myself and a responsible research professional. At LBB, chemical derivatization, grafting process, and CA were performed by myself. Pascale Chevallier helped me with plasma equipment and performed the XPS analyses. I wrote the draft of the article and the analysis of the results was done in collaboration with Éléonore C. Michel and Pascale Chevallier. All the authors contributed to the corrections in the final version.

Note: Tables 4.1, 4.3 and 4.4 were modified from the published version to add the error of the measurements and to adjust the significant figures. The rest of the content is presented in this thesis as published.

Chapter 5: Influence of chitosan-based coating process on antibacterial activity.

Authors: Juliana Miguel Vaz, Thiago B. Taketa, Jacobo Herandez-Montelongo, Pascale Chevallier, Monica A. Cotta, Diego Mantovani and Marisa Masumi Beppu.

Article history:

Journal: Applied Surface Materials

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The third article had as objective to validate the antibacterial action of chitosan coatings using PTFE surfaces treated by plasma-grafting employing *Xylella fastidiosa* as a model bacterium. The results obtained were used as the basis for selecting the chitosan-coated PTFE films that showed the most promising results to perform the antibacterial tests using *E. coli*, *P. aeruginosa* (Gram-negative) and *S. aureus* (Gram-positive). The experiments were carried out by myself and, Jacobo Hernandez-Montelongo helped me with *Xylella fastidiosa* tests. I wrote the draft of the article and all the authors contributed to the corrections in the final version.

In **Chapter 6**, is presented the general discussion containing the results of the three articles and unpublished results and the perspective for future works. **Chapter 7** presents the conclusion of this study and **Chapter 8** contains the list of bibliography used to base this research project. In **Chapter 9** is presented Appendix with additional information.

1. INTRODUCTION

1.1 General context

In recent decades, significant progress has been observed in the field of materials due to the development of new methodologies and technologies applied for the treatment and modification of surfaces [1–3]. Several approaches have been used, such as plasma treatment [4], grafting of molecules [5], layer-by-layer (LbL) [6] and UV irradiation [7] among others. These surface modifications have allowed the modulation of the contact interface of materials. Thus, they can present a dynamic and adequate behavior in response to the environment in which they will be inserted [5].

Despite the current and constant evolution in this area, the susceptibility of these materials to the action of microorganisms continues to be a serious problem. Microbial contamination, especially bacterial contamination, can lead to simple problems as stains and bad odors, as well as deterioration of food and, can lead to grave problems also, as material failures, transmission of disease, spent billions of dollars and, ultimately, deaths [8–11].

In the hospital environment, Healthcare-associated infections (HAIs), in other words, infections acquired while receiving medical treatment in a healthcare, are the major cause of morbidity and mortality, as well as a significant financial burden. These infections can reach around 5-10% of hospitalized patients in North America and Europe and more than 40% of hospitalizations cases in parts of Africa, Asia and Latin America [12,13], where the low hygiene conditions contribute to the spread of microbial contamination.

Generally, HAIs are caused by opportunistic microorganisms that can attach to the material surfaces. In the most part of the time, these microorganisms are bacteria (about 90% cases), which can adapt to different conditions, colonizing the material and forming a resistant biofilm. In this condition, bacteria are less susceptible to host defense mechanisms and systemic antibiotics [12-14].

Thus, the development of antibacterial coatings constitutes an important area of research within the field of materials which can be a viable solution to reduce the

contaminated surfaces and the risks posed by pathogenic microorganisms, providing an additional, complementary barrier to pathogen transmission, while acting in jointly with normal cleaning and disinfection procedures.

1.2 Bacterial contamination: a social and economic problem in the human history

Microorganisms are present in our life since the beginning of human evolution. Bacteria, virus, yeast, fungi, and algae dominate us in number and mass, the proportion of microbial cells and body cells is 10:1. And, we only survive because of a natural relation of equilibrium between human body and these microorganisms [4,15,16]. These associations often bring many benefits, such as intestinal bacteria that help the digestion process and the yeast used in the fermentation of food and beverage processes [6].

However, the uncontrolled microbial proliferation or the presence of pathogenic microorganisms have been causing serious health problems. In Figure 1.1, it is possible to see examples of different microorganism colonies.

a) b) c)

Figure 1.1 - Examples of different microorganisms: a) households, b) biofouling and, c) *Pseudomonas aeruginosa* [16].

The human history presents several remarkable episodes where bacteria caused the death of thousands of people, such as Black Death, typhus, and tuberculosis, among others. This continues nowadays, despite considerable efforts of research and development from multidisciplinary knowledge fields. Bacteria possess great adaptation capacity and they are becoming multi-drug resistant, posing serious challenges for humans [2].

In the USA, the estimated number of HAIs per year exceed of 1.5 million cases, amongst which 100 000 resulted in deaths and in spending of $7 billion USD. In Canada, 220 000 people develop HAIs each year and $106.4 million CAD have been spent. In Brazil, per year, 2.4 million patients will develop HAIs and 100 000 of them will die [12–17].

1.3 Transmission of bacterial contamination

Two ways can be pointed as being responsible for the spread of pathogenic bacterial contamination: by contact or non-contact. Pathogenic microorganisms can originate by direct contact from an infected host (either human with skin cuts, for example) or indirectly from the environment, via contaminated surfaces or droplets. By non-contact through airborne, insect or animals or common vehicles, such as water or food, which also plays an important role [16,18].

At the hospital environments, bacterial infections are more commonly passed on by direct contact, usually by the hand of healthcare workers, patients, and visitors or by indirect contact, by the surface of materials [19,20,25].

Microscopically, infections are related to the attachment of bacterial cells to a surface depending on several factors such as physical and gravitational forces, electrostatic charge, chemical composition, roughness, porosity and hydrophobic interactions to form biofilm followed by colonies which are often resistant to the methods of cleaning, sterilization, as well as, antibiotics [2,15,18].

Biofilms constitute a protected mode of growth that allows survival of bacteria in a hostile environment. Its formation process (Figure 1.2) begins with the adhesion of planktonic cells on material surfaces and interfaces. The proliferation of bacteria into multi communities as well as bacterial biofilm maturation (development of three-dimensional communities encapsulated) is accompanied by the formation of a self-generated extracellular polysaccharide-based (EPS) matrix (granting additional cell protection) [18]. Literature has described that about 80% of chronic bacterial infections are related to microorganisms in biofilms [22, 26–27].

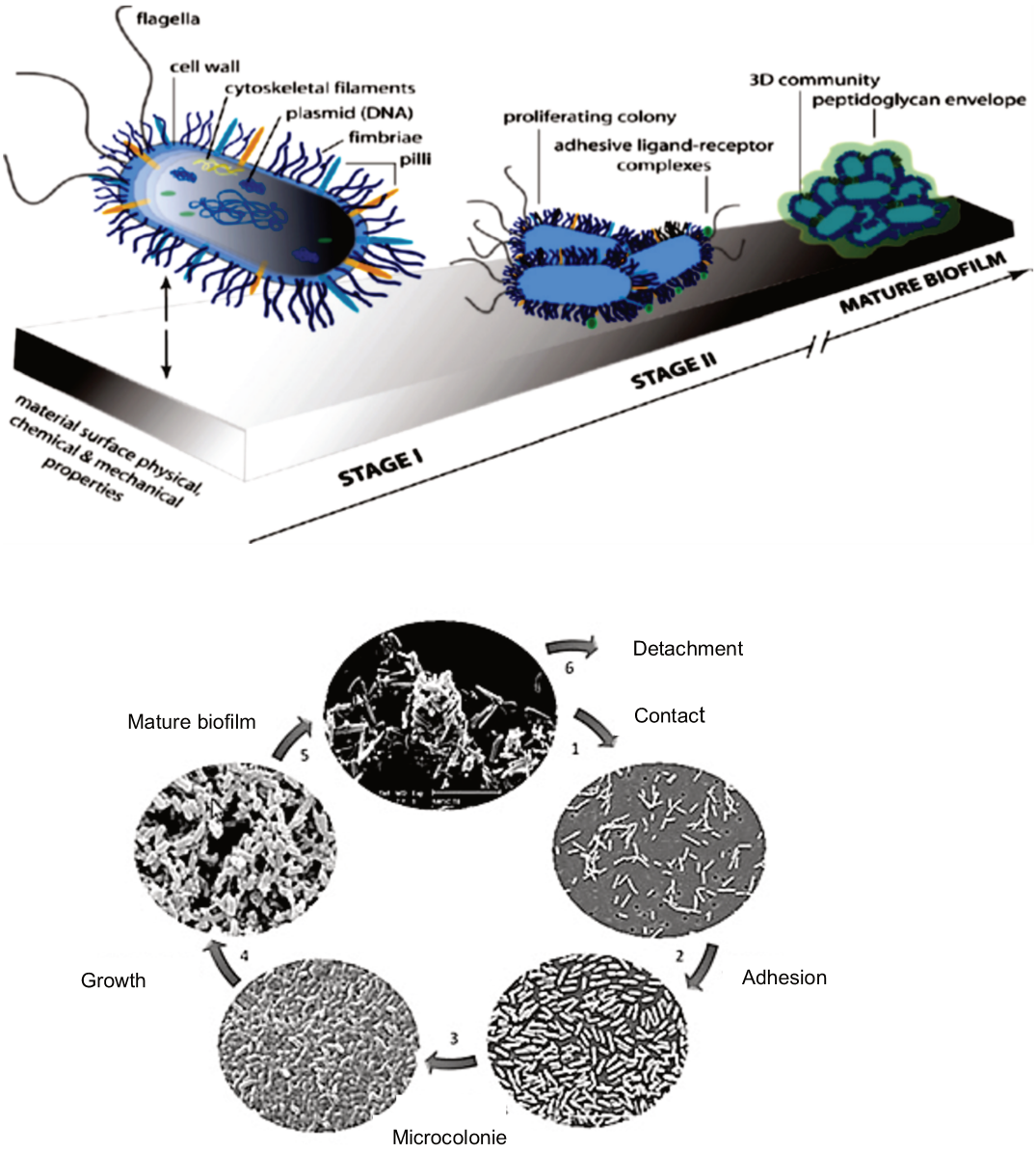


Figure 1.2 - The stages of biofilm development [18].

In this context, the development of antibacterial surfaces may be an interesting alternative to control the spread of bacterial contamination. Different strategies have been proposed to develop these surfaces, however, great challenges cannot be omitted as because bacterial contamination generates irreversible physicochemical, molecular and cellular interactions and its removal after biofilm formation is extremely difficult [2,16,22,23].

1.4 Antibacterial surfaces: strategies and design

As mentioned previously, the bacterial biofilm formed on the surface of the material is hard to remove once it is installed, which creates major challenges in the process of development of effective surfaces against these pathogenic microorganisms. The literature has pointed out several strategies for the development of antibacterial surfaces and among these, three are widely used (Figure 1.3):

- Adhesion resistance/repelling [18,24,25]: consists in developing a surface capable of repelling microbial cells by mechanisms which impair adhesion, thereby preventing the advanced stages of adhesion of microorganisms that lead to the formation of stable biofilm. It can be achieved through superhydrophobic surfaces, superhydrophilic surfaces or surface topography modifications.

- Contact-killing activity [2,16,18]: this mechanism of action aims to eliminate or hinder the growth of microorganisms stably adhering to the surface of the material stably via conjugation of its surface materials with antibiotic functional groups.

- Incorporation/release of antimicrobial compounds [26,27]: metal ions, peptides and other antibiotic compounds may be incorporated in the material constituting the surface to give it antimicrobial functionality. In this case, the material should be designed to be favorable to the development and release of these compounds. In the biocide leaching, cytotoxic compounds are released and diffused from the material surface to the surroundings and consequently, bacteria do not adhere to the surface.

The three previously mentioned strategies can be explored individually or synergistically, aiming to enhance the antimicrobial properties of a surface [16,27]. However, independently of the strategy employed and consequently, of the surface design produced, common characteristics are required, such as easy and inexpensive synthesis, long-term stability (temperature, solubility, etc.), non-toxicity (no irritating and no allergenic), easy regeneration upon loss of activity and, broad spectrum against undesirables microorganisms [6,28-33].

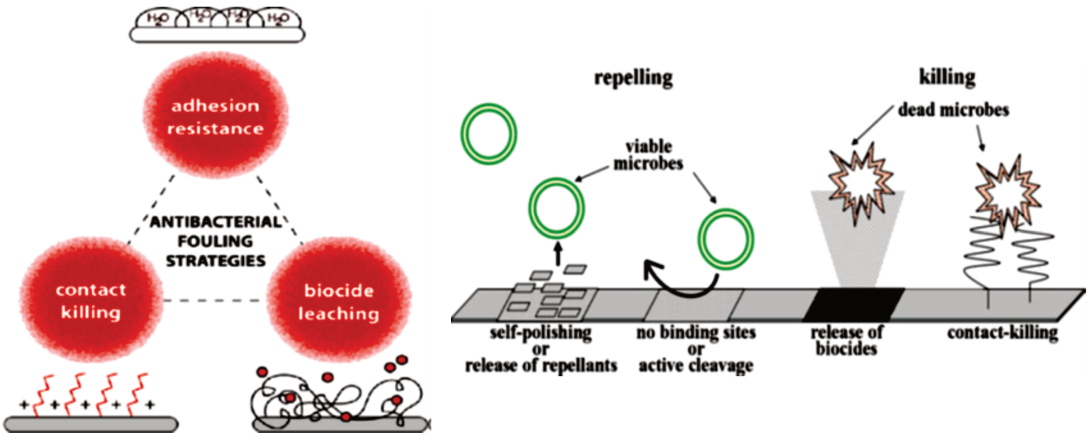


Figure 1.3 - General strategies for the development of antibacterial surfaces [16,18].

1.5 Technological development of antibacterial surfaces

Several surface modification techniques such as UV irradiation, plasma, layer-by-layer, and wet chemical synthesis have been studied aiming to control wetting and adhesion of antibacterial surfaces. The different techniques of surface treatment with their advantages and disadvantages are shown in Table 1.1 [29,31–37].

These techniques can be consolidated with the use of several chemicals which possess antimicrobial activity. Antibacterial materials can also be obtained by administering the metabolic poisons, toxic by ingestion of cumulative elements such as antibiotics, iodine, heavy metals, and by grafting antibacterial polycations containing quaternary ammonium groups, phosphonium, pyridine, antimicrobial peptides, natural polymers (chitosan, nisin, pectin) or natural lipids (such as essential oils) or physical techniques through a photocatalytic process, for example [35,37–54].

Several substrates such as glass, polyethylene (PE), polypropylene (PP), nylon, polyethylene terephthalate (PET) treated with quaternary ammonium salts, nylon fiber coated with antibacterial compounds by chemical vapor deposition (CVD), and stainless steel substrates covered with polymer materials have been employed as antibacterial surfaces [26,34,55].

Table 1.1 - Techniques of surface modification/treatment of the surface.

Technique	Advantages	Disadvantages	Reference
Wet chemical (piranha)	Do not require specialized equipment, the power of penetration is high	Hazardous chemical, irregular surface etching	[57–59]
Dip coating	Simple, cheap and, fast	May show a low adhesion to substrate	[57,60,61]
Layer-by-layer	The combination of different polymers	Mechanism of film formation is complicated; may show a low adhesion to substrate	[57,62,63]
Spray coating	Allows to deposit thin films	May show a low adhesion to substrate	[57,64,65]
Spin coating	Simple, cheap and, fast	Works only for flat geometries; may show a low adhesion to substrate	[57,66,67]
UV irradiation	Ability to tailor the depth of surface reactivity by varying wavelength.	Affect the optical properties, UV light can be blocked by particles, which may affect treatment consistency	[41,57,68]
Plasma treatment	Do not require solvents, less degradation and roughening; allows to deposit thin films and highly adherent; functionalizing the surface with chemical groups	Results depend on many parameters, complications for a continuous operation in large scale	[5,15,57,69]
Grafting	Form covalent bonds between the surface and the coating offering better stability	The homogeneity of the coating depends on the functionalization of the surface	[5,57,70,71]

Composites based on chitosan have interesting mechanical and biological properties for the development of antimicrobial materials, where they are used as

antimicrobial agents for applications in the areas of medicine, food, and textile, for the fabrication of carpets, fabrics, gloves, etc. [24,27,35,51,55,73].

It is important to observe that to be applied onto polymeric materials; antimicrobial compounds should be efficient regarding their antimicrobial activity at low concentrations, present broad antimicrobial spectrum and act selectively on undesirable microorganisms. They must also meet the requirements of regulatory organisms in order to be harmless both to the manufacturer and consumer, as well as have a reduced environmental impact. These compounds should yet be easy to apply, consistent with other chemical processes involved in material finishing, have low cost and do not negatively affect the properties of the material [74].

However, many of the chemicals used are toxic to humans and do not easily degrade in the environment. The industry continues to look for eco-friendly processes that avoid the use of toxic chemicals. In this context, the plasma-grafting treatment with chitosan can be an excellent candidate to produce an eco-friendly material which minimizes microbial effects.

1.6 Chitosan

With several interesting characteristics such as biocompatibility, biodegradability, -low toxicity and large antimicrobial spectrum, chitosan is a biopolymer that can be employed in different application fields [75,76].

Structurally, chitosan is a polycationic with a linear chain consisting of *N*-acetyl-*D*-glucosamine and *D*-glucosamine units linked by *β*-(1→4) glycosidic bonds with three types of reactive functional groups: an amino group at the C_2 position, as well as primary and secondary hydroxyl groups at the C_3 and C_6 positions, respectively, in each repeating unit (Figure 1.4) [5,76,77].

The relative amount of these two monomers (units) in the chitosan may vary, giving samples of different degrees of deacetylation (DDA) between 40-95% and molecular weights (Mw) between 50-2000 kDa. The ratio of *D*-glucosamine units to the total number of units per chain is called the degree of deacetylation (DDA). These structural parameters have a directly influence the properties of this polymer [79–81].

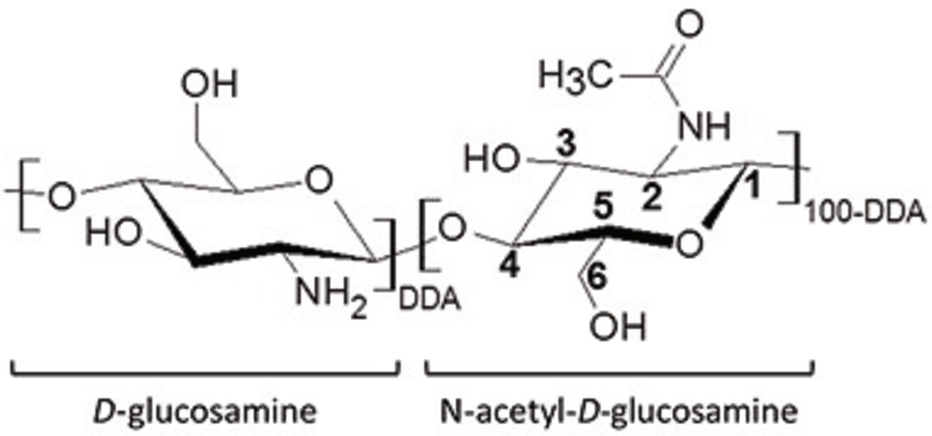


Figure 1.4 - Chemical structure of chitosan and denomination of carbon position (C_1-C_6).

From a mechanical point of view, the adhesion and stability of chitosan coatings are dependent on the chain length as well as on the number of hydroxyl groups (-OH) and amino groups (-NH_2) that could be attached to the substrate by covalent bonds. The absence of covalent bonds to the material could lead to the rapid delamination of such coatings [5,29,82].

Chitosan (pKa~6.3) is insoluble in water, in alkaline medium and even in organic solvents. However, water-soluble salts of chitosan may be formed by neutralization with organic acids (1-10% aqueous acetic, formic, succinic, lactic, glutamic and malic acids) or inorganic acids such as hydrochloric acid. The pH dependent solubility of chitosan is attributed to its amino groups (-NH_2), which become protonated upon a dissolution at pH 6 or below to form cationic amino groups (-NH_3^+), increasing intermolecular electric repulsion and resulting in a polycation soluble, with a large number of charged groups [5,29,82,83].

Biocompatibility is an important biological property for biomaterials. Chitosan is well tolerated by living tissues, including skin, ocular membranes, bones as well as the nasal epithelium, and is an important alternative for a wide range of biomedical applications. The low toxicity of chitosan is considered as an attractive characteristic when compared to other natural biopolymers. *In vivo* toxicity studies demonstrated its safety in terms of inertness and low/no toxicity [76].

Even though chitosan has low toxicity toward mammalian cells, this polymer has a wide spectrum of activity against fungi, yeasts and Gram-positive and Gram-negative bacteria. The mechanism of antibacterial activity of chitosan is not completely known but several factors may have an influence on it. Studies evaluating the antimicrobial activity of chitosan against different groups of microorganisms suggested three main mechanisms of inhibition of microbial growth [34,85,86].

The basic mechanism proposed for chitosan antimicrobial activity is that the interactions between the amino groups of chitosan, positively charged, increases the permeability of negatively charged cell membrane, causing disruption and release of intracellular compounds. Two other mechanisms have been also identified: chelation of metals in trace amounts by inhibiting the enzyme activity thereof and, in the case of yeast cells, going through the cell membrane inhibiting RNA synthesis [5,79,80,88].

Literature has shown that antimicrobial activity of chitosan is influenced by its Mw and DDA. Chitosan presents an increase of antibacterial activity when molecular weight increases. Moreover, chitosan with a high DDA is more effective than those with a low deacetylation degree in inhibiting bacterial growth. In these two cases, the increases of the antibacterial activity of chitosan are related to the higher percentage of amino groups. The antimicrobial activity of chitosan is inversely affected by pH, with higher activity observed at lower pH value. However, is important to remarke that the chitosan action will be different when it is in solution (dissolved) or in dry state such as in film, membrane or coating [80,90–94].

1.7 Polymeric and textile substrates

In general, polymers are materials easily processed, with good mechanical and physicochemical properties and, are not relatively expensive [95,96]. They are widely accepted in various application areas and new technologies have permitted the development of a wide range of high added-value product options to the non-conventional application sectors (Table 1.2).

The textile term is associated with a wide range of polymeric materials that can be processed into yarns and consequently in fabrics, with polymers such as poly(propylene), poly(ethylene), poly(tetrafluoroethylene) etc. These application

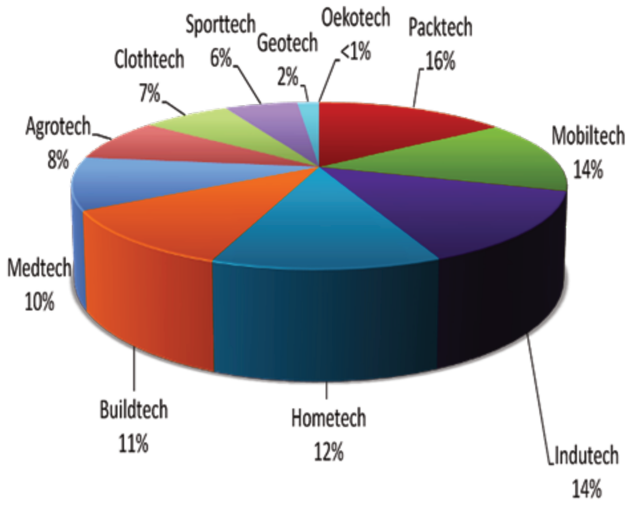
sectors range from specific technical and biomedical demands to simple transient fashion demands (Figure 1.5) [95,96].

Table 1.2 – Some polymers and their applications.

Polymer	Applications	Reference
Poly(propylene) (PP)	Antimicrobial surfaces, biomedical devices, textiles, hemocompatible materials, active packaging	[7,99,100]
Poly(ethylene) (PE)	Drug delivery, biomedical devices, textiles, biocompatible materials, active packaging	[101,102]
Poly(ethylene terephthalate) (PET)	Antimicrobial surfaces, textiles, hemocompatible materials, active packaging	[59,103]
Poly(tetrafluorethylene) (PTFE)	Biosensors, biomedical devices, hemocompatible materials, immobilized enzymes, textiles, non-sticking coatings	[5,104]

However, these materials, natural or synthetic, are often vulnerable to microbial attacks. In the food industry, the growing concern over possible contamination by microorganisms has led to the development of containers using antibacterial nanocomposites or silver compounds or chitosan in order to prevent the adhesion of bacteria and prolonging the shelf life of food [40,69,74].

Packaging and environment fields have also sought alternatives to reduce the negative effects of disposable plastic packaging, such as the current increasing trend of using reusable packaging, also known as eco bags. However, recent researches have shown that the use of reusable bags can favor the formation of an environment which induces the growth of potentially pathogenic microorganisms, indicating the possibility of a serious public health problem [105,106]. Regarding the clothing applications, there is a current demand from consumers for “active wear”, which creates a substantial market for antimicrobial textile products. It became popular in sportswear, women's wear, and aesthetic clothing to impart anti-odor or biostatic properties [28,74,96].



Packtech: bags, sacks, food wrappings, etc.

Indutech: separating, purifying, cleaning gases and effluents, etc.

Mobiltech: seat covers, seat belts, car carpets, air bags, etc.

Hometech: carpet, (fragrant) curtains, waddings, etc.

Buildtech: concrete reinforcement, roofing materials, insulations, etc.

Medtech: absorbents, bandages, surgical materials, artificial organs, and skins, etc.

Agrotech: agriculture, forestry, fishing, etc.

Clothtech: functional, insulating, military, shape memory, luminous, astronaut, etc.

Sporttech: suits, sports materials, etc.

Geotech: bridges, roads, engineering projects, etc.

Oekotech: environmental protection, oil spillages, separate water and oil, etc.

Protech: heat-resistant clothing, fire suits, clothing against chemical, biological particles, etc.

Figure 1.5 - Technical textile sectors [107].

In a hospital environment, studies have strongly suggested that textiles can contribute to air contamination by spreading pathogens. Health professionals may be

contaminated by touching infected surfaces and transmit pathogens to patients via skin and surfaces contact. Thus, contaminated textiles such as sheets and pijamas can directly infect people who work in this environment, even if they are wearing protective equipment such as gloves. These professionals can contaminate patients and transfer germs to other surfaces, such as door knobs, etc., continuing the contamination cycle [108,109].

In this regard, it is expected that antimicrobial treatment of polymer materials acts quickly in order to be effective to prevent contamination by pathogenic microorganisms and control infestations of microbes, reducing the formation of bad odor, protecting from deterioration and decreasing infections spread by these products [95,108,110].

2. THE PROJECT *"Development and characterization of chitosan-coatings by plasma-grafting for antibacterial surfaces"*

This research project investigated the problematic related to the development of antibacterial surfaces. The aim was to minimize the contamination by preventing bacterial adhesion and/or growth on polymeric surfaces. Several fields can be benefited with antimicrobial products. However, in this study, the performance of the coating was investigated for materials for medical and daily life applications.

Thus, in this doctoral project, it was proposed to combine the process the functionalization of surfaces by plasma with the immobilization of chitosan by grafting employing three different spacer molecules. In this perspective, the work carried out led to the study: characteristics of chitosan, plasma and grafting techniques for providing a coating with antibacterial properties.

2.1 General objective and hypothesis

The **main objective** of this research was **to study antibacterial coating of chitosan via plasma-grafting, which remains attached covalently to the substrate**.

The proposed strategy in this thesis was to combine the properties of plasma treatment following grafting of spacer molecules and to create an antibacterial chitosan coating. In this regard, this work was organized in three steps as shown in Figure 2.1.

The hypothesis that supported this research project was based on a balance of the availability of the $-NH_2$ groups present on chitosan structure.

"Antibacterial activity of coatings increases with the number of free amine groups available the chitosan coating".

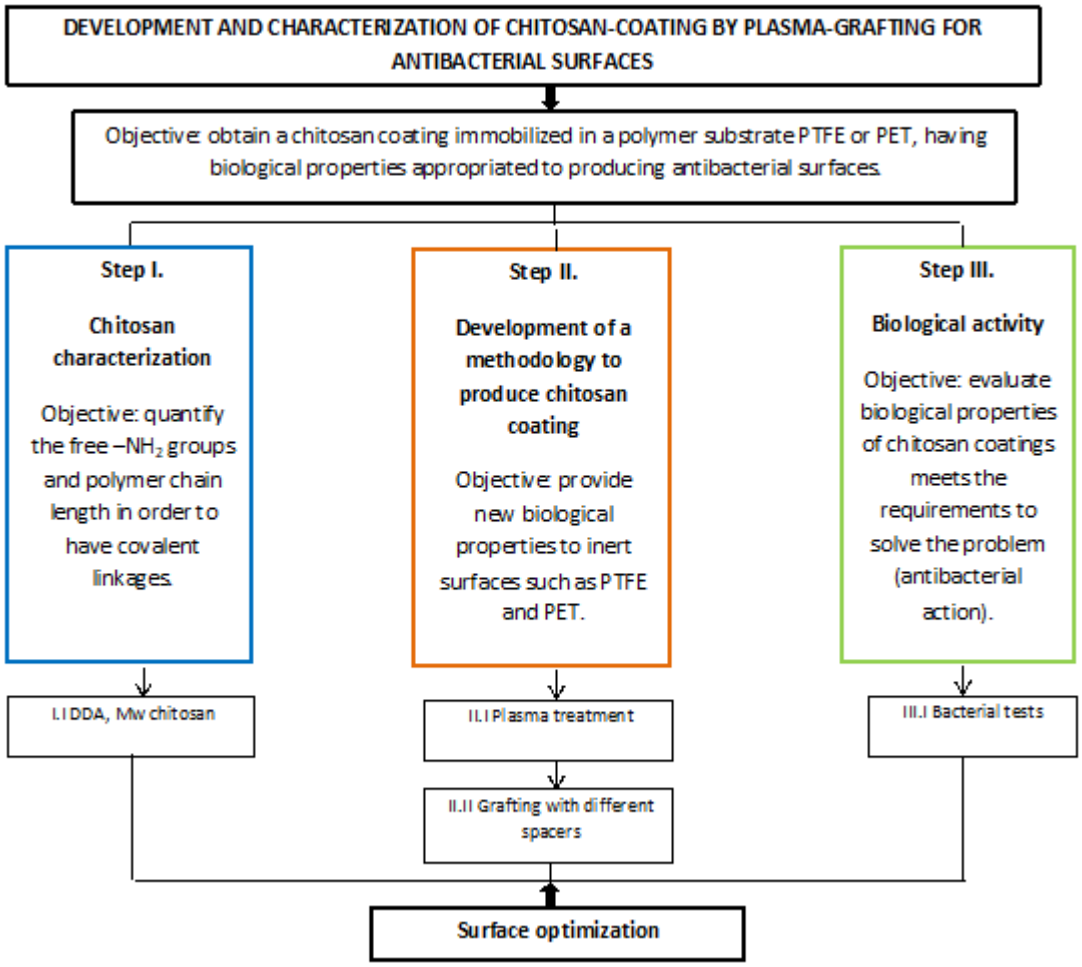


Figure 2.1 – General scheme of the project.

2.1.1 Specific objectives

To achieve the general objective of this study, this research project was organized in three steps, each one representing a specific goal.

In the **first step**, the characterization of three different types of commercial chitosan (CHIOS, CHILW and CHIMW) was performed to determine the degree of deacetylation (DDA) and molecular weight (Mw) values of these samples, seeking to validate the information presented by the supplier and based also, on the literature. These results were presented in **Chapter 4**.

In the **second step** of this research work, also presented in **Chapter 4**, the following points were studied:

- Development of a methodology to produce a chitosan coating covalently linked to the substrate using plasma and grafting techniques;

- Evaluation of the influence of the different characteristics of the spacer molecules on the physicochemical properties of the coatings;

- Evaluation of the influence of the degree of deacetylation and the molecular weight of the chitosan on the physicochemical properties of the coatings.

In the **third step** of this research work, presented in **Chapters 5 and 6**, the following points were studied:

- Performing antibacterial tests with *Xylella fastidiosa* (Gram-negative), as a model bacterium, using the PTFE substrate;

- Coating of PET textiles using the methodology developed (plasma-grafting) and evaluation of the coating on these surfaces;

- Antibacterial tests with bacteria *Pseudomonas aeruginosa* and *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) to evaluate the antibacterial response of these coatings on the different substrates (PTFE and PET).

2.2 The pertinence of this research project

In this context, chitosan coating was attached on the polymeric substrate using a DBD plasma (dielectric barrier discharge plasma) system for the functionalization of the surface, followed by a grafting process. DBD plasma is widely employed in industries because it is a low-cost technique with easy applicability, high versatility. This system can provide the functionalization of polymer surfaces allowing to obtain of stable and adherent coatings through the formation of covalent bonds formed through grafting of spacer molecules. These several important aspects are discussed below.

2.2.1 Why chitosan?

Chitosan was selected to produce the functionalized coating onto synthetic polymer surfaces because it is a semi-natural and not expensive polysaccharide with intrinsic antimicrobial activity against fungi, Gram-positive and Gram-negative bacteria, and yeasts, with low toxicity for mammalian cells and large application in several fields, as previously described. Moreover, microorganisms are not able to develop resistance against this biopolymer, a large advantage if compared to other antibacterial agents.

Another reason why chitosan was chosen is related to the presence of accessible functional groups in its chain. The primary amino group at the C_2 position and the primary and secondary hydroxyl groups at the C_3 and C_6 positions, respectively, can readily react and/or subjected to chemical derivatization, to form new functional groups. This possibility could be considered as an important alternative in the case if it was necessary to improve the physicochemical and biological properties of the coating.

In this context, these characteristics have been making chitosan particularly interesting for the development and application of new functionalized materials and encouraging its use in this thesis.

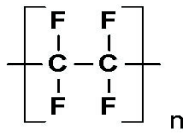
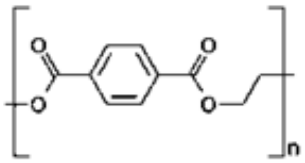
2.2.2 Substrates

PTFE polymeric film was used in this study because it is a widely polymer used in the biomedical field, as implants and vascular prostheses, etc. As well as, the use of PTFE films brought other interesting issues, such as its ease of handling, simpler geometry and, the presence of fluorine in its composition.

PET textile was chosen because it is a polymer largely employed in diversified applications as a textile (sports clothing, bags and active packaging and in the medical field as vascular prostheses, among others).

Another reason because these two substrates were chosen was its inertness, stability and, easy functionalization by plasma to immobilize the chitosan coating by grafting. Table 2.1 shows the chemical structure and dimensions of the samples.

Table 2.1 – Polymeric samples employed in this project: their chemical structure and dimensions.

Polymer	Chemical structure	Area/thickness/fabric weight
PTFE		3.0 cm X 3.0 cm; 250 μm
PET		3.0 cm X 3.0 cm; 225 g/m^2

2.2.3 Functionalization and immobilization of chitosan coatings

2.2.3.1 Generalities - plasma

Plasma can be considered as the fourth physical state of matter where there is a balance between the thermal energy of its particles (radicals, electrons, ions, atoms and/or molecules) and the binding force among them [4,20,31,112].

The parameters of plasma such as power, pressure, gas, equipment configuration and the substrate nature determine the type of surface treatment produced, such as functionalization, deposition, substitution or an ablation (Figure 2.2). These different types of surface modification can be employed for materials with different configurations and different types of the surface [4].

Plasma technology is versatile, fast and offers a lot of advantages, because this technique allows the surface modification without changing the inherent properties of the material (bulk properties). Moreover, this technique is eco-friendly and has great industrial importance in modifying polymer surfaces, as it can be performed in many different ways, for example with simple replacement of the gas used (argon, oxygen, nitrogen, ammonia, fluoride, fluorinated hydrocarbons, carbon dioxide, water vapor and air dioxide), and can produce surfaces with properties desired for different applications, without to need of replacing the whole plasma system [74,113–115].

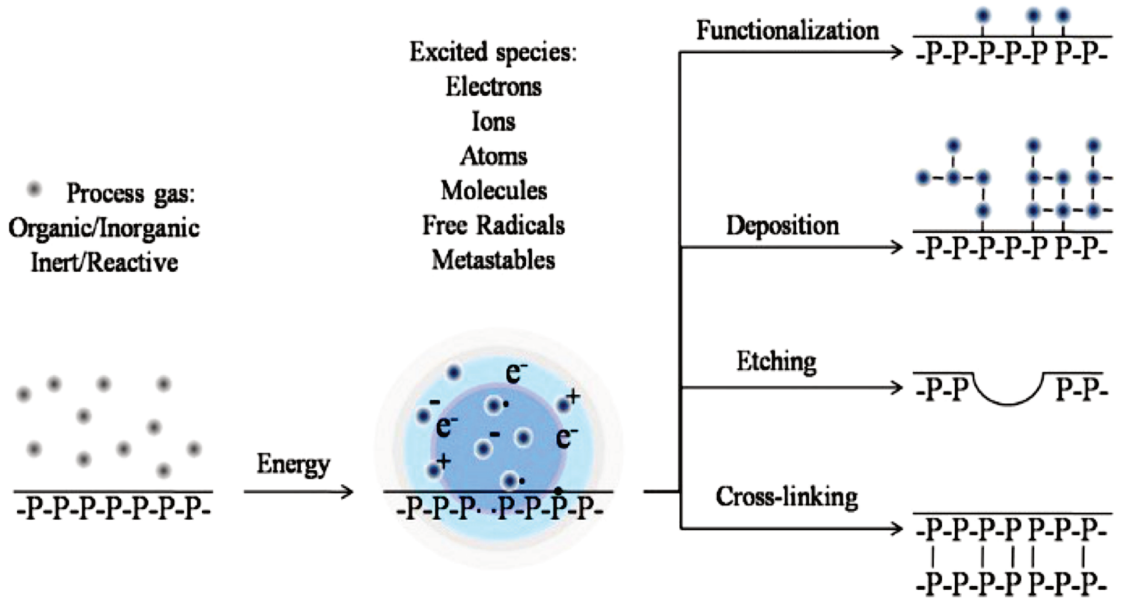


Figure 2.2 - Mechanisms of surface modifications that can achieve by plasma [69].

A special type of plasma is the dielectric barrier discharge plasma (DBD plasma). In this equipment, the discharge occurs between two planar or cylindrical electrodes provided that at least one of them is covered by a dielectric layer. As a consequence, after the gas breakdown takes place, the charge accumulation on the dielectric surface prevents the corona-to-arc transition. Figure 2.3 shows a scheme of DBD plasma.

The DBD plasmas have many applications ranging from surface functionalization and grafting, improvement of adhesion and hydrophilicity, sterilization and, processing of textile and fabrics [5,116,117] .

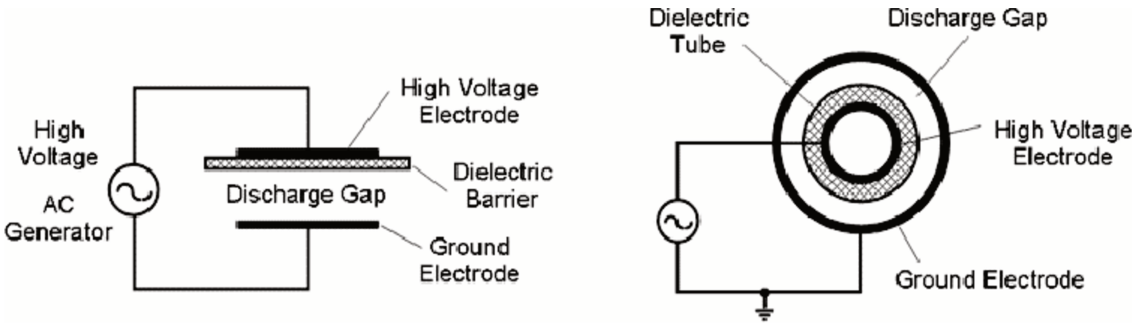


Figure 2.3 – Scheme of DBD plasma configuration, adapted of [117].

DBD plasma is widely used in industry due to its simplicity of operation. It is a type of plasma that has been a promising technology in the past few years regarding the modification of surface properties of polymers. Usually, voltages of a few kV and frequencies ranging from 5 to 500 kHz are used [114]. The mean electron energy in DBD plasma is in the range of 0-10 eV [119], while the chemical binding energy of polymers is less than 10 eV. Therefore, energetic particles in DBD can break the chemical bonds of polymers, without dramatic degradation of the substrate. In addition, to modify the surface properties of polymers without expensive vacuum system is another advantage of DBD.

2.2.3.2 DBD plasma in this research project

In this study, plasma technique prepared (functionalized) the substrate for the posterior grafting, as described in **Section 2.2.3.3**. Plasma parameters were previously based on Sarra-Bournet *et al.*, 2009 [120] and optimized to provide a sufficient number of amino groups and to minimize damage to the substrate.

During plasma functionalization, employing 95% N_2 + 5% H_2 gas flow, 3 kHz, 10 kV, 1 mm and 45 s as parameters (described in detail in **Chapter 4**), numerous nitrogen species were introduced at the surface of the samples. However, amino groups (-NH_2) are the most important reactive groups introduced by this treatment due to its capacity to react by covalent linkages with the carboxylic groups from spacer molecules.

In order to differentiate the primary amines from the other nitrogen compounds, a chemical derivation has been carried out, where amino groups present on the previously treated substrate, react with 5-bromosalylaldehyde, as shown in Figure 2.4.

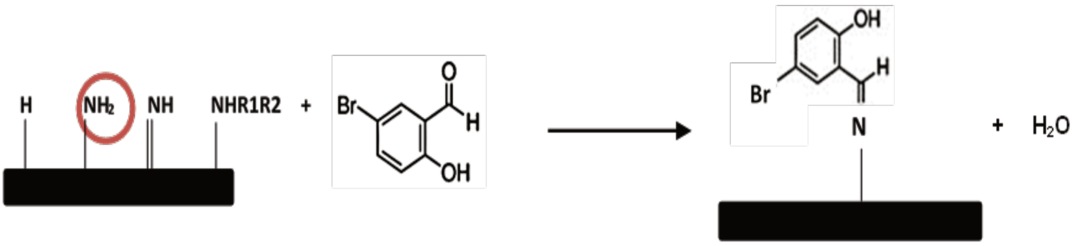


Figure 2.4 - Scheme of chemical derivation reaction, where R1≠R2≠H_2.

As presented in the literature, results of the surface chemical composition of the sample with 2-3% amines are equivalent to 0.5-2 amino groups per nm^2, an amount largely sufficient to graft high molecular weight molecules [5,121].

2.2.3.3 Grafting of spacers and chitosan

One the main challenges of achieving “smart” materials are increasing the surface adhesion in order to obtain a durable coating. In order to avoid the short durability of the coating, the interfacial interactions between the material surface and the coating could be effectively improved by the introduction of new chemical groups to the substrate surface followed by the grafting [4,5,74].

The grafting is a technique that can be accomplished by different mechanisms; one way is to produce chemically irreversible covalent bonds. These interactions are stables and restrained the delamination of the coating [5,29,59,122].

In this work, grafting technique was suggested after plasma treatment because it is possible to obtain covalent bonds which lead to more stable links between the functionalized surface and the coating. Figure 2.5 shows the steps of surface modification used in this study.

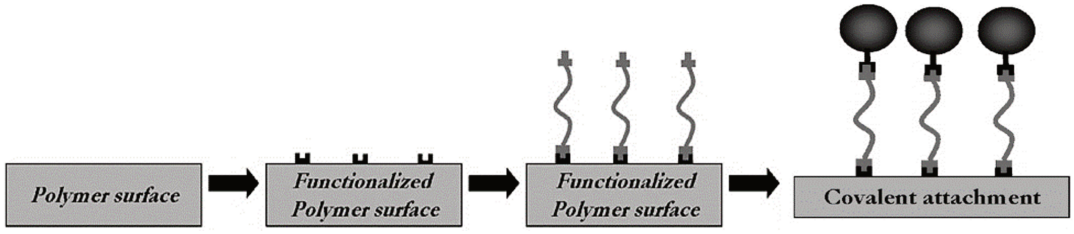
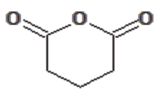
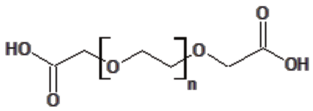
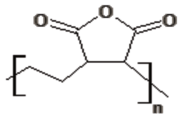


Figure 2.5 - *S*teps of surface modification used in this study (adapted of [57]).

In this context, the first grafting step was based on reactions between the $-NH_2$ groups on the substrate and spacer molecules. Three different anchors (glutaric anhydride, poly(ethylene glycol) bis (carboxymethyl) ether and poly(ethylene-*alt*-maleic anhydride)) were selected for this study.

Table 2.2 shows the characteristics of the spacer molecules employed in this study and their advantages and disadvantages.

Table 2.2 – Name, characteristics of the anchors and their advantages and disadvantages (adapted of [32])

Spacer (Mw)	Chemical structure	Advantages	Disadvantages
Glutaric anhydride **GA** (114 Da)		Low molecular weight which possibly allows a more effective grafting (lower steric mobility greater proximity of the substrate) can provide more stability 2 anchor points/molecule	Possible formation of non-uniform coating
Poly(ethylene glycol) bis(carboxymethyl) ether **PEGb** (600 Da)		Anti-adhesive property, good biological response 2 anchor points/molecule	Possible formation of non-uniform coating, possible dual-link of PEGb molecules to the treated substrate
Poly(ethylene-*alt*-maleic anhydride) **PA** (100-500 kDa)		Known to induce high crosslinking so should increase stability of the coating Many anchor points/molecule	Crosslinking with all amino groups from chitosan leading to inexistent biological response

The reaction between GA and the amino groups on the previously functionalized substrate occurred in acetone solution, where the glutaric anhydride rings are opened, resulting in the formation of carboxylic groups in the extremities of the molecule. These groups were initially available to react with amino groups on the substrate. The same conditions were used for PA grafting, however, the washing with water was not done to order to preserve the reactivity of the anhydride functional groups.

For PEGb grafting, the reaction occurred in PEGb solution, previously activated with EDAC (*N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride). Carboxyl groups present in the extremity of the PEGb molecules reacted with amino groups present on the surface treated by plasma, forming amides bonds, it was the same for GA and PA shown before.

As literature shows several options for an activating agent, such as dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC) and, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), etc. However, in this study, EDAC was chosen as the activating agent in the grafting procedure due to its convenience in handling in an aqueous medium and, a good index of molecules activation [82,121].

The activation reaction occurs when EDAC react with carboxyl groups forming an intermediate ester able to react with -NH_2 groups forming amide linkages. Second grafting step was based on reactions between the anchor molecules and the chitosan coating. Prior to chitosan grafting, the carboxylic acid functional groups of GA and PEGb grafted films were activated in a solution of EDAC. These GA and PEGb activated films and PA films (which are able to react directly with chitosan amino groups) were immersed in chitosan solutions as previously determined. Figure 2.6 shows the activation by EDAC for GA and PEGb.

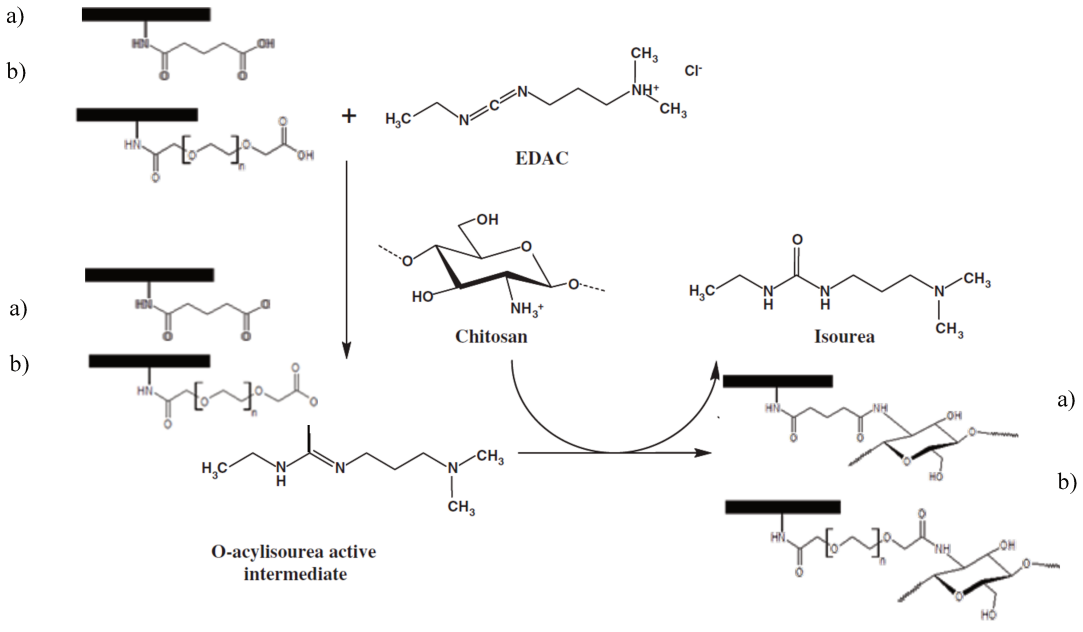


Figure 2.6 – Activation of EDAC for GA (a) and PEGb (b) for the chitosan grafting.

The conditions used to produce the chitosan solution were determined by an optimization procedure. It was defined the basic parameters such as concentration, temperature, immersion time aiming easy handling, the stability of the chitosan solution, and better coverage area. Thus, in this work, 2% (w/v) chitosan solutions (aqueous solution of acetic acid 1% (v/v)) were prepared at room temperature and were placed under mild stirring for 3 hours.

2.2.4 Characterization analyses

2.2.4.1 Chitosan characterization

The first step of this study was organized as seen in Figure 2.7.

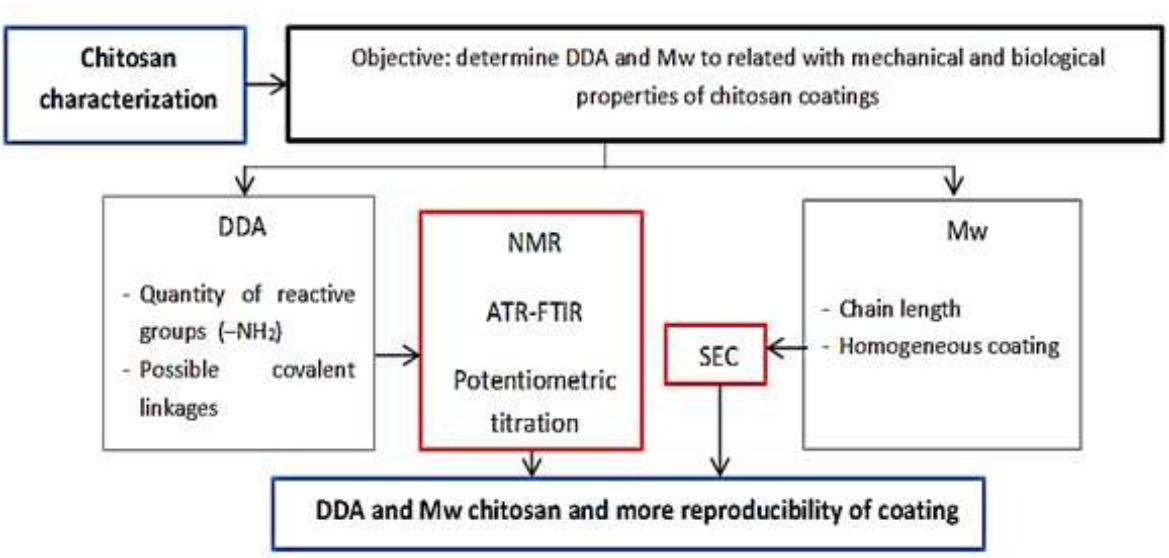


Figure 2.7 - Step I of the project: chitosan characterization.

The characterization of three different types of commercial chitosan (CHIOS, CHILW and CHIMW) was performed for the degree of deacetylation (DDA) and molecular weight (Mw), seeking to validate the information presented by the supplier and based on the literature. Table 2.3

Table 2.3 – Chitosan characterization analyses.

Analyses	Information	Reference
Nuclear Magnetic Resonance (NMR)	DDA	[123,124]
Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR)	DDA	[125–127]
Potentiometric Titration	DDA	[128]
Size Exclusion Chromatography (SEC)	Mw	[129,130]

2.2.4.2 Physicochemical surface characterization

Physicochemical analyses and morphological characterizations were conducted to evaluate the characteristics and coatings properties. Thus, X-ray Photoelectron Spectroscopy (XPS) and colorimetry UV-VIS were used to determine the surface composition of the new coatings. The surface morphology and texture were evaluated by Profilometry and Scanning Electron Microscopy (SEM). The surface hydrophobicity was evaluated by contact angle (CA) measurements, described in Table 2.4.

Table 2.4 - Characterization analyses for the surface-untreated and treated.

Analysis	Information	Depth of analysis
Chemical derivatization	Relative concentration of $-NH_2$ groups after plasma treatment	-
UV-Vis	Presence of $-NH_3^+$ groups on the chitosan coating	-
XPS	Chemical composition of the F, C, O, and N on the surface indicating the presence of anchors and chitosan coating	< 5 nm
CA	Surface wettability	< 1 nm
Profilometry	Surface roughness measurements	20 nm
SEM	Surface morphology	~ 1 µm

The second step of this work was organized as seen in Figure 2.8.

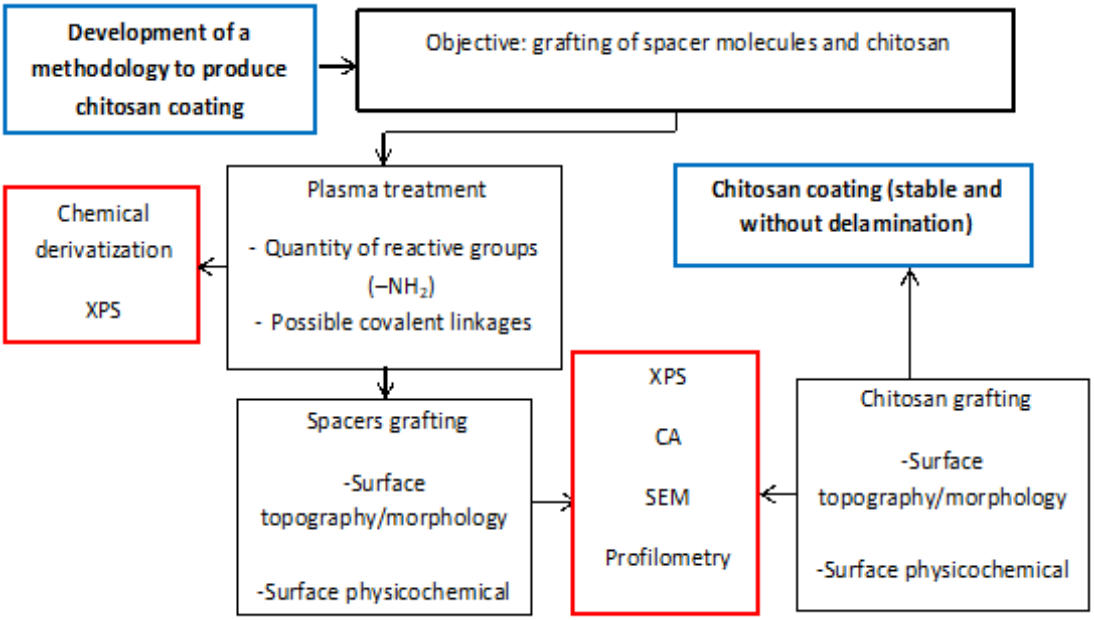


Figure 2.8 - Step II of the project: Development of a methodology to produce chitosan coating.

2.2.4.3 Antibacterial tests

The third step of this work was organized as seen in Figure 2.9.

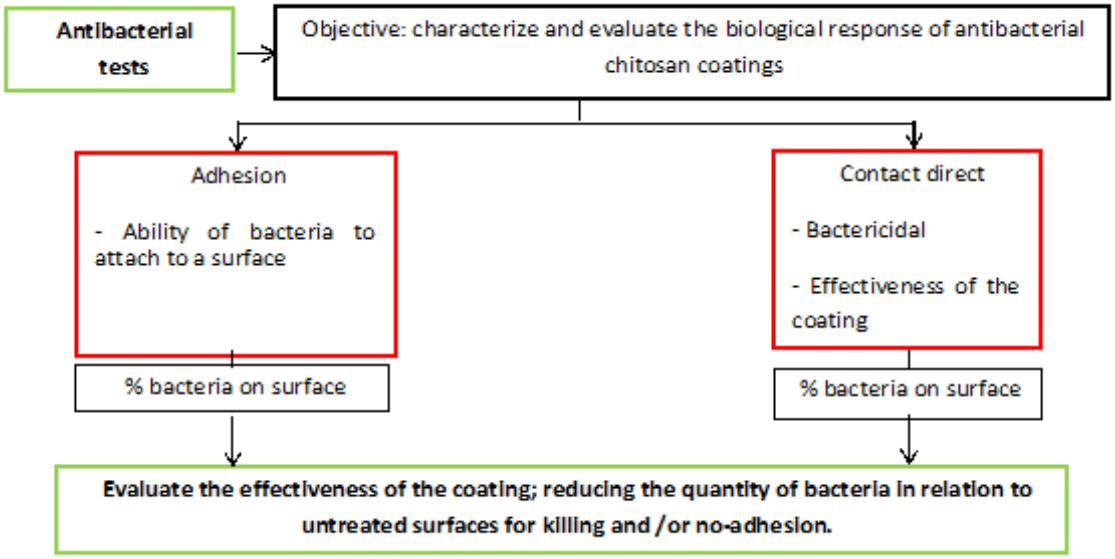


Figure 2.9 - Step III of the project: biological tests.

The performance of biological assays allowed the examination of the antibacterial behavior of these coatings, providing vital information to its use as an antibacterial coating.

Thus, the antibacterial effectiveness of the chitosan coatings was evaluated with biological tests, previously with *Xylella fastidiosa* (Gram-negative) and after with bacteria *Escherichia coli*, *Pseudomonas aerugionosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive).

Bacterial tests were performed by qualitative and quantitative methodologies, as shown in Table 2.5.

Table 2.5 – Bacterial tests

Test	**Information**
Adhesion	Ability of surface to prevent bacterial attachment
Determination of antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions	This method is used to quantitatively assess the efficacy of a sample treated with non-diffusible antimicrobial agent by stirring in a suspension with an microorganism.

3. ANTIBACTERIAL COATINGS BASED ON CHITOSAN FOR PHARMACEUTICAL AND BIOMEDICAL APPLICATIONS

This Chapter was submitted in Current Pharmaceutical Design

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Résumé

Le risque de colonisation bactérienne sur des surfaces abiotiques de dispositifs biomédicaux pose des défis importants pour les domaines des sciences pharmaceutiques et des biomatériaux. Dans ce contexte, des revêtements antibactériens ont été développés, en utilisant un certain nombre de molécules et de matériaux différents. Parmi eux, le chitosane est un biopolymère biocompatible non cytotoxique présentant une activité antimicrobienne inhérente qui a déjà été utilisé dans une grande variété d'applications médicales et industrielles. Dans ce cadre, les revêtements antibactériens à base de chitosane sont étudiés de manière critique, en mettant particulièrement l'accent sur leurs méthodes de production, leurs applications pharmaceutiques et biomédicales, leurs avantages et leurs inconvénients et enfin en soulignant les principaux défis à relever et les perspectives futures dans ce domaine.

Resumo

O risco da colonização bacteriana em superfícies abióticas de dispositivos biomédicos coloca desafios importantes para os campos da ciência farmacêutica e dos biomateriais. Neste cenário, os revestimentos antibacterianos têm sido desenvolvidos, usando um número de diferentes moléculas e materiais. Entre eles, a quitosana é um biopolímero biocompatível, não citotóxico, com uma atividade antimicrobiana inerente que já tem sido utilizada numa ampla variedade de aplicações de cuidados de saúde e industriais. Aqui, os revestimentos antibacterianos à base de quitosana são criticamente pesquisados, com especial ênfase nos seus métodos de produção, aplicações farmacêuticas e biomédicas, juntamente com os seus prós e contras, e finalmente destacando os principais desafios a enfrentar e as perspectivas futuras neste domínio.

Abstract

The risk of bacterial colonization of abiotic surfaces of biomedical devices poses important challenges for the pharmaceutical and biomaterials science fields. In this scenario, antibacterial coatings have been developed, using a number of different molecules and materials. Among them, chitosan is a non-cytotoxic, biocompatible biopolymer with an inherent antimicrobial activity that has been already used in a wide variety of healthcare and industrial applications. Herein, chitosan-based antibacterial coatings are critically surveyed, with a special emphasis on their production methods, pharmaceutical, and biomedical applications, along with their pros and cons, and finally highlighting the key challenges to be faced and future perspectives in this field.

Keywords: chitosan, antibacterial coatings, biomedical devices, biofilm, contact-killing surfaces, biocompatibility, physisorption, grafting.

3.1. Introduction

Bacterial contamination of material surfaces represents an extremely serious issue in biomedical device development owing to the associated health, social and economic expenses [1]. In this context, antibacterial coatings are an increasingly studied area of research. The tremendous progress in material design and processing, and surface modification techniques have in fact prompted the development of surfaces that are able to prevent bacterial adhesion and proliferation and eventually biofilm formation, thus minimizing the risks of biomedical device-related infections.

Several strategies for the design of antibacterial coatings have been reported, such as antimicrobial agent release, contact-killing and adhesion resistance/bacteria-repelling [2], each aiming to overcome the limitations of the customary administration of antibiotics.

Chitosan is a natural-derived polymer (*i.e.*, a biopolymer) with acknowledged antimicrobial properties that, due to its many favorable biological properties, such as high biocompatibility, low immunogenicity and allergenicity, ease of processing, represents a very attractive material for the development of surface coatings with inherent antibacterial activity and that can be further loaded with other, more selective antimicrobials [3].

Up to now a plethora of applications in the biomedical field has been proposed for chitosan-based antibacterial coatings, ranging from wound healing [131] to intraocular lenses [132], from dental implants to orthopedic prostheses [133–142], from sutures [143] to catheters [144].

This survey aims to review the most striking developments in chitosan-based antibacterial surface coatings for pharmaceutical and biomedical applications. After pointing out in brief the issue of microbial contamination of abiotic surfaces and the main strategies adopted for the development of antibacterial coatings, the foremost properties, and applications of chitosan and chitosan derivatives will be thoroughly described. Finally, the techniques developed for the production of chitosan-based antibacterial coatings will be reviewed and critically discussed.

3.2. Bacterial contamination: a challenge for the biomedical field

In this section, we will discuss the concerns related to bacterial contamination relevant to the biomedical field. The main mechanisms of bacterial colonization on material surfaces and the several factors related to this phenomenon will be described, and the socio-economic impact of healthcare-associated infections (HCAIs) examined.

3.2.1 Some basics on bacteria

Microorganisms such as bacteria are constantly present in large quantity in the human body, in an approximate proportion of 10 microbial to one body cell, ordinarily establishing beneficial relations. In fact, a physiologically stable equilibrium exists between tissues/organs and guest microorganisms, such as the resident intestinal and oral flora, the skin bacteria, etc. [23,145,146].

There are two common ways to classify bacteria based on phenotypic features (Figure 3.1). The first one relies on their shape, which depends on the types of cytoskeletal proteins and their organization: bacteria can be spherical (cocci, *e.g.*, *Staphylococcus aureus*), cylindrical (bacilli, *e.g.*, *Escherichia coli*), spiral-shaped (spirilla, *e.g.*, *Spirillum volutans*), and comma-shaped (vibrio, *e.g.*, Vibrio cholera).

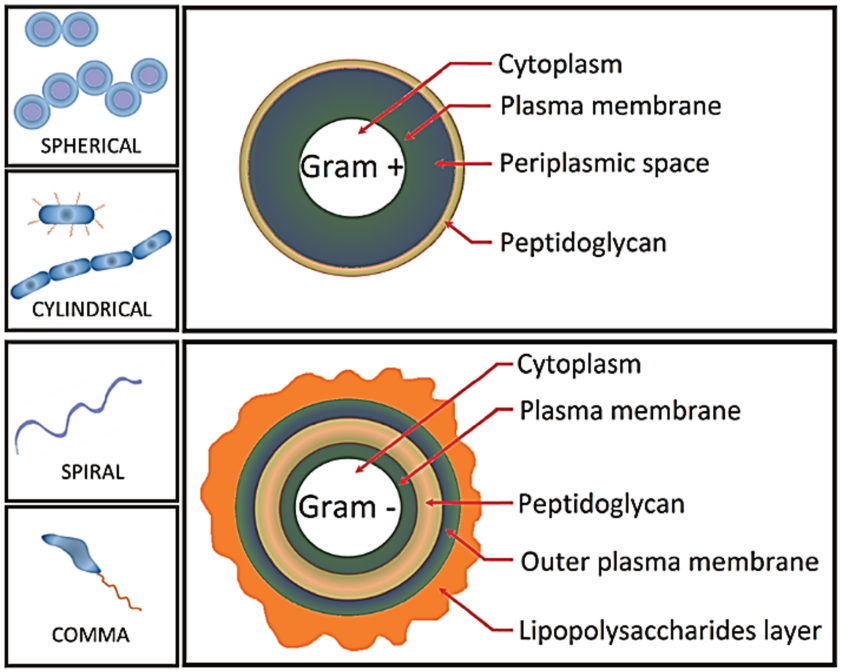

Figure 3.1 - Representation of bacteria classification by shape (spherical cocci, cylindrical bacilli, spiral-shaped spirilla and comma-shaped vibrio).

Besides, based on the cell wall composition and structure, bacteria can be categorized as Gram-positive and Gram-negative. Gram-positive bacteria, such as *Staphylococcus aureus*, have an outer cell wall composed of several thick layers of peptidoglycans. Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli*, present a more complex cell wall composed of a single thin layer of peptidoglycans sandwiched between the inner (cytoplasmic) cell membrane and an outer membrane rich in lipopolysaccharides (LPS) and lipoproteins [18].

Bacteria can exist as planktonic cells (*i.e.*, isolated, free-floating cells) or sessile aggregates (*i.e.*, attached to a surface or living within a biofilm). Planktonic cells are responsible for the rapid proliferation and spread of microorganisms to new sites, while sessile cells characterize localized bacterial colonization and are often related to chronic pathological conditions [147,148]. Worthy of note is the ability of bacteria to switch back and forth between these two states.

3.2.2 Mechanism of bacterial colonization of surfaces

In general, the bacterial colonization process of a surface begins with the reversible adhesion of planktonic bacteria on the surface (Figure 3.2). Bacteria approach surface through Brownian motion or in directed mode, by means of flagella, the lash-like appendages of locomotion. When the microorganism and the surface reach a critical proximity (in the range of ≈ 1 nm), its adhesion depends on the balance of attractive and repulsive forces between their surfaces. At this stage, non-specific physicochemical interactions are involved, including hydrogen bonds, electrostatic, van der Waals and hydrophobic interactions. On the other hand, adhesins, unique proteins present on the bacterial surface, mediate the adhesion to the substrate [147,149,150]. When the bacterial density increases, by proliferation or recruitment of other cells, the quorum sensing process stimulates the proliferation and the exopolysaccharide matrix (EPS or slime) production, leading to the formation of the biofilm (Figure 3.2). The biofilm is a viscous layer, forming mushroom-like structures, composed of a variable fraction of 5-35% of bacteria, of the total biofilm volume, surrounded by EPS, permeated by water channels for delivery of nutrients, and removal of metabolites. This structure constitutes a protected mode of growth for bacteria populations, allowing their survival in hostile environments [21,147,151]. Finally, in certain conditions, such as when the environment is no more favorable or

because of a cellular programming for virulence, the detachment of planktonic cells or cell groups bound by the EPS occurs. This phenomenon leads to biofilm spreading and to the colonization of new sites [21,147,151,152].

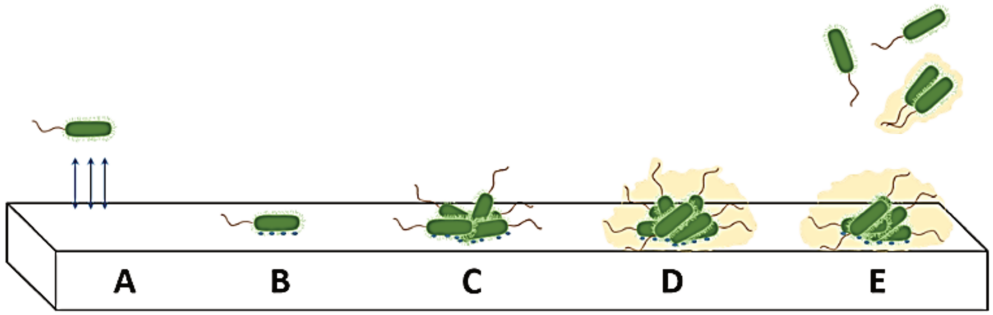

Figure 3.2 - The model of stages of bacterial colonization surfaces. The process begins through (A) physicochemical, non-specific and labile interactions followed by (B) stable attachment to the surface mediated by adhesins. (C) The bacteria density increases and (D) the colony produces an exopolysaccharide matrix (EPS) finally forming the biofilm. When the environment is no more favorable, (E) bacteria detach to colonize new sites.

When considering devices implanted into the body, the interactions and reversible/stable adhesion between bacteria and the abiotic surface may be influenced by the implantation site. In fact, several molecules present in physiological fluids such as proteins (*e.g.*, albumin, immunoglobulins (Ig), fibrinogen, fibronectin, etc.), proteoglycans, polysaccharides and lipids interact nonspecifically with the surface through electrostatic, Van der Waals and hydrophobic forces leading to reversible adsorption on the material and to the formation of the so-called conditioning film that modify the physicochemical properties of the surface and thus possibly affecting bacterial adhesion and the following colonization process [36,147,153,154]. The bacterial adhesion and biofilm formation on a material surface is a complex phenomenon, being influenced by three main factors: the characteristics of the bacteria strain, the physical, chemical and biological properties of the microenvironment, and the type of substrate, and each one of these factors is strictly related to many sub-parameters [154–156], as summarized in Figure 3.3. Once formed, biofilms are often resistant to the popular cleaning methods, sterilization as well as to antibiotics. These features make them very difficult to eradicate and, when associated with implanted biomedical devices, they can cause severe health problems [1].

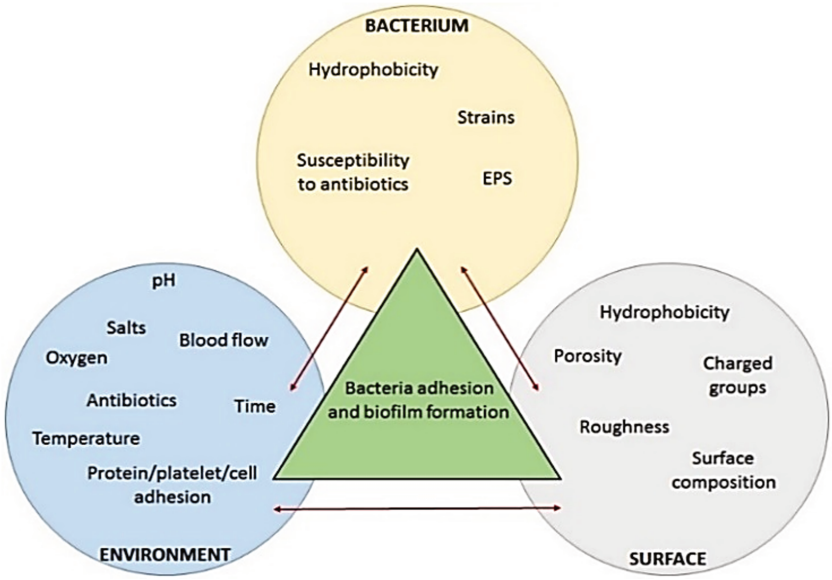

Figure 3.3 - Parameters and sub-parameters related to bacterial adhesion and biofilm formation on a biomedical device.

3.2.3 Bacterial contamination: a socio-economic problem

Despite the considerable progress in the treatment of microbial infections, in fact, bacterial contaminations in healthcare environments can cause serious health concerns and this is particularly relevant when considering the surface of a biomedical device that must be implanted or used in direct contact with body tissues [11,21,111]. In healthcare environments, pathogenic bacteria usually arise from an infected host or directly from the environment, through common vehicles such as contaminated air or water (Figure 3.4). In this regard, the contamination of biomedical devices and surgical tools is considered a major issue in medical interventions [1,11,110,155].

HCAIs are not pre-existential infections developed after a patient is exposed to healthcare facilities or biomedical devices contaminated by pathogenic microorganisms. HCAIs are considered as very serious safety threats in healthcare today because of their high incidence and of their related social and economic expenses. In fact, HCAIs can dramatically prolong the hospitalization period, affect patients after discharge and, the most important, when associated with surgical interventions and to the use of invasive biomedical devices they can lead to implant failure and reintervention, and if not properly treated, even to death.

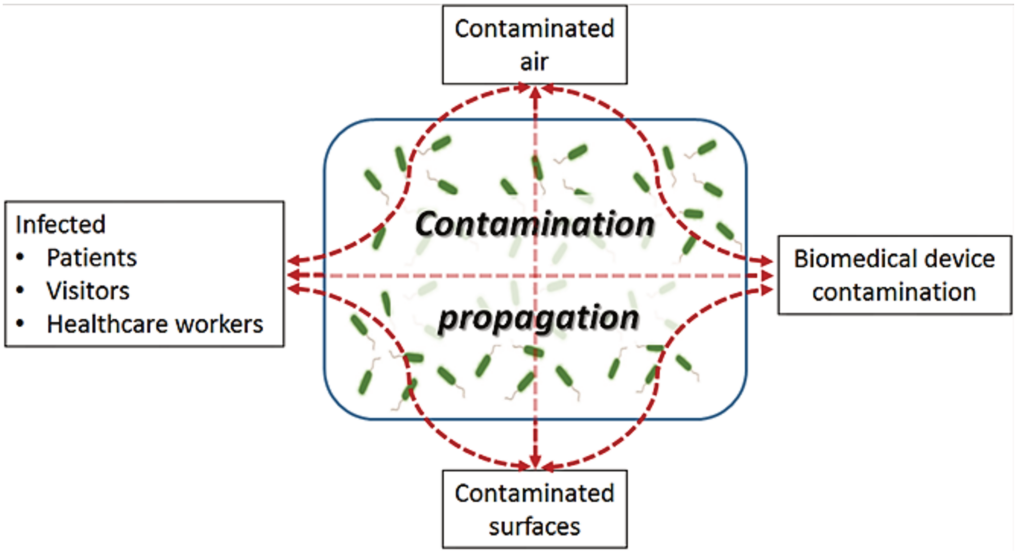

Figure 3.4 - Routes of the bacterial contamination spread in healthcare environments. The contamination can be spread by infected patients, visitors, and healthcare workers, through contaminated surfaces or air.

It is estimated that, worldwide, HCAIs will be responsible for 10 million deaths in 2050 [30]. Currently, in the USA alone, the contamination caused by microorganisms is responsible for around 722,000 HCAIs, resulting in nearly 75,000 deaths, and the associated yearly costs are estimated between $4.5 and $11 billion. In Canada, every year 220,000 people develop HCAIs, 8,000-12,000 patients die and more than $1 billion is spent. The estimated incidence in Europe is 4.1 million affected patients yearly, with a burden of 37,000 direct deaths and 117,000 indirectly related deaths each year. The situation is much worse in Latin American and African countries due to poor infrastructures and inadequate hygiene conditions [2,12,13]. Another serious problem related to HCAIs is the increased and widespread antibiotic resistance of microorganisms. HCAIs caused by methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and *Clostridium difficile* are becoming progressively more contagious and hard-to-treat [20].

In order to minimize the cases of HCAI, hand washing is the main strategy that is applied by healthcare employers worldwide. Although this is crucial to control and to prevent infections, it does not avoid the problem of cross-contamination. For example, an employee that has cleaned his hands can contaminate a patient after touching a contaminated surface. In addition, regular surface cleaning and hygiene procedures in hospitals remain less effective when a bacterial biofilm is already formed. Therefore,

the prevention of surface bacterial contamination is a great challenge in the biomedical field [19,44], particularly relevant when considering implantable biomedical devices [14] as evidenced by Table 3.1 that shows a general retrospect about bacterial infection incidence connected to some of the most commonly employed biomedical devices.

Table 3.1 - Most common device-related pathogens and infection incidence.

Device	Infection agent	Infection incidence	Reference
Breast implants	*S. aureus*, CoNS*, *S. pyogenes*, *Propionibacterium* spp.	0.8-1.7%	[158]
Cardiac pacemakers	*S. aureus*, CoNS, *Streptococcus* spp, *Candida* spp.	0.1-7%	[159]
Central venous catheter	CoNS, *S. aureus*, *Enterococcus* spp., *Candida* spp., *K. pneumoniae*	2-10%	[22]
Cochlear implants	*S. aureus, P. aeruginosa, H. influenzae, Streptococcus* spp.	1.7-3.3%	[160]
Contact lenses	*S. aureus, P. aeruginosa, Bacillus* species	0.3-5.2%	[161]
Coronary stents	*S. aureus*, CoNS, *P. aeruginosa*, *Candida* spp.	0.4%	[162]
Dental implants	*Streptococcus* spp., *Actinomyces* spp., *Porphyromonas* spp., *Prevotella* spp.	5-10%	[147,154]
Fracture fixation devices	*S. aureus*, CoNS, *Propionibacterium* spp., *Streptococcus* spp., *Corynebacterium* spp.	5-10%	[147]
Hip/knee implants	*S. aureus*, CoNS, *Streptococcus* spp., *Enterobacteriaceae*	0.5-4%	[22,147]
Intraocular lenses	*S. epidermidis*	0.01-0.3%	[164]
Mechanical heart valve	*S. aureus*, CoNS, *Streptococcus* spp., *Enterococcus* spp.	1-3%	[147]
Penile implants	*Staphylococcus* spp., *Pseudomonas* spp., CoNS, *Enterobacter* spp.	1-3%	[22]
Sutures	*S. aureus*, *P. aeruginosa*, *S. epidermidis*, CoNS	2-5%	[8,165]
Urinary catheter	*E. coli, Enterococcus* spp	10-30%	[167]

* CoNS coagulase-negative staphylococci

On this ground, the development of inherently antibacterial surfaces is a very promising approach to prevent HCAIs. The practical strategies proposed by investigators to this purpose are described in the section herein below.

3.3. Antibacterial surfaces: strategies and designs

As mentioned in Section 3.2, once formed on the surface of materials, biofilms are very difficult to strip out. The development of effective antibacterial surfaces has thus become a major challenge in biomaterial design and antibacterial coatings have become a very active field of research, strongly stimulated by the increasing urgency of identifying viable alternatives to the prophylactic administration of antibiotics [2].

An extensive literature survey points out three main strategies for the development of antibacterial surfaces, each of them with some pros and cons (Table 3.2), that are antibacterial agent release, contact-killing, and adhesion resistance/bacteria-repelling. Such lines of research that aim to enhance the antimicrobial properties of a surface can be implemented individually or synergistically [2,18,20,23,27,168]. However, regardless of the strategy and surface design, common features for antibacterial surfaces are required, such as biocompatibility, non-cytotoxicity, and reproducible production methods [168].

3.3.1 Antibacterial agent release

Antimicrobial agents such as metal ions, peptides, and antibiotics can be incorporated within and/or grafted on the surface of biomaterials to provide them with antimicrobial properties [170]. The material composition should favor the release of these compounds in a controlled fashion allowing antimicrobials to kill both adherent and adjacent planktonic bacteria with a long-lasting effect (Figure 3.5) [23,27,168]. The release of incorporated antibacterial agents can be achieved by simple diffusion from the implantation site to the surrounding aqueous medium, by material degradation, or hydrolysis of covalent bonds [2,168].

This general approach has some drawbacks (Table 3.2). Indeed, as it is difficult to predict the type and number of infecting bacteria at the implantation site, the load and the selection of the appropriate antimicrobial agent is challenging. The load may be too low to allow it to diffuse to the surroundings at the effective concentration, thus

failing in its proper function. If the agent is an antibiotic, it can face the problem of the host developing sensitivity to it and it can be inefficient toward antibiotic-resistant bacteria [171]. Furthermore, the lifetime of the antibacterial activity is limited by the antimicrobial agent reservoir (Table 3.2).

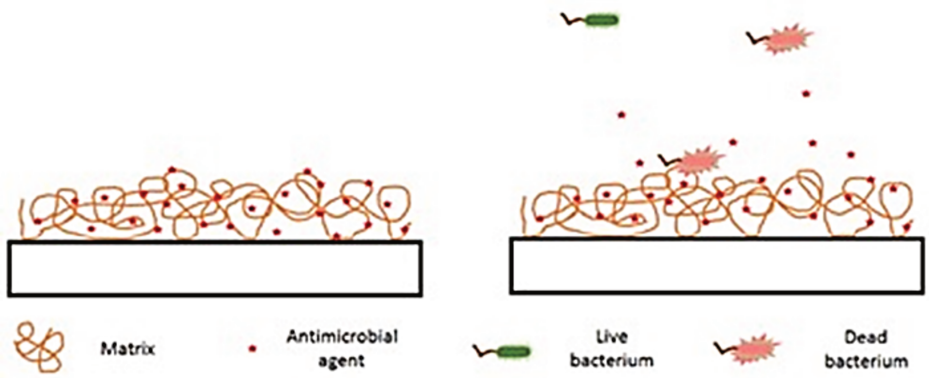

Figure 3.5 - Agent release strategy for the development of antibacterial surfaces.

3.3.2 Contact-killing

The contact-killing approach aims to eliminate or, at least, to blunt the proliferation of microorganisms adhering to the material surface via the covalent tethering of antimicrobials through polymeric spacers (Figure 3.6) [18,20,23,168] that permit the penetration of the biocides into the cell wall [16,73] and consequently lead to bacterial death. Due to the net negative surface charge that bacteria typically display [172], the most effective compounds for contact-killing coatings are cationic chemicals (quaternary ammonium compounds, chitosan, antimicrobial cationic peptides, etc.) or enzymes, such as lysozyme and other proteases [2,173].

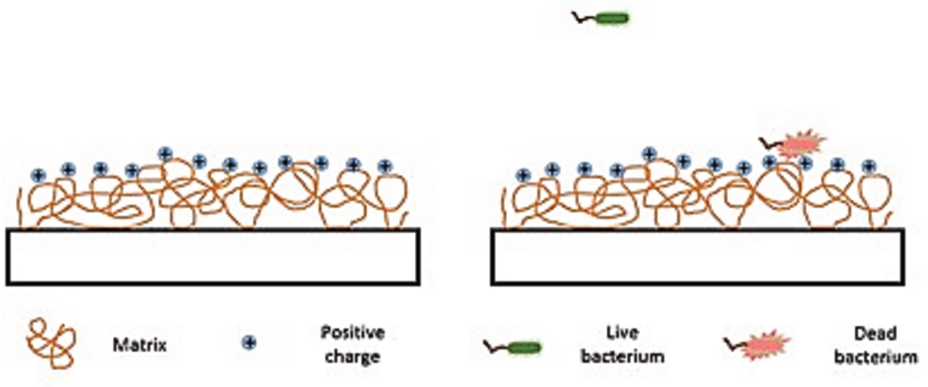

Figure 3.6 - Contact-killing strategy for the development of antibacterial surfaces.

Differently from the antibacterial release, in the contact-killing approach, the antimicrobial agent does not exhaust its effect over time, retaining its activity for a long time. However, its range of action is restricted to the area of the device that has been tethered (Table 3.2).

3.3.3 Adhesion resistance/bacteria-repelling

Anti-adhesive surfaces aim to avoid the early attachment of microorganisms to the material, thus finally preventing the formation of stable biofilms [20,23,24,168], by means of different surface modification strategies (Figure 3.7).

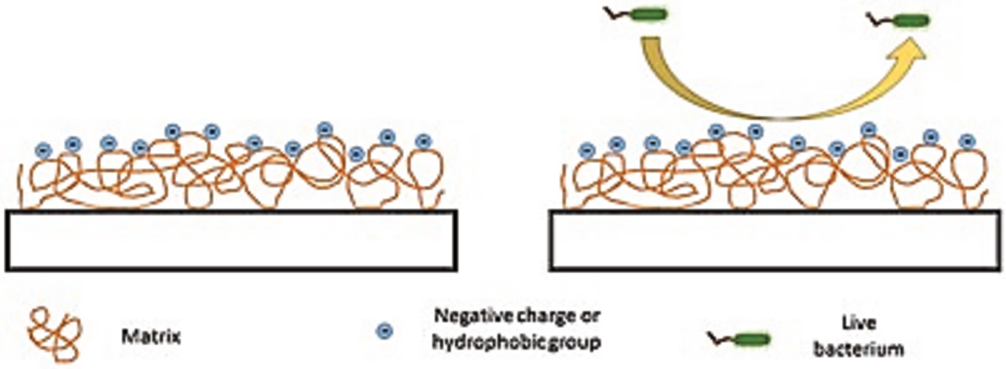

Figure 3.7 - Adhesion resistance/bacteria-repelling strategy for the development of antibacterial surfaces.

One of the approaches is to functionalize surfaces with molecules that can resist protein adsorption, such as polyethylene glycol (PEG) and zwitterions, or with superhydrophobic coatings (e.g., chitosan-based hydrophobic nanoparticles) [174], that demonstrated important anti-adhesion properties.

Even though stability concerns are often raised, these are generally considered as the standard approaches for the production of bacteria-repelling coatings. Despite evidence of the efficiency of the modified surface to prevent or limit bacterial adhesion, the main problem is related to the inability of such coatings to kill bacteria if the adhesion has already taken place (Table 3.2) [147].

Among the different design strategies developed for the production of antibacterial surfaces for biomedical applications, chitosan-based coatings represent a versatile, safe and promising approach due to the ease of processability and

functionalization of chitosan, its inherent antibacterial activity and to its many other favorable properties. In the following sections, after summarizing the main physicochemical and biological properties, and the many applications of chitosan and its derivatives, the techniques developed for the production of antibacterial chitosan coatings are reviewed in detail, with special emphasis on their advantages, drawbacks and on their range of pharmaceutical and biomedical applications.

Table 3.2 - Main advantages and disadvantages of the three strategies for the development of antibacterial surfaces.

Strategy	Advantages	Disadvantages
Antibacterial agent release	Extensive action, possibility to deliver a high amount of antibacterial agent Localized action, without exceeding systemic toxicity limits	Effect of bacterial inhibition temporary limited by the reserve of antibacterial agents Possible toxicity of the biocidal agent Possible induction of bacterial resistance
Contact-killing	Long-term functionality	Action restricted to the area of the modified surface
Adhesion resistance/bacteria-repelling	Non-cytotoxic mechanisms Bacterial colonization prevented at the first step of contamination	Action restricted to the functionalized surface No bacterial killing

3.4 Chitosan: a peculiar eclectic material

Due to its chemical structure, especially to the presence of amino groups in its saccharide chain, chitosan is a versatile biopolymer very interesting for many biomedical applications. Indeed, it is biocompatible, biodegradable and low toxic and its antimicrobial activity is acknowledged. In this section, the sources of chitosan, the production process, its physicochemical and biological properties are described, with a special focus on the biomedical and pharmaceutical fields.

3.4.1 History, source, and production

Chitosan is a semi-natural polymer derived from chitin, a polysaccharide of major importance. Chitin is the second most abundant natural polymer after cellulose and, excluding proteins, is the natural compound with the largest nitrogen content [77]. Historically, chitin and chitosan appeared for the first time in the world research context in 1811 with H. Branconnot. This French professor treated mushrooms with an alkali solution and obtained a white fibroid residue that he called fungine. Branconnot believed that this material was a cellulose derivative [175,176]. In 1823, A. Odier obtained a similar substance from the elytra of beetles and called it “chitin”, based on Greek etymology, which means “A Coat Of Mail”. In 1843, J.L. Lassaigne demonstrated that chitin composition displays nitrogen, in contrast with cellulose. In 1859, C. Rouget boiled chitin in a concentrated alkali solution and rendered it soluble in organic acids. This chitin derivative was named “chitosan” only in 1894 by Hoppe-Seyler [175,177]. However, it was only in the 1950’s that the chemical structure of chitosan, featuring deacetylated residues, was defined.

The chitosan sources, which depend on the chitin source, are various and are summarized in Table 3.3.

Table 3.3 - Sources and content of chitin in nature.

<table>
<tr><th>Sea animals</th><th>Chitin %</th><th>Arthropods</th><th>Chitin %</th><th>Micro-organisms</th><th>Chitin %</th></tr>
<tr><td>Annelida</td><td rowspan="2">20-38</td><td>Scorpions</td><td rowspan="11">20-60</td><td>Green algae</td><td rowspan="11">trace-45</td></tr>
<tr><td>Archiannelida
Chaetopoda
Hirudinea</td><td>Locusts</td><td rowspan="2">Brown algae</td></tr>
<tr><td></td><td></td><td>Flies</td></tr>
<tr><td>Mollusca</td><td rowspan="3">3-26</td><td>Spiders</td><td>Yeast (β-type)</td></tr>
<tr><td>Polyplacophora
Gastropoda
Scaphopoda
Cephalopoda</td><td>Butterflies</td><td>Chytridiaceae</td></tr>
<tr><td></td><td>Beetles</td><td>Ascomydes</td></tr>
<tr><td></td><td></td><td>Mosquitos</td><td>Blastocladiaceae</td></tr>
<tr><td>Cnidaria</td><td rowspan="2">3-30</td><td rowspan="2">Silkworm chrysalis</td><td>Fungi</td></tr>
<tr><td>Hydrozoa
Scyphozoa
Anthozoa</td><td>Ascomycetes
Basidiomycetes
Phycomycetes</td></tr>
<tr><td>Crustaceans</td><td rowspan="2">58-85</td><td>Ants</td><td rowspan="2">Mycelia penicillium</td></tr>
<tr><td>Lobster, crab, shrimp, krill</td><td>Cockroaches</td></tr>
</table>

They are mainly divided into the following categories: sea animals, arthropods, algae, and fungi. Indeed, chitin is found in the cell wall of fungi belonging to Zygomycetes, in the green algae Chlorella Spirulina, in yeast, in radulae of mollusks as well as in exoskeleton of arthropods [178,179]. Nowadays, the main commercial production of chitosan, 10^9-10^{10} tons per year, is based on crustacean shells, due to their high content and ready availability. Chitosan market size was valued over $1.52 billion in 2015 and is forecast to experience gains exceeding 16% compound average growth rate between 2016 and 2024.

The industry frequently uses as raw material crustacean shell wastes, coming from by-products of the seafood industries. Thus, this process is ecological-friendly and economically viable. The chitosan fabrication process used is a multi-step procedure:

- Chitin extraction: chitin is bound to proteins and mineral salts, thus, to recover the polysaccharides, a deproteinization step with alkali treatment at high temperature, and a demineralization step, in diluted chloride acid solution, are first performed.

- Bleaching process: to remove coloration from pigments present in the isolated chitin, oxidizing agents such as $KMnO_4$, H_2O_2 and hypochlorite solution or solvent extraction are carried out.

- Deacetylation: the common procedure is the addition of sodium hydroxide solution at 40-50% (w/v in water), under stirring at high temperature ranging from 80 to 150°C for several hours.

- Neutralization and purification: the previous basic solution is neutralized, and then chitosan purification is performed by solvatation/precipitation and filtration.

However, the properties of obtained chitosan in terms of purity, viscosity, the degree of deacetylation, molecular weight (Mw) and polymorphous structure vary considerably with the process parameters such as temperature, reaction time, products used for deproteinization, demineralization, neutralization, and the purification steps. In order to avoid all this harsh procedure using acidic and basic solutions that induce partial depolymerization, other more reproducible and controlled

biotechnological procedures such as fermentation and enzymatic treatments are under investigation.

3.4.2 Physicochemical properties of chitosan

The presence of primary amino groups in the chitosan structure differentiates it from chitin and gives to this polymer many peculiar properties. At acidic pH, chitosan is a polycation with a linear chain consisting of *N*-acetyl-*D*-glucosamine and *D*-glucosamine units linked by *β* (1→4) glycosidic bonds (Figure 3.8).

Depending on the source and on the chitin extraction process, it is possible to produce chitosan with different ratios of *D*-glucosamine units over the total number of units per chain [5].

This ratio is called degree of deacetylation (DDA) that is usually between 60 and 95%. The Mw, instead, can range from five to 8,000 kDa, meaning from oligomers to very high Mw polymers. These two chemical properties are key parameters, influencing the distribution and amount of protonated amino moieties ($-NH_3^+$) in the chitosan chain. Indeed, *N*-acetyl-*D*-glucosamine units affect the intrinsic viscosity and solubility of chitosan in aqueous solutions, and influence also the biodegradability and biocompatibility behaviors, the antibacterial activity, and many other properties, as summarized in Table 3.4 [5,76,81].

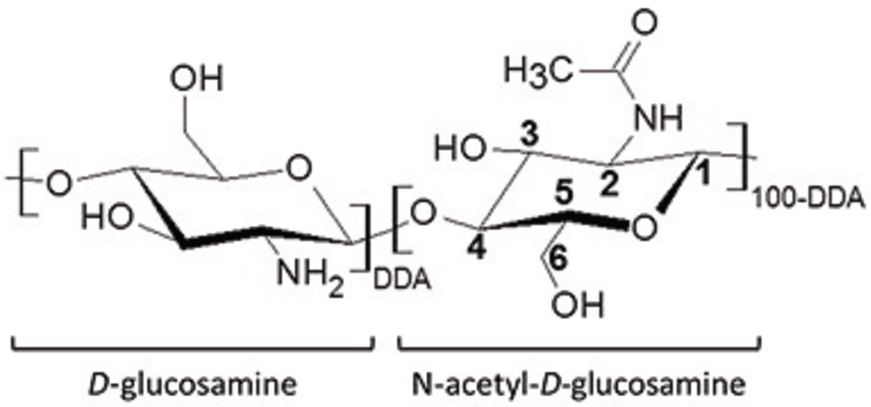

Figure 3.8 - Chemical structure of chitosan and denomination of carbon position (C1-C6). The N-acetyl-D-glucosamine and D-glucosamine units are indicated; DDA indicates the deacetylation degree.

The crystallinity of chitosan is another key parameter that influences the biodegradability and its biological properties [181]. Higher DDA leads to greater crystallinity, which reaches the maximum when chitosan is fully deacetylated. On the other hand, crystallinity increases while decreasing the Mw [182].

A large number of analytical tools have been used to quantify these important structural features. The DDA can be evaluated by different spectroscopic techniques such as FTIR, UV, NMR, but also by conventional titration methods, equilibrium dye adsorption, elemental analysis, acid degradation coupling with HPLC, and thermal analysis. The Mw and its distribution are usually assessed by light scattering spectrophotometry, gel permeation chromatography and viscometry [77,80,83,184].

Chitosan is poorly soluble/insoluble in (neutral) water, in alkaline medium and even in organic solvents. The pH-dependent solubility of chitosan relies on its amino groups, which become protonated upon dissolution at pH ≤ 6.5 (pKa~6.3) to form cationic amino moieties, increasing intermolecular electric repulsion and resulting in a soluble polycation [5,29,82,83]. Chitosan forms viscous solutions with pseudoplastic and viscoelastic properties, which are affected by DDA, Mw, concentration, type of solvent, pH, ionic strength, and temperature [76,83,186].

Table 3.4 – Relationships between chitosan structural parameters (DDA and Mw) and its properties.

Property	Structural features	
	DDA	Mw
Physicochemical		
Solubility	Increase	Decrease
Viscosity	Increase	Increase
Crystallinity	Increase	Decrease
Biological		
Biodegradability	Increase	Increase
Biocompatibility	Increase	Increase
Antimicrobial	Increase	Decrease

3.4.3 Biological properties of chitosan

Biocompatibility and biodegradability are important properties for applications in the pharmaceutical and biomaterial fields. In a general way, biomaterials produced with chitosan are well tolerated by living tissues, including skin, ocular membranes, bones as well as the nasal epithelium, and this is an important feature for a wide range of

biomedical applications such as tissue engineering (*e.g.*, bone, cartilage, cardiac, nerve, etc.), wound healing, and delivery systems (*e.g.*, drug, proteins, peptides, antibiotics, etc.). In vivo toxicity studies, with chitosan, demonstrated its safety profile in terms of inertness and low toxicity for mammalian cells [187]. However, the purity degree of chitosan and its origin should be carefully checked as they may have some dramatic toxic effects (residual byproducts from the extraction procedure and traces of proteins from seafood) [76].

Chitosan has been reported as highly biodegradable because the break of glycosidic bonds can be easily achieved through a chemical or an enzymatic hydrolysis. This characteristic is crucial for drug delivery systems and tissue regeneration applications [188]. Chemical biodegradation refers to chitosan hydrolysis in acidic gastric milieu [189–191], whereas the enzymatic hydrolysis occurs by means of some enzymes such as lysozyme, which is found in mucosal surfaces, and chitinases, which is produced by the intestinal flora. The biodegradation of chitosan induces its depolymerization, and this, in turn, leads to the production of non-toxic oligosaccharides. This process is of key importance in regard to biomedical applications as such short oligosaccharides can be easily processed by regular metabolic pathways or excreted by renal clearance due to their size [190]. The chitosan biodegradation is related to its crystallinity degree, Mw and DDA: when chitosan crystallinity decreases, its biodegradation rate increases [179,192]. Besides, it can be assumed that smaller chitosan chains will be more rapidly degraded than chitosan with higher Mw [179].

Interestingly, chitosan displays useful mucoadhesive properties which are directly related to the DDA. Indeed, free amino groups from chitosan interact with the mucoadhesive membranes, made of a negatively charged glycoprotein called mucin [179,193]. Therefore, the higher the DDA of chitosan, the greater the number of cationic charges carried, the stronger the interaction with anionic mucous membranes [92]. The interaction of the polycationic chitosan with the negatively charged cell membranes gives also rise to very interesting biological properties, such as hemostatic and analgesic effects. In this regard, the interaction of red blood cells, as well as platelets, with chitosan allows to speed up clot formation and hemostasis [70,195], thus taking part in the coagulation and cicatrization process [93,94,196]. In the same matter,

the analgesic effects of chitosan can be ascribed to the proton release by the cationic *D*-glucosamine residues in the area of inflammation [77].

Despite its non-cytotoxicity towards mammalian cells, chitosan has a wide spectrum of antimicrobial activity against fungi, Gram-positive and Gram-negative bacteria, and yeasts [197]. The mechanism of antimicrobial activity of chitosan is not completely known but several factors can influence it. Studies evaluating the antimicrobial activity of chitosan against different groups of microorganisms suggested that three main mechanisms of inhibition of microbial growth are involved [34,85]. The first and basic mechanism proposed relies on the cationic amino groups of chitosan that increase the permeability of negatively charged outer cellular layer, causing disruption and release of intracellular components (Figure 3.9A). Two other synergist mechanisms have been also identified: the inhibition of intracellular enzymes activity through the chelation of metals (Figure 3.9B) and cytoplasmic DNA/mRNA complexation causing the inhibition of protein syntheses (Figure 3.9C) [79,86].

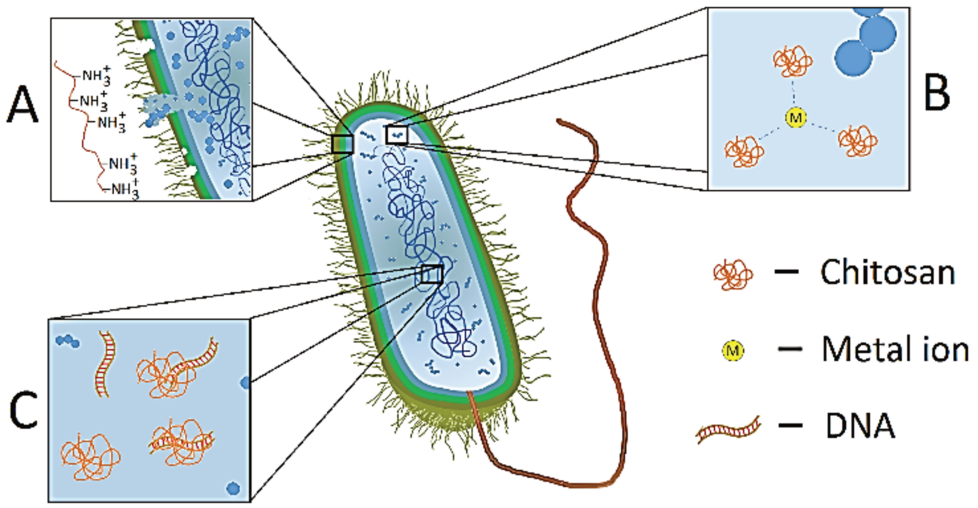

Figure 3.9 - Mechanisms of antibacterial action of chitosan: (A) cell wall charge disruption; (B) chelation of metals in trace; (C) complexation with DNA.

The antimicrobial activity of chitosan is influenced by its Mw. Low Mw chains were found more effective than longer ones, probably due to the higher mobility of small chains that can better penetrate the bacterial membrane [3,179,199]. In the same way, chitosan with high DDA and thus with highly cationic character, are more effective

than those with a low DDA in terms of bacterial growth inhibition. Taken together, the antimicrobial activity of chitosan is due to the presence of protonated amino groups. In this regard, the pH thus strongly affects the antibacterial effectiveness of chitosan: the lower the pH (below the pKa value of ≈ 6.3), the higher the antibacterial activity displayed by the aminopolysaccharide [80,90-94].

3.4.4 Immunogenicity, allergenicity, and genotoxicity of chitosan

Chitosan has been tested for safety and toxicity in a number of animal species, and by various routes of administration [200]. In this regard, Kitozyme and Primex Corporations have compiled comprehensive information as part of self-certifications to support a “generally recognized as safe” (GRAS) status as it has been recently and comprehensively reviewed by some authors [191,201]. Overall, chitosan is widely regarded as being a non-toxic, biologically compatible polymer [202] and it is approved in Japan, Italy, and Finland for dietary applications [203] and it has been FDA approved for use in wound dressings [204].

3.4.4.1 Immunogenicity and antigenicity

While immunogenicity is the ability of a particular substance, such as an antigen or epitope, to provoke a humoral and/or cell-mediated immune response in the body of a human or animal, antigenicity is the capacity of some (bio)chemical factors, such as antigens or haptens, to be specifically recognized and bind T cell receptors or antibodies (IgG-binding) that are the products of adaptive immunity. Antigenicity was more commonly used in the past to refer to what is now known as immunogenicity, and the two are still often used interchangeably.

The use of chitosan for pharmaceutical and medical applications requires highly purified GMP-grade material comprising carbohydrate containing little or no residual protein and chitosan-based products should comply with appropriate pharmacopoeial tests [200]. Since proteins are significantly more immunogenic than polysaccharides, purified chitosan is considered non-immunogenic and thus non-antigenic. On the other hand, chitosan has been shown to be involved in the production of IgM by the immune system in response to antigens. It stimulates in vitro IgM production but not that of IgG or IgA by HB4GS cells and human lymphocytes [205].

3.4.4.2 Allergenicity

Allergenicity is defined as the capacity of a given substance to elicit an IgE immune response upon animal or human exposure. Allergenicity is thus the potential of a material to cause sensitization and allergic reactions and is mediated through immunological mechanisms such as IgE antibody binding. Because some individuals are allergic to shellfish, some scientists have been prompted to investigate the presumed relationship between allergy and the presence of chitin in shellfish.

It is worthy of note that isolated chitin is a biopolymer deeply different from that present in vivo, that is part of a complex structure with other inorganic and organic and components responsible for its allergenic potential. Instead, isolated, pure and ultrapure chitin and chitosan, are plain polysaccharides devoid of any residual proteins [206]. In this regard, a number of researchers have demonstrated the absence of any allergic response in subjects with shellfish allergy following oral challenge with shellfish-derived glucosamine [207,208]. Further evidence on the anallergic properties of chitosan comes from the absence of allergic reactions, or any other adverse event, following the use of chitosan dressings, even in people allergic to shellfish [209].

3.4.4.3 Genotoxicity

Genotoxicity describes the property of chemical agents that damages the genetic information within a cell causing mutations, which may lead to cancer. The antigenotoxic activity of chitosan, assessed using the sister chromatid exchange assay following adsorption of different mutagens [210] showed that this biopolymer did reduce the genotoxicity of such chemicals, suggesting that it may play a protective role against environmental mutagens [211].

3.4.5 Chitosan modifications

The deacetylation process gives to chitosan a new reactive functional group when compared with chitin: a primary amino at the C_2 position. This amino and the primary and secondary hydroxyl groups at the C_3 and C_6 positions, respectively, are responsible for one of the most important features of chitosan: the ease of chemical modification under mild conditions. Indeed, these reactive groups are readily subjected

to chemical derivatization, to allow chitosan new functionalities and properties, as described in Table 3.5.

Such modifications are also used to provide chitosan, and chitosan-derivatives, with new mechanical and physicochemical properties: for instance, the solubility of chitosan at neutral pH has been improved by tethering water soluble, hydrophilic moieties to it [212], or its mechanical behavior has been heightened by controlled acetylation [5,76,77,178,213].

Table 3.5 – Some typical modifications of chitosan

Modification	Function	Reference
Methylpyrrolidinone chitosan	Hydrophilic chitosan-based scaffolds for bone regeneration	[214]
2-N-/6-O-/2-N,6-O-sulfated chitosan	Enhance the activity of Bone morphogenetic protein-2	[215]
N,N,N-trimethyl chitosan	Antibacterial activity	[216]
Sulfonated chitosan	Hemocompatibility	[195]
Chitosan-g-PVA	Hemocompatibility	[217]
N-hexanoyl chitosan	Hemocompatibility	[218]
N,O-/N-succinyl chitosan	Hemocompatibility	[219]
Chitosan-g-PEG-folate	Gene carrier	[220]
O-/N,O-carboxymethyl chitosan	Antibacterial activity	[221]
Phosphorylcholine-chitosan	Drug delivery / gene therapy	[99]
	Nanocarriers with protein-repelling proteins	[222]
	Substrate for endothelial progenitor cells culture	[223]
O-carboxymethyl chitosan	Hemocompatibility	[224]
O-stearoyl chitosan	Hemocompatibility	[101]
Chitosan-g-PEG	Antibacterial activity	[225]
Chitosan-g-caffeic acid	Antioxidant activity	[226]
Chitosan-g-lysozyme	Antibacterial activity	[173]
6-O-/3,6-O-sulfated chitosan	Hemocompatibility	[227]
N-octyl-O-sulfate chitosan	Drug carrier	[228]
Chitosan-g-poly(glycidyl methacrylate)	Drug delivery	[229]
Chitosan-g-poly(2-(furan-2-carbonyl)-acrylonitrile)	Antibacterial activity	[230]
Chitosan-g-imidazole	Bone regeneration	[231]

3.4.6 Chitosan applications

The study and use of chitosan and of its derivatives have been constantly growing over the last four decades, as demonstrated by the steep increase in the number of papers indexed in Scopus database from 1975 (16 documents) to 2016 (5,045 documents) related to chitosan and its derivatives. The peculiar chemical and biological properties of chitosan, together with the possibility to process it in multiple forms (powders, solutions, gels, sponges, beads, fibers, scaffolds, nanoparticles, films, porous and dense membranes) [195,232–238], in fact have opened the way towards a number of applications in different fields, such as cosmetics, pharmaceutical, medical, agricultural, water treatment, food, and textiles, as summarized in Table 3.6.

Table 3.6 - Main applications of chitosan and derivatives in different fields

Field	Applications	Reference
Cosmetics	Skin, hair, and oral care products; Lipsticks; Deodorants	[193]
Pharmaceutical	Controlled drug release; pills coating and stabilizer; antibacterial, antitumor, antioxidant and anticoagulant agent; nutritional aid for weight loss	[70,75,76,88,195,241–242]
Biomedical	Wound dressing; thromboresistant and antimicrobial coatings; scaffolds for tissue engineering; cell delivery systems; gene delivery	[88,,243–246]
Agriculture	Microbial infection prevention; biofungicide; plant growth promoter	[88,90]
Food industries	Food shelf life improver; preservative; thickener; moisture loss prevention	[88,91]
Textile industries	Antimicrobial coatings; moisture control; dye absorption	[77,88,92]
Wastewater treatment	Coagulation and flocculation agent; removal of heavy metal ions	[88,93]

This review focuses on chitosan-based antibacterial coatings for biomedical applications. The main applications of chitosan and its derivatives in cosmetics and in pharmaceutical and biomedical fields are summarized hereinafter in brief.

3.4.6.1 Cosmetics applications

Owing to its biodegradability, biocompatibility, viscosity, and moisture holding properties chitosan is widely used in cosmetics for skin, hair, dental and oral care products, lipsticks, and deodorants [193].

3.4.6.2 Pharmaceutical applications – Drug delivery

There are many examples in the literature of the use of chitosan and its derivatives as pharmaceutical products, for example in drug delivery [3].

Chitosan is commonly used as an excipient in tablet formulations for oral medication. In fact, viscous high Mw chitosan can delay the release of the active component, thus prolonging the duration of drug activity and improving the therapeutic efficiency and reducing the side effects related to high peak doses [250].

Biocompatible and biodegradable chitosan-based microspheres and hydrogels can be employed for the delivery of a wide variety of drugs in a controlled/sustained manner. As comprehensively reviewed by Mitra and Dey [251], chitosan microspheres can be prepared by different techniques such as self-assembly of positively charged chitosan with polyanions (*i.e*. ionotropic gelation), emulsion cross-linking, thermal cross-linking, coacervation/precipitation, spray drying and sieving, among others. Hydrogels are three-dimensional (3D) hydrophilic polymeric networks in which the solid phase typically represents less than 10% of the total volume of the gel and that can thus absorb huge volumes of water, as recently reviewed by Ahmadi and colleagues [252]. Chitosan-based hydrogels can be prepared by physical crosslinking, exploiting ionic interactions (*e.g*., ionically cross-linked and polyelectrolyte complexed chitosan hydrogels), secondary interactions (*e.g*., entangled chitosan-based hydrogels [253]), or by chemical cross-linking, allowing to obtain a wide variety of assemblies with specific mechanical, thermal and biological properties that can be tuned by changing the composition of the gel. Specific medical applications of chitosan microspheres and hydrogels involve gastrointestinal, colon, ophthalmic, oral, nasal, transdermal and vaginal drug delivery [251,254].

Interestingly, injectable thermosensitive hydrogels combining chitosan with glycerophosphates or other weak bases such as sodium hydrogen carbonate, sodium phosphate dibasic or phosphate buffer have been developed [255]. They do behave

liquid-like at temperatures between 4 and 20°C but, upon injection into the body at 37°C they form semi-solid gels allowing local controlled drug delivery [256].

In addition, chitosan and their derivatives can be formulated in micelles for the delivery of poorly soluble pharmaceuticals, mainly anticancer drugs such as paclitaxel, Mytomycin C, doxorubicine, and camptothecin [257,258].

Chitosan has also been thoroughly investigated as highly cytocompatible non-viral gene delivery vector in gene therapy approaches [246]. Gene delivery consists in the introduction into cells of nucleic acids that can therapeutically act as gene substitutes, gene inhibitors and gene vaccines [259]. Chitosan is positively charged in solution at slightly acid pH, thus interacts electrostatically with nucleic acids and self-assemble with them to form nano/micrometric complexes named polyplexes that are capable to enter eukaryotic cells and deliver their content. The gene delivery activity of native chitosan has been demonstrated both in vitro and in vivo [260,261] but its efficiency is lower as compared to other polymeric non-viral vectors. Several chitosan derivatives have thus been synthesized by grafting to the chitosan chain other cationic polymers more effective for this purpose, such as low Mw polyethylenimine (PEI) [262] and hydrophilic, hydrophobic, pH-sensitive, thermosensitive and cell-specific moieties [263].

3.4.6.3 Biomedical applications

The versatility and the many favorable biological properties of chitosan and its derivatives greatly widen the number of their biomedical applications they are used in, spanning from wound healing to tissue engineering, from gene delivery to antibacterial coatings.

Owing to their high water content, biodegradability, biocompatibility, porosity, tunable properties, and ability to promote cell adhesion and proliferation, chitosan-based hydrogels have been widely employed as scaffolds for tissue engineering purposes and as (thermosensitive) injectable cell delivery systems for tissue regeneration [179]. Specifically, they are investigated for engineering/regenerating various tissues such as bone, cartilage [264], skin [265], blood vessels [266], and nerves [267].

All the aforementioned properties make chitosan also very suitable for wound dressing/healing. Several commercial wound care products working as effective antibacterial barriers are already on the worldwide market, even if not all are EMA and FDA approved (*e.g.* HemCon®, ChitoFleX®, ChitoGauze®, Chitodine®, Tromboguard®, Tegaderm™). Chitosan, processed in different forms such as sponges, films, and nanofibrous porous and non-woven membranes, in fact, can provide a hydrated 3D matrix for tissue growth that allows for high gas exchange, and protect from microbial infections [268]. Furthermore, chitosan has been demonstrated to promote the activity of macrophages, leukocytes, and fibroblasts, thus stimulating and enhancing extracellular matrix (ECM) deposition along with possibly preventing excessive scar formation [269].

3.5 Main techniques to obtain antibacterial chitosan coatings: advantages, limitations, and applications

As previously mentioned, pathogenic microorganisms can adhere to the surfaces of medical devices, causing serious infections and clinical complications (Section 3.2). Antibacterial chitosan coatings can meet the current urgent need of biomaterials with inherent antibacterial properties, to which microorganisms cannot develop resistance as it often happens with antibiotics. Furthermore, as described in section 3.4, chitosan coatings are promising due to their biocompatibility, antimicrobial activity at low concentrations and their broad antimicrobial spectrum. This section is thus dedicated to the description and evaluation of the main methodologies employed to produce antibacterial chitosan coatings, their advantages, their limitations and their main applications in the biomedical field.

Several techniques can be used in order to produce chitosan coatings with antibacterial properties. However, to immobilize these coatings on biodevice surfaces, two main approaches are commonly used: physical deposition based on surface secondary interactions such as electrostatic and Van der Walls forces and hydrogen bonding, or surface grafting leading to stable covalent attachment [29,59]. The approach of choice will depend on the specific application: for example, if the coating should be stable at long-term, as expected for devices such as vascular catheters, orthopedic prosthesis, dental implants and other implantable devices, the covalent attachment will be preferred. However, if biodegradation is expected such as in drug

delivery systems and tissue engineering scaffolds, or if the antibacterial activity is needed only at short term as for wound dressing applications, adsorption/deposition approach based on physical interactions will be preferred [5,59,121].

Independently of the target application, the production of chitosan coatings basically consists of two steps: material surface preparation/activation and immobilization of the chitosan coating.

3.5.1 Material surface preparation/activation

Before the deposition of any type of coating, the material surface must first be cleaned, usually by several washing steps in a solvent or/and aqueous solution, followed by physical or chemical activation to improve the coating adhesion. Cleaning steps are required in order to ensure that grafting or coating is done on materials and not on contaminants which would lead to premature detachment. Pre-treatments of biomedical device surfaces could be done by physical modification, polishing or blasting, by physical/chemical techniques such as plasma treatment, anodization, and chemical modifications such as acid etching and surface functionalization by using for example silanol, phosphates, phosphonates, dopamine, acrylic acid, etc., as summarized in Table 3.7. These techniques will be briefly detailed thereafter.

3.5.1.1 Physical pre-treatments

Physical surface pre-treatment techniques are commonly employed for metallic substrates.

Mechanical polishing

Mechanical polishing is performed by using abrasive papers [142], (sand, silicon carbide, diamond papers), using different grits to obtain the desired finishing or roughness.

Table 3.7 - Surface treatments employed before the deposition of chitosan-based coatings. Graft. and Ads. indicate, respectively, grafted and adsorbed coatings.

Type of modification	Technique		Effect on the surface	Surface	Device/Application	Ref.
	No modification	Ads		Metal	Ni/Ti orthopedic	[139]
Physical	Mechanical polishing	Ads	Morphology Roughness	Metal	Ni/Ti implant for dental, orthopedic, Stainless Steel implants Bone replacement and regeneration applications	[140] [142]
	Blasting	Ads		Metal		[271]
Physical and chemical	Plasma - He/ Ar/Air	Ads	Etching	Polymer	PEEK* for regenerative medicine and orthopedic PP nets - hernia repair	[272] [273]
	- $He/O_2/H_2O$	Ads	Functionalization	Polymer	Biomedical devices	[274]
	- Air	Ads	Functionalization	Polymer	PTFE* - Prosthetic devices	[5]
	- N_2/ H_2	Grft	Functionalization	Polymer		
	Electrochemical anodization	Ads	Controlled porous oxide layer	Metal	Titanium nanotube arrays for bone applications	[275]
Chemical	Acid etching (HF, HNO_3, HCl)	Ads	Morphology Activation /Etching	Silicon/Glass Silicon/Glass	Protective coatings for medical devices Surface activation used before silanes and dopamine	[58] [276] [133]
	Phosphates	Ads		Polymer SS316L	Biomedical applications	[277] [278]
	Phosphonates	Ads	Functionalization	Cortical bones	Modified periosteum	
	N-halaminos	Ads	Functionalization	Polymer	Polyglycolide biodegradable suture	[143]
					Ti implant – Orthopedic	[134,138]
	Poly(ethylene imine) - PEI	Ads	Functionalization	Metal		[132]
				Polymer	PDMS*/silicone materials for intraocular lenses (cataract surgery Prevent infections on catheters and tracheal tubes	[144]
				Glass	General biomedical applications	[9]
	Dopamine	Grft	Functionalization	Metal	Ti implants for dental and orthopedic applications SIBS* / Drug-eluting stents	[133,137,141] [279]
				Polymer	PU* membrane for wound healing	[131]
	Silane	Grft	Functionalization	Metal	Ti implants for dental and orthopedic applications Stainless steel-based medical implants and devices	[133–136,276,280] [281]
	Methacrylic acid	Grft	Functionalization	Polymer	General biomedical applications	[282]

* PEEK poly(ethylene ether ketone), polyurethane (PU),poly(tetrafluoroethylene) (PTFE), polypropylene (PP), poly(styrene-b-isobutylene-b-styrene) (SIBS), polyvinylfluoride (PVF)

Blasting

Blasting process is performed in order to increase the surface roughness to increase the adhesion strength of the coating. It is done by driving, under high pressure, a stream of abrasive particles (sand, alumina, glass etc.) onto the surface. For example, Song et al. used alumina particles of 380 µm under 0.4-0.7 MPa on Ti alloy (Ti-6Al-4V), before spraying their chitosan coating [271].

3.5.1.2 Physical/chemical pre-treatments

Among physical/chemical pre-treatments, anodization is also performed only on metallic substrates, whereas plasma techniques are usually performed for surface modification of polymers such as PEEK, PU, PTFE, PP, etc.

Anodization

In the anodization process, the substrate to be modified is used as the anode in an ionic solution, an oxidation reaction occurs at the surface increasing the thickness of the natural oxide layer. The oxide layer is often porous allowing the reaction with the underlying metal and the incorporation of bioactive agents [283-286] and to improve the adhesion of the successive chitosan coatings [275].

Plasma technique

In a general way, plasma can be defined as the fourth fundamental state of matter, where a totally or partially ionized gas is obtained by electrical discharges originating radicals, electrons, ions, neutral atoms and/or metastable and unstable molecules. Several chemical and/or morphological modifications can be generated to the material surfaces when exposed to plasma, depending on its composition and energy. Plasma allows to modify only the first layer of materials without changing its bulk properties, is eco-friendly (no solvent) and versatile, furthermore it is already an industrial technique [4,31]. By varying parameters such as power, pressure, treatment time, but also the feed gas, the effect of plasma on the substrate can be easily changed and adapted depending on the desired surface modification [31,112-115]. As pre-treatment for chitosan coatings, two main types of effects can be obtained:

- Surface etching by using argon or helium leads to an increase in the coating adhesion [272],

- Surface functionalization with water vapor, air, carbon dioxide, nitrogen/hydrogen, etc., permit to introduce functional groups on the surface, used for their hydrophilic character, allowing a better surface wettability and an increase in the coating/surface adhesion through Van der Walls or hydrogen interactions [273,274,287]. When used for the grafting approach, instead, these functional groups act as anchor points for chitosan grafting, leading to the formation of covalent stable bonds [5].

3.5.1.3 Chemical modifications

In wet chemistry, the surface modifications are obtained by employing chemical solutions aiming to introduce functional groups or charges, or to change the surface hydrophilicity, morphology and roughness (e.g., etching by acid-basic solutions) of the substrate biomaterials [59]. The wet chemistry used for biomaterials are often classic laboratory approaches, not requiring specialized equipment, this being considered an advantage. Furthermore, chemical modifications offer many different strategies to functionalize material surfaces, thus they can be considered as a versatile platform.

In some case, pre-treatments with harsh acidic solution such as piranha one ($H_2SO_4/H_2O_2/H_2O$) or Kroll's reagent ($HF/HNO_3/H_2O$) are done in order to increase the density of hydroxyl groups (Figure 3.10A), on the metallic or glass surface [59,133,276] before further functionalization with dopamine, silanol, phosphates, etc. Although, these pre-treatments are very efficient for the removal of organic impurities and to promote changes in the roughness of the material surface, depending on the composition and the shape of the substrate, the control over the reactions can be limited, and since it is not always possible to tune the intensity and the efficiency of the chemical reactions on the surfaces, non-uniform wettability and roughness are obtained [58].

Regarding surface functionalization, it can be done by using various reagents, such as harsh acid solution (piranha), halamino, phosphates or phosphonates derivatives, dopamine, silanol and methacryl acid, as shown in Table 3.7, leading to functionalized surfaces with different chemical groups (Figure 3.10), which are then

used as anchor points for ionic interactions for adsorption techniques or for chemical grafting of chitosan or its derivatives.

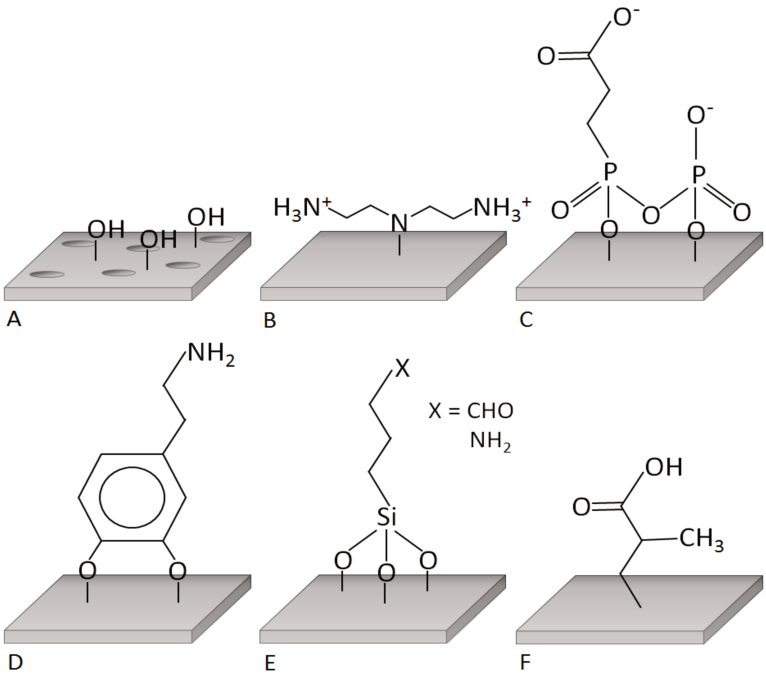

Figure 3.10 - Schematic representation of surface activation by (A) harsh acid solution (e.g., piranha), (B) halamino, (C) phosphates or phosphonates derivatives, (D) dopamine, (E) silanol, and (F) methacryl acid.

In order to have ionic interactions, the surface of the device can be functionalized with chemical functionalities leading to a highly-charged surface: to obtain a positive charge, ammonium derivatives (Figure 3.10B) [13,132,134,138,143,144] are commonly used whereas for a negative charge (Figure 3.10C), anionic reagents, such as phosphates [277], carboxylic acids [278] have been investigated. To maximize the interactions with the antibacterial chitosan-based coatings, polycations and polyanions can also be used:

- Polycationic agents such as halaminos (Figure 3.10B) [143], poly(ethyleneimine) (PEI) [13,132,134,138,144] can be adsorbed on the surface after its activation (*e.g*. Piranha);

- The most employed polyanions are polyphosphates (Figure 3.10C) such as sodium tripolyphosphate (TPP), sodium pyrophosphate (PP), and sodium hexametaphosphate (HMP) that are anchored on the preactivated surface (*e.g*.,

oxidation) [277]. The phosphonate approach can also lead to free carboxylic end groups by using 11-phosphonoundecanoic acid (PUA), for example [278].

The grafting approaches for chitosan-based coatings aim at creating stable covalent linkages between the surface and the antibacterial coating. The most commonly used approaches are:

- Dopamine: the reactivity of dopamine is based on its catechol moieties known to form stable bonds with surfaces (Figure 3.10D), through its oxidation to quinone in alkaline conditions (pH 8.6, in aqueous buffer solutions). Furthermore, its capability to polymerize on various substrates, from metals, to organic polymers (polyethylene, polystyrene, polyethylene terephthalate and polycarbonate) and to inorganic materials (SiO_2 and Al_2O_3) [70,137,173,288–290] makes dopamine functionalization a very attractive strategy in stable coating deposition. Indeed, biomedical devices can be easily fully and uniformly covered by a dopamine layer, which can thereafter react with amino groups present on chitosan by Michael addition or Schiff base reaction to produce covalently attached antibacterial coatings [70,131,133,137,141,279].

- Silanization is another very attractive surface activation technique due to the versatility of the various silanol derivatives available. Indeed, silanol moieties react with the hydroxylated substrate leading to a stable covalent link (Figure 3.10E), whereas the terminal end groups remain available for the chemical grafting of the chitosan-based-coating. For example, it is possible to introduce aminos by using (aminopropyl)triethoxysilane (APTES) [6–8,81] and aldehydes with triethoxsilylbutyraldehyde (TESBA) [135,136,280]. However, oppositely to dopamine, this reaction can be performed only on metallic surfaces, and furthermore, it should be done in the anhydrous condition in organic solvents such as toluene.

- Polymeric surfaces can be functionalized with carboxylic reactive groups by using methacrylic acid, as shown by Lv et al. [282] (Figure 3.10F). After pre-activation of the polymeric substrate by ozone plasma leading to peroxide, the surface was reacted with the methacrylic acid in solution leading to a covalent graft. Free carboxylic acids were then used to anchor the chitosan-based antibacterial coating.

3.5.2 Chitosan coating deposition/grafting techniques

3.5.2.1 Deposition techniques by physisorption

3.5.2.1.1 Simple adsorption

The simplest way to produce chitosan coating is to cast a chitosan solution over the substrate, or immerse the substrate in the solution and let the solvent evaporate. This process occurs at the liquid-solid interface and it is based on the interface charges [291]. The main advantages of this technique are its simplicity and facility to perform at low cost. Another important issue is this it is a reversible process, without chemical changes [291] between the substrate and chitosan coating, in this regard, the antibacterial activity of the coating can be increased due to higher the availability of free amines (no chemical bond with the surface). However, the quality of chitosan coatings produced by simple adsorption is limited, control over the coating properties is difficult and delamination can easily occur.

An example of the application of chitosan coating obtained by simple adsorption is reported by Sulek and colleagues that developed chitosan-based antimicrobial coatings for orthopedic implants, previously mechanically ground and etched by sulfochromic acid. Tests with *S. epidermidis* and *S. aureus* showed that the number of bacteria attached to the surface coated with chitosan was lower if compared to bare surfaces [292]. Moreover, cotton gauzes were functionalized by carboxymethyl chitosan-calcium alginate solution coating to produce a wound healing dressing featuring moisture holding and antibacterial activity that was demonstrated on *E. coli* and *S. aureus* [293]. In another recent work, Vicryl, a commercial absorbable suture was directly coated with hydroxypropyl trimethyl ammonium chloride chitosan dissolved in type I collagen solution and it was compared with Vicryl Plus, a similar suture with antibacterial properties. Methicillin-resistant *S. epidermidis* and *S. aureus* were employed to perform the antibacterial tests evaluating the bacterial adhesion and biofilm formation on the sutures and human skin-derived fibroblasts cells were used to test the cytocompatibility. Results showed that hydroxypropyl trimethyl ammonium chloride chitosan-coated Vicryl sutures exhibited antibacterial activity comparable to that of Vicryl Plus sutures together with good cytocompatibility [294].

3.5.2.1.2 Dip coating

The dip coating is a simple and low-cost technique to cover surfaces with a thin film usually of high quality. Similarly, to simple adsorption method, the chitosan coatings obtained by dip coating can present a high number of free amino groups but, unfortunately, delamination process can occur at the long term. In a general way, this technique consists of three steps [60,61]:

- Immersion and dwell time: the substrate is dipped into the solution and the dwell time should be sufficient to let occur interactions between the surface and the solution;

- Deposition and drainage: the substrate is pull up with a constant speed and the excess solution is drained from the surface;

- Evaporation: the solvent evaporates forming a thin film on the surface.

For example, chitosan/poly(vinyl pyrrolidone) (CHI-PVP) coatings obtained by dip coating on pre-activated PET devices demonstrated a decrease in the adherence of *S. aureus* and *E. coli* together with bactericidal activity. Furthermore, no cytotoxicity was observed in human umbilical vein endothelial cells (HUVEC) seeded on the modified surfaces [297]. Chitosan nanoparticles-polymethylacrylate and chitosan colloids-polymethylacrylate were also dip coated on glass substrates. These surfaces exhibited antibacterial behavior against *S. aureus*, with superior activity demonstrated for chitosan colloids-polymethylacrylate coatings [295].

In another work, Ignatova et al. produced electrospun fibers dip-coated with quaternized chitosan and k-carrageenan, leading to significant antibacterial activity against *S. aureus* and *E. coli* but also with antioxidant activity due to the combined presence of quaternized chitosan and k-carrageenan. These fibers with dual effect are promising for wound healing dressings [296].

3.5.2.1.3 Layer-by-layer (LbL)

Chitosan coatings can also be produced by layer-by-layer (LbL) deposition. In this technique, the substrate is previously electrically charged through a functionalization technique (*e.g.*, plasma, piranha solution, etc.) and is then, sequentially dipped in polyelectrolyte solutions with opposite charges, as shown schematically in Figure 3.11 thus depositing successive layers of opposite charge.

Importantly, each deposited layer must have a minimum charge density to attract the other polyelectrolytes with opposite charge.

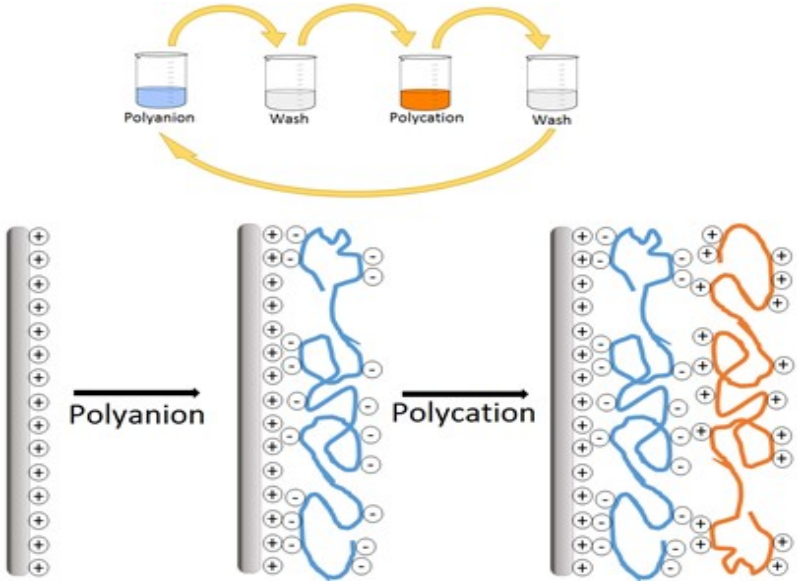

Figure 3.11 - Layer-by-layer approach: steps 1 and 3 represent the adsorption of oppositely charged polyelectrolytes and steps 2 and 4 represent washing steps.

The repetition of the adsorption sequence forms the final multi-layered coating [62–63]. LbL technique allows the utilization of different polyelectrolytes such as synthetic polymers (*e.g.*, polyacrylic acid (PAA), polyethylenimine (PEI), etc.) or natural polymers (*e.g.*, chitosan, alginate, hyaluronic acid, etc.) to cover different types of substrates with irregular shapes and sizes, in an easy and versatile coating production process. Some examples of the use of LbL technique to obtain antibacterial chitosan coatings are briefly described below.Heparin/chitosan LbL coatings were deposited on aminolyzed PET substrates. The antibacterial and antiadhesive properties of the coatings were evaluated using *E. coli* and it was shown that a superior bacterial reduction could be obtained for layers prepared at low pH (*i.e.*, pH 3.8) with respect to slightly acidic pH (i.e., pH 6.0), owing to the higher amount of electronically charged chitosan chains present on the surface of the coating [24].

Richert and colleagues [270] thoroughly investigated the process of deposition of chitosan/hyaluronic acid films by LbL demonstrating that low MW chitosan and high ionic strength (*i.e.*, 150 mM NaCl) allow a faster film growth. Interestingly the films obtained at high ionic strength demonstrated a significant resistance to bacterial

adhesion (80% reduction using *E. coli*) but also eukaryotic cell adhesion was impaired [270]. The antibacterial properties of chitosan/hyaluronic acid LbL films were confirmed by another study where silicon (Si) wafers pre-treated with PEI were employed and a reduction of up to 99% of bacterial colonies (*S. epidermidis*) adhered to the functionalized substrates was observed [249]. Recently, aiming to optimize the LbL deposition of chitosan/hyaluronic acid nanofilms and to maximize their antibacterial activity, Hernandez-Montelongo and collaborators [13] investigated the relation between the pH of the polysaccharides solutions and both the growth of the nanofilms and their antibacterial effect. In this study, a single PEI pre-layer was deposited onto the bare surfaces, and the results showed that deposition at pH 3 led to the maximal exposure of chitosan chains (and their free ammonium groups) on top layer of the surface and consequently to improved antibacterial activity, with a reduced cell density of 5 orders of magnitude against *S. aureus* (Gram-positive). Furthermore, it was shown that surface charge density and antibacterial activity increased by increasing the number of bilayers. However, unexpectedly, only a limited antibacterial effect was observed for *P. aeruginosa* (Gram-negative), these results suggest a bacterium-specific activity of these chitosan coatings and a lower efficiency against Gram-negative bacteria, possibly owing to their outer cell membrane [13]. Chitosan/hyaluronic acid LbL coatings were also employed to coat intraocular lens, pre-coated with a single PEI layer. The results showed that chitosan could provide antimicrobial activity against *E. coli* and *S. aureus* by two ways, reducing the bacterial adhesion and killing the bacteria attached to the substrate. On these premises, the authors propose this coating for the prevention of post-cataract surgery infectious endophthalmitis [132].

A particular strategy to produce all-chitosan-based LbL films was proposed by Bulwan *et al.* that employed cationic and anionic chitosan derivatives (a cationic polyelectrolyte based on chitosan substituted with quaternary amines and an anionic polyelectrolyte based on chitosan substituted with sulfonate groups) for the deposition on Si and glass surfaces pre-treated with piranha solution. Noteworthy, the developed surfaces demonstrated antifouling, antibacterial and anticoagulant properties making them promising versatile protective coatings for medical devices and tools that come into contact with blood [58].

To prevent bone allografts suffer failure due to poor integration and infection, chitosan-heparin coatings were deposited via LbL on pre-activated cortical bones. Results showed that the deposition of a PUA pre-layer enabled the formation of chitosan-heparin layers resulting in the complete coverage of the surface and significant antibacterial activity against *S. aureus* and *E. coli*. Surprisingly, notwithstanding the presence of the coating bone cells could probably still interact with adhesion ligands presents on the surface of the bone and the adhesion of mesenchymal stem cells was not inhibited [278].

3.5.2.1.4 Spray coating

Spray coating is a deposition technique that employs gas flow to separate a fluid into small droplets and direct them onto the surface of a substrate where a film is deposited (Figure 3.12).

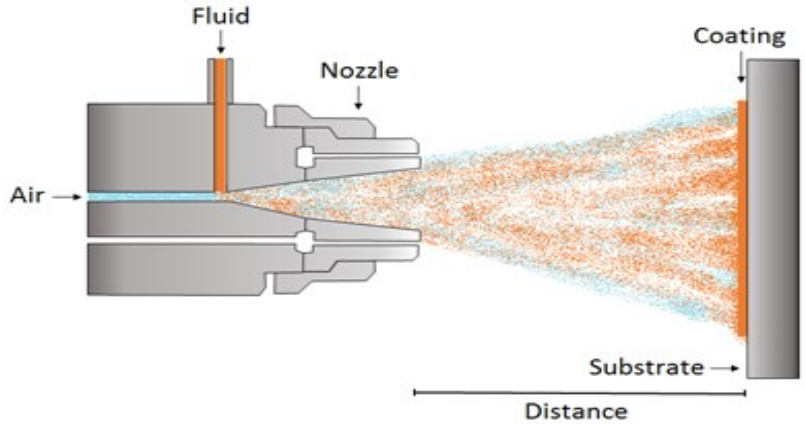

Figure 3.12 - Schematic representation of the spray coating system.

This technique allows obtaining homogeneous coatings in short deposition times on substrates with various geometries [64–248]. Furthermore, electrostatic forces can be exploited to improve the adherence of the coating and to produce stable polyelectrolyte multilayers. However, the deposition parameters, such as, distance from the sample, nozzle, needle opening (fluid flow), pressure and spraying time play a determinant role in obtaining a quality coating.

Mitra and collaborators coated polymeric and metallic substrates with quaternized chitosan. For the two treated substrates, it was observed antibacterial activity against *S. aureus* and *P. aeruginosa* and no cytotoxicity on 3T3 mouse

fibroblast cells. Besides, the coatings were highly stable to wiping [277]. In another study, glass substrates were coated with polyelectrolyte multilayers of chitosan and hyaluronic acid functionalized with cateslytin (an antimicrobial peptide). Interestingly, antimicrobial activity increased with the number of deposited bilayers, up to the complete inhibition of the development of *S. aureus* and of the fungus *Candida albicans*, combined with a limited fibroblasts adhesion on these coatings which have been thus proposed for applications such as catheters or tracheal tubes where tissue growth is not desired and infections should be prevented with extreme care [144].

3.5.2.1.5 Spin coating

The spin-coating technique involves the application of a solution containing the compound to be deposited (usually polymers) on a flat substrate, followed by rotation at high speed, causing the liquid to undergo centrifugal acceleration so that it spreads throughout the substrate. When the excess liquid is ejected from the substrate, the solvent evaporates leaving a uniform thin film, as shown in Figure 3.13. The thickness of the coating is a function of rotation speed, viscosity of the solution, concentration of the deposited agent and of the type of solvent. Despite this technique is widely employed, among the main disadvantages, there are material wasting and the limited type of geometries that can be processed [66].

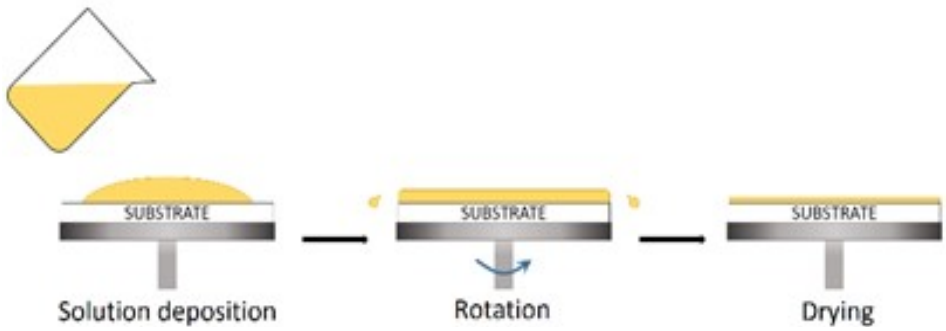

Figure 3.13 - Schematic representation of the spin coating system.

A representative application of this technique is reported by Sutha and co-workers. They coated stainless steel implants for orthopedic applications with a chitosan solution blended with Si-doped hydroxyapatite powders. Remarkably, the antibacterial activity against *E. coli* and *S. aureus* increased the amount of substituted

Si in hydroxyapatite, possibly owing to a different amount and distribution of the negative surface charges of the Si-doped hydroxyapatite component [67].

In another work, in order to develop titanium implants with anticancer and antibacterial properties, TiO_2 nanotubes were produced to work as selenium nano-reservoirs and coated by a chitosan layer. Results demonstrated that this material could inhibit the proliferation of cancerous osteoblasts while promoting that of healthy osteoblasts and at the same time it exhibited antibacterial properties when tested against *E. coli* [275].

3.5.2.1.6 Electrospraying

Electrospraying is a technique that exploits electrical forces to atomize fluids. The fluid, flowing through a capillary nozzle maintained at a high potential, is forced by the electric field to be dispersed into droplets that are directed towards a grounded and heated substrate where the macromolecules they carry are deposited upon evaporation of the solvent. The size of the droplets can be finely tuned by changing the process parameters with radii from few nanometers to hundreds of micrometers, thus allowing to obtain coatings with different topographies [240].

The use of electrospraying has been often proposed in combination with chitosan to obtain antibacterial coatings for biomedical devices, especially in the orthopedic and dental fields. To improve the properties of NiTi alloys for orthopedic applications, chitosan blended with gold nanoparticles was electrodeposited on these surfaces. Results showed that the coating reduced the Ni release, improved the corrosion resistance and possessed fast and long-lasting antibacterial effectiveness against *S. aureus* [139]. Similarly, also, chitosan/Ag composite coatings were deposited by electrospray on NiTi alloys, showing once again good antibacterial activity [140].

3.5.2.1.7 Electrophoretic deposition

In the electrophoretic deposition, the charged colloidal particles, in suspension, migrate under the action of an electric field and are deposited on an electrode. In the case of chitosan coatings, the substrate material is cathodically polarized and the deposition is due to the local pH variation caused by electrochemical decomposition of

water; in fact, a high pH region is produced at the cathode where the loss of charge of the chitosan amino groups lead to the formation of an insoluble deposit [240]. This technique is not expensive and versatile, enabling to produce homogeneous coatings on complex geometries, without using expensive apparatuses. However, the production of high-quality chitosan coatings with the desired thickness and homogeneity properties by this technique can be complicated if variables as pH and temperature are not finely controlled. In particular, H_2 bubbles are formed at the cathode and their presence can affect the smoothness of the surface. In addition, electrodeposited chitosan coatings are not stable in acidic conditions [239]. Examples of proposed applications of electrodeposited chitosan come once again from the orthopedic field. Recently, titanium surfaces were coated with chitosan-vancomycin by cathodic electrophoretic deposition. The coating reduced the number of *S. aureus* colonies due to chitosan action, further improved by the antibiotic vancomycin, and did not show any adverse effect on biocompatibility when it was tested with osteoblast-like cells [194]. In another similar study, electrophoretic deposition was used to produce coatings composed of bioactive glass particles and chitosan on stainless steel and TiAl4V6 alloys. An important antibacterial effect against *E. coli* was observed for all the developed coatings. The coating was homogeneous and displayed high adhesion to the substrate by tape test on planar samples, but it was not possible to produce homogeneous coatings on complex geometries (metal foams made from TiAl4V6 alloy) [185].

3.6 Grafting of chitosan coatings on biomaterial surfaces

Grafting of chitosan on the surface allows the strong attachment of the coating by covalent bonds and can be accomplished by different approaches, as summarized in the Figure 3.14:

- The coating is linked directly to the surface (Figure 3.14 A, B) due to chemical functionalities present in the material structure or introduced by previous chemical modification of the surface (as described above in 3.5.1.3);

- The grafting occurs through a linking arm (*e.g.*, glutaric anhydride, glutaraldehyde, bifunctional poly(ethylene glycol), etc.), used to indirectly link the coating to the pre-functionalized substrate (Figure 3.14C) [5,29,59,122];

- The grafting occurs by using chitosan modification (Figure 3.14D), leading to reactive groups able to react with the functionalized surface [42].

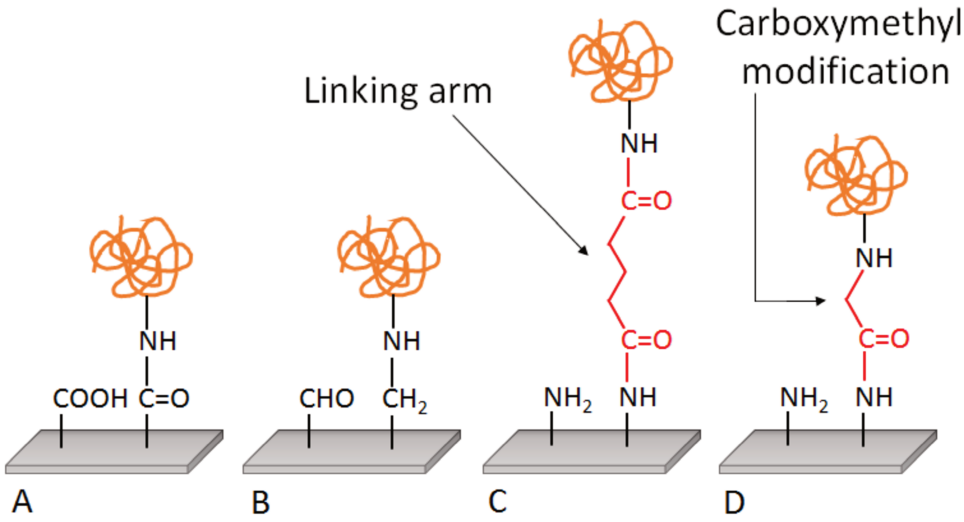

Figure 3.14 - Schematic representation of the approaches employed for the grafting of chitosan on biomaterial surfaces. Chitosan is directly linked to the (A) COOH or (B) CHO groups present on the surface; chitosan is grafted to the NH_2 groups present on the surface by (C) a bifunctional linker or (D) directly by exploiting chemical functionalities introduced in the chitosan structure, in the example carboxymethyl chitosan is reported.

3.6.1 Directly tethering chitosan coatings on biomaterial surfaces

The directly covalent immobilization of chitosan coatings onto the substrate can be performed by several ways. Among them, the most employed is the surface functionalization by aldehydes and carboxylic groups. These functionalities are able to react with nucleophilic groups from chitosan, such as amines and alcohols, leading to a covalent bond, originating a stable chitosan coating. However, due to the higher reactivity of amines, the reaction with amino moieties occurs preferentially thus leading to loss of free amines for antibacterial activity.

Chitosan grafting on surfaces pre-activated with carboxylic groups

As described in section 3.5.1.3., carboxylic groups can introduce on the surface, for example, by acrylic acid (AA) grafting. Using this approach, Lv and collaborators [260,264] grafted chitosan coatings onto PU surfaces by methacrylic acid linkages, which presented a good biocompatibility when tested with mammalian cells and a long action against *S. epidermidis*, *S. aureus*, and *E. coli*. Furthermore, it was possible to incorporate in the coatings anionic antibiotics (*e.g.*, rifampin), extending their release

time and thus improving the long-term antibacterial behavior [282]. Chitosan was also grafted on acid-activated nonwoven PP, useful for the production of pads and fabrics used in hospitals, demonstrating antibacterial activity towards *P. aeruginosa* [7]. In another work, the grafting of chitosan-Rose Bengal (CHRB) trough chitosan amino groups and carboxylic acid of PDMS activated substrates was performed in order to reduce the risks of bacterial infection during surgical application. The CHRB coating antibacterial activity was investigated using *E. coli* and *S. aureus* and results suggested a preferential bactericidal activity against Gram-positive bacteria [97].

Chitosan grafting on surfaces pre-activated with aldehyde groups

Aldehyde-functionalized surfaces can be obtained by using, for example, triethoxysylilbutyraldehyde (TESBA) as described in section 3.5.1.3. These terminal aldehydes are very reactive towards amines and alcohols. For instance, titanium dental implants previously activated by aldehydes led to a stable chitosan coating due to covalent grafting, exhibiting great scratch resistance, and also a significantly higher adherence than a simple chitosan deposition, as evidenced by indentation and scratch tests. Furthermore, the chitosan grafted titanium implants exhibited good biocompatibility to NIH3T3 fibroblasts, strong inhibition of *Actinomyces naeslundii* growth; nonetheless they showed a non-significant inhibition against *Porphyromonas gingivalis*, which can be explained by the lower antibacterial activity of chitosan against Gram-negative bacteria [280].

3.6.2 Tethering chitosan coatings on biomaterial surfaces using a linking arm or chitosan modification

The covalent immobilization of chitosan coatings employing a linking arm occurs when the pre-activated surfaces exhibit terminal amino groups, as described in subsection 3.5.1 for dopamine, aminosilane (APTES) and N_2/H_2 plasma treatments. Indeed, amino groups present on the substrate cannot react directly with amino moieties of chitosan, therefore it is necessary to use a homo or hetero-bifunctional linking arm, that possesses terminal carboxyl or derivatives (*e.g.*, N-hydroxysuccinimide, NHS), or aldehyde reactive groups (*e.g.*, glutaraldehyde) [135]. This approach permits to obtain covalently bonded stable chitosan coatings, but it adds a step in the surface processing and makes the procedure longer. However, the

reactions with linking arms are often easily performed in buffer solutions. Furthermore, the use of specific spacer-containing linking arms, such as PEG spacers of different length, could be helpful regarding antibacterial coatings, as PEG is known to be anti-fouling thus possibly permitting to combine bacteria-repelling behavior with the bactericidal properties of chitosan [5].

Another way to graft chitosan directly on previously aminated surfaces is to modify chitosan reactive groups (NH_2, CH_2OH) with other functionalities, such as carboxymethyl moieties [133], able to directly react with the amino functionalized surface. This approach induces chemical modification of chitosan, which should be well controlled, and also introduces the need for further purification steps. However, as described in section 3.4.5 (Table 3.5), this chitosan modification can be also an added value as it can improve biological properties: for example, carboxymethyl chitosan has been shown to induce hemocompatibility properties, but also to have a high antibacterial activity.

Chitosan grafting on pre-activated surfaces by using a linking arm

In a preliminary study, Vaz and coworkers [5] evaluated the influence of three linking arms, exploiting the reaction between carboxylic and amino groups, on the grafting of chitosan on plasma-aminated PTFE substrates, aiming to identify structure-property relationships. The short glutaric anhydride linker (Mw: 114 Da), was used due to its low steric mobility and led to chitosan coatings with high proximity to the substrate. Poly(ethylene-alt-maleic anhydride) was used as long, high Mw linker (Mw: 100-500 kDa). This anchor molecule allowed multiple linking points with both the substrate and the chitosan molecules. Finally, a PEG-based bifunctional linker, poly(ethylene glycol) bis(carboxymethyl) ether (Mw: 600 Da), with carboxymethyl groups at both the extremities, was used aiming to combine the PEG antiadhesive properties with the bactericidal ones of chitosan. Chitosan coatings showed good stability and the type of anchor used influenced the quality of the obtained coatings, with the glutaric anhydride-based coatings being the most homogeneous. Unfortunately, no antibacterial tests were performed, thus hindering to draw structure-activity relationships [5]. In another study, chitosan-lysozyme coatings were grafted on stainless steel surfaces activated by dopamine with glutaraldehyde as a homo-bifunctional linker. Bioconjugation of chitosan with the antibacterial enzyme lysozyme

was aimed at improving the antibacterial efficiency of the coating, especially under non-acidic conditions. Results showed that lysozyme moieties could further enhance the antibacterial activity of chitosan coatings against *S. aureus* under neutral pH conditions [173].

Modified hydrophobic chitosan coatings on magnetic nanoparticles have been recently proposed for an interesting biomedical application: the removal of bacteria and biofilms from contaminated surfaces [98]. Chitosan was modified by grafting with dodecyl hydrophobic tails and then deposited on the surface of magnetic nanoparticles activated by aminosilane through glutaraldehyde grafting. The developed hydrophobically modified nanoparticles were able to capture and coagulate Gram-negative bacteria (*E. coli*, capturing capacity: 1.38×10^{8} cells/mg), that could not be captured at all by nanoparticles coated with plain chitosan.

Chitosan grafting on aminated surfaces by chitosan modification

Carboxymethyl chitosan is often used as chitosan derivative to coat aminated surfaces, due to its chemical reactivity and to the preserved antibacterial activity. As a practical example, medical silicone surfaces were pre-treated with dopamine and then modified with carboxymethyl chitosan. Antibacterial assays showed that the coating could significantly reduce the adhesion of *E. coli* and *Proteus mirabilis*, even if without an outstanding efficiency (*i.e.*, ca. 90%), and cytotoxicity was not observed when treated surfaces were tested with fibroblasts [42].

In a comparative study, it was evaluated the stability of carboxymethyl chitosan coatings grafted onto Ti surfaces using dopamine and aminosilane as surface pre-functionalization agents. The carboxymethyl chitosan coatings efficacy against S. epidermis was demonstrated, however, surprisingly, chitosan coatings anchored with aminosilane presented a decreasing stability after contact with 70% ethanol treatment, autoclaving, and immersion in PBS [133]. These results demonstrated the need for performing systematic characterizations of the coatings not only in terms of antibacterial activity but also of stability in different milieus, upon sterilization and cleaning techniques, aspects that are often underestimated and overlooked in the development of chitosan-based antibacterial coatings.

3.7 Conclusion

The development of antibacterial coatings for biomedical devices has been strongly prompted in the last 20 years owing to the increasing awareness and understanding of the healthcare-associated infections and particularly of biomaterial-associated infections together with the parallel growth of antimicrobial resistant pathogens that limit the usefulness of the traditional antibiotic-based approaches.

A wide variety of approaches has been investigated, relying mainly on coatings for antibacterial agent release, contact killing, and adhesion resistant surfaces. Even if the release of antibacterial agents can take advantage of compounds with recognized strong activity such as antibiotics, the release kinetics must be finely tuned to obtain the desired effects, pathogens can develop or have already developed resistance toward the antibiotics and, most importantly, the lifetime of the antibacterial activity offered by this strategy is limited. In this light, the development of adhesion resistant and especially of contact killing surfaces, with inherent antibacterial properties, has gained more and more interest in the biotechnology and biomaterial fields. In this light, in this review, we focused on chitosan as naturally-derived biocompatible antibacterial material for the development of surface coatings for pharmaceutical and biomedical applications.

Chitosan in fact, in addition to its intrinsic antimicrobial activity, features a series of beneficial properties for application in the biomedical field since it is highly biocompatible, non-immunogenic and non-allergenic, it is quite inexpensive and it can be easily processed in different forms such as gels, films, membranes, sponges, nanoparticles with tunable characteristics. On these premises chitosan has often been considered as the material of choice for the development of antibacterial coatings, not only in the biomedical field but also in food and textile industry.

Several strategies, involving adequate surface preparation followed by physiosorption or chemical grafting, can be exploited to produce chitosan-based coatings, generally on metal and polymeric surfaces, for a number of applications that range from dental implants to catheters, from orthopedic prostheses to intraocular lens. However, it is difficult to identify general rules or coatings with superior “universal” properties since (i) many parameters, other than antibacterial efficiency, should be taken into account simultaneously (*e.g.*, mechanical, physical and chemical stability, biodegradation rate, mechanical properties, thickness, morphology, etc.), (ii) the

characteristics of the coating must be tailored and optimized for the specific application, and (iii) comparative studies are often missing, still making not clear how deposition techniques and chemical grafting can affect the antibacterial properties of the chitosan molecules. Furthermore, there is a general lack of information on the performance of chitosan antibacterial coatings in vivo, owing to the expensiveness of these tests, and currently employed in vitro experiments are performed in exemplified models that do not adequately mimic the huge number of factors acting in vivo (*e.g.*, inflammation, pH, presence of enzymes, adsorption of proteins, cyclic mechanical solicitations, etc.).

A more systematic research, involving both thorough material physicochemical characterization and biological evaluation, preferably also *in vivo*, would be necessary to draw reliable structure-activity relationships that could guide the design of chitosan antibacterial coatings optimized for the specific application. More standardized evaluation protocols would also be beneficial to allow the easier and trustworthy comparison among the results obtained across different laboratories.

Nevertheless, the positive results usually obtained by chitosan coatings against both Gram-positive and Gram-negative bacteria (and sometimes also against fungi), despite their known preferential bactericidal activity against Gram- positive cells, and their almost always demonstrated cytocompatibility are very promising cornerstones for the future translation into clinics of these technologies. In particular, multifunctional systems, combining different mechanisms of action against pathogens are emerging as the next generation chitosan-based coatings.

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4. COVALENT GRAFTING OF CHITOSAN ON PLASMA-TREATED POLYTETRAFLUOROETHYLENE SURFACES FOR BIOMEDICAL APPLICATIONS

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Résumé

La modification de la surface des polymères permet une amélioration des propriétés des biomatériaux et le développement de matériaux optimisés qui ont des réponses mécaniques et biologiques adaptées ou adaptables à l'environnement où elles seront implantées. Le chitosane est considéré comme un biopolymère qui présente des propriétés biodégradables, biocompatibles et antimicrobiennes, ce qui le rend particulièrement intéressant pour le développement et l'application de nouveaux matériaux fonctionnalisés. Cependant, ses propriétés et ses applications dépendent fortement de leur degré de désacétylation (DDA) et de leur poids moléculaire (Mw). Dans cette étude, trois types de chitosane ont été utilisés et ont été caractérisés selon leur DDA et Mw par RMN ^{13}C, FTIR, titrage potentiométrique et SEC. Ensuite, trois bras d'ancrage ont été étudiés afin de greffer ces trois types de chitosane sur les surfaces de PTFE aminées: l'anhydride glutarique, le poly (éthylène glycol) bis (carboxyméthyl) éther et le poly (éthylène-*alt*-anhydride maléique). L'efficacité du greffage a été démontrée par XPS, avec une augmentation des bandes caractéristiques du chitosane dans les spectres à haute résolution et par des mesures d'angle de contact indiquant le caractère hydrophile des surfaces. L'influence de la procédure de greffage a entrainé des changements topographiques qui ont également été observés par MEB. Les résultats montrent le potentiel de conjuguer les traitements de plasma avec des techniques de greffage pour le développement de revêtements optimisés à base de chitosane pour une large gamme d'applications pour les biomatériaux et les matériaux pour la santé.

Resumo

A modificação superficial de materiais poliméricos permite a melhoria das propriedades da superfície do biomaterial, possibilitando o desenvolvimento de materiais otimizados que tenham respostas mecânicas e biológicas adaptadas ou adaptáveis ao meio onde serão implantadas. Quitosana é conhecida como um biopolímero que apresenta propriedades biodegradáveis, biocompatíveis e antimicrobianas, tornando-se particularmente interessante para o desenvolvimento e aplicação de novos materiais funcionalizados. No entanto, suas propriedades e aplicações dependem fortemente do seu grau de desacetilação (DDA) e do peso molecular (Mw). Neste estudo, utilizaram-se três tipos de quitosana e caracterizaram-se completamente de acordo com o seu DDA e Mw por ^{13}C RMN, FTIR, titulação potenciométrica e SEC. Em seguida, três moléculas ancoradoras foram investigados para enxertar esses três tipos de quitosana em superfícies de PTFE aminadas: anidrido glutárico, poli (etilenoglicol) bis (carboximetil) éter e poli (etileno-*alt*-anidrido maleico). A eficiência do grafting (enxerto) foi evidenciada por XPS, com aumento das bandas características de quitosana em espectros de alta resolução e por medidas de ângulo de contato que indicavam o caráter hidrofílico das superfícies. As alterações topográficas após o grafting também foram observadas por SEM com evidências da influência desse procedimento. Os resultados mostram o potencial das técnicas de tratamentos de plasma com grafting para o desenvolvimento de revestimentos otimizados à base de quitosana para uma ampla gama de aplicações em biomateriais e materiais para a saúde.

Abstract

Surface modification of polymeric materials allows biomaterial surface properties improvement, enabling the development of optimized materials that have mechanical and biological responses adapted or adaptable to the environment where they will be implanted. Chitosan is claimed to be a biopolymer which presents biodegradable, biocompatible and antimicrobial properties making it particularly interesting for the development and application of new functionalized materials. However, its properties and applications strongly depend on its degree of deacetylation (DDA) and molecular weight (Mw). In this study, three types of chitosan were used and fully characterized according to their DDA and Mw by ^{13}C NMR, FTIR, potentiometric titration and SEC. Then, three linking arms were investigated in order to graft these three chitosan types on aminated PTFE surfaces: glutaric anhydride, poly(ethylene glycol) bis (carboxymethyl) ether and poly(ethylene-*alt*-maleic anhydride). The grafting efficiencies were evidenced by XPS, with an increase of chitosan characteristic bands in high resolution spectra, and by contact angle measurements which indicated the hydrophilic character of the surfaces. Topographic changes after grafting were also observed by SEM with evidences of the influence of the grafting procedure. Results show the potential of conjugating plasma treatments with techniques for grafting techniques toward the development of optimized chitosan-based coatings for a wide range of applications for biomaterials and materials for health.

Keywords: chitosan characterization, chitosan coatings, grafting, plasma treatment, surface modification.

4.1. Introduction

Over the past decades, artificial implants, stents, heart valves, vascular grafts, orthopedic fixation screws, accesses, and catheters have been widely used to save and to restore the quality of life for many people [20]. Despite considerable research and development efforts, several problems remained in term of biological complications such as inflammatory or thrombogenic responses and opportunistic infections [19]. In order to prevent such problems, coatings have been used to alter the surface properties of the biomaterial. The common techniques used are blending of different molecules, immobilization of small or large molecules on the surface, systems with quaternary ammonium compounds, layer-by-layer self-assembled polyelectrolytes, and polyamine films, among others [6,59,78,121].

Among these strategies, chitosan has emerged has a promising candidate for the development of functionalized coatings for several biomaterials [77,84,187]. Chitosan is a linear cationic polysaccharide derived from deacetylation of chitin which exhibits interesting properties such as biocompatibility, biodegradability, chemical stability, mechanical strength and antimicrobial action [111,126]. Structurally, chitosan is formed by β-(1-4)-linked *D*-glucosamine and *N*-acetyl-*D*-glucosamine units randomly distributed. The proportion of *D*-glucosamine units regarding the total number of units per chain is called the degree of deacetylation (DDA). This structural parameter, in conjunction with the molecular weight (Mw), directly influences the properties of the polymer [79,80,81]. Chitosan is hydrophobic in its deprotonated state (pH>6.3) and becomes water soluble in its protonated state (pH<6.3), due to the protonation-deprotonation equilibrium of amino groups ($–NH_2$). Its antibacterial action depends on the number of deacetylated amino groups positively charged ($-NH_3^+$) [122]. This increases the permeability of negatively charged cell membrane, causing disruption and release of intracellular compounds [126]. From a mechanical point of view, the adhesion and stability of chitosan coatings are dependent on the chain length as well on the number of hydroxyl groups (-OH) and amino groups ($-NH_2$) that could be attached to the substrate by covalent bonds. The absence of covalent bonds to the material could lead to the rapid delamination of such coatings and few studies address the question of stability and performance of coated antibacterial polymer layer [29,82].

Plasma treatments are versatile processes which enable the surface modification of polymers without its degradation and consequent loss of mechanical properties [4,31]. Furthermore, grafting may be used to increase the adhesion of the coating onto the substrate through the formation of covalent bonds [59].

In this context, Teflon (PTFE) films were functionalized by first inserting amino groups with an N_2/H_2 atmospheric plasma treatment. Three spacer molecules, glutaric anhydride, poly(ethylene glycol) bis (carboxymethyl) ether and poly(ethylene-*alt*-maleic anhydride) were then tested in order to maintain chitosan's structure, its biological properties and increase the adhesion of the coating onto a substrate. To evaluate the influence of the molecular weight and degree of deacetylation three different types of chitosan were studied.

4.2. Materials and methods

4.2.1 Materials

Polytetrafluoroethylene (PTFE) film with a thickness of 250 µm was purchased from Goodfellow (Cambridge, England). After being cut to size (3.0 X 3.0 cm^2), the samples were cleaned in successive ultrasonic baths (Branson Ultrasonics, 1200, USA) of acetone, deionized water, and methanol for 10 minutes each and dried with particle-free compressed air and kept under vacuum prior to use. Chitosan of various degree of deacetylation (DDA) and molecular weight were purchased from Sigma-Aldrich (Oakville, ON, Canada): chitosan oligosaccharide lactate (CHIOS; DDA > 90%; $M_n \approx 5$ kDa; 60 % of oligosaccharides content), chitosan low molecular weight (CHILW; DDA = 75-85%) and chitosan medium molecular weight (CHIMW; DDA = 75-85%). Chemical reagents were all of the analytical grade and were used without further purification: hydroxide sodium (NaOH), hydrochloric acid (HCl), methanol and acetone were purchased from Laboratoire MAT (Quebec, QC, Canada); acetic acid, 5-bromosalicylaldehyde, potassium bromide (KBr), glutaric anhydride (GA; M_w = 114 Da), poly(ethylene glycol) bis (carboxymethyl) ether (PEGb; M_w = 600 Da) and poly(ethylene-alt-maleic anhydride) (PA; M_w = 100-500 kDa), buffer 2-(N-morpholino)ethanesulfonic acid hydrate (MES) and N-(3-dimethylaminopropyl)-N'-ethylcarboidiimide hydrochloride (EDAC) were obtained from Sigma-Aldrich (Oakville, ON, Canada).

4.2.2 Methods

4.2.2.1 Chitosan characterization

The degree of deacetylation (DDA) of CHIOS, CHILW and CHIMW is defined in this work as the proportion of deacetylated (*D*-glucosamine) units to the total number of units per chitosan chain and was determined from three different methods: Nuclear Magnetic Resonance spectroscopy (solid state ^{13}C CP/MAS NMR), Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR) and potentiometric titration.

Method 1: solid state ^{13}C CP/MAS NMR

NMR spectra were recorded on a Bruker AC 300/P spectrometer (Germany) at ambient temperature. For each scan, approximately 1 g of each solid sample was compacted in a 7 mm zirconium oxide rotor. Measurements were acquired at a frequency of 75.47 MHz, using a combination of cross-polarization, proton decoupling and magic angle spinning (CP/MAS) at 10 KHz. The DDA of chitosan samples were determined using Equation 4.1, from the ratio of the intensity of the methyl group carbon (CH_3) signal of the *N*-acetyl-*D*-glucosamine units to the average of the intensities of anhydroglucose ring carbons (C1-C6) signals as shown in Figure 4. [123].

$$\boldsymbol{DDA}\ (\%) = \mathbf{100} - \left(\frac{\mathbf{100} \cdot \boldsymbol{I_{CH_3}}}{\frac{\mathbf{1}}{\mathbf{6}} \boldsymbol{\Sigma I_{C_{1-6}}}} \right) \qquad \text{Equation 4.1}$$

with I_X the intensity of the resonance signal of group X.

Method 2: ATR-FTIR

Infrared spectra were recorded on an FTIR spectrophotometer Nicolet 6700 (Thermo Scientific, Madison, Wisconsin, USA). Chitosan samples (2 mg) were dried overnight at 60°C under reduced pressure and mechanically blended with 100 mg of KBr. Pellets were produced and desiccated for 24 h at 110°C under reduced pressure and the IR spectra were recorded in transmittance determined between 400 and 4000 cm^{-1}, by the accumulation of at least 32 scans with a resolution of 4 cm^{-1}.

The DDA of chitosan samples was assessed by measuring the ratio of the absorbance of amide I band at 1655 cm^{-1}, from the acetylated amino, and the hydroxyl band at 3450 cm^{-1}, as described in Equation 4.2 [125,127].

$$\boldsymbol{DDA}\ (\%) = \mathbf{100} - \left(\mathbf{115}.\frac{\boldsymbol{A_{1655}}}{\boldsymbol{A_{3450}}}\right) \qquad \text{Equation 4.2}$$

where 115 is the correlation factor between DDA and the measured absorbance, estimated from standards of chitosan.

Method 3: potentiometric titration

For potentiometric titration, chitosan (0.20-0.23 g) was dissolved in standard HCl solution (25 mL; 0.02 $mol.L^{-1}$) for 24 hours to protonate the available amino groups. The resulting solution was titrated with NaOH (0.1 $mol.L^{-1}$). This method allowed us to differentiate the amount of base used to neutralize free protons from the one used to protonate amino groups. The DDA of the different types of chitosan was calculated with Equation 4.3.

$$\boldsymbol{DDA}\ (\%) = \frac{C_{NaOH}.(V_2\text{-}V_1).161}{m_2}.\mathbf{100} \qquad \text{Equation 4.3}$$

with: C_{NaOH} ($mol.L^{-1}$) which is the concentration of the NaOH titrant solution, V_1 and V_2 (L) are respectively the volume of NaOH used for neutralizing the excess of HCl and the chitosan protonated amino groups, 161 ($g.mol^{-1}$) is the molecular weight of chitosan monomer unit, and m_2 (g) the mass of dry sample used for titration.

The molecular weights of CHIOS, CHILW and CHIMW were determined by Size Exclusion Chromatography (SEC) on Waters chromatographic system with two columns Ultrahydrogel 250 (6 µm; 7.8 mm X 300 mm, 1-80 kDa) and Ultrahydrogel 500 (10 µm; 7.8 mm X 300 mm, 10-400 kDa) which allows the detection of molecular weights in the range 1 a 500 kDa. The system was equipped also with a Guard column Ultrahydrogel (Waters). The mobile phase used was acetic acid 0.33 $mol.L^{-1}$ set up at pH = 3.9 ± 0.2 with NaOH 0.1 $mol.L^{-1}$ and at a flow rate of 0.8 $mL.min^{-1}$. For analysis, chitosan samples were dissolved in the mobile phase (1mg.m L^{-1}) with subsequent filtration of the mixture through a syringe filter with a pore diameter of 45 µm. Then, the molecular weight calculation was done with a dn/dc = 0.173, related to the mobile phase and its temperature.

4.2.2.2 Plasma treatment

In order to introduce amino groups on the PTFE surfaces, an atmospheric plasma treatment was carried out. Samples were placed in a conventional parallel-plate dielectric barrier discharge (DBD) reactor on the grounded electrode [120]. Gas flow (95% N_2 + 5% H_2) was introduced directly between the electrodes through a diffuser and was maintained constant at 5 $L.min^{-1}$. The frequency, applied voltage, gas gap and treatment time were kept constant (3 kHz, 10 kV, 1 mm and 45 s). These parameters were previously optimised in order to provide a sufficient amount of amino groups and to minimize damage to the film's surface [120]. Before and after each plasma treatment, the plasma chamber was purged during 5 minutes to ensure homogeneity and gas purity for the discharge and to avoid post-plasma oxidation reactions with free radicals or unstable functional groups present on the surface.

4.2.2.3 Grafting

4.2.2.3.1 GA and PA grafting

In a glovebox, purged with dry nitrogen, plasma-treated PTFE films were immersed in acetone and 0.3 $g.mL^{-1}$ of GA was added three times at 0, 20 and 40 minutes. After 1 hour of reaction, the films were washed three times with acetone, five times with deionized water and then air-dried and stored under vacuum before use. The grafting procedure for PA was the same but without the last step of washing with water in order to maintain the reactivity of anhydride functions.

4.2.2.3.2 PEGb grafting

PTFE films treated by plasma were immersed in 0.1 $g.mL^{-1}$ PEGb solution (pH 4.75, MES buffer), previously activated with EDAC (3 $mg.mL^{-1}$ every 10 min for three times, the reaction was complete after 30 minutes). After 1 hour of reaction, the films were washed three times with MES buffer, five times with deionized water and then dried and stored under vacuum before use.

4.2.2.3.3 CHIOS, CHILW and CHIMW grafting

The different chitosan grafting approaches are illustrated in Figure 4.1.

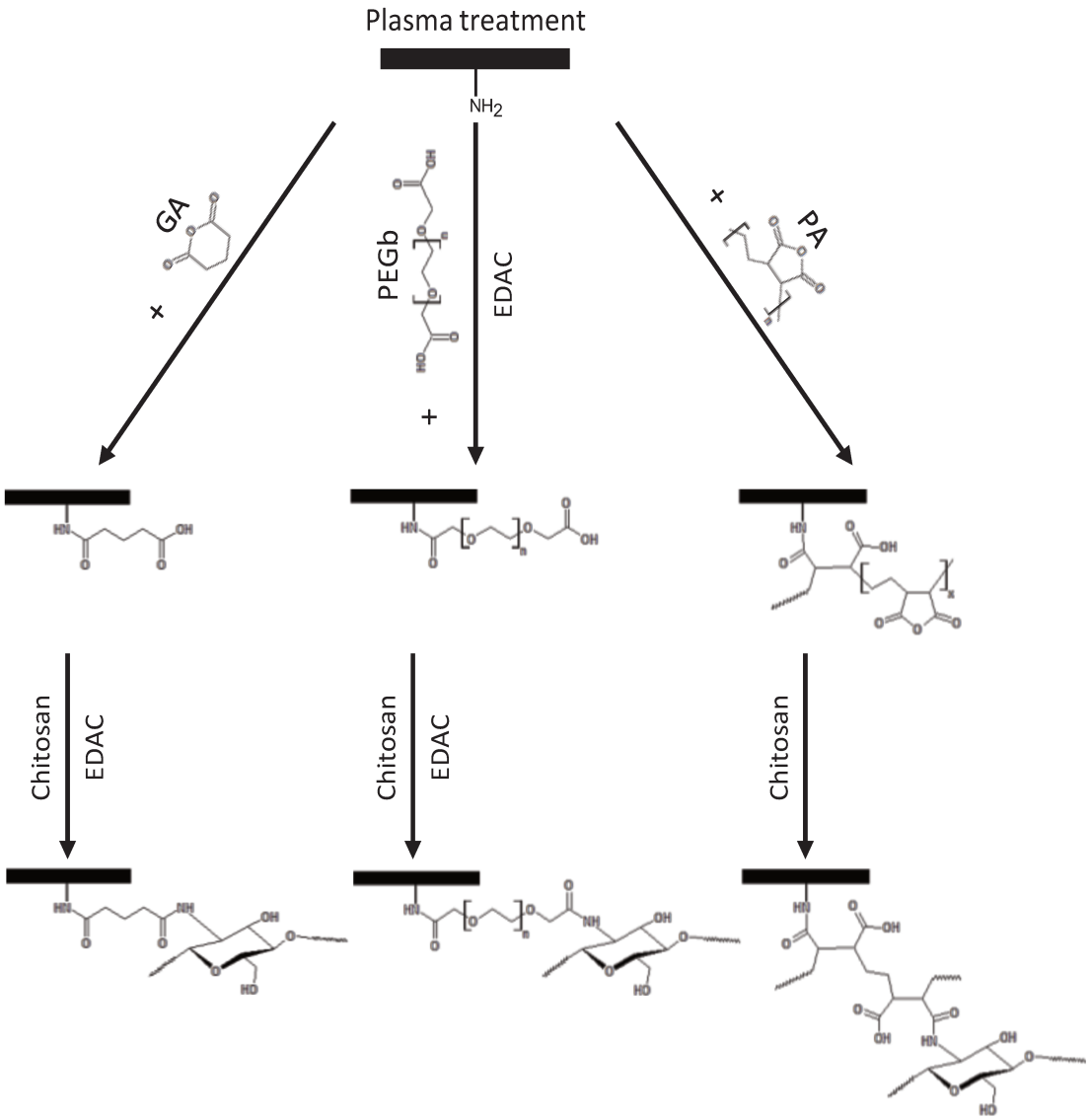

Figure 4.1 - Schematic representation of the grafting methodology for GA, PEGb and PA spacers and CHIOS, CHILW and CHIMW.

Prior to chitosan grafting, the carboxylic acid functionalities of GA and PEGb grafted films should be activated by opposite to anhydride functions obtained on PA grafted films. The activation step was done in a solution of 3 $mg.mL^{-1}$ of EDAC in MES buffer (0.1 $mol.L^{-1}$, pH 4.75). Two subsequent additions of 3 $mg.mL^{-1}$ of EDAC were made every 10 minutes to minimize the effect of the water-induced hydrolysis of the activator on grafting efficiency. The reaction was performed at room temperature and under stirring. After 30 minutes of reaction, these films were washed with MES buffer and used immediately.

The activated films as well as the PA ones were then immersed in chitosan solutions (CHIOS, CHILW and CHIMW) at 2% (w/v) in aqueous solution of acetic acid

1% (v/v), at room temperature for 3 hours and under stirring. The samples were then washed five times with ultrapure water (>18.0 MΩ-cm resistivity) and then dried and kept under vacuum.

4.2.3 Surface characterization

The concentration of amino groups (-NH_2) on the film surface after plasma treatment was determined by chemical derivatization with 5-bromosalylaldehyde followed by XPS analysis [120]. The concentration of the amino groups on the surface was quantified by using Equation 4.4.

$$\%\,\boldsymbol{NH_2} = \frac{\%\boldsymbol{Br}}{\mathbf{100}-(\mathbf{9}\,.\,\%\boldsymbol{Br})}\,.\mathbf{100} \qquad \text{Equation 4.4}$$

with: %Br, the atomic percentage of Br determined by XPS.

The chemical composition of the after each surface modification was investigated by XPS analyses using a PHI 5600-ci system (Physical Electronics, Eden Prairie, MN, USA). A standard aluminum (Al Kα 1486.6 eV) X-ray source was used with a charge neutralizer to record survey spectra whereas high-resolution spectra were recorded by using a standard magnesium anode (Mg Kα 1253.6 eV) without charge compensation. The detection was performed at 45° with respect to the surface normal and the analyzed area was 0.005 cm^2. The spectrometer work function was adjusted to give 285.0 eV for the main C (1s) peak. Curve fittings for both the C (1s) survey and the high resolution were determined by means of least squares minimization procedure employing Gaussian-Lorentzian functions and a Shirley-type background. At least, three measurements per sample were recorded on three different samples to ascertain the homogeneity and reproducibility of the surface chemistry.

The wettability of the functionalized films was determined by the sessile drop method using a Video Contact Angle System (VCA-2500 XE™, AST products Inc., Billerica, MA, USA). The analyses were carried out at 25°C, with 1µL ultrapure water droplets. The CA were measured from, at least, five drops per sample, randomly deposited, and followed by triplicates. The angle value was taken as an average of the number of measurements taken for right and left angles.

The surface and cross-section morphologies of bare and coated PTFE films were characterized using a scanning electron microscope (LEO Electron Microscopy, Leo 440i, Cambridge, England) at an accelerating voltage of 15 kV. To study the cross section, the films were immersed in liquid nitrogen for 5-10 seconds and fractured. Before measurement, the samples were sputter-coated with a thin layer of gold (~ 3 nm). The images were collected at 3500X magnification in order to find characteristics and significant surface features.

4.3. RESULTS AND DISCUSSION

4.3.1 Chitosan characterization

As the properties of chitosan are strongly dependent on their chemical structure, CHIOS, CHILW and CHIMW DDA were determined by NMR, ATR-FTIR and potentiometric titration and their molecular weight were evaluated by SEC.

Figure 4.2 presents the chemical structure of CHIOS, CHILW and CHIMW and their NMR spectra. CHILW and CHIMW exhibited similar RMN spectra, with very close chemical shifts of the carbon groups: 105, 83, 75, 61 and 57 ppm attributed to the anhydroglucose C1-C6 carbons unit's and the carbons of the remaining acetamide functions were detected at 174 and 23 ppm, for the C=O and the CH_3, respectively. All chemical shifts were in agreement with the results presented in various works [123,125]. Nevertheless, CHIOS spectrum showed some differences compared to the two other types of chitosan.

According to the supplier, the purchased product contained only 60% of oligo-chitosans, which were obtained by ultrafiltration enzyme hydrolysis. This method to obtain oligomers is known to have a poor yield in oligosaccharides formation [94] and to give several by-products such as lactacte counter-ions (observed at 182 ppm (C=O) and 22 ppm (CH_3)), remaining higher molecular weight chitosan or chitin and monosaccharides, which explained the peak splitting observed in the CHIOS NMR spectrum. These by-products in addition to the lower molecular mass of the chitosan (i.e. ≈ 5 kDa) may explain the chemical shifts observed for CHIOS which were 175, 101-97, 80, 75, 72, 60, 57 and 24 ppm related to C=O, C1, C4, C5, C3, C6, C2 and CH_3, respectively.

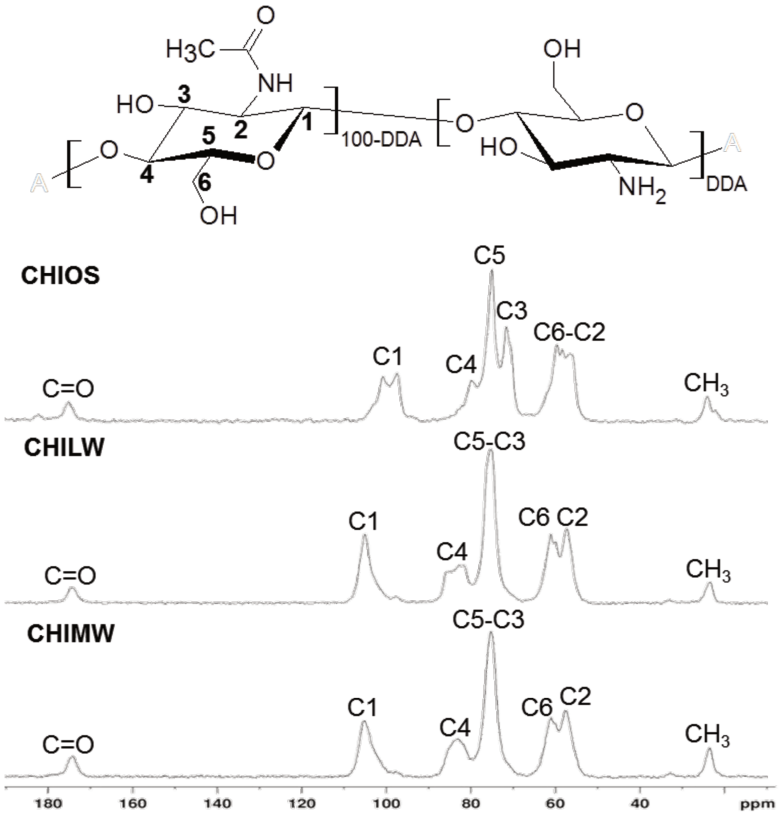

Figure 4.2 - Top: General chitosan chemical structure with carbons denominated C1, C2, C3, C4, C5, and C6. Bottom: ^{13}C-CP-MAS-NMR spectra obtained from CHIOS, CHILW, and CHIMW.

In the same way, FTIR analyses (Figure 4.a) showed highly similar spectra for CHILW and CHIMW, with the expected characteristic bands of chitosan, in particular, the sugar unit at 1151-1028 cm^{-1} from C-O-C stretching. The stretching band of OH and NH were noticed between 3480-3440 cm^{-1}, and the one from CH, CH_2, CH_3 vibration at 2918-2877 cm^{-1}. The amide characteristic bands were amide I (C=O) and amide II (NH(CO)) at 1656 and 1595 cm^{-1} respectively. The band at 1420 cm^{-1} was associated to CN deformation and the on at 1378 cm^{-1} to CH_3 deformation from acetylamide. Though, the spectrum of the CHIOS exhibited some differences such as a specific band at 1730 cm^{-1}, associated to C=O vibration from lactate ion, as well as shifts in the amides band. Indeed, amide I (C=O) and amide II (NH(CO)) were detected at 1630 and 1528 cm^{-1}, respectively, whereas they were at 1656 and 1595 cm^{-1} in the CHILW and CHIMW.

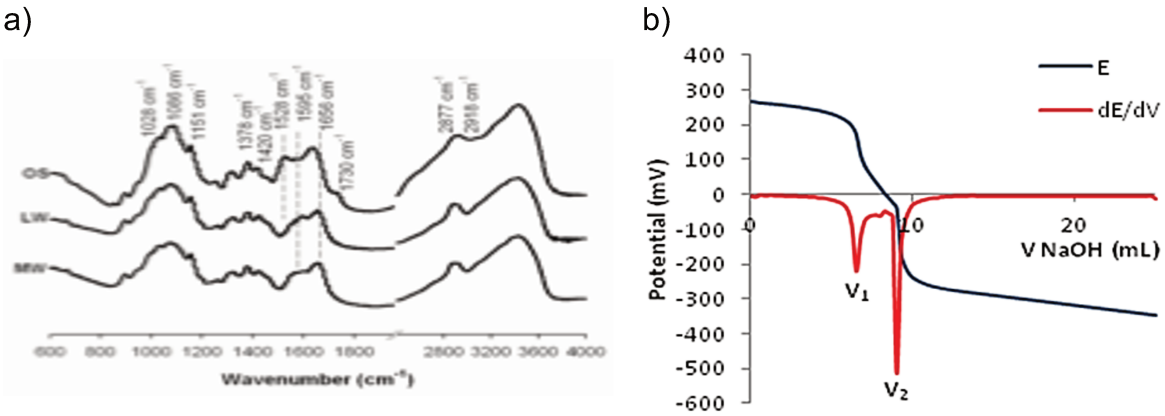

Figure 4.3 – (a) ATR-FTIR spectra obtained from CHILW, CHIMW and CHIOS and (b) Typical chitosan potentiometric titration curve.

These shifts could be explained by the fact that amide bands are very sensitive to the chemical environment such as inter and intra-molecule interactions. Moreover, as stated previously, the CHIOS material contained some by-products that could influence the amide bond vibrations.

Figure 4.b presents the general characteristic curve of chitosan potentiometric titration as well as its derivative. The first inflection point (V_1) of the curve is due to the neutralization of free protons from the excess of HCl acid and the second one (V_2) is related to the titration of protonated amino groups of deacetylated chitosan units. The three chitosan presented very similar titration curves. Indeed, it can be noticed that the presence of by-products did not seem to influence the amount of acid titrated or their influence was negligible towards the actual H^+ and $–NH_3^+$ titration. The titration confirmed the presence of amino groups available on chitosan chains for further grafting. As stated previously, the NMR, ATR-FTIR and potentiometric titration led to the determination of CHIOS, CHILW and CHIMW's DDA values, as showed in Table 4.1. The values obtained with the three methods gave similar results for each chitosan. As expected, a higher variance was observed for DDA determined by titration, as this method involved a higher uncertainty compared to the other ones. Furthermore, it can be noticed that the calculated DDA were in accordance with those provided by the supplier. Although CHIOS showed a slightly higher DDA than CHILW and CHIMW, all chitosan DDA were in the same range.

Table 4.1 - DDA values of CHIOS, CHILW and CHIMW obtained by ^{13}C-CP-MAS-NMR, ATR-FTIR and potentiometric titration.

Sample	^{13}C NMR [%]	FTIR-ATR [%]	Potentiometric titration [%]
CHIOS	90.3	92.4	87 ± 4
CHILW	84.8	83.1	86 ± 4
CHIMW	85.1	83.8	88 ± 4

Another important parameter regarding the chitosan structure and properties, it is its average molecular weight in number (M_n), in weight (M_w), its polydispersity index (M_n/M_w) and its hydrodynamic radius (R_h) [21, 22], which are presented in Table 4.2

The values acquired for number average and weight average for the three types of chitosan were consistent with what was expected, in that they increased from CHIOS to CHIMW. Nevertheless, it can be noticed that the CHIOS molecular weight was much higher than the value provided by the supplier (*i.e*. $M_n \approx 5$ kDa).

Thus, it appeared that CHIOS could no longer be considered as an oligomer and that there was not a considerable difference in the molecular weight of the three chitosan samples, although CHIMW showed slightly longer chains compared to CHILW and CHIOS. As from FTIR and NMR analyses, the heterogeneity of the CHIOS material was also observed in the molecular mass distribution (data not shown).

Indeed, while CHILW and CHIMW exhibited a distribution close to a Gaussian, typical for a natural polysaccharide, CHIOS sample showed the presence of multiple sharp populations of lower mass molecules (34-65 kDa), distinct from the average molecular weight distribution. However, the polydispersity index of CHIOS, CHILW and CHIMW remained in the same range and presented satisfying values for natural polymers [129], which suggested that the disparities observed for CHIOS had no substantial influence on its characteristic parameters. Indeed, as for molecular weights, hydrodynamics radius indicated the same tendency, increasing from CHIOS to CHIMW.

As the same solvent was used for the preparation of chitosan solutions during grafting process and SEC analyses, chain configuration and orientation would not be influenced by the solvent.

Table 4.2 - Number average molecular weight (M_n) and average molecular weight (M_w), polydispersity index (M_w/M_n) and the hydrodynamic radius (R_h) of chitosan samples.

Sample	M_n [kDa]	M_w [kDa]	M_w/M_n	R_h [nm]
CHIOS	91	152	1.68	9.49
CHILW	97	168	1.73	12.36
CHIMW	166	264	1.59	19.18

4.3.2 Surface characterization

The different steps of PTFE surface modifications were followed by XPS analyses and contact angle measurements with water. The results were summarized in Table 4.3.

Table 4.3 - Surface chemical concentrations obtained from XPS survey spectra and contact angle measurements for PTFE films before and after plasma treatment as well as after GA, PEGb, and PA graftings.

Samples	C [%]	O [%]	N [%]	F [%]	CA [°]
PTFE	33.3	-	-	66.6	120 ± 3
PTFE – NH_2	52.8 ± 0.4	2.5 ± 0.7	6.7 ± 0.5	38.1 ± 0.6	ND
PTFE - GA	58.5 ± 0.7	7 ± 1	5 ± 1	30 ± 3	88 ± 5
PTFE - PEGb	55 ± 1	9.4 ± 0.6	5.1 ± 0.2	30.2 ± 0.6	75 ± 5
PTFE - PA	61.4 ± 0.7	23 ± 2	3.1 ± 0.6	13 ± 2	68 ± 3

ND = non-determined.

XPS is a useful technique permitting to assess the relative surface concentrations of carbon, fluorine, nitrogen, and oxygen atoms, which granted essential information on the surface modification efficiency.

The elemental composition of untreated PTFE films, determined by XPS, was two fluorine atoms per carbon, as expected. After the N_2/H_2 plasma treatment, 6.7% of nitrogen was detected with a decrease of fluorine meaning that the treatment was successful. However, these N-containing species introduced on PTFE films could be amines, amides, nitriles and imines [120,124]. Thus, as the linking arms used should react with amino moieties, the presence, as well as the amount of these groups, should be assessed. Chemical derivatization technique was therefore used to determine the concentration of amino groups on the plasma treated PTFE films [120,121], which was measured at 2.5 ± 0.4%. According to literature, 2.0-3.5% of NH_2 relative surface concentration, corresponding to 0.5-2 molecules NH_2/nm^2 [120], which is a sufficient

density to further graft high molecular weight molecules such as polysaccharides and proteins.

The GA, PEGb, and PA grafting onto the aminated PTFE surface was confirmed by the significant decrease in fluorine and nitrogen concentrations and the increase in the oxygen and carbon components, associated with linking arms structures studied in this work. Furthermore, surface composition differences were noticed depending on the linking arm chemical structures, in particular, the chain length. Indeed, the PA which exhibited the highest molecular weight (100-500 kDa) led to a surface mainly composed of carbon and oxygen, whereas for the other linking arms, GA and PEGb of 114 Da and 600 Da respectively, the main components were carbon and fluorine, with no significant difference between GA and PEGb despite the higher molecular weight of PEGb. Since PEGb is only composed of O and C atoms, a higher percentage of these atoms would be expected on the PEGb grafted PTFE film composition. Due to PEGb molecular weight (600 Da), some steric hindrances may be induced, leading thus to unfavorable grafting with other amino moieties. This phenomenon was not observed for PA grafting. Indeed, even though PA has a high molecular weight, it also has numerous available anhydride functions for grafting compared to PEGb, which only exhibits two carboxylic functions per chain. The introduction of polar functional groups, carboxylic and/or anhydride moieties induced a hydrophilic character to the surface, as evidenced by contact angle measurements (Table 4.3), compared to the untreated PTFE, which is highly hydrophobic. It occurred that PA grafted PTFE presented the highest hydrophilicity. This observation was in accordance with previous XPS results. Indeed, PA grafted surfaces exhibited the highest amount of oxygen, leading to hydrophilic character, with the lowest amount of fluorine associated to the hydrophobic one. However, despite a quite similar chemical composition in XPS, PEGb surfaces exhibited slightly lower contact angle values compared to GA, mainly due to the well-known PEGb hydrophilic behavior.

Once the grafting efficiency of the different linking arms had been evidenced, the chitosan grafting step could be investigated. In one hand, after the reaction with the aminated PTFE film, GA and PEGb exhibited a carboxylic functionality, which has to be activated by EDAC before reacting with free amines on chitosan chains. In the other hand, PA grafted surfaces led to high concentrations of anhydride moieties which can further react with amines as well as alcohol functions (without any additional

activation step). It is important to keep in mind that the free amino groups of chitosan were correlated to the DDA, previously characterized. Indeed, higher the DDA is, greater is the availability of free amines for further chitosan grafting. Therefore, it was expected that the DDA of CHIOS, CHILW and CHIMW will have an influence on the graftings, which were characterized by XPS and contact angle measurements. The results were summarized in Table 4.4.

Table 4.4 - Surface chemical concentrations obtained from XPS survey spectra and contact angle measurements for chitosan grafted films.

Samples	C [%]	O [%]	N [%]	F [%]	CA [°]
PTFE-GA-CHIOS	60.3 ± 0.5	19.2 ± 0.3	5.7 ± 0.2	14.8 ± 0.6	54 ± 6
PTFE-GA-CHILW	62.1 ± 0.5	23 ± 2	6.0 ± 0.6	9 ± 3	58 ± 4
PTFE-GA-CHIMW	60 ± 2	20 ± 2	6.2 ± 0.4	13 ± 4	59 ± 6
PTFE- PEGb-CHIOS	58 ± 1	20 ± 3	6.0 ± 0.4	16 ± 4	54 ± 2
PTFE- PEGb-CHILW	58 ± 1	20 ± 2	7 ± 1	15 ± 4	53 ± 3
PTFE- PEGb-CHIMW	59 ± 1	17.3 ± 0.9	6.7 ± 0.1	17 ± 2	51 ± 4
PTFE-PA-CHIOS	64 ± 2	26 ± 3	7 ± 4	3.3 ± 0.8	51 ± 3
PTFE-PA-CHILW	63.6 ± 0.7	29.4 ± 0.7	5.9 ± 0.3	2.1 ± 0.4	55 ± 3
PTFE-PA-CHIMW	64.1 ± 0.7	28 ± 1	5.2 ± 0.1	2.3 ± 0.4	53 ± 2

The XPS survey results clearly demonstrated that chitosan grafting occurred with every linking arms or initial types of chitosan investigated. Indeed, in each case, the oxygen percentage, characteristic from chitosan chemical structure, increased significantly with an important decrease of fluorine component, coming from PTFE film. Concerning the influence of the chitosan molecular weight as well as DDA on the grafting efficiency, it appeared that CHILW exhibited a slightly higher one towards its surface composition as the samples exhibited the highest oxygen percentage with the lowest fluorine atomic concentration, for all GA, PEGb, and PA spacers. Regarding the linking arm influence on the chitosan grafting yield, PA-CHI exhibited the better surface coverage, as revealed by the very low amount of fluorine detected in XPS (≈ 2-3%). Surprisingly, GA-CHI showed a lower fluorine concentration than PEGb-CHI, assuming that chitosan grafting was more efficient or displayed a better surface coverage with GA. This observation could be explained by the effect of the linking arm size. Indeed, GA (114 Da), after its grafting, led to a free carboxylic group with only 3 CH_2 as spacer groups from the surface, whereas PEGb of 600 Da (≈ 10 units of PEG) could adopt a 3D conformation involving steric hindrance thus leading to the unavailable reactive group for further grafting steps. Furthermore, a lower amount of PEGb chains and available carboxylic groups may occur as stated previously. Despite these differences,

the final chitosan grafted surfaces exhibited similar contact angle values, between 51-59°, due to the hydrophilic character of chitosan. It should be mentioned that contact angles are also strongly influenced by the surface roughness induced for example by an inhomogeneous coating.

Therefore, in order to gather more information on the grafted surfaces, high-resolution XPS were carried out on C1s, giving the type of bonds present on samples surfaces (Figure 4.). Once again, the chitosan grafting with the different linking arms was confirmed by the significant increase of C-N and C-O bonds signal (286.5 eV) compared to their respective reference (GA, PEGb, and PA). Indeed, this increase was mainly due to C-O bonds from saccharide structure. It could also be noticed that the contribution of the signal at 288.5 eV (carboxyl and amide bonds) were slightly increased after chitosan grafting for GA and PEGb.

Hence, the results obtained in C1s high resolution corroborated the previous XPS survey results (Table 4.4) by showing differences between chitosan coatings. Furthermore, the detection of PTFE substrate, CF_2 bonds at 291.5 eV, suggested a chitosan layer thickness of less than 5 nm (XPS analysis depth) and/or an incomplete coverage of the PTFE surface (XPS surface analysis of 0.5 mm^2). This late observation could explain the contact angle measurements which were not significantly different between the chitosan types used.

Therefore, the surface coverage was estimated, from specific bands one associated to bare PTFE, CF_2, and one from saccharide CO, by calculating CF_2/CO ratio (Figure 4.4d).

Lower CF_2/CO ratios mean that less CF_2 (PTFE) and/or more CO from chitosan were detected. As seen in Figure 4.4d by the CF_2/CO ratio, the linking arm had an influence on the surface coverage whereas the DDA or/and the molecular weight of different chitosan types apparently had a lower effect. Indeed, there was an important decrease of CF_2/CO ratios from linking arms to chitosan grafted films for CHIOS, CHILW, CHIMW whereas there were slight variations between the different chitosan types for a specific linking arm: for example, for CHILW grafting, CF_2/CO ratio decreased from 0.75, 0.89, 0.49 (only spacers) to 0.18, 0.25, 0.03 for GA, PEGb and

PA, respectively, whereas the variations noticed inside a specific linking arm was only of 0.1-0.2 for each chitosan.

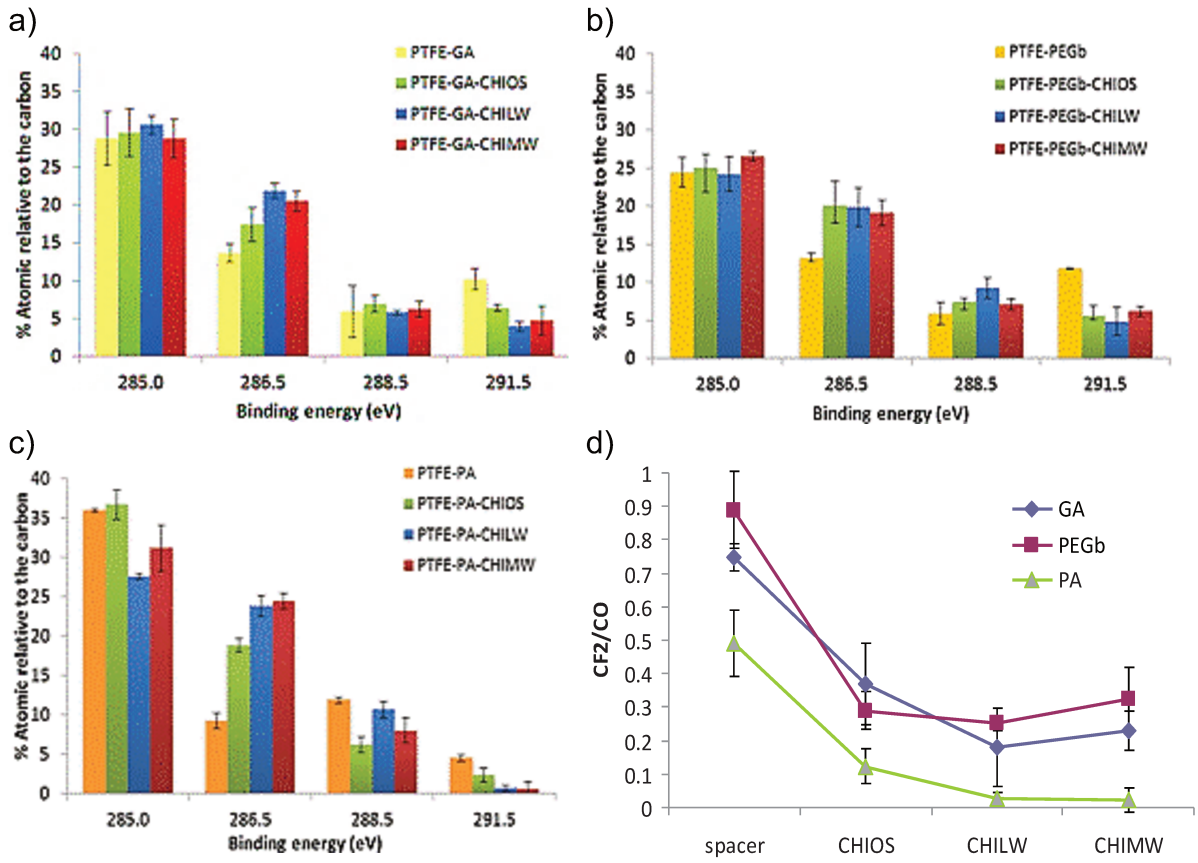

Figure 4.4 - Relative percentage from XPS high resolution on C1s for chitosan grafted films through the different linking arms: (a) GA (b) PEGb and (c) PA with the following bonds: C-C/C-H (285.0eV); C-N/C-O (286.5eV); NC=O/HOC=O (288.5eV) and CF_2 (291.5eV) d) Surface coverage estimated by CF_2/CO ratio.

Finally, it was difficult to bring out, from the XPS analyses, the influence of chitosan's molecular weight and DDA on the grafting efficiency. This was mainly due to the fact that no big differences in chitosan chemical composition were found between the three chitosan types, as evidenced in Tables 1 and 2. Although chitosan grafted surfaces gave similar results for each linking arm, these latest seemed to have an influence on the chitosan grafting efficiency.

This influence has been also investigated through the surface morphology of chitosan-grafted PTFE films by using SEM analyses as presented in Figure 4.5.

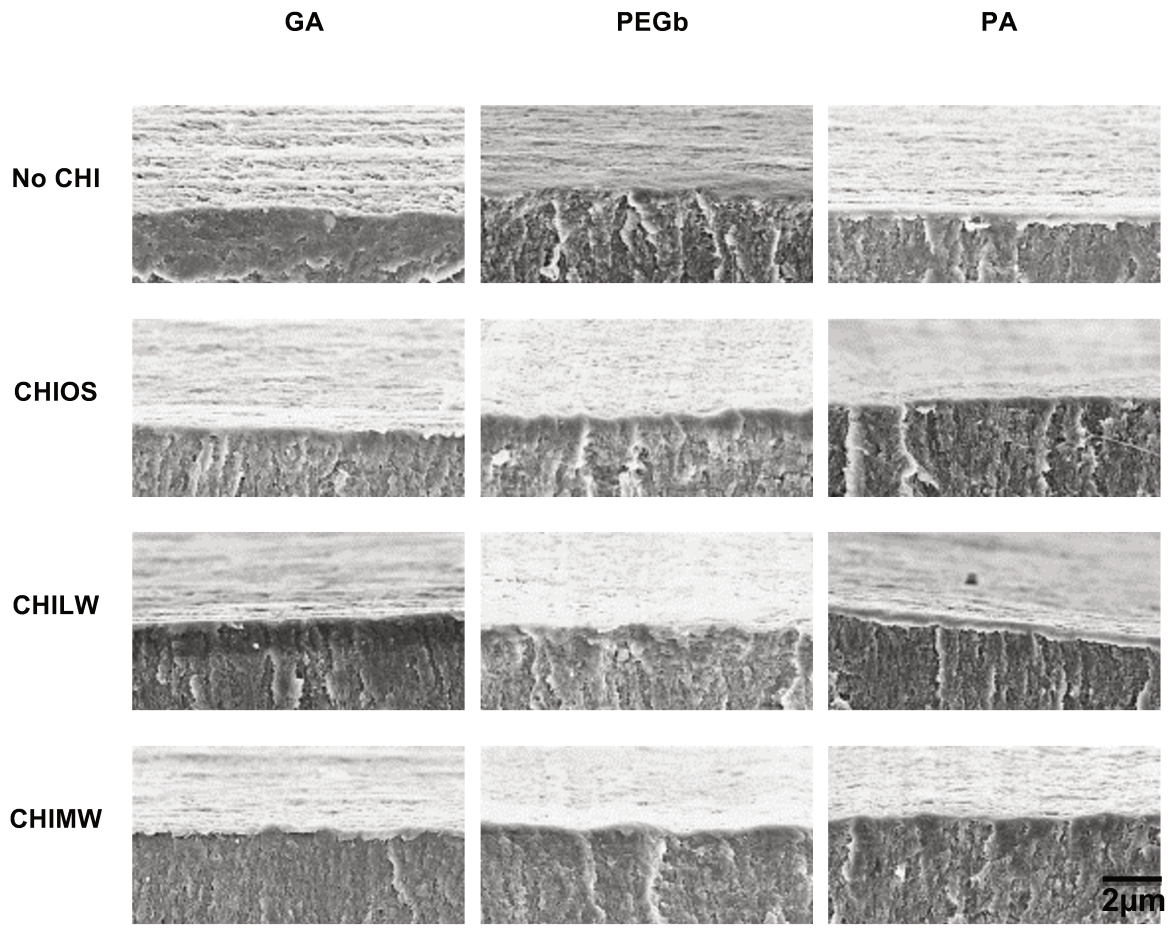

Figure 4.5 - SEM images of PTFE films grafted with GA, PEGb and PA and chitosan samples (CHIOS, CHILW and CHIMW) at magnification 3.500X. The top represents the coating and the medium the PTFE film with an appearance of rippling scales.

SEM images clearly showed the coatings formed on the surface of PTFE, which appeared to be quite homogeneous, meaning without the formation of domains or clusters on the surface.

For PTFE surfaces only grafted with linking arms, GA samples exhibited a very thin and heterogeneous top coating as well as a very rough surface, close to PTFE roughness, compared to PEGb and PA. Indeed, PA grafted surfaces exhibited a smooth surface and the coating thickness was noticeable on the cross section. These first observations were quite consistent with the results obtained in XPS and contact angle analyses, with a minor exception for PEGb: PEGb coating seemed to provide a better coverage of the PTFE surface compared to GA, yet some ripple could be observed, reflecting variation in the coating thickness. As stated previously for XPS and contact angle, when considering chitosan grafting for each linking arm, a

significant difference was noticed between GA with and without chitosan grafting: CHI-GA samples provided smoother and thicker coatings compared to GA alone. No significant morphology change was observed for PEGb and PA, before and after chitosan grafting. Furthermore, chitosan characterization previously revealed that CHIOS, CHILW and CHIMW had similar molecular weight and DDA, which explains why no difference was noticed between the different chitosan types for each linking arm. This also corroborated the results presented in Figure 4.4, in which chitosan grafted samples exhibited similar chemical composition for each spacer.

One of the aims of this work was to study the influence of linking arms on the grafting of different chitosan types. The analyses of the surface chemical composition already revealed that for all chitosan types, the grafting with PA exhibited the best coverage and/or the highest coating thickness. However, it was difficult to state which spacers led to a higher amount of grafted chitosan. For each chitosan types, small or no differences were observed between chitosan grafted with GA and PEGb, whereas grafted with PA, samples presented smoother surface and thicker coatings on SEM images. However, the increase in coating thickness from chitosan grafted with GA and PEGb to PA, could not only be assigned to a higher amount of grafted chitosan. Indeed, CHI-PA coatings may be an entanglement of PA and chitosan chains instead of two distinct layers, meaning the thickness observed could be due to both PA and chitosan grafting.

4.4. Conclusion

As chitosan biological properties are strongly dependent on its degree of deacetylation and molecular weight, the different chitosan types used have been fully characterized. RMN, FTIR analyses and potentiometric measurements have confirmed the chitosan DDA values given by the supplier, whereas the molecular weight for the oligosaccharide (OS) was found to be close to those of CHILW and CHIMW. The slight differences observed in chitosan structures led to minor variations in the chitosan grafted surfaces, as exhibited by XPS survey and C1s high-resolution analyses, contact angle values as well as SEM images. Whereas, it appeared that the linking arm used had more influence on the chitosan grafting efficiency. Results showed the potential of plasma treatments and conjugation techniques for grafting toward the development of optimized chitosan-based coatings for a wide spectrum of applications

in biomaterials and materials for health. Thus, in order to further investigate linking arms and chitosan structures, coatings biological properties will be evaluated and antibacterial tests will be performed.

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5. INFLUENCE OF CHITOSAN-BASED COATING PROCESS ON ANTIBACTERIAL ACTIVITY

This article is submitted in Applied Surface Science

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Résumé

Le chitosane est un polymère semi-naturel avec des propriétés antibactériennes connues, qui dépend de son poids moléculaire (Mw) et de son degré de désacétylation (DDA). Cependant, lorsqu'elles sont greffées sur des surfaces en tant que revêtement, l'efficacité antibactérienne du chitosane dépend fortement de la conformation de la chaîne de polymère en surface, ce qui signifie que les groupes amine ($-NH_2$) doivent être exposés et disponibles pour le contact avec des bactéries. Dans ce travail, des chitosanes de même DDA mais Mw différents ont été greffés sur des surfaces aminées par plasma à travers de trois de bras de liaison de longueurs différentes : l'anhydride glutarique (GA), le poly (éthylène-glycol) bis (carboxyméthyl) éther (PEGb) et le poly(ethylene-*alt*-anhydride maléique) (PA). Les changements sur le substrat greffé ont été évalués par spectroscopie photoélectronique de rayons X (XPS), angle de contact (CA). Les caractéristiques morphologiques ont été évaluées par des analyses de profilométrie. Ces modifications de surface avec le chitosane ont ensuite été corrélées à une activité antibactérienne contre *Xylella fastidiosa*, utilisée comme modèle bactérien, qui a été évaluée par fluorescence. Les résultats ont mis en évidence l'influence du bras d'ancrage et le poids moléculaire du chitosane à la fois sur l'efficacité du greffage et sur le comportement antibactérien. Sur la base de ces résultats, le développement de revêtements à base de chitosane peut être étendu à une large gamme d'applications dans le domaine des matériaux antibactériens.

Resumo

A quitosana é um polímero semi-natural com propriedades antibacterianas conhecidas, que dependem da sua massa molecular (Mw) e do seu grau de desacetilação (DDA). No entanto, quando enxertada em superfícies como revestimento, a eficiência antibacteriana da quitosana depende fortemente da conformação da cadeia polimérica na superfície, o que significa que os grupos amino (-NH_2) devem estar expostos e disponíveis para contato com bactérias. Neste trabalho, quitosanas de mesmo DDA, mas diferentes Mw, foram enxertadas em superfícies aminadas por plasma através de três diferentes moléculas ancoradoras: anidrido glutárico (GA), poli (etilenoglicol)bis(carboximetil) éter (PEGb) e poli(etileno)-*alt*-anidrido maleico) (PA). As alterações no substrato enxertado foram avaliadas por espectroscopia fotoelétrica de raio X (XPS), ângulo de contato (CA) e as características morfológicas foram avaliadas por análises de profilometria. Essas modificações de superfície com quitosana foram então correlacionadas à atividade bacteriana da *Xylella fastidiosa*, usada como bactéria modelo, que foi avaliada por fluorescência. Os resultados evidenciaram a influência do tipo de molécula ancoradora e da massa molecular da quitosana na eficiência do *grafting* e no comportamento antibacteriano. Com base nestes resultados, o desenvolvimento de revestimentos à base de quitosana pode então ser estendido à uma ampla gama de aplicações no campo de materiais antibacterianos.

Abstract

Chitosan is a semi-natural polymer with recognized antibacterial properties, which are strongly dependent on its molecular weight (Mw) and its degree of deacetylation (DDA). However, when grafted on surfaces as a coating, chitosan antibacterial efficiency is also strongly dependent on the polymer chain conformation on the surface, suggesting that the amino groups (-NH_2) require to be exposed to be available for contact with bacteria. To elucidate this behavior, in this work, chitosans of same DDA but different Mw were grafted onto plasma aminated surfaces through three different lengths of linking arms: glutaric anhydride (GA), poly(ethylene-glycol) bis(carboxymethyl) ether (PEGb), and poly(ethylene-*alt*-maleic anhydride) (PA). The surface modifications and the grafting efficiency were evaluated by X-Ray Photoelectron Spectroscopy (XPS) and Contact Angle (CA), while morphological features were assessed by profilometry analyses. The antibacterial activity of these chitosan-based coatings was then assessed against *Xylella fastidiosa*, used as model bacterium. Results evidenced a clear influence of the anchor arm length and of the Mw of chitosan both on the grafting efficiency and on the antibacterial behavior. Based on these results, the development of chitosan-based coatings can then be extended to a wide range of antibacterial applications in the biomedical field.

Keywords: chitosan-based coatings, grafting, surface modification, interface characterization, antibacterial tests, *Xylella fastidiosa*.

5.1. Introduction

In the recent years, increased efforts have been allocated from academy and industry in the research for solutions to prevent and solve the undesirable problems caused by the contamination of materials by microorganisms [1,2,8]. In general, both natural and synthetic materials are vulnerable to microbial attacks. Problems and complications caused by bacteria are as simple as bad odor or substrate stains, but might be as severe as breaks from corrosion in bridges, ships and pipelines, or as alimentary contaminations, loss of crops (*e.g.* orange, olives, etc.), or, at the worst, the transmission of infectious diseases causing worldwide deaths each year [19,22,111,163,166]. In this context, bacterial contamination is considered a current and important socio-economic burden.

Different approaches for designing and optimizing antibacterial surfaces are reported in the literature. Among them, three strategies are well known and often pursued: 1) antibacterial agent releasing; 2) adhesion resistance/repellency, and 3) contact killing [18,23]. These strategies rely on the fine tuning of chemical composition, wettability, topography, and morphology of the surface and aim to minimize the capacity of microorganisms to modulate a dynamic response of surface colonization once the environmental conditions are unfavorable [13,169].

In the antibacterial agent release coatings, metal ions, peptides, or antibiotics can be incorporated to provide specific antimicrobial property [9]. Ideally, the material composition should allow to regulate their release thus allowing antimicrobials to kill both adherent and adjacent planktonic bacteria with a long-lasting effect. However, this approach presents the disadvantage that the effect of bacterial inhibition is inherently temporarily restricted by the reserve of antibacterial agents [23,27,168].

Anti-adhesive surfaces aim to avoid the early attachment of microorganisms to the material, thus preventing the formation of stable biofilms [20,23,27,147], by means of different surface modification strategies. Despite evidence of the efficiency of the modified anti-adhesive surfaces to prevent or limit bacterial adhesion, the main problem is related to the inability of such coatings to kill bacteria once the adhesion takes place [24].

The contact-killing approach aims to eliminate or, at least, to blunt the proliferation of microorganisms adhering to the material surface via the covalent tethering of antimicrobial agents through polymeric spacers [18,20,27] that permit the penetration of the biocides into the cell wall [16,73] and consequently lead to bacterial death. Due to the net negative surface charge that bacteria typically display [172], the most effective compounds for contact-killing coatings are cationic chemicals such as chitosan. This approach presents a considerable advantage since the antimicrobial agent does not exhaust its effect over time, retaining its activity for a long time.

As previously mentioned, chitosan appears as an attractive candidate to produce antibacterial contact-killing coatings. It is a semi-natural biopolymer, presents low toxicity for the mammalian cells and exhibits antimicrobial activity [23,144], with a wide spectrum of activity against fungi, yeasts and Gram-positive and Gram-negative bacteria.

The basic mechanism proposed for its antimicrobial activity is that the interactions between the amine groups of chitosan, which are positively charged, increases the permeability of negatively charged cell membrane, causing disruption and release of intracellular compounds [5,34,79]. Two other mechanisms have been also identified as synergists: 1) chelation of metal trace amounts by chitosan functional groups thus inhibiting bacterial enzyme activity and, in the case of yeast cells, 2) chitosan segments are able to penetrate the cell membrane and inhibit the RNA synthesis [34,79].In this light, chitosan-based coatings have been widely applied for antimicrobial purposes [7,42,57,194].

Obtaining stable and durable antibacterial coatings involves the use of grafting processes in order to increase its adhesion to the substrate by the formation of stable covalent bonds [5,80,220].

In that way, the substrate surface should be activated in order to allow the covalent graft of the chitosan coatings. For that purpose, the treatment of surfaces by plasma appears as an attractive technique for different reasons: no organic solvents are needed, making it an environmentally friendly process, and also plasmas are highly versatile procedures [4,112]. For example, with a simple change of the feed gas (argon, oxygen, nitrogen, ammonia, carbon dioxide, water vapor, air, etc.), different functional

groups could be easily created and modulated depending on the target applications [113–115]. Importantly, plasma technique modifies only the top outmost layer (few Angstroms depth), thus limiting material degradation and loss of mechanical properties [4,5].

After surface activation, and depending on the present functional active groups, chitosan can be grafted by using an intermediate molecule called linking arm. For example, glutaraldehyde, anhydride derivatives such as succinic, glutaric or maleic ones, homo or hetero-bifunctional molecules, *e.g.* poly(ethylene glycol) derivatives. However, since differences in the chitosan structure and conformation may result in a coating with different antimicrobial properties [77,178], parameters such as chitosan chain conformation, molecular weight (Mw) and degree of deacetylation (DDA), but also the coating assembly (influence of the linking arm, the anchor points, etc.) can play a major role.

In this context, the goal of this work was to investigate and correlate the chemical composition of the coatings (i.e. chitosan Mw and linking arm chemical structure and length) with surface properties such as morphology and wettability and finally with their antibacterial activity. PTFE surfaces were first activated by N_2/H_2 plasma leading to amino groups, used as anchor point for further grafting.

These moieties were then reacted with different linking arms, chosen for their size/length, their reactive functional groups and their capability to graft chitosan molecules. For instance, glutaric anhydride (GA) and poly(ethylene-glycol) bis(carboxymethyl) ether (PEGb) after being grafted on the surface led to one terminal carboxyl group, whereas poly(ethylene-*alt*-maleic anhydride) (PA) led to numerous anhydride functionalities capable of linking chitosan multiple sites (Figure 5.1). The influence of chitosan Mw (low and medium) on the antibacterial activity of the coatings was also investigated.

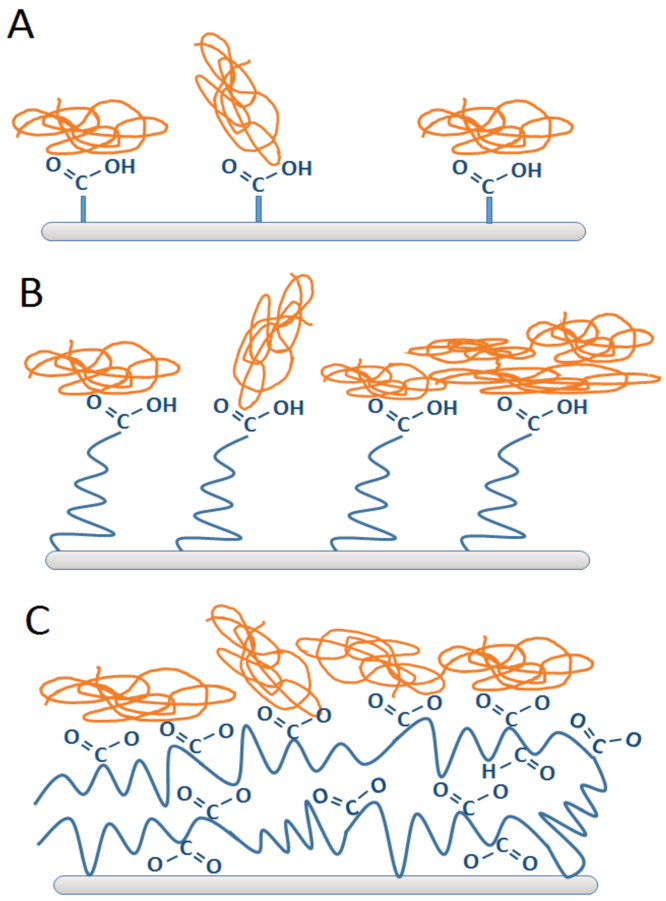

Figure 5.1 – Scheme of chitosan immobilization: a) glutaric anhydride (GA) and b) poly(ethylene-glycol) bis(carboxymethyl) ether (PEGb) after being grafted on the surface with one terminal carboxyl group, c) poly(ethylene-*alt*-maleic anhydride) (PA) with numerous anhydride functionalities capable of linking chitosan multiple sites.

5.2. Materials and methods

5.2.1 Materials

All reagents were analytical grade and were used without further purification. Chitosan low Mw (CHILW; DDA = 75–85%; Mw = 97 kDa), chitosan medium Mw (CHIMW; DDA = 75–85%; Mw = 166 kDa), acetic acid, glutaric anhydride (GA; Mw = 114 Da), poly(ethylene glycol) bis(carboxymethyl) ether (PEGb; Mw = 600 Da), poly(ethylene-*alt*-maleic anhydride) (PA; Mw = 100–500 kDa), buffer 2-(*N*-morpholino)ethanesulfonic acid hydrate (MES) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), Rose Bengal (RB), and phosphate buffer solution (PBS) were purchased from Sigma-Aldrich (Missouri, USA). Methanol and acetone were purchased from Laboratoire MAT (Quebec, Canada). All solutions were

prepared using Milli-Q® water (18.2 MΩcm). The polymeric substrate used was polytetrafluoroethylene (PTFE) films (Goodfellow, England) with a thickness of 250 µm.

5.2.2 Methods

5.2.2.1 Chitosan solution

The 2% (w/w) chitosan solution was prepared by dissolving 2 g of chitosan in 97 mL of distilled water, followed by addition of 3 mL of acetic acid and stirred for 24 h.

5.2.2.2 Preparation and functionalization of PTFE films with chitosan

PTFE samples were cleaned in acetone, water, and methanol in ultrasonic baths for 10 min in each solution, then dried with particle-free compressed air before used.

In order to introduce amino groups on PTFE surfaces, an atmospheric plasma treatment was carried out. Samples (3 X 3 cm^2) were placed in a conventional parallel-plate dielectric barrier discharge (DBD) reactor on the grounded electrode [120]. Gas flow (95% N_2 + 5% H_2) was introduced directly between the electrodes through a diffuser and was maintained constant at 5 $L.min^{-1}$. The frequency, applied voltage, gas gap and treatment time were kept constant (3 kHz, 10 kV, 1 mm and 45 s). Before and after each plasma treatment, the plasma chamber was purged for 5 minutes to ensure homogeneity and gas purity for the discharge and to avoid post-plasma oxidation reactions with free radicals or unstable functional groups present on the surface.

Thereafter, plasma-treated PTFE substrates were grafted with three different linking arms, chosen for their different length, by reaction with the free available -NH_2 groups of the surface. The grafting processes were already described elsewhere [5]. Briefly, plasma treated substrates were immersed in acetone containing 0.3 $g.mL^{-1}$ of GA and reacted for 1 hour before washing thoroughly with acetone and water. The grafting procedure for PA was the same but without the last step of washing with water in order to maintain the reactivity of anhydride functions. In the case of PEGb, the substrates treated by plasma were immersed in 0.1 $g.mL^{-1}$ PEGb solution (pH 4.75, MES buffer), previously activated with EDAC (3 $mg.mL^{-1}$). After 1 hour of reaction, the

films were thoroughly washed with water and then dried and stored under vacuum before use.

Unlike anhydride functions obtained on PA grafted films, prior to chitosan grafting, the carboxylic acid functionalities of GA and PEGb grafted films should be activated. The activation step was done, as described above, by EDAC in MES buffer. Then, the treated substrates were immersed in 2% (w/v) chitosan solutions (CHILW and CHIMW), at room temperature for 3 hours and under stirring. The samples were then washed five times with water and then dried and kept under vacuum before characterization.

5.2.2.3 Surface characterization

The efficiency of surface modifications was assessed by X-Ray Photoelectron Spectroscopy (XPS) and Contact Angle (CA), and the morphological features were assessed by profilometry analyses. These surface modifications with chitosan were then correlated to antibacterial activity against *Xylella fastidiosa*, used as model bacterium, which was evaluated by fluorescence.

5.2.2.3.1 Surface chemical composition

Chemical composition was obtained by XPS analyses using a PHI 5600-ci system (Physical Electronics, Eden Prairie, MN, USA). Survey spectra were performed with a standard aluminum X-ray source (Al Kα, 1486.6 eV) with charge neutralization whereas high-resolution spectra were recorded by using a standard magnesium anode (Mg K α, 1253.6 eV) without charge compensation. The detection was performed at 45° with respect to the surface normal and the analyzed area was 0.005 cm^2. The spectrometer work function was adjusted to give 285.0 eV for the main C (1s), as a reference for the calibration of the binding energies (BE). Curve fittings for high-resolution C1s and N1s were determined by means of least squares minimization procedure employing Gaussian-Lorentzian functions and Shirley-type background. At least three different areas per sample were analyzed on three different samples for each coating type to ascertain the homogeneity and the reproducibility of the surface chemistry.

5.2.2.3.2 Free amino group measurements

To measure the free amino groups on the surface, the PTFE samples were immersed in an aqueous solution of Rose Bengal (0.01 mol.L^{-1}, pH 7). After 15 min samples were extensively washed with Milli-Q® water for 2 min twice consecutively and dried under N_2 flow. The samples were placed in a 1mL solution of 1 mol.L^{-1} NaOH to dissolve the incorporated dye. Absorbance readings were taken with the HP-8453 spectrometer (Hewlett-Packard, USA), at the wavelength of 567 nm. At least, three measurements per sample were made on three different samples to ascertain the homogeneity and the reproducibility of the free amino group evaluation.

5.2.2.3.3 Surface wettability

Contact angle measurements were used to determine the surface wettability. Analyses were carried out at 25°C, with 1µL ultrapure water droplets employing a Video Contact Angle System (VCA-2500 XE™, AST products Inc., Billerica, USA). The angle value was taken as an average of the number of measurements taken for right and left angles, five drops per sample, randomly deposited, in triplicate.

5.2.2.3.4 Surface roughness

The surface topography analysis of the coating, meaning roughness and homogeneity, were assessed by using a surface profiler (Dektak 150, Veeco), by using a 12.5 µm stylus with an applied force of 1 mg. The scan area was 1 X 1 mm^2 and the mean variance Rq was obtained by using Vision software. At least, three measurements per sample were made to ascertain the homogeneity.

5.2.2.4 Bacterium strain and growth

Genetically modified *Xylella fastidiosa* have been chosen as model bacterium due to its fluorescence traceability. Briefly, *Xylella fastidiosa* is a Gram-negative phytopathogen bacterium that grows slowly with duplication times over several hours and produces biofilms featuring a mushroom-like structure [338]. Owing to its easy handling and visualization in fluorescence, in the last years, this bacterium has often been used as a model to study the surface role in the process of cell adhesion and biofilm formation [89,157,166,169,180].

The isolation process of *Xylella fastidiosa* strains was carried out according to Muranaka et al. (2012) [163]. Strains were obtained from petioles of Citrus variegated chlorosis (CVC) symptomatic sweet orange trees hosted in a greenhouse and incubated in Periwinkle Wilt medium (PW). Subsequently, the harvested cells were re-suspended in 500 µL of sterile Phosphate-Buffered Saline (PBS). The bacterial concentration was evaluated via optical density at 600 nm (OD600) and was adjusted to OD600 = 0.3. Afterward, the cells were transferred to a 250 mL Erlenmeyer flask containing 50 mL of PW broth and incubated at 28°C in a rotary shaker at 180 rpm for seven days.

5.2.2.5 Antibacterial tests

Bacterial culture protocol was adapted from Janissen *et al*. (2015) [89]. Bacterial inocula with a concentration of $2x10^7$ CFU mL^{-1} in PW medium were used for the experiments. The substrates were incubated in triplicate for 4 days in a bacterial incubator (410/3NDR, Nova Ética, Brazil) at 28°C without culture media replacement. After the growth time, culture media were removed in order to interrupt the growth; the samples were subsequently washed three times with deionized water to remove the constituents of the culture media and the non-attached cells. In a final step, the samples were dried under a mild N_2 flow.

The antimicrobial activity of PTFE samples coated with chitosan was evaluated by quantifying, from fluorescence images, the bacterial surface density after 4 days of growth. The data were obtained from 30 widefield fluorescence microscopy images acquired in different regions on each sample. Image processing was obtained using freely available software (ImageJ V. 1.32j), and data were statistically analyzed by analysis of variance (ANOVA) with subsequent Tukey post-hoc test using the Statistica 12.0 software; p-values of 0.05 or less were considered statistically significant.

5.3. Results and discussion

Chitosan-based coatings have been extensively applied to antimicrobial surfaces and several studies have shown the influence of parameters such as the source, the DDA, and the Mw of chitosan on the antibacterial activity of the obtained coatings. However, also the coating grafting strategy may play an important role in the final functionality of the surface, affecting chitosan chain configuration and thus the

availability of the amino groups associated with the chitosan antibacterial properties. In this light, in this study low and high molecular-weight chitosans were grafted on plasma-aminated PTFE substrates through three different linking arms (Figure 5.1). Before evaluating the antibacterial behaviors of the different coatings, the grafting efficiencies were ascertained by XPS analyses, the coating homogeneity was evaluated by stylus profilometry and the wettability by contact angle measurements.

5.3.1 Surface characterization

5.3.1.1 Chemical Composition

XPS survey analyses provided the atomic surface composition (5 nm depth) and allowed to evaluate the efficiency of each surface modification step, starting from bare PTFE up to the final chitosan coating. Bare PTFE films presented carbon (C) and fluorine (F) atoms with a 1:2 ratio, as expected. The plasma (N_2/H_2) functionalization step was confirmed by the increasing of C, the appearance of N (represented by the variation of the elemental ratio N/C from 0 to 0.9) and a decrease of F (F/C from 2.0 to 0.7) on the treated PTFE samples (%C = 53 ± 1; %O = 1.9 ± 0.3; %N = 5.0 ± 0.5; %F = 39 ± 1). The achievement of the first grafting step, with GA, PEGb, and PA anchors led to an increase of C and O atomic concentrations since they are the main two chemical elements presents in the linker composition used in this study. These results were also confirmed by the decrease of the percentage of N and F on the PTFE surfaces (data not shown; [5]).

Regarding the grafting of chitosan, second step of the coating procedure, the effectiveness of the three strategies (three linking arms) was clearly evidenced by a decreasing of F (Figure 5.2b) and the increasing of C, O, and N elements present in the composition of chitosan compared to the chitosan-free surfaces (Figure 5.2a, 5.2c, and 5.2d). It should also be noticed that PEG and GA exhibited a similar behavior towards chitosan grafting efficiency, as seen by XPS results. Indeed, a similar decrease in fluorine (Figure 5.2b) and a comparable amounts of nitrogen species (~ 4%; Figure 5.2d) were observed. Conversely, due to its high molecular weight, PA alone displayed a better covering (%F around 5% in comparison to ~ 30% for GA and PEG ones; Figure 5.2b). Further, its various functional groups induced a higher

capability to retain chitosan molecules, as evidenced by the amount of nitrogen detected on PA-chitosan coating (between 6.5 to 7 %, Figure 5.2d).

These features are also observed in high-resolution spectra of C1s (Figure 5.3). Indeed, the peak related to the CF_2 bond (at 291.5 eV), characteristic from PTFE substrate, vanished after the chitosan covalent grafting for PA spacer whereas it is still noticeable on GA and PEG linking arms (less than 10%). Further, the presence of the carbon peaks related to C-N, C-O-C bonds (286.5 eV) confirms the presence of chitosan (glucosidyl, amine, acetamide and hydroxyl functions), which is once again higher for PA samples.

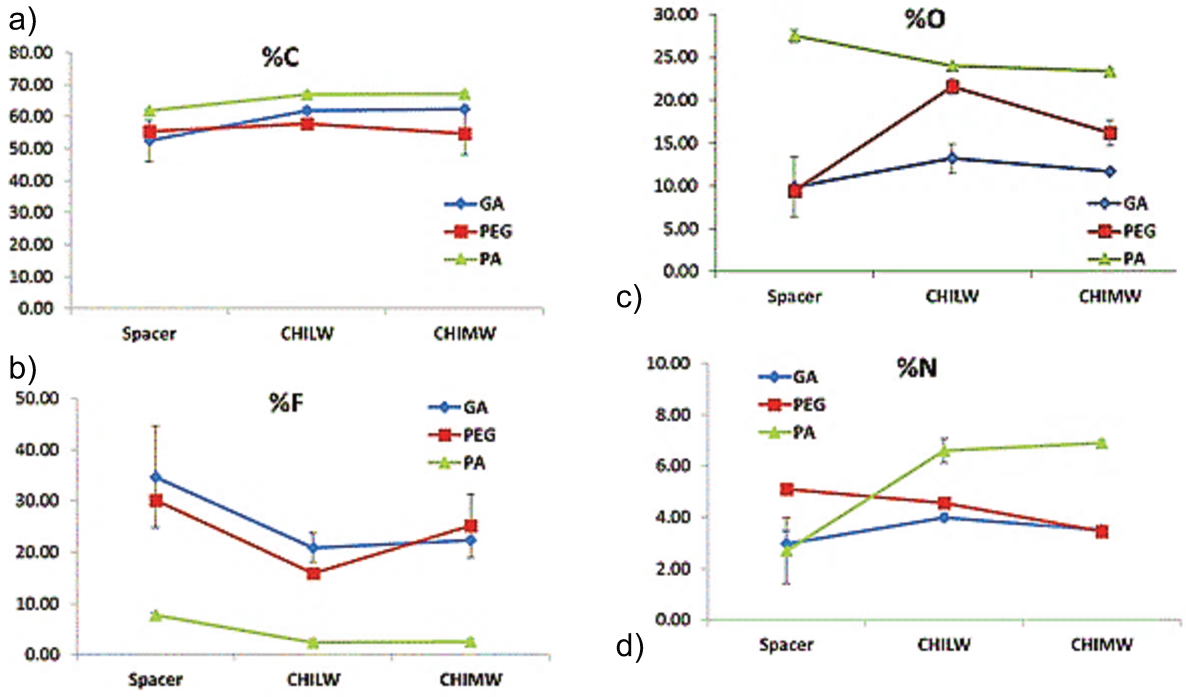

Figure 5.2 - Relative percentage of each element %C (a), %F (b), %O (c) and %N (d), inferred from XPS survey analyses, at the different grafting steps.

It should be also emphasized that CHILW coatings for GA and PEG apparently exhibit a better covering than the CHIMW ones (Figure 5.3), based on CF_2 peak percentage: respectively, 6 ± 1% vs. 7.5 ± 0.3% for GA, and 5.2 ± 0.1% vs. 9 ± 2% for PEG. This tendency is not discernable for PA coatings as no CF_2 is detected.

These results clearly evidence that there is an influence of the linking arm used on the grafting efficiency and the coating covering. This can be explained by the fact that PA is a high molecular weight polymer (Mw = 100–500 kDa), compared to PEG

(Mw = 600 Da) and GA (Mw = 114 Da), and PA has further a high density of reactive functional groups (anhydrides) thus increasing the number of reactive sites where chitosan can be grafted, by opposite to PEG and GA with just one terminal carboxylic group (Figure 5.1).

Regarding the influence of the chitosan Mw on the coating, no difference was observed for PA, whereas the CHILW coatings seem to be improved for GA and PEG spacers. This behavior may be explained by a steric hindrance due to high molecular weight of CHIMW thus hiding the neighbor carboxylic group available from GA and PEGb whereas for PA due to the high density of reactive groups the molecular weight has no real influence on grafting efficiency.

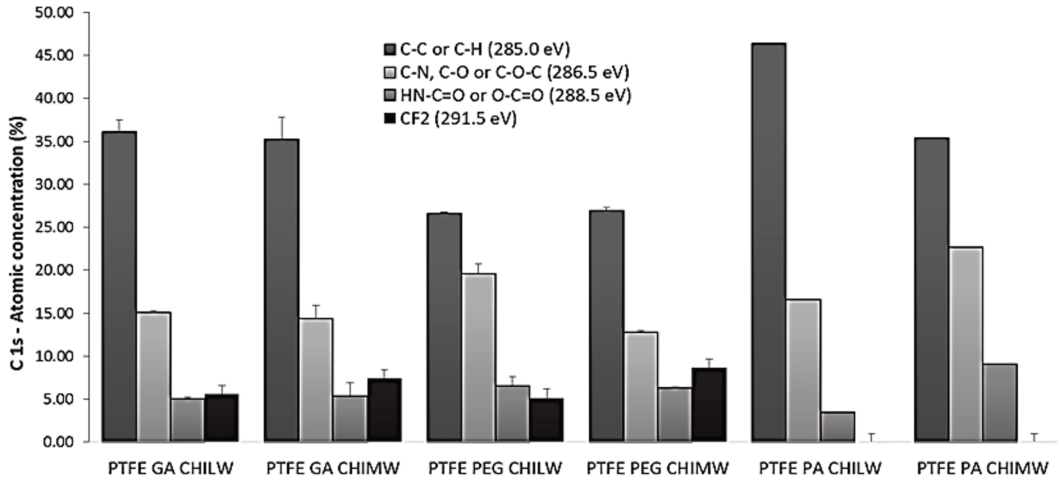

Figure 5.3 - C1s XPS high-resolution results of the different chitosan coatings.

5.3.1.2 Evaluation of chitosan amine density

In order to have a better understanding of the chitosan coating structures towards bacteria, and since the chitosan antimicrobial property is strongly dependent on the ammonium groups available on the coating surface, high resolution of N1s has also been investigated (Figure 5.4a). The $-NH_3^+$ peak is shifted to higher binding energy values (401.5 eV), due to charge effect, compared to the other nitrogen species, such as C-N and NH_2 (399.6 eV) [31]. The $-NH_3^+$ amount detected is quite low for both chitosan coatings when grafted through GA (~ 0.8%; Figure 5.4a), whereas as previously noticed in XPS survey data and C1s HR ones, PEG CHILW exhibits a slight higher percentage of $-NH_3^+$ peak with respect to PEG CHIMW (1.3% vs. 0.9%,

Figure 5.4a). Once again, the chitosan coatings with PA, as linker, displayed the highest amount of ammonium moieties, between 1.7 to 2.2% for CHILW and CHIMW, respectively. However, it should be noticed that these amounts remain very low compared to the other nitrogen species, associated with the peak at 399.6 eV (Figure 5.4a).

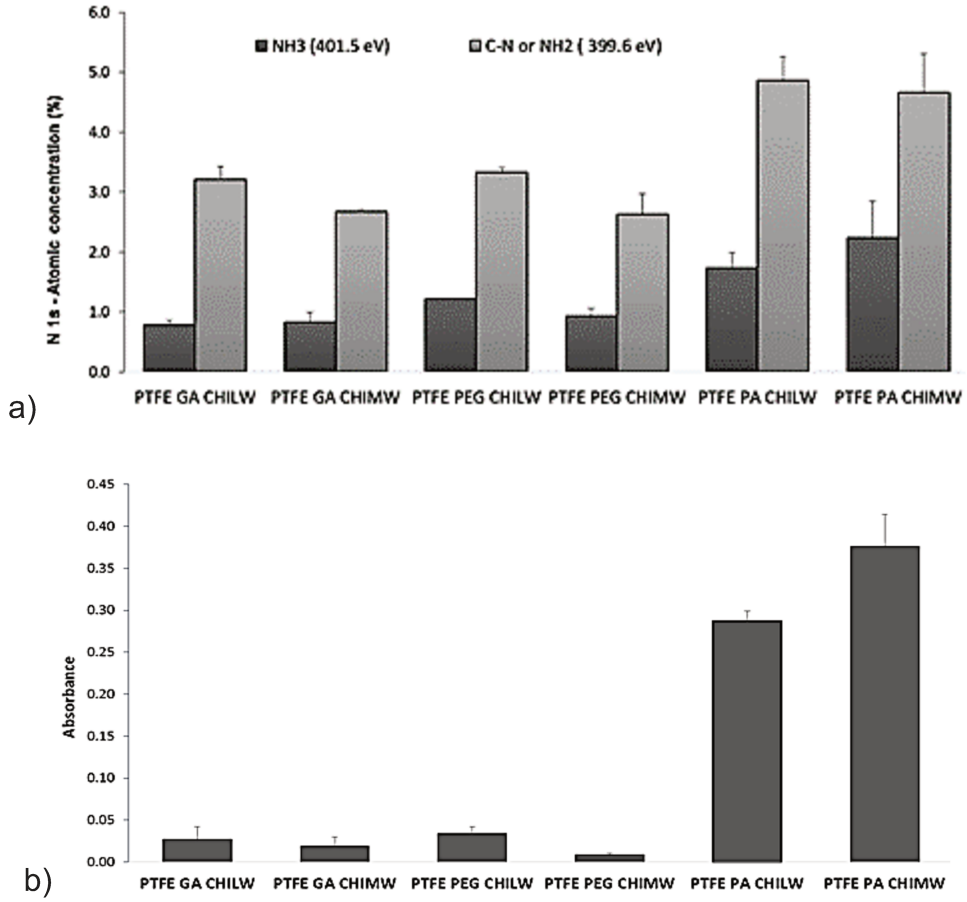

Figure 5.4 - N1s XPS high-resolution results (a) and amino quantification in solution by using Rose Bengal (b) of the different chitosan coatings.

It is worth to note that XPS analyses are performed under high vacuum, thus in dried state inducing some polymer chain reorganization compared to the hydrated state. Therefore, another quantification procedure, using Rose Bengal, has been done in aqueous solution. This dye, known to interact with the amino groups [31], can provide further information regarding free $–NH_3^+$ group on chitosan coatings, capable to interact with bacteria. The results are shown in Figure 5.4b, and followed the same trend observed by N1s HR XPS results (Figure 5.4a).

Indeed, chitosan coatings with PA exhibited the higher absorbance, indicating that more free NH_2 groups were present, in accordance with the N1s HR XPS data (Figure 5.4a). This corroborates to the fact that due PA chemical structure, more chitosan was grafted, as previously suggested by XPS results (Figures 5.2 and 5.3). Also, chitosan coating with GA and PEG incorporated a lower amount of Rose Bengal, as expected. Besides, the influence of the molecular weight of chitosan on free amino groups follow the same trend as described previously: for both GA and PEG films, CHILW coatings exhibit a slight higher absorbance than CHIMW: (respectively, 0.0027 versus 0.0019 for GA and 0.0034 versus 0.009 for PEGb), whereas it was the opposite for PA ones. These results they clearly highlight that the linking arm plays an essential role in final the extent of chitosan coating and that the CHI molecular weight can also affect the coating composition.

Before investigating the chitosan coating antibacterial activities, other key parameters should be taken into account such as the surface topography and hydrophilicity, both known to have a strong effect regarding bacteria adhesion.

5.3.1.3 Surface morphology

The surface topography was evaluated by profilometry analyses and the hydrophilicity by contact angle measurements. The results are shown in Figure 5.5. Regarding the roughness Rq, once again, the GA and PEG exhibit the same trend. Indeed, the Rq values are higher for CHILW coatings, around 1.2 μm, and lower for CHIMW ones (~ 0.8 μm).

By opposite, chitosan coatings with PA are not influenced by the chitosan molecular weight, around 0.7 μm for both chitosan coatings. Furthermore, it is surprising that despite the high PA molecular weight, the roughness is lower than those of GA and PEG surfaces. Concerning, the hydrophilic character of the surfaces, contact angle was very similar for all of them, between 50 and 60° (Figure 5.5), without specific tendencies.

However, the PEG CHILW coating, despite a high Rq value (1.2 ± 0.3 μm; Figure 5.5) exhibits the lowest contact angle value, 50 ± 2°, which may be induced by uncovered PEG areas, known for their hydrophilic character. This hypothesis is further

supported by C1s HR spectra (Figure 5.3) where C-O bonds detected at 286.5 eV, associated to PEG structure, reached a value of 19.6%, close to the PA CHIMW value (22.7%), whereas it was only less than 15% for PEG CHIMW. In the PA CHIMW, this value is mainly associated to chitosan (C-O-C), due to its high amount detected in Rose Bengal test (absorbance of 0.377 and for PEG CHILW only 0.034; Figure 5.4).

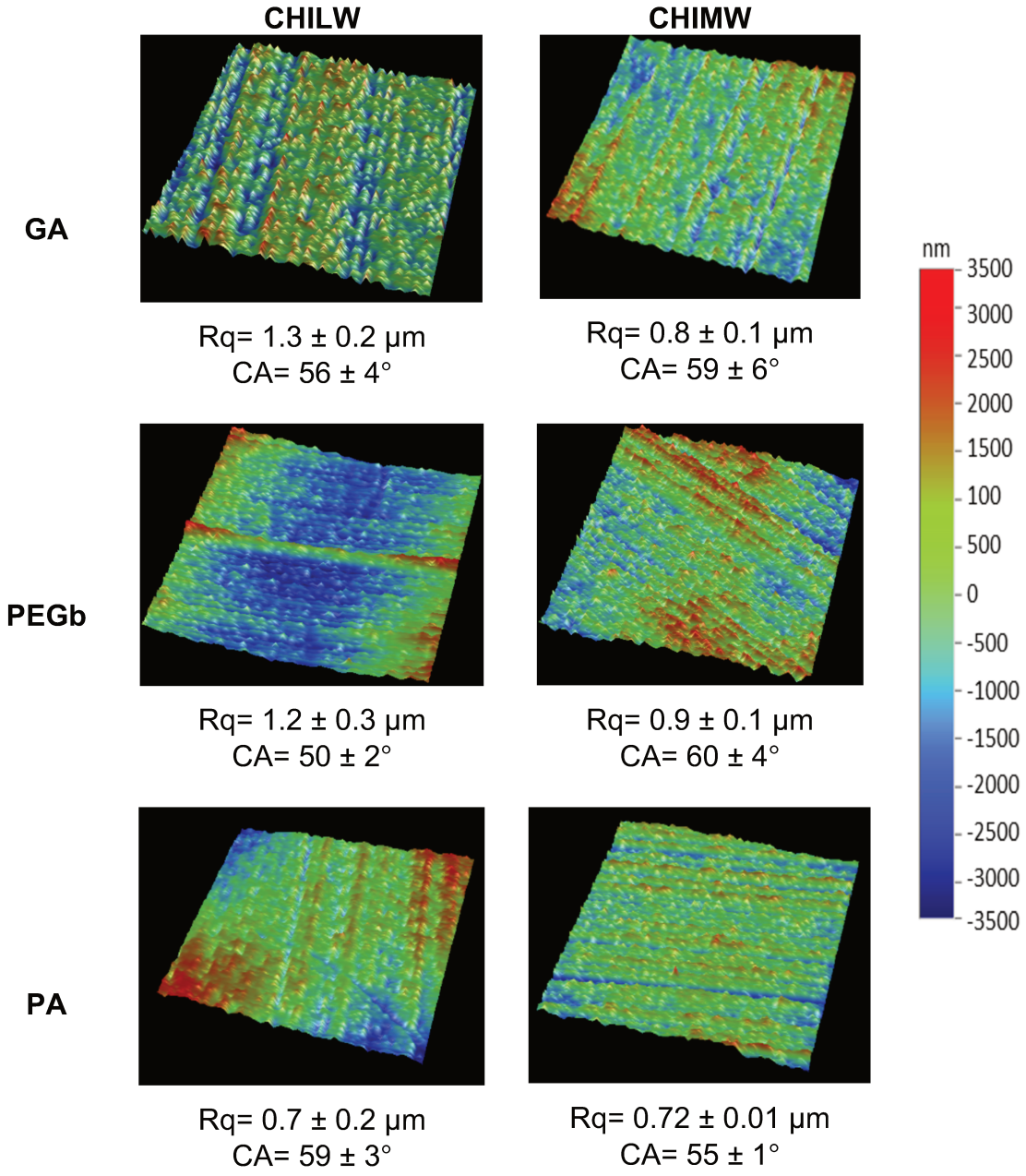

Figure 5.5 - Profilometry images (1 x 1 mm^2) of the different chitosan coatings and their respective roughness Rq and contact angle values.

This difference in PEG CHI coating morphology could be explained by the capability of CHIMW capability to react with different carboxylic moieties from PEGb, thus inducing a better covering of underneath PEG chains.

5.3.2 Antibacterial tests

The antibacterial activity of PTFE surfaces coated with CHILW and CHIMW, was evaluated against the bacterium *Xyllela fastidiosa*, a phytopathogenic, Gram-negative bacterium, genetically modified for fluorescence visualization, and widely used in studies aiming to understand bacterial colonization and biofilm development processes [89,157,166,180]. After 4 days of incubation, *Xyllela fastidiosa* activity was evaluated through widefield fluorescence microscopy images and analyses. An incubation time of 4 days was chosen because this bacterium possesses a late growth physiology (over 10 hours to start its duplication) if compared with bacteria such as *E. coli* and *S. aureus* [61,166,225].

Regarding the widefield fluorescence microscopy images and their respective histograms (Figure 5.6) on the bare PTFE, employed as the control, the surface was practically covered by *Xylella fastidiosa* cells.

For samples coated with chitosan, Figure 5.6 revelated two different tendencies. Indeed, PTFE-plasma-GA-CHI and PTFE-plasma-PEGb-CHI exhibited a higher antibacterial activity for the samples coated with chitosan low molecular weight (CHILW), whereas for PTFE-plasma-PA-CHI samples it is the opposite behavior. In fact, for PA-CHI samples, a higher anti-bacterial action was observed for samples coated with CHIMW. These results corroborating with Figure 5.7.

Results presented in Figure 5.7, show that PTFE samples coated with CHILW and CHIMW exhibited a clear antibacterial activity against *Xylella fastidiosa* when compared to untreated PTFE substrate. This different trend could be mainly associated with the linking arm length and reactive functional groups, inducing then different coating configuration known to play a major role towards antibacterial activity. The literature indicates that factors such as the homogeneity of the coating, accessibility of amino groups, roughness and, its degree of hydrophilicity could influence the development of the bacterial biofilm in different ways [89,166,180].

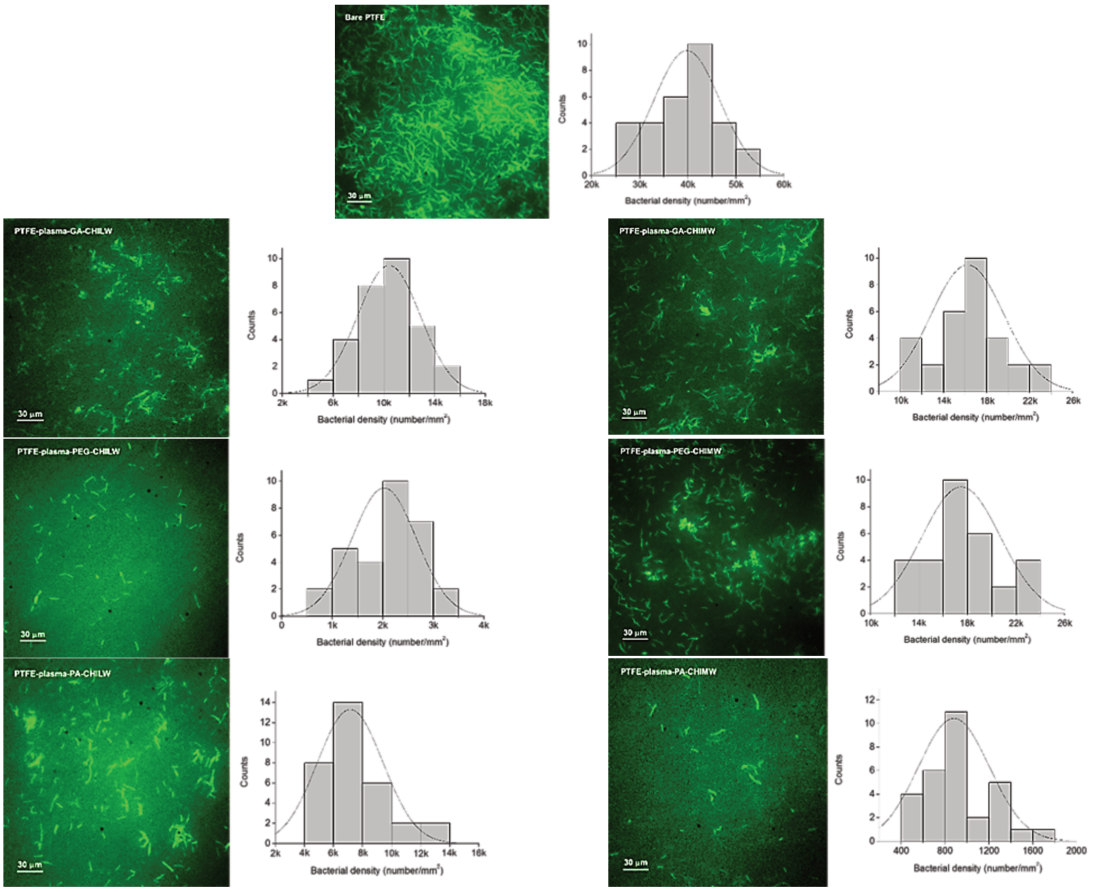

Figure 5.6 - Widefield fluorescence microscopy images of *Xylella fastidiosa* grown on samples for 4 days and their respective histograms of the bacteria surface density.

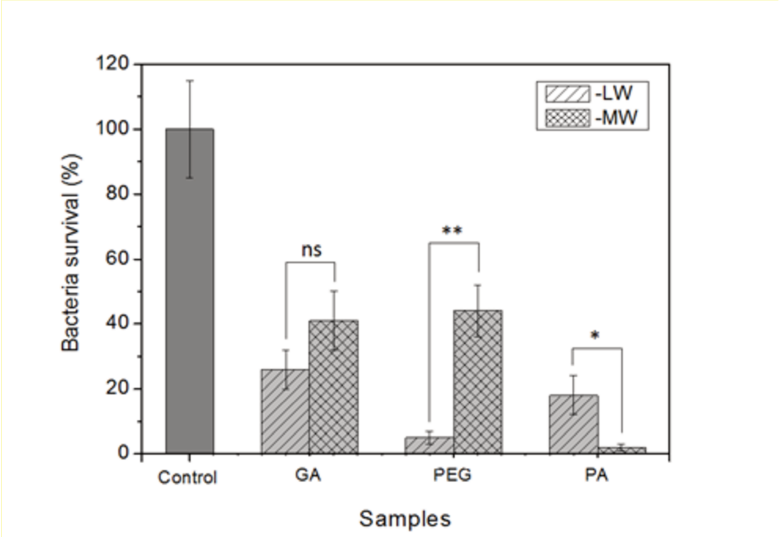

Figure 5.7 - Comparison of bacterial survival among samples. Results were statistically analysed by ANOVA with subsequent Tukey post-hoc test. * and ** denote a significant difference with p-value <0.05. ns indicates no statistical difference.

Indeed, GA and PEGb have one carboxylic group per molecule capable of binding to the -NH_2 functional group of chitosan. On the other hand, PA has in each of its molecules numerous anhydride functions which are capable of binding to the various free amino groups of the chitosan, as previously presented and showed in Figure 5.1.

For the samples containing GA as the anchoring molecule, the quite low values of bacterial reduction on chitosan coated surfaces are possibly related to the fact that GA has the lowest molecular weight among the anchor molecules. Thus, these short molecules made the coatings of chitosan to be very close to the PTFE substrate. This proximity of the chitosan chains to the substrate may influence the configuration of the coatings that possibly presented several inaccessible chitosan-functional groups, thus limiting its functionality (Figure 5.1).

Taking this aspect into account for the coatings of chitosan using PEGb as an anchoring molecule, it can be assumed that because it has a higher molecular weight than GA (six-fold greater), this anchoring arm has a higher mobility, which in this case allowed the configuration of the chitosan coatings to present more accessible amino groups of CHILW. This feature, when added to the action of PEGb, allowed to obtain a coating with antiadhesive properties, which hinder bacterial adhesion, and contact killing, since the functional groups of chitosan can interact with the bacterial membrane, which is negatively charged, causing its leakage [34].

For PTFE-plasma-PA-CHILW and PTFE-plasma-PA-CHIMW samples, a bacterial survival of 18% and 2% was observed, respectively (Figure 5.7). Due to its multiple anhydride groups, PA was able to bind to a large number of amino groups, resulting in a coating rich in chitosan. These segments were able to interact with the cell wall of *Xyllela fastidiosa*, disrupting them. Due to the interactions between the positively charged amino groups of chitosan and the negatively charged cell membrane, the action of the -NH_3^+ chemical groups present in these substrates was a key factor for achieving a greater antimicrobial action.

As previously explained, coatings using PA as the anchoring molecule were more homogeneous, forming a coating with a higher amount of chitosan and amine groups. By varying the molecular weight of chitosan, it was evidenced that chitosan

Mw affects the structure and organization of the resulting chitosan coating, with higher Mw molecules allowing to increase the interaction between free GlAc units and the bacterial cell wall.

Based on these aspects, the behavior presented for samples containing PA can be explained by the presence of a more homogeneous coating, as indicated by the lower amount of F in the surface, through the XPS analysis when compared to the substrates PTFE-plasma-GA. The same trend was observed for the samples containing PEGb, which despite having less homogeneous coatings than PTFE-plasma-PA samples, have anti-adhesive properties, which is widely reported in the literature [34,197] as a mechanism to prevent bacteria adhesion and possibly hinder the development of a more pronounced biofilm.

It is important to note also, that the different types of bacterial strains may present different abilities to adhere to the surface of the substrates. In theory, hydrophobic bacterial strains will more likely adhere to materials with hydrophobic surface properties and, correspondingly hydrophilic species will preferentially adhere to hydrophilic surfaces [56,87,157,180].

However, *Xylella fastidiosa* secretes a conditioning film to decrease the degree of hydrophilicity of the surface to start colonizing the substrate [180]. In this study, contact angle results showed that PEGb-CHILW and PA-CHIMW samples presented a more hydrophilic character than other samples, and this feature may have contributed to later bacterial growth on these surfaces, being less favorable to the bacterium adhesion.

In relation to *Xylella fastidiosa* adhesion and roughness results, it is important to emphasize that bacterial adhesion can be not exactly linked to the increase and decrease of roughness, but to the formation of surface patterns that facilitate the adhesion of the microorganism [183], explained the best results for PEGb-CHILW and PA-CHIMW against *Xylella fastidiosa*, even if these samples have different roughness.

Thus, these results demonstrated the potential of chitosan-based coatings as antibacterial surfaces, indicating the importance of fine-tuning the composition and structural organization of the chitosan coating by playing with linking arm length and chemistry and grafted chitosan Mw.

5.4. Conclusion

Chitosan has gained great interest in the past 30 years due to its versatility to bind with other molecules and its intrinsic antibacterial properties. Here, we explored the possibility to functionalize plasma-treated PTFE substrates with chitosan using linker molecules. Due to the simplicity and versatility of this technique, it was possible to verify how different spacers bind to chitosan to form an antibacterial surface. Another important point is that the biofunctionality of chitosan is related to its 3D conformation on the surface, so we also evaluated how different values of molecular weight affected the coating morphology. The XPS analyses showed that chitosan was successfully grafted onto PTFE surfaces. When PA was used as spacers, a lower amount of fluorine was observed, indicating a thicker coating of the surface, due to the high length of the linking arm. In addition, a greater amount of amines was shown in PA-based samples owing to the higher number of reactive functionalities present on PA and to the consequent higher amount of grafted chitosan. This surface composition was suitable to act as an antibacterial surface since the amino groups interacted with the *Xylella fastidiosa* cell wall, resulting in a better response in comparison to GA and PEGb samples.

Acknowledgements

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6. GENERAL DISCUSSION

The aim of this thesis was the development of a stable antibacterial chitosan coating immobilized by covalent bonds, employing plasma-grafting techniques.

Thus, the surface modifications by plasma-grafting allowed the dual balance between grafted and free amine groups from chitosan, in such a way to guarantee joint stability and bactericidal effects of the coatings. The platform, herein developed and studied, offers the flexibility to adapt the process to different antibacterial applications.

Thus, in this Chapter, we discuss the results previously shown in **Chapters 3**, **4**, and **5**, as well as some unpublished results. This Chapter will present also the limitations of this study and of the chitosan coatings. Moreover, it will be pointing some perspectives for the future works.

6.1. Role and contribution of each step explored in this study

Due to the complex and multidisciplinary aspect of this study, an extensive and comprehensive literature review was done and presented in **Chapter 3**, which served as base and directive of the research work. Antibacterial coatings in biomedical field has strongly grown in the last 20 years due to two main reasons: increasing awareness and understanding of the HAIs and the parallel growth of antimicrobial-resistant pathogens that limits the use of antibiotic-based approaches [1,8,141,147,149,150,155,167]. Despite a wide variety of existing approaches and tools, three main strategies were identified for the development of antibacterial coatings, as described in **Sections 1.4** and **3.3**. Besides, as shown in the literature, chitosan is one of the materials considered for the development of these coatings [9,42,154,287]. Multifunctional systems, combining different mechanisms of action against pathogens, are emerging as the next generation chitosan-based coatings, not only in the biomedical field but also in food and textile industry [10,46,55,91,178,199].

Although this study was based on an encouraging theory, there was a great challenge to be overcome. Part of $-NH_2$ groups would be linked by covalent bonds to the substrate to produce stable chitosan coatings. However, it was also necessary to have a sufficient number of free $-NH_2$ groups to present a satisfying antibacterial

response. These two issues were investigated and resulted in two publications presented in **Chapters 4** and **5**.

The methodology to produce chitosan coatings was presented in **Chapter 4**. The initial parameters were based on the others studies developed at the LBB and LIS (Laboratoire d'Ingénierie de Surface) involving proteins, dextran for cardiovascular applications and plasma treatment, respectively [45,70,120,121]. Thus, the study presented in this thesis, (focused on the production of chitosan coatings) confirmed that the use of plasma pre-treatment to functionalize the polymeric substrate, in combination with grafting techniques to covalently immobilize biomolecules, allowed the development of a standard protocol for the achievement of optimized chitosan-based coatings.

In **Chapter 5**, the antibacterial tests employing *X. fastidiosa*, as a model bacterium, were presented and evidenced the influence of the spacer molecule and the molecular weight of chitosan, both on the antibacterial behavior of the coatings. On the other hand, these results allowed to study the different surface mechanisms (contact killing and anti-adhesive), as previously presented [2,18,27]. Results showed that surface modifications by plasma-grafting allowed the dual balance between grafted and free amine groups from chitosan, where antibacterial activity is increased with the number of free amine groups available the chitosan coating, corroborating with several studies presented in the literature [5,72,166]. Although these studies did not employ the techniques of plasma-grafting to produce the coating, the importance of the presence of free protonated amino groups from chitosan, against bacterial action [13,43,68,166]. In this thesis, it confirmed by the bacterial reduction of 82-98% on the chitosan coated surfaces in relation of bare PTFE, encouraging the tests with human pathogenic bacteria. The most promising samples were tested against *E. coli, P. aeruginosa* and *S. aureus*.

After the validation of the antibacterial chitosan coatings on PTFE films, the plasma-grafting methodology was extended to the PET textile substrate and physicochemical characterizations, such as XPS, CA, and antibacterial tests with *E. coli, P. aeruginosa* and *S. aureus* were performed confirming the antibacterial potential of these coatings.

6.2 Development of a methodology to produce chitosan coatings covalently linked to the polymeric substrate by plasma-grafting

6.2.1 The importance of characterization analyses when biopolymers are employed in technological issues

In this study, three commercial chitosan samples, with different DDA and Mw, were employed to verify the influence of these two structural parameters on the grafting of the coating and on its antibacterial activity. Indeed, these two chemical properties are key parameters, influencing the distribution and amount of protonated amino moieties ($-NH_2$ and $-NH_3^+$) in the chitosan chain [5,128,178].

These parameters are important because they allow to predict the number of amino groups in each of the chitosan samples and their chain length. In this study, this information was fundamental because part of these functional groups was used to anchor the coating and the other part available (after the linkage to the functionalized substrate) was responsible for the antibacterial activity [38-39,43]. Predicting these chemical characteristics of chitosan assisted also in the choice and study of the behavior of the different spacer molecules used herein.

Thus, these results evidenced the importance of conducting effective analyses for the characterization of biopolymers, corroborating with the literature [43,124,130]. The DDA analyses, with different precision degrees, cost and easiness implementation, showed the reproducibility of these different methods. It was remarkable the heterogeneity of the chemical composition of the CHIOS sample by these analyses and it could be also observed in the mass distribution through the SEC analysis.

Based on the results, CHIOS could not be considered completely pure when compared to the CHILW and CHIMW samples, possessing compounds that could influence its physicochemical and biological behavior. Thus, CHIOS was evaluated in the primary analyses for the development of the methodology but not considered for the other tests of this work.

6.2.2 Choice of the substrate

As previously presented in **Section 2.2**, two polymeric substrates were employed aiming to attend the objectives of this study: poly(tetrafluoroethylene) (PTFE) and poly(ethylene terephthalate) (PET). These substrates were chosen due to their large and diversified applications as textile and in the medical field.

However, the use of PTFE films brought other interesting issues, such as its ease of handling, simpler geometry and, the presence of fluorine in its composition. The decrease of this element was important indicative of the successful modification of the substrate in each treatment step. As previously presented in **Chapter 4**, the decrease of F on the surface indicated that PTFE films were coated by the anchors and/or chitosan, composed basically of C and O. The concentration of F was also used to indicate the most homogeneous coating. It is important to clarify that these results were complemented by other analyses. Several studies in the literature have been reporting this behavior using different functionalization strategies [70,121].

Once the method for chitosan coatings on PTFE films was validated, PET textile fibers were used aiming to apply the methodology in a system having a more complex geometry. The parameters used for PTFE served as the basis for PET functionalization.

The instability of PET was a concern, once this polymer is a polyester and the plasma treatment could degrade this substrate. However, using the same parameters for PTFE, as shown in **Chapter 4** (plasma treatment) it was possible to functionalize PET fabrics without significant damages [120]. However, literature describes the relationship between treatment time and PET substrates damages, indicating that this issue can be more explored [120,354].

6.2.3 Plasma-grafting treatment

Plasma technique was used to prepare the substrate, functionalizing the surface by the insertion of amino groups for grafting different types of anchors molecules, which worked as linking spacers to covalently graft the chitosan coating onto the substrate [5,121]. It should be noted that the plasma treated samples led to amino groups stable for 3 months when protected from oxygen. Indeed, some graftings were made late (after 3 months) and the surfaces had exhibit similar compositions, close to the one obtained just after plasma treatment.

For the grafting process, two methodologies were evaluated in order to optimize a part of the process, *i.e.*, the graft was made using two different ways. The first one, called “grafting to”, was the anchoring of the spacer molecules (GA, PEGb, and PA) to the chitosan chain before surface grafting. The second one, called “grafting from”, was first the grafting of these spacer molecules onto the surface and then the grafting of chitosan onto the previously modified surface [32]. The second strategy suggested, “grafted from”, was used in this study, since it presented a better coating. In the case of “grafting to”, the yield of the chitosan coating adhered on the surface was low, possibly due to the higher reaction of chitosan amino groups with the spacer molecules, which may have induced crosslinking, thus leading to less available functionalities for further reaction with the amino groups of the functionalized surface.

Results discussed in **Chapters 4** and **5** showed clearly that the type of spacer molecule plays an essential role regarding chitosan coating efficiency and coverage and that the chitosan molecular weight also has an influence on the chitosan layer. For the anchors, PA has further a high density of reactive functional groups (anhydrides) thus, improving chitosan facilities to be grafted, by opposite to PEG and GA with just one terminal carboxylic group.

Regarding the influence of the chitosan molecular weight on the coating, no difference was observed for PA, whereas the CHILW coatings seem to be improved for GA and PEG spacers. This behavior may be explained by a steric hindrance due to high molecular weight of CHIMW thus hiding the neighbor carboxylic group available from GA and PEGb whereas for PA due to the high density of reactive groups the molecular weight has no real influence on grafting efficiency. Chitosan coatings with PA, as linker, display the highest amount of ammonium moieties, when compared to GA and PEGb. Despite these differences, the final chitosan grafted surfaces exhibited similar contact angle values, between 50-60°.

The plasma-grafting procedure was extended to textile PET substrate and validated by physicochemical and antibacterial analyses, showing the effectiveness of the methodology employed, which can be further extended to different substrates. By XPS and CA, the chitosan grafting success on PET textile was evidenced by the chemical changes in the surface (increase of the amount of nitrogen on the surface)

and, the increase of the wettability (PET 118 ± 6; PET-plasma-PA-CHILW 43.6 ± 0.7; PET-plasma-PA-CHIMW 44.1 ± 2.1) [118,120], respectively (Figure 6.1a, b and d).

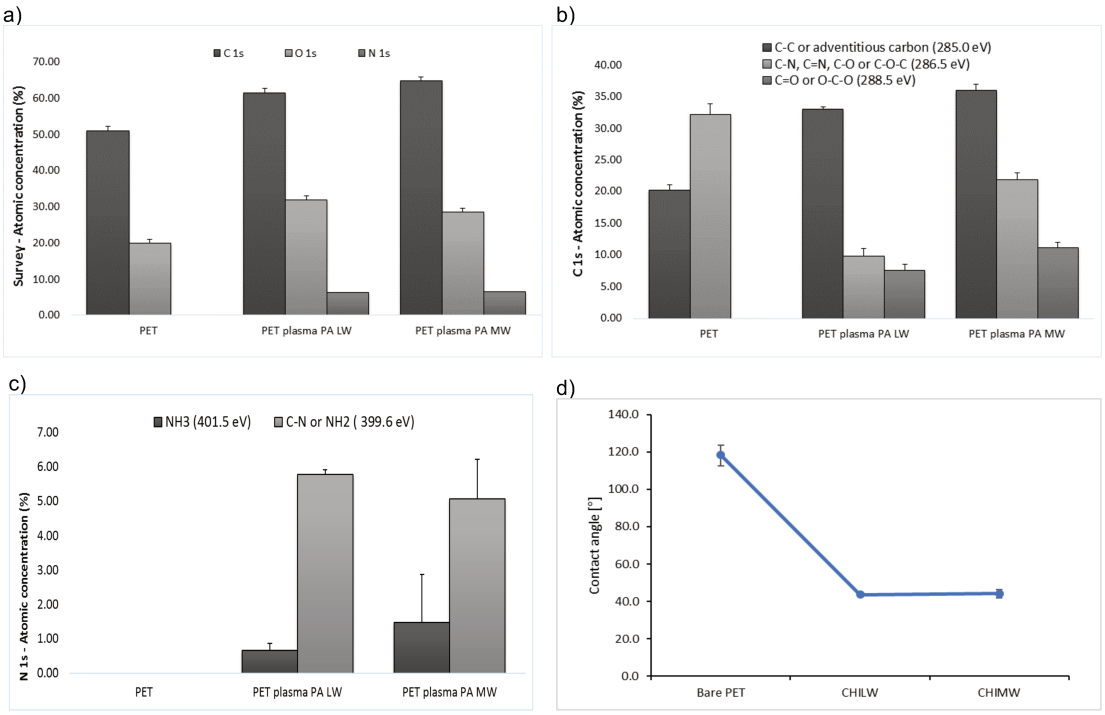


Figure 6.1 - a) Surface chemical concentrations obtained from XPS survey spectra b) C1s XPS high-resolution results of the different chitosan coatings, c) N1s XPS high-resolution results and d) contact angle.

In Figure 6.1c is possible to observe the N1s XPS high-resolution results, corroborating with PTFE results, where PA-CHIMW surfaces presented a greater quantity of NH_3^+ groups if compared to PA-CHILW, as presented in **Chapters 4** and **5**. In this context, PET-plasma-PA-CHIMW samples were selected for the antibacterial tests.

6.3 Antibacterial tests

The performance of biological assays allowed the examination of the antibacterial behavior of these coatings, providing vital information to its use as an antibacterial coating for the applications aimed in this work.

In **Chapter 5**, the antibacterial tests employing *X. fastidiosa*, as a model bacterium, showed itself a good alternative to study the bacterial behavior on the functionalized surfaces, due to its slow time growth, the direct and easy counting procedure, and the absence of contamination risks for humans (**Appendix A.1**).

Through these tests, it was possible to show that the samples containing PEGb and CHILW can offer a bacterial reduction up to 92% due to an antibacterial mechanisms combination (presented in **Chapters 1** and **3**): anti-adhesion action from PEGb and contact killing from chitosan [2,18].

When PA was used as spacer molecule, a lower detection of fluorine was observed, indicating a better cover up of the surface. And after the chitosan deposition, a higher amount of N was verified. PTFE-plasma-PA-CHI samples acted by contact killing action, due to the best homogeneous chitosan coating with a greater number of $-NH_3^+$ groups. Coatings formed with higher molecular weight chitosan showed more effective antibacterial action. Hernandez-Montelongo *et.al* [9], working with LBL, showed that the amino groups present on the surface and their availability is fundamental for antibacterial action.

The most promising samples were tested in contact with pathogenic human bacteria (**Appendix A.2**) (*E. coli, P. aeruginosa* and *S. aureus*): PTFE-plasma-PEGb-CHILW, PTFE-plasma-PA-CHILW and PTFE-plasma-PA-CHIMW, confirming again the antibacterial action by the greater number of $-NH_3^+$ groups [9,292]. For PTFE-plasma-PEGb-CHILW (Figure 6.2a and 6.2b) the bacterial reduction decreased to 55, 48 and 62% for *E. coli, P. aeruginosa* and *S. aureus*, respectively. These results are presented in relation to bare PTFE, used as a control.

Possibly, the anti-adhesion action (from PEGb) was not efficiently for 4 hours in contact with these bacteria. Literature reports that bacterial populations having an extremely rapid growth, like *E. Coli, P. aeruginosa* and *S. Aureus,* can double the number of their population in 30 minutes [13,42]. In this case, the rapid growth may have caused a possible accumulation of these bacteria and organic material (from died bacteria) on the surface to have prevented accessibility to PEGb that had previously presented a repellency action in conjunction with contact killing for *X. fastidiosa*. Results after 8 hours of contact corroborate with the results previously presented, where there was presented a bacterial reduction about 25-30%. Employing PA spacer and by varying the molecular weight of chitosan, it was possible to confirm that amino groups, derived from biopolymer, played the major role in the antimicrobial effect of the samples. PTFE-plasma-PA-CHIMW (Figure 6.3a and 6.3b) sample presented the best action reducing 95% (*S. aureus*) of the bacteria on the surface.

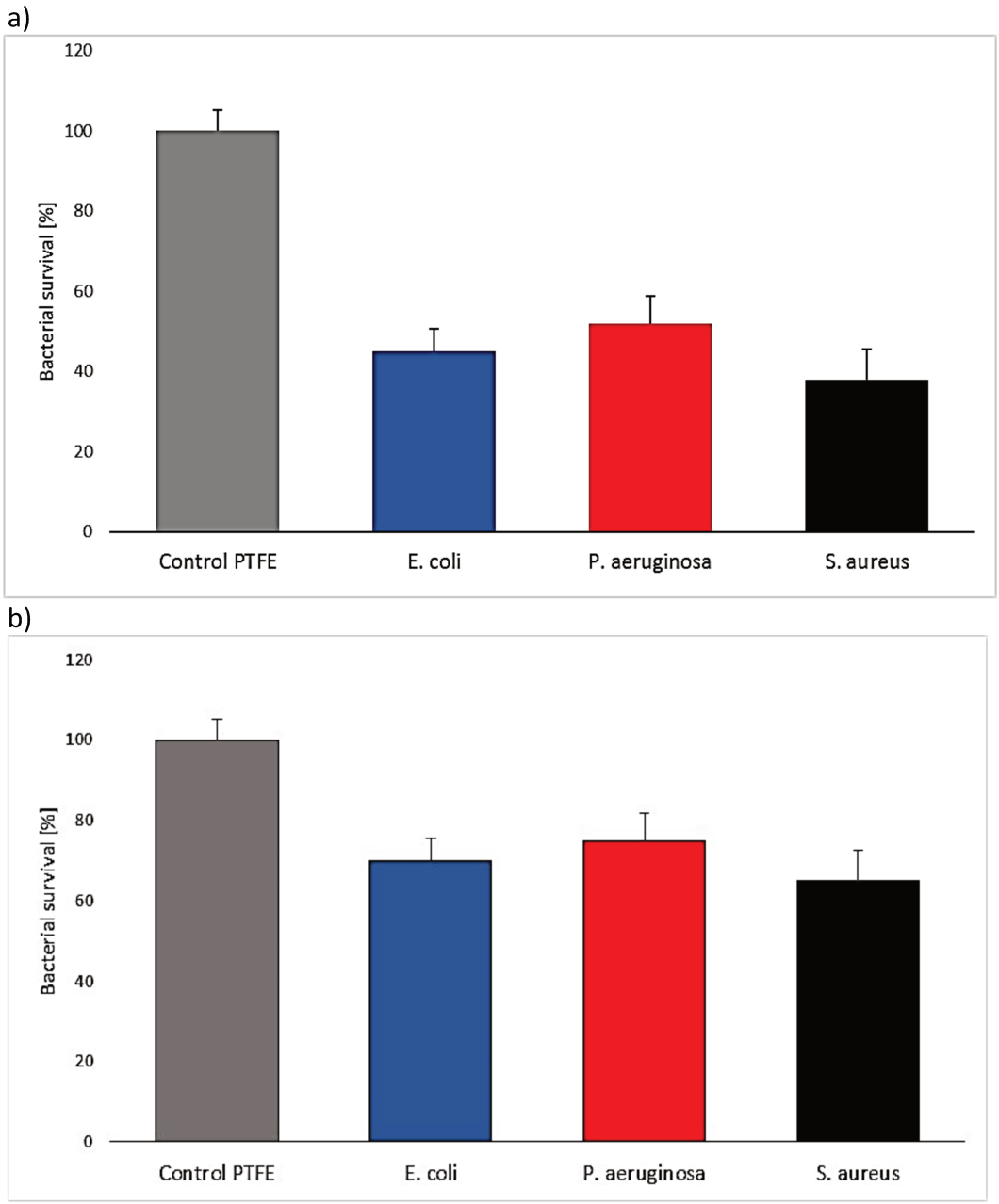
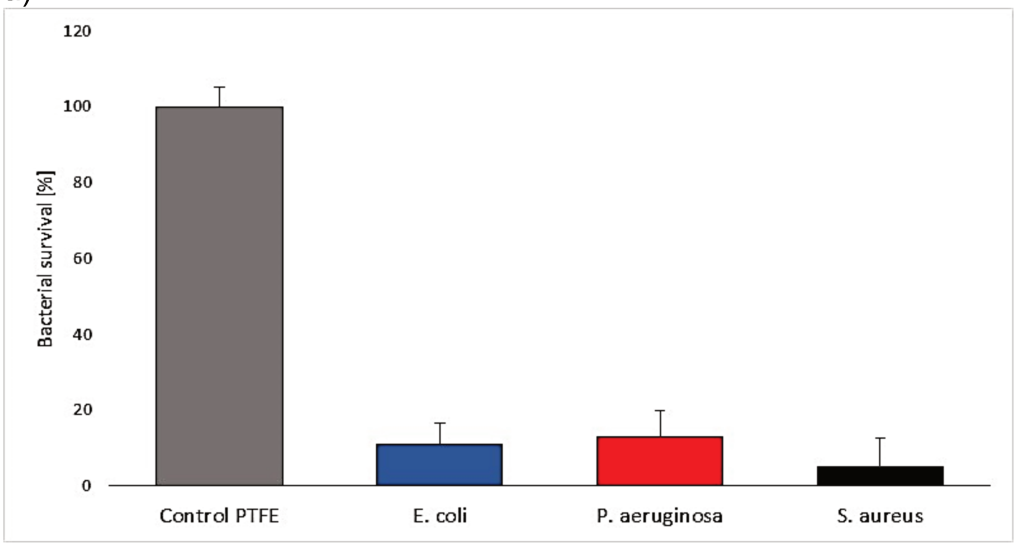


Figure 6.2 – Results for antibacterial adhesion for 4 a) and 8 b) hours for PTFE-plasma-PEGb-CHILW.

Coatings with CHIMW had a better antibacterial response compared to the coatings formed with CHILW probably due to a large quantity of positively charged amino groups responsible for the inactivation of the bacterium by rupturing the negatively charged wall of the microbial cell. It was perceived in these samples a great influence of the type of anchor used, with PA, which has several points of anchorage, allowing the formation of a more homogeneous coating when compared to GA and PEGb.

a)



b)

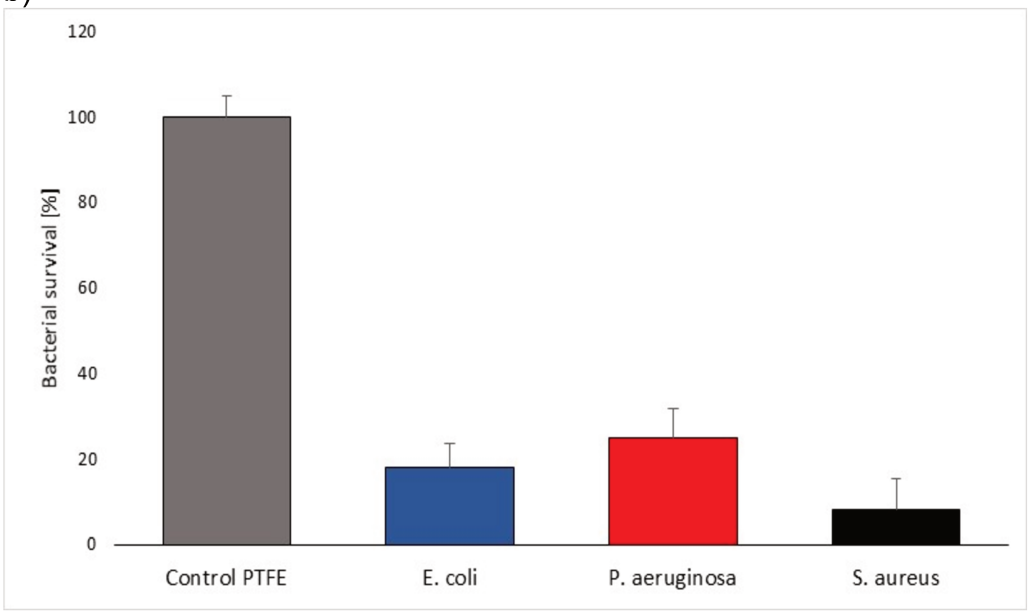


Figure 6.3 – Results for antibacterial adhesion for 4 a) and 8 b) hours for PTFE-plasma-PA-CHIMW.

In addition, the molecular mass of CHILW and CHIMW also had an influence where it is possible to detect a better coating formed for the CHIMW samples. Evaluating the type of bacteria, it was possible to observe that PET-plasma-PA-CHIMW presented the best results for *S. aureus.* This behavior can be explained due to the difference in bacterial cell wall composition, which is simpler for Gram-positive bacteria, which is also reported in the literature [9,18,72,197]. Suggesting that these coatings are more effective for Gram-positive bacteria.It is important to note that the different types of bacterial strains may present different abilities to adhere to the surface of the substrates. In theory, hydrophobic bacterial strains will more likely adhere to materials with hydrophobic surface properties and, correspondingly

hydrophilic species will preferentially adhere to hydrophilic surfaces. And some papers report that rougher surfaces facilitate bacterial adhesion [56,87,157,180].

However, in this study, it was also possible to verify that PACHIMW samples have close contact angle values, being the samples hydrophilic and with a similar roughness, indicating that the antibacterial action of these coatings is primarily linked to the amount and availability of the $-NH_3^+$ groups.

PET substrates were coated with the chitosan that presented a more successful behavior in preventing the adhesion of bacteria to its surface due to a greater number of $-NH_3^+$ groups and this was evidenced by Strip test (Figure 6.4) (**Appendix A.3**).

In the Strip test, it is observed that there is no bacterial growth (neither for *E. coli* nor for *S. aureus*) in the samples treated with CHIMW, when compared to the control (bare PET).

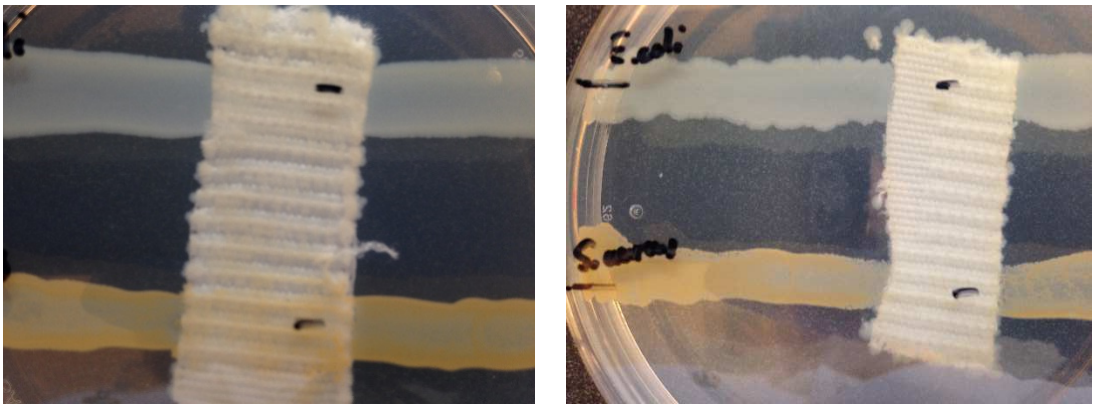


Figure 6.4 – Strip test a) Bare PET and b) PET-plasma-PA-CHIMW.

Results were confirmed by the test “Determining the antimicrobial activity of immobilized antibacterial agent under dynamic contact conditions” ASTM E2149 (Figure 6.5) (**Appendix A.4**).

PET-plasma-PA-CHIMW presented the best results for *S. aureus*. This behavior can be explained due to difference in bacterial cell wall composition, which is simpler for Gram-positive bacteria [2,9,39,143,221].

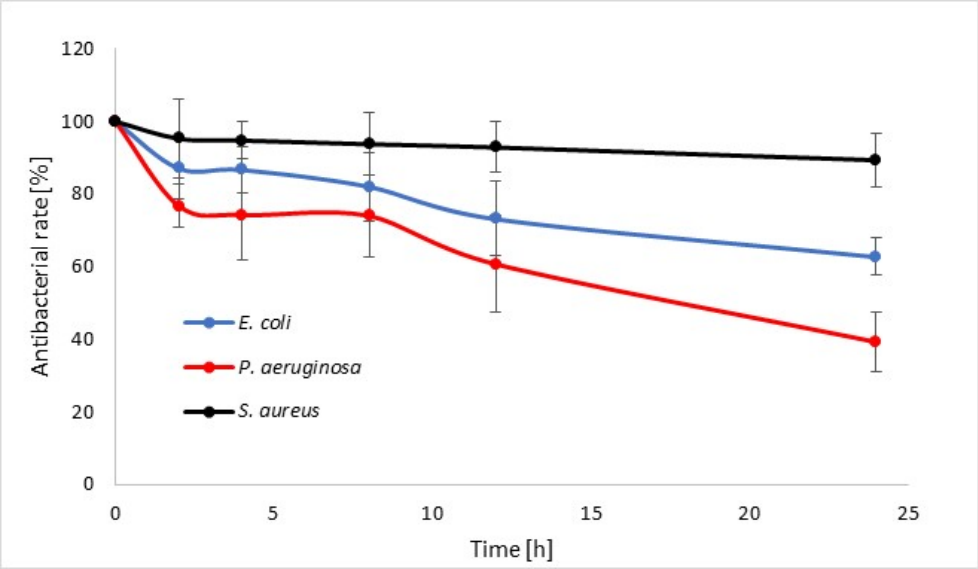


Figure 6.5 - Determining the antimicrobial activity of immobilized antibacterial agent under dynamic contact conditions for PET-plasma-PA-CHIMW.

6.3.1 Desinfection methods

Two types of desinfection process were used for samples coated with chitosan: 70% alcohol and UV light [298]. For these two cases, samples remained stable and kept their antibacterial activity.

6.4 Limitation and perspectives

This study brought a joint methodology to produce antibacterial coatings of chitosan, which can be used in the most diverse applications. However, analyses directly related to each type of application should be carried out allowing a more realistic evaluation of the use of these coatings. Five key issues can be explored:

Mechanical tests:

- should be performed based on the type of application in which this coating will be applied, for example, microscratch and pull off, for PTFE films. The microscratch is employed for characterizing the mechanical properties of films and thin surface coatings, such as adhesion properties, fracture and deformation. Thus, microscratch could be employed to evaluate the adhesion strength of chitosan coatings. The presence of delamination could be observed by optical microscopy and scanning

electron microscopy. Pull-off test could be performed to have a quantitative measurement of the adhesion strength;

- should be performed for a better investigation of the stability and durability of these coatings. The wear-resistance of the PTFE films coated with chitosan can be estimated by micro-abrasion. After this test the samples can be inspected for surface damages and degradation with optical microscopy or scanning electron microscopy;

- antibacterial response of these coatings should be tested before and after these tests, in different time intervals.

For biomaterials applications should be performed:

- flow tests in a bioreactor that simulates the body conditions in order to verify the influence of the shear of a liquid on the surface of these materials. The damages and degradation with optical microscopy or scanning electron microscopy;

- cytotoxicity test can be made by an indirect method using resazurin. Resazurin is a blue dye that is weakly fluorescent and is used to determine the cell activity. This dye can be irreversible reduced by mitochondrial enzymes to produce resorufin, which is pink colored and present a highly intense red fluorescence. blood contact tests should be performed with the intent extending this methodology for antibacterial-wound healing applications. Platelet adhesion and clotting time can provide important information about the efficacity on the control of coagulation process.

For textile applications:

- tests that simulate cleaning, washing conditions and abrasion resistance can be performed. The durability of the treated films against repeated launderings will be evaluated by washing treated subtracts.

- antibacterial response of these coatings should be tested before and after these tests, in different time intervals.

Two major limitations of this project can be pointed out. The first one is that the plasma treatment just allowed modification in only one side of the sample, which may limit the antibacterial action of the treated sample.

Another point was the low standardization of tests for biological tests. More standardized evaluation protocols would also be beneficial to allow the easier and trustworthy comparison among results obtained across different laboratories.

Despite some issues, a remarkable trend shows that multifunctional systems, combining different mechanisms of action against pathogens are emerging as the next generation chitosan-based coatings, for example, as antibacterial-wound healing.

7. Conclusion

The constant threat of bacterial contamination, its social-economic damages, and the increasing of the number of antibiotic-resistant pathogens bacteria have led to a search of new alternatives of infection control, as the antibacterial coatings.

This thesis aimed to develop coatings based on chitosan for antibacterial applications. Based on an extensive literature review, the biological aspects of bacterial contamination and the main techniques targeting to obtain chitosan antibacterial coatings were evaluated and assisted to guide the development of practical works previously presented in this thesis.

Exploratory characterization analyses showed the importance of conducting effective investigations when chitosan is employed for technological applications aiming to respect the uniformity among the samples.

These coatings, covalently linked to the substrate, showed the potential of plasma treatments and conjugation techniques for grafting. The physicochemical characterizations showed that type of spacer molecule used had more influence on the chitosan grafting then the molecular weight. Antibacterial chitosan coatings were produced by a new methodology via plasma-grafting. These analyses also showed that samples containing PA as anchor presented a greater number of available $-NH_3^+$ groups. These functional groups interacted with the bacteria cell wall, resulting in an effective antibacterial response on the surface when tested against *X. fastidiosa, E. coli, P. aeruginosa* and *S. aureus*. Thus, by this work, it was possible to develop antibacterial polymeric substrates, which represent an alternative for a wide spectrum of applications where the antibacterial action is desired, by reducing microbial loads on a surface. Therefore, these coatings can provide an additional, complementary barrier to pathogen transmission, while acting in conjunction with normal cleaning and disinfection procedures. However, it is important to remark that the main contribution of this work is the technology here developed that comes possibilities to explorer the production of new materials.

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Appendix

A.1 Main studies employing *Xylella fastidiosa*

A.1 – Some studies that employed *Xylella fastidiosa* as a model to know the effect of surface modification on its biofilm formation and colonization.

Objective	Principal analyses	Principal results/Conclusion	Reference
Adhesion and biofilm evolution of *Xylella fastidiosa*: film formation and role of surface chemical	Bacteria strain and growth conditions ATR-FTIR AFM Electron microscopy Contact angle	General case: hydrophobic surfaces with roughness increase cell attachment and biofilm evolution. *Xylella fastidiosa*: adhesion only occurs after roughness and hydrophobicity are minimized (formation of a conditioning film). Chemical surface changes are extensively involved in facilitating biofilm growth, which correlates well with current models for *Xylella fastidiosa* cell adhesion. Phosphate groups as a regulator for the secretion of surface proteins, essential for biofilm formation.	Lorite *et al.*, 2011 [157]
Adhesion and biofilm formation of *Xylela fastidiosa* : surface physicochemical properties (micro and nano scales)	Bacteria strain and growth conditions AFM Optical and electron microscopy Contact angle	Different adhesion mechanisms are active along the biofilm life cycle representing an adaptation mechanism. Stiffer and electrically more homogeneous surfaces with larger surface potential exhibit enhanced *Xylella fastidiosa* adhesion and proliferation, likely due to a stronger cell-surface interaction under these circumstances.	Lorite *et al.*, 2013 [180]

Adhesion and biofilm formation of *Xylela fastidiosa*	Bacteria strain and growth conditions Spinning disk confocal fluorescence microscopy Confocal Laser Scanning Microscopy	Experimental validation of important steps of *Xylella fastidiosa* biofilm formation, starting at single adhesion until biofilm maturation. Different compositions of EPS (extracellular polymeric substance) and their roles during biofilm development.	Janissen *et al.*, 2015 [89]
Nanofilms of hyaluronan/chitosan assembled LbL; their application as a potential antimicrobial material was demonstrated for the phytopathogen *X. fastidiosa*, a Gram-negative bacterium, used as a model.	FTIR UV-Vis AFM Contact Angle Bacteria strain and growth conditions	The best antibacterial effect of $3.0^{pH}/0.10^{IS}$ can be explained by its larger number of nitrogenated groups exposed in the surface, which included a higher concentration of free protonated ammonium groups ($-NH_3^+$), the antibacterial killer agent, which was mainly 3 generated by the synthesis pH.	Hernández-Montelongo *et al*., 2016 [166]

A.2 Evaluation of antibacterial activity of PTFE films coated with chitosan coatings by plasma-grafting

As previously explained in Chapters 5 and 6, PTFE-plasma-PEGb-CHILW, PTFE-plasma-PA-CHILW, and PTFE-plasma-PA-CHIMW were selected for testing with *E. coli, P. aeruginosa* and *S. aureus*.

Bacterial adhesion

The microorganisms *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 25923) were purchased from Coleção de Culturas Tropical (Fundação André Tosello, Campinas, Brazil) and broth and agar Mueller-Hinton from Medix, USA.

Bacterial adhesion assays were performed based on the work of Montelongo *et al*. (2016) [9]. The bacteria were, subsequently, re-suspended in a concentration of 10^6 cells/mL. PTFE samples were incubated in triplicate for in an oven with air circulation at 37°C. After 4 h and 8h, the culture medium was removed to stop growth. The samples were washed extensively with sterile Milli-Q water to remove traces of culture medium as well as poorly adhered bacteria on the surface of the sample. In order for the cells present on the surface of the film to be counted, the samples were sonicated in PBS for 10 minutes to enable the bacteria to detach from the buffer solution. From 0.1 mL of this solution, serial dilutions were made in 1:9 (v/v) PBS. Aliquots of 0.1 mL at dilutions were plated on agar MH. After 24h of incubation at 37°C in forced circulation air oven, the number of bacterial colonies was counted and the result, after multiplication by dilution factor, was expressed in colony forming units per cm^2 of the sample (CFU/cm^2).

A.3 Evaluation of antibacterial activity of PET coated with chitosan coatings by plasma-grafting

For evaluation of the enhanced antimicrobial activity of PET fabrics, a qualitative method, based on the parallel streak method (AATCC Test Method 147-2011), was investigated. *Escherichia coli* (ATCC 11229) and *S. aureus* (ATCC 6538) were used in this study. The strains were maintained in tryptic soy broth (TSB) with 10% glycerol and stored at −20°C. With a loop, the frozen bacteria were scraped and streaked onto a Tryptic Soy Agar (TSA) and incubated for 24 h at 37°C. A colony was then culture agitation at 150 rpm in TSB at 37°C for 24h. The bacteria were, subsequently, re-suspended in TSB medium to a concentration of 10^6 cells/mL. The optical density of diluted suspension was used to estimate the bacteria concentration. For each kind of bacteria, one loop-full of the diluted suspension was streaked onto the Petri dish in parallel line. The PET samples (1 cm × 5 cm) were disinfected UV light for 30 min prior to experiment. They were then placed and gently pressed transversely across the streak. The plates with the PET samples were incubated for 24 h at 37°C.

A.4 Evaluation of antibacterial activity of PET coated with chitosan coatings by plasma-grafting

ASTM E2149 - Antimicrobial Surface Test - "Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents Under Dynamic Contact Conditions".

This method is used to quantitatively assess the efficacy of a non-diffusible antimicrobial agent treated sample in contact with a bacterial suspension. The microorganism is grown in liquid medium. The concentration of the bacterial suspension is standardized (usually at 10^6 cells/mL). 50 mL of standardized microbial culture is placed in 3 flasks. One flask receives only bacterial suspension, another receives the sample to be tested with antimicrobial treatment, the latter receives a control sample (a sample similar to the sample to be tested, without the antibacterial agent). The microbial concentration in the liquid of all containers at "zero time". All flasks are shaken by the action of an agitator for some time, usually 1-24 hours, at 37°C. In this study, aliquots were withdrawn at times 2, 4, 8, 12 and 24 hours and the number of bacterial colonies was counted and the result, after multiplication by dilution factor, was expressed in colony forming units per cm^2 of the sample (CFU/cm^2) and related to the control (bare PET).