

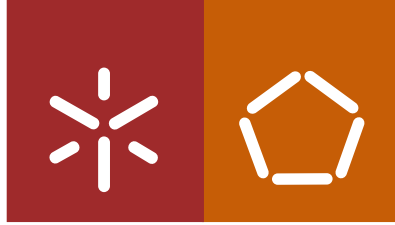


Universidade do Minho
Escola de Engenharia

Tugce Caykara

**Development of Innovative Anti-adhesive
Materials in Diagnosis Tools**

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Materials in Diagnosis Tools**



Universidade do Minho
Escola de Engenharia

Tugce Caykara

Development of Innovative Anti-adhesive Materials in Diagnosis Tools

PhD thesis
Ph.D. in Chemical and Biological Engineering

Work developed under supervision of
Doctor Carla Joana Santos Marinho Silva
and
Professor Doctor Lúgia Raquel Marona Rodrigues

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STATEMENT OF INTEGRITY

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Development of Innovative Anti-adhesive Materials in Diagnosis Tools

ABSTRACT

Spread of infections is a growing concern in the healthcare sector, leading to high medical expenses, antibiotic resistance and even death. One recently explored alternative to reduce the spread of infections is by using anti-adhesive surfaces. Different strategies can be used to develop such anti-adhesive surfaces, as for example manipulating the surface wettability. Polyethylene terephthalate (PET) is one of the most widely used polymers in the healthcare sector. Although it provides advantages including excellent chemical, physical and mechanical properties and biocompatibility, its anti-adhesive properties still need to be improved. In this thesis, PET surface was modified to improve its anti-adhesive properties using three different approaches, namely (1) rendering the surface hydrophilic by chemically grafting a natural polysaccharide (gum Arabic); (2) rendering the surface hydrophilic by UV polymerization of acrylates (sodium acrylate and 3-sulfopropyl acrylate potassium salt); and (3) rendering the surface superhydrophobic by *in situ* growth of nanoparticles and low surface energy molecules. Determination of contact angle, AFM, FTIR, XPS and UV-VIS spectrophotometer analysis on treated surfaces clearly showed that the abovementioned modifications were successfully accomplished. The hydrophilic modified PET surfaces were found to be anti-adhesive, while the superhydrophobic one was contrarily promoting bacterial adhesion which was thought to be due to bacterial orientation on the ununiformly structured surface, when evaluated for their anti-adhesiveness against an *Escherichia coli* expressing YadA (*Yersinia enterocolitica* adhesin) herein used as a model adhesive microorganism. Finally, the performance of the modified materials was assessed regarding crystal formation using artificial urine. Although, the modified materials did not perform better than the unmodified PET regarding crystal formation, the gum Arabic modified PET and the acrylate modified PET could still be useful for intermittent catheterization due to their anti-adhesive properties, besides exhibiting a great potential also for other applications, such as diagnosis tools.

Keywords: anti-adhesive surfaces; bacterial adhesion; grafting; polyethylene terephthalate; surface modification.

RESUMO

A propagação de infecções é uma preocupação crescente no setor da saúde, pois acarreta elevados custos para o setor hospitalar, bem como potencia o aumento da resistência a antibióticos e pode mesmo levar à morte da pessoa infetada. Uma alternativa recentemente explorada para reduzir a propagação de infecções é o uso de superfícies anti-adesivas. Para desenvolver estas superfícies podem ser usadas diferentes estratégias, como por exemplo manipular a molhabilidade da superfície. O polietileno tereftalato (PET) é um dos polímeros mais utilizados no setor de saúde. Embora apresente vantagens como excelentes propriedades químicas, físicas, mecânicas e biocompatibilidade, as suas propriedades anti-adesivas ainda precisam ser melhoradas. Nesta tese, a superfície do PET foi modificada para melhorar as suas propriedades anti-adesivas, usando três abordagens diferentes, nomeadamente (1) tornando a superfície hidrofílica através do enxerto químico de um polissacarídeo natural (goma arábica); (2) tornando a superfície hidrofílica através da fotopolimerização de acrilatos (acrilato de sódio e sal de potássio de acrilato de 3-sulfopropilo); e (3) tornando a superfície super-hidrofóbica pelo crescimento *in situ* de nanopartículas e moléculas de baixa energia superficial. As superfícies tratadas foram caracterizadas por ângulo de contato, AFM, FTIR, XPS e análise por espectrofotometria UV-VIS, evidenciando claramente que as modificações acima mencionadas foram realizadas com sucesso. As superfícies hidrofílicas do PET modificado demonstraram propriedades anti-adesivas, contrariamente à superfície super-hidrofóbica, que promoveu a adesão bacteriana, pensando-se ser devido à orientação das bactérias na superfície irregular do PET, quando foi efetuado o teste de adesividade usando uma *Escherichia coli* expressando *YadA* (adesina *Yersinia enterocolitica*), como um microrganismo adesivo modelo. Por fim, avaliou-se o desempenho dos materiais modificados quanto à formação de cristais, utilizando urina artificial. Embora os materiais modificados não tenham tido um desempenho significativamente melhor que o PET virgem quanto à formação de cristais, o PET modificado com goma arábica e o PET modificado com acrilatos pode ser extremamente útil para o cateterismo intermitente devido às suas propriedades anti-adesivas, para além de apresentar um enorme potencial para outras aplicações, como a utilização em kits de diagnóstico.

Palavras-chave: superfícies anti-adesivas; adesão bacteriana; enxerto; tereftalato de polietileno; modificação da superfície.

OUTPUTS

Articles in peer reviewed journals

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LIST OF ABBREVIATIONS

AF	Acrylate polymer blend
AFM	Atomic Force Microscopy
ANOVA	Analysis of variance
aTc	Anhydrotetracycline
ATR-FTIR	Attenuated total reflectance - Fourier Transform Infrared
ATRP	Atom transfer radical polymerization
BP-QAS	Benzophenone group terminated cationic quaternary ammonium salts
CAE	Constant Analyser Energy
CHI	Chitosan
CMC	Carboxymethylcellulose
Cs	Cationic chitosan
DBD	Dielectric Barrier Discharge
DCC	N,N'-dicyclohexylcarbodiimide
DEDA	N,N-diethylethylenediamine
DLS	Dynamic Light Scattering
DLVO	Derjaguin-Landau-Verwey Overbeek
DNA	Deoxyribonucleic Acid
DoE	Design of experiments
DS	Dermatan sulfate
ECDC	The European Centre for Disease Prevention and Control
eDNA	Extracellular deoxyribonucleic acid
EDS	Energy dispersive spectroscopy
EF	Epoxy polymer
ePET	Expanded polyethylene terephthalate
EPS	Extracellular polymeric substances
ePTFE	Expanded polytetrafluoroethylene
FimA	Adhesin from fimbriae type 1
GMA	Glycidyl methacrylate
HA	Hyaluronic acid

HAI	Hospital Associated Infections
HTCC	Hydroxypropyltrimethyl ammonium chloride chitosan
IC	Intermitted catheters
IPTG	β -D-1-thiogalactopyranoside
LAMA	2-lactobionamidoethyl methacrylate
LB	Luria Broth
KAc	3-Sulfopropyl Acrylate Potassium Salts
MPEO	Methoxy polyethylene oxide
NAc	Sodium acrylate
NP	Nanoparticle
OFAT	One-factor-at-a-time
OWRK	Owens-Wendt-Rabel&Kaelble Model
PANI	Polyaniline
PapC	Adhesin type from pili
PBS	Phosphate buffer saline
PC	Polycarboxybetaine
PCCs	Phosphorylcholine–chitosan
PDMAEMA	Poly(2-(dimethylamino)ethyl methacrylate)
PEG	Polyethylene glycol
PEI	Polyethylenimine
PET	Polyethylene terephthalate
PG	Peptidoglycan
PHA	Polyhexylene adipate
PS	Polysulfobetaine
PTFE	Polytetrafluoroethylene
PU	Polyurethane
RAM	Rhizome <i>Atractylodes macrocephala</i>
RIF	Rifampicin
ROS	Reactive oxygen species
SD	Sulfadimehoxine
SEM	Scanning electron microscopy
SfAS	Adhesin type from fimbriae S

TFPDM	2,3,5,6-tetrafluoro-p-phenylenedimethanol
TRI	Triclosan
UV-VIS	Ultraviolet – visible spectrophotometer
UV	Ultraviolet
VAN	N-(4-hydroxy-3-methoxybenzyl)-acrylamide
WCA	Water contact angle
XPS	X-Ray Photoelectron Spectroscopy
YadA	<i>Yersinia enterocolitica</i> adhesin

GENERAL INTRODUCTION

Context and motivation

Research aims

Outline of the thesis

CONTEXT AND MOTIVATION

Spread of infections is a growing health problem and the number of infected people is increasing constantly every year in healthcare facilities [1]. Antibiotics are used to treat infections, however, the increase in antibiotic resistance is a great concern. Indeed, 30% of the bacteria found in healthcare facilities have been found to be antibiotic resistant [2]. Biofilms are ideal for the development of such resistance as they protect bacteria conferring them resistance [3]. Thus, for material-associated infections, which are common in the medical scenario, it is important to prevent the initial bacterial adhesion, that ultimately leads to biofilm formation and infection. Inhibition of planktonic bacteria adhesion [4] can be achieved by modifying materials properties that affect adhesion. Some of the strategies that have been reported to prevent bacterial adhesion include the development of:

- resistant hydrophilic surfaces, where a hydration layer on the top of surface is formed that prevents bacteria from approaching to the surface [1,4,5,8];
- repellent superhydrophobic surfaces, where air pockets are created on surfaces to prevent bacteria from approaching and to enable their easy cleaning [5];
- bactericidal surfaces, where bacteria approaching the surface are killed by antimicrobials which are either bonded to the surface or released from it [6];
- resistant and bactericidal patterned surfaces, where surface patterns can be designed in a way that either reduces bacterial adhesion or kills the adhered bacteria [7,8].

Since the bacteria deterrence strategies with hydrophilic surfaces and superhydrophobic surfaces are based on anti-adhesion, the spread of infections in these surfaces would be reduced, and consequently also the unnecessary use of antimicrobials, limiting the occurrence of antimicrobial resistance.

PET is one of the most used polymeric materials in the healthcare sector due to its desirable properties such as biocompatibility, high uniformity, mechanical strength and resistance against chemicals and/or abrasion. However, PET surfaces are prone to bacterial adhesion [4]. Therefore, the main goal of this thesis was to modify PET surfaces to improve its surface wetting properties towards bacterial adhesion prevention. Furthermore, to assure a durable surface modification, several methods including “grafting to”, “grafting from” and “*in situ* nanoparticle growth” for covalent bonding the different studied molecules to the surface were implemented. All modified surfaces were characterized by contact angle measurement, FTIR, AFM, XPS and UV-Vis spectrophotometer analysis and further bacterial adhesion tests were conducted with a modified *Escherichia coli*.

RESEARCH AIMS

This thesis aims to develop PET surfaces presenting anti-adhesive properties against bacteria. The modified PET materials are expected to be used in biomedical applications such as hospital utilities, diagnosis tools and medical devices, such as urinary catheters, among others.

Specific aims include:

- Development of anti-adhesive PET surfaces with hydrophilic properties through a “grafting to” and a “grafting from” process and with superhydrophobic properties through modification with nanoparticles functionalised with fluorinated carbon chains.
- Characterisation of the obtained surface properties via contact angle, AFM, SEM, FTIR, UV-VIS.
- Evaluation of the stability and durability of the obtained surfaces after several washing cycles and ageing tests.
- Evaluation of the anti-adhesive properties of the obtained surfaces against a modified gram-negative bacterium (*E. coli* expressing YadA)
- Simulation of the application of the modified surfaces in an artificial bladder model using artificial urine.

OUTLINE OF THE THESIS

This thesis describes the main outcomes of the research work developed at Centre of Nanotechnology and Smart Materials (CENTI) (Vila Nova Famalicão, Portugal), Centre of Biological Engineering (CEB), University of Minho (Braga, Portugal) and Hydrumedicals (Guimarães, Portugal) under the supervision of Dr. Carla Silva and Prof. Ligia Rodrigues. This thesis is organized in 7 chapters that cover the abovementioned research aims as follows:

- **Chapter 1**, includes a description of the bacterial adhesion concept and the parameters that are relevant for the modification of a biomaterial. Also, it explores the strategies that can be used to modify polymeric materials in order to prevent bacterial adhesion, envisaging its ultimate application in urinary catheters.

- **Chapter 2** summarizes the methodologies that can be used to modify PET surfaces to render them anti-adhesive and anti-bacterial properties.

- **Chapter 3** focuses on the PET modification with a natural substance, gum Arabic. The modified PET surfaces were characterised by water contact angle measurements as it indicates the

surface hydrophilicity. Moreover, the potential of the modified materials to inhibit bacterial adhesion was assessed.

- **Chapter 4** investigates the combination of atmospheric plasma and UV polymerisation of sodium acrylate and 3-sulfopropyl acrylate potassium salt in the “grafting from” technique. The modified PET was characterised by water contact angle measurement. Finally, the modified PET ability to inhibit bacterial adhesion was further evaluated.

- **Chapter 5** describes a surface modification method using *in situ* formed nanoparticles with fluorocarbon chains to develop a superhydrophobic surface. Characterisation of the surface properties and its effect on bacterial adhesion were performed.

- **Chapter 6** addresses the use of the modified materials in a simulated urinary catheter environment. The modified PET surfaces were immersed under artificial urine for a given period of time to evaluate the occurrence of crystallisation/encrustations. This initial trial focuses on the comparison between treated and untreated PET and discusses their use in urinary environments.

- **Chapter 7** presents the main conclusions of this thesis and discusses some future perspectives in this field of research.

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CHAPTER 1

Biomaterials surface engineering towards anti-adhesive urinary catheters

Hospital Associated Infections (HAI) have been growing in number over the years, representing a concern that requires an increased attention. The most frequent infections are observed in the urinary tract and 80 % of them are catheter related. These infections cause problems ranging from mild discomfort to severe health implications and even death. Being the bacterial adhesion onto the material surface the first step of many of these catheter-related infections, the ability to modify those materials, conferring them anti-adhesive features, is seen as an important strategy towards the prevention and/or reduction of HAI. Bacterial adhesion is a complex phenomenon mediated by several factors such as biological, physical and environmental factors. Bacteria have morphological differences like different shape and cellular wall composition, surface appendages, surface hydrophilicity/hydrophobicity and surface charge and the complexity of bacterial adhesion increases when they interact with material surfaces and under variable environmental conditions. This chapter describes the state of the art on the design of novel materials for urinary catheters based on the main factors that affect bacterial adhesion and biofilm formation. Strategies being developed to confer these urinary catheters biodegradability and antimicrobial properties will also be discussed.

Keywords: Biomaterials; urinary catheters; anti-adhesive; surface modification; bacterial adhesion.

This chapter is based on the following publication:

Caykara T, Rodrigues LR, Silva C (2022). Biomaterials surface engineering towards anti-adhesive urinary catheters. (to be submitted)

1.1. INTRODUCTION

Bacterial adhesion is a concern in several fields including hospitals, implants, diagnostic tools, membranes, bioreactors, food and marine industry, among others [1]. In the healthcare sector, dealing with bacterial adhesion and consequent biofilm formation through the design of novel anti-adhesive materials can help to decrease expenditure [2], antibiotic resistance and deaths resulting from material-related infections [2]. Over 90 % of implants contain pathogens due to the contact with patients' skin, medical personal clothing, and medical equipment, or due to their presence in the patient's body when implanted [3]. In 2017, it was estimated that 8.9 million registered infection cases occurred in hospitals and long-term care facilities only within Europe [4], while this number in 2012 was estimated to be more than 4 million [2]. As also it can be seen from the infection cases, the global rise of human infectious disease outbreaks is a current well-known concern [5].

The most frequent infections are observed in the urinary tract. Urinary tract infections account for 30 – 40 % of total HAI worldwide and 80 % of those infections are associated with urinary catheters. Moreover, it is important to notice that these statistics have been drawn for developed countries and that for less developed countries numbers are expected to be 3 – 5 times higher [6]. Both catheters and stents offer a simple method to maintain internal drainage of the urinary tract. However, these biomedical devices provide an ideal place for bacterial colonisation and biofilms formation leading to infections and playing a critical role in morbidity [7]. Catheters can be used for short-term (up to 7 days) or long-term (28 days or longer) procedures [8]. Indeed, urinary catheters can stay in place for up to 12 weeks. However, this is not common due to encrustation and bacterial infection, which can block catheters and cause complications ranging from mild encrustation and bladder stones to severe septicemia, endotoxic shock and pyelonephritis [9].

Factors that prevent urinary infections in healthy people are (1) the repeated sloughing of urethral epithelial cells, (2) innate mucosal immune function and (3) the periodic flushing action of urine expelled from the bladder. As mentioned, with catheters, the material surface offers a spot for bacterial colonisation, followed by biofilm formation and encrustation [8]. As a solution to overcome this problem, intermittent catheters (IC) can be used, however, not all patients are eligible to use them, particularly those lacking the ability to take care of their own needs. Nevertheless, the occurrence of infections is the main issue arising from catheter's use independently of being for long-term or short-term procedures. Indeed, all catheter types and brands are somehow susceptible to bacterial adhesion leading ultimately to infections [9].

The infection and bacterial colonisation of the catheters start with the reversible attachment of

planktonic bacteria to material's surfaces. Afterwards, the bacteria can either go back into the bulk medium or strengthen its bond with the surface, resulting in an irreversible adhesion [1]. When proper conditions are provided, the bacteria will divide and form microcolonies, and then, those microcolonies will grow and form a mature biofilm [10]. A biofilm is a microbial community surrounded by a polymer matrix composed of polysaccharides, secreted proteins, lipids and extracellular DNA [11]. However, at this stage, continuous growth of the microbial colonies can become unsustainable due to environmental limitations such as low levels of oxygen and nutrients. Hence, bacteria can be dispersed into the liquid solution and these cells can again attach to (other) surfaces and form new biofilms [10]. Biofilms provide several advantages for bacteria survival including a greater access to nutritional resources, improved survival to biocides, enhanced organism productivity and interactions and greater environmental stability [1]. Bacteria within biofilms are resistant to antibiotics and it has been shown that bacteria collected from both urine and stents are resistant to antibiotics resulting from their biofilm state as can be inferred by the expression of specific biofilm-related genes. Therefore, it is crucial to prevent bacterial adhesion and biofilm formation onto these biomedical devices [12]. Indeed, understanding bacterial adhesion is a key step to further design novel anti-adhesive materials for many applications. In this review, we will discuss the interactions and conditions affecting bacterial adhesion and the design of new materials for urinary catheters.

1.2. FORCES MEDIATING BACTERIAL ADHESION

When bacteria are moving from liquid to a solid surface, *Van der Waals*, electrostatic (or *Coulomb*) and acid-base interactions are the first physicochemical forces that bacteria experience [10]. *Van der Waals* forces are the most long-ranged ones with distances up to 1 μm and these forces become gradually stronger when surfaces become closer [13]. *Van der Waals* interactions are generally attractive, while electrostatic interactions are modulated by the ionic strength and the pH of the liquid environment, and acid-base hydrophobic interactions can be attractive or repulsive depending on the environment, bacterium and surface chemistries [10]. While *Van der Waals* interactions are more dominant close to the surface, the electrostatic ones become more dominant at a distance [14]. These bacterial adhesion forces are measured in the picoNewton and nanoNewton ranges [15]. Stronger forces ($> 10 \text{ nN}$) can cause bacterial cell wall damage and bacterial cell death [13].

Considering the forces applied on a surface, different theories have been developed to explain physicochemical interactions between bacteria and interacting surfaces, namely the *Derjaguin-Landau-Verwey Overbeek* (DLVO) theory, the thermodynamic theory and the extended DLVO (XDLVO) with

thermodynamic approach [16]. However, all these theories do not completely describe adhesion since the bacterial surface is complex, as well as structurally and chemically heterogeneous. Furthermore, extracellular polymeric substances (EPS) and proteinaceous cell appendages bridge between bacteria and the substrate, directly affecting bacterial adhesion and the theories are unable to model it. While EPS is important for biofilm formation, the presence of proteinaceous cell appendages are often essential for the initial bacterial adhesion [17]. Thus, experimental bacterial adhesion studies and design of new anti-adhesive materials remain of critical importance [16].

1.3. FACTORS AFFECTING BACTERIAL ADHESION

Adhesion of bacteria onto surfaces depends on three main factors (Figure 1.1), namely bacteria itself (so-called biological factors), material surface (so-called physicochemical factors) and the surrounding environment (so-called environmental factors) [10] which must be considered when engineering materials to confer them anti-adhesive properties.

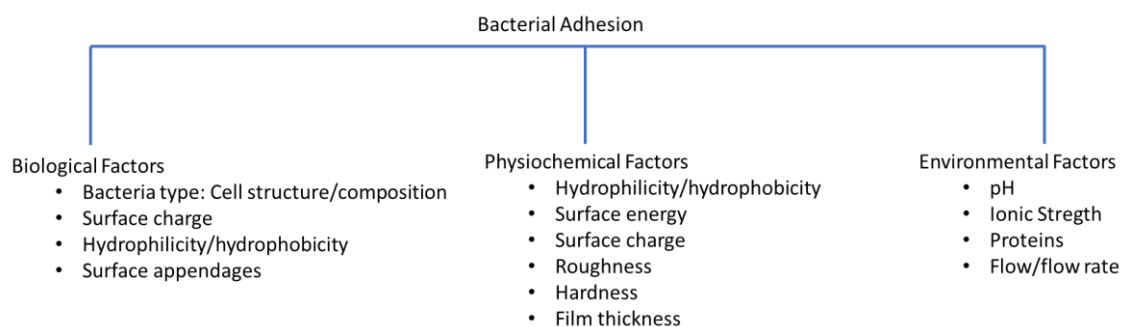


Figure 1.1. Factors affecting bacterial adhesion onto material's surfaces.

1.3.1. Biological factors

Urinary infections are caused by both internal microflora and external contamination [9]. The most common bacteria found in catheter-related infections are *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Escherichia coli*, *Citrobacter freundii*, *Providentia rettgeri*, *Candida albicans*, *Morganella morganii*, *Burkholderia cepacia*, *Providencia sp.*, *Providencia stuartii* [8,9,18]. From all those bacteria, *E. coli*, *E. faecalis*, *S. epidermidis* are frequent in short-term device applications, while *P. mirabilis*, *P. aeruginosa*, *P. stuartii*, *M. morganii* and *K. pneumoniae* usually occur in long term applications [18,19]. In indwelling catheters, *P. mirabilis* biofilm is the main concern, while in

intermittent catheters, the main infections that occur are caused by *E. coli* due to the microflora of meatus being pushed into the bladder [9].

Bacteria can be divided into Gram-negative and Gram-positive depending on the cell wall composition [20]. Although both bacterium types are seen, gram-negative bacteria seem to be the dominant bacteria type in urinary infections [8,9,18]. One of the main differences in these two types of bacteria is the thickness of the peptidoglycan layer in the cell wall. Gram-positive bacteria have a thick peptidoglycan layer above the cell membrane, while gram-negative ones have a thin peptidoglycan layer between the cell membrane and outer membrane layer, and it includes a gel-like region which is called periplasmic space [20]. Thus, gram-negative bacteria have a less rigid surface compared to the gram-positive ones and, the more flexible cell wall of gram-negative bacteria can promote a better adhesion onto rough surfaces [21]. Furthermore, the outer cell wall in gram-negative bacteria consists of lipopolysaccharides, whereas in gram-positive bacteria teichoic and lipoteichoic acids in the wall are attached to the peptidoglycan layer [20].

Additionally, bacteria are almost always negatively charged [20]. Bacteria cell surface charge originates from dissociation or protonation of carboxyl, phosphate and amino groups and consequently depends on the environmental pH [22]. The negative bacterial charge is mainly due to phosphate groups in teichoic acids for gram-positive bacteria [23] and phosphoryl and 2-keto-3- deoxyoctonate carboxylate groups in gram-negative bacteria [20]. However, the bacterial cell is highly heterogeneous and contains various exposed proteins, lipids and exopolysaccharides, that exhibit different charges and hydrophobicity depending on the growth conditions such as environmental pH and ionic strength, to provide adhesive adaptability [10,24]. Surface charge is also influenced by the bacteria age and surface structure. A high surface charge usually goes together with hydrophilic bacteria [25]. The hydrophobicity of bacteria differs according to the bacterial species, and it is also influenced by growth, medium, bacteria age and surface structure [26]. Microbial cell hydrophobicity can be determined by contact angle measurements and the values for some of the abovementioned bacteria can be found in literature [27].

A great variety of structural surface appendages like fimbriae or flagella can be found on the top of the peptidoglycan layer in gram-positive bacteria and on the top of the outer membrane in gram-negative bacteria [22]. The surface appendages are extracellular structures which are important in bacterial growth and survival in diverse environments [28]. Filamentous protein extensions and surface appendages from the cell surface, including flagella, fimbriae, curli and pili, are involved in the non-specific initial adhesion to abiotic surfaces [10]. On the other hand, non-fibrillar adhesins on the surface

of bacteria can play a role in the close contact between bacterial cell and substrate, as well as in the maturation of interactions and irreversible adhesion [10,24]. It has been shown that adhesins like Fimbriae type 1 (FimA), Fimbriae S (SfaS), PapC (which forms pili) [29] and extracellular polysaccharides [24,30,31] and eDNA (extracellular DNA) [32] also promote adhesion. Moreover, bacterial cells are highly dynamic, and they can adsorb ions and macromolecular components. Charged groups may associate or dissociate upon changes in pH or ionic strength of the surrounding fluid and even when approaching a charged surface. This might induce changes in the conformation of different kinds of surface appendages as fimbriae and flagella [22]. Additionally, bacteria can reach a surface by active and/or passive movement. While some bacteria have swimming motility, some are subjected to physical forces like Brownian motion and gravitational forces to bring them close to the surface. In active movement, bacteria have flagella, which are responsible for the swimming ability by generating a propulsive force. They can also play a role in reversible and irreversible adhesion. Flagella can direct the swimming towards a surface in response to the cues in environment like chemical signals, light, temperature, magnetic fields and oxygen [10,24].

1.3.2. Physical factors

Several physicochemical properties of the surfaces affect bacterial adhesion, namely, wettability, surface energy, surface charge, roughness and others like hardness and film thickness [33,34]. The hydrophobicity effect on bacterial adhesion is mostly governed by the hydrophobicity of the bacterial cell [10]. Hydrophobic bacteria show higher adhesion compared to hydrophilic bacteria, while hydrophilic materials were found to be more resistant to bacterial adhesion than hydrophobic materials. Large numbers of bacteria adhere to hydrophobic surfaces with little or no surface charge, however, this number is smaller for hydrophilic surfaces [26]. The number of bacteria becomes even smaller if the hydrophilic surfaces are negatively charged [25]. A study suggests that bacteria adhere with only few strongly binding macromolecules on hydrophilic surfaces whereas bacteria adhere with many weakly binding macromolecules leading to high adhesion with low variability on hydrophobic surfaces. [35]. Anti-adhesive materials via hydrophilic surface modification will benefit from a hydration layer formed on the material surface which acts as a barrier to the bacteria approaching [36]. Although there is an increased interest in hydrophilic surfaces, the bacterial inhibition observed on some natural surfaces like lotus leaves, dragonfly wings and shark skin has inspired the scientific community to also work on the design of superhydrophobic surfaces. These surfaces, due to their superhydrophobic nature, form air pockets on the surface of the materials, preventing bacteria from approaching and consequently

adhering [10] and, they are also easier to clean due to a weaker binding at the interface [37].

An additional parameter affecting bacterial adhesion is surface energy [38]. Katsikogianni *et al.* showed that bacterial adhesion was negatively correlated with the total surface free energy and its polar component according to dispersive-polar approach, where total surface free energy is expressed as the sum of polar and dispersive components [39]. Adhesion free energy becomes more negative resulting in an increased bacterial adhesion strength with the increasing hydrophobicity of a surface [40]. However, some studies have shown that low surface energies (20 – 30 mN/m) have the lowest adhesion [34]. This may be due to the chemical and physical properties of bacteria, substrates and water solution used [38]. However, since surface energy values are usually calculated using contact angle values, which are an indication of hydrophilicity and hydrophobicity, conflicting results can arise from using these wetting properties of the materials [37].

Another factor influencing bacterial adhesion is the material surface charge. As previously mentioned, bacterial cells generally exhibit a net negative charge. As most surfaces are naturally negatively charged, bacteria experience electric double layer repulsion [34]. However, positively charged surfaces can be used to kill bacteria by attracting them and damaging their cell wall [33].

Roughness is another parameter showing conflicting results [41]. The general opinion in the literature reports that irregularities on polymer surfaces promotes bacterial adhesion, while ultra-smooth surfaces show lower bacterial adhesion. This surface behaviour has been explained with the increase of favourable sites on the surface area for bacterial attachment [34]. However, other studies showed that roughness had no effect or even inhibited the adhesion of bacteria [42] in cases of creating nano roughness [42]. This kind of conflicting results may be due to the bacteria used, as bacteria type (gram type) [21] and fibril structures like flagella [43] can make a difference. Moreover, it should also be noticed that the surface roughness can affect its hydrophilicity and hydrophobicity. Surface wetting can either be homogeneous or heterogeneous, which have different impacts on bacterial adhesion. The *Wenzel*'s phenomenon suggests that both hydrophilicity and hydrophobicity are enhanced by an increasing roughness on homogeneously wetted surfaces, meaning that a hydrophilic surface will become more hydrophilic, and a hydrophobic surface will become more hydrophobic [44]. The porous surfaces behave according to another phenomenon, the so-called *Cassie-Baxter* phenomenon. In this state, the water droplet heterogeneously wets the surface causing air pockets and affecting the wettability [45]. In this way, hydrophobic surfaces can prevent bacterial adhesion.

Additionally, micro-texturing has also been found to be beneficial on surfaces where bacteria width is bigger than the gap between micro-textures. This reduces the bacterial adhesion by reducing

the surface area [34]. Thus, it is important to tune the dimensions and shapes of textures according to the bacterial cell to control bacterial cell adhesion [46,47]. Micro and nano texture of the surfaces can also be arranged in a way to mechanically rupture bacterial cell wall, thus providing antibacterial properties while avoiding antimicrobial resistance. However, this mode of action works better for gram-negative bacteria, due to its flexible cell membrane which allows stretching and tearing upon surface adsorption [48].

In addition to the factors discussed above, other factors like hardness and film thickness have attracted attention. While it had been found that increased hardness reduced bacterial cell adhesion in polymers [26,49], there has been reports that soft materials were more resistant to bacterial adhesion [50]. On the other hand, the film thickness was important for soft materials and thicker films reduced bacterial adhesion, which was thought to be due to the stiff substrate having a higher effect on thin films [50].

1.3.3. Environmental factors

Environmental effects caused by the presence of proteins, ions, pH and flow rate can also play a role in bacterial adhesion. Human urine consists of 91 – 96 % water and the remaining parts comprise inorganic salts, urea, organic compounds and organic ammonium salts [51]. It was found that pH and the ionic strength of the surrounding buffer affect the cell surface and material surface's hydrophobicity [52]. The pH of urine is between 4.5 - 8 [19]. A problem that arises due to increased pH in the medium is encrustation. It may cause blockage of the catheter leading to damage of the bladder, ureters and kidneys [9]. Encrustation can occur due to metabolic dysfunction but generally, it is due to bacteria as urease-producing bacteria (*P. mirabilis*, *P. vulgaris* and *P. rettgeri*). Urease hydrolyses urea into ammonia and carbon and with increased ammonia, the pH of the urine increases above 8.0, causing calcium and magnesium phosphate to crystallise. However, some urease forming bacteria species do not form crystals due to low levels of urease production. Some of these species include *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. coli*, *M. morganii*, and *P. stuartii*. Nevertheless, other bacteria can still produce mucoid which can also cause catheters blockage. Thus, catheters should be resistant to bacterial adhesion, biofilms formation and encrustation [9]. On the other hand, ionic strength is one of the factors affecting bacterial adhesion. At low ionic strength (≤ 20 mM) adhesion can be driven by electrostatic repulsion, while *Van der Waals* and hydrophobic interactions can be primary driving forces at higher ionic strengths (50 – 100 mM) [14]. The ionic strength of urine is mainly determined by sodium and chloride due to their abundance [53] and its physiological ionic strength is 150 mmol L⁻¹

[54]. Due to the relatively high ionic strength value, it can be said that the adhesion will be driven by Van der Waals and hydrophobic interactions thus, the design of new materials should be more focused on wetting properties of the materials rather than on surface charge. Moreover, other ions like calcium can also enhance bacterial adhesion [14]. While a low amount of iron and manganese weakens bacterial adhesion, phosphate limitations enhance bacterial adhesion [10].

Furthermore, a conditioning film is formed on the surface of the urinary catheter when it enters the body [19]. This conditioning film consisting of proteins, polysaccharides [55] and urine can make the surface more susceptible to bacterial adhesion [8,56]. Tamm-Horsfall glycoprotein and other proteinaceous molecules like serum albumin, fibrinogen, collagen and fibronectin can be found in conditioning films [57]. Tamm-Horsfall, fibrinogen and fibronectin can promote bacterial adhesion [8,58] while albumin inhibits it [59,60] and collagen has been shown to promote and inhibit bacterial adhesion depending on the experimental conditions [61,62]. Urine also includes proteins from urothelial cells, as well as damages at the urethral lining caused during insertion of catheters [19]. Electrolytes, ions, mineral and other organic molecules will also be present on the conditioning film [19,63–65]. Furthermore, initial bacterial adhesion stages in alkaline environments can cause the formation of microcrystals on the catheter surface which are also proven to support bacterial adhesion (Figure 1.2) [8,66].

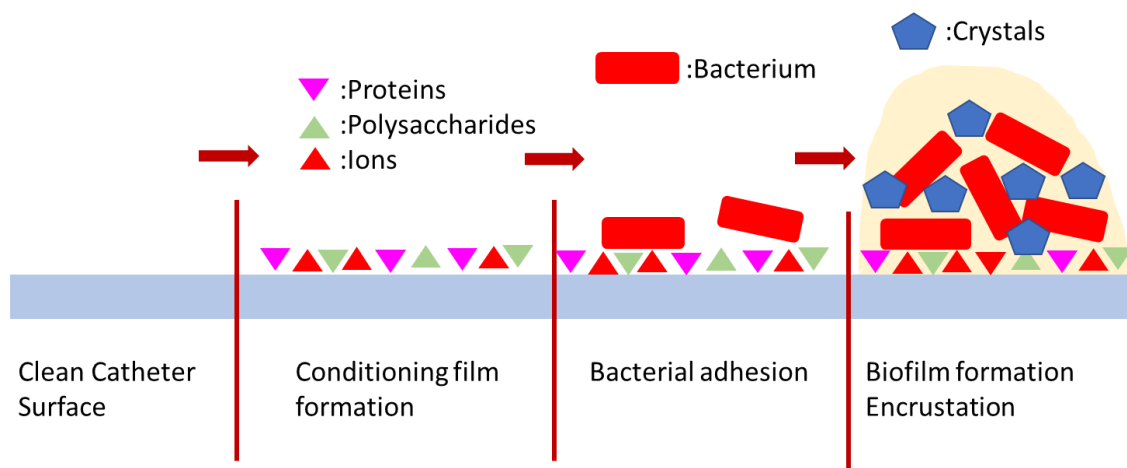


Figure 1.2. Stages of biofilm formation on catheter surfaces [adapted from 9, 19, 67].

Other important factors to prevent bacteria from reaching and adhering to the surface are flow and shear. If the flow rate is high, it would cause a lower boundary layer, thus bacteria near to the surface are affected by higher shear and at high velocity, limiting the bacteria approach to the surface [10]. Furthermore, antimicrobial substances can be depleted from the surface in a shorter time compared to static conditions. Therefore, catheters should be studied under flow [68]. Unfortunately, the lack of standard testing methods makes the development and study of new materials harder to

compare, due to the absence of well-documented data on bacterial adhesion onto different materials [19,69].

1.4. NEW MATERIALS FOR URINARY CATHETERS

Catheters are one of the most used medical devices [9] and bacteria with antibiotic resistant genes found in biofilms and in urinary samples of patients, raises even more concerns [7]. Thus, there is a great need to develop better materials that can prevent or reduce bacterial adhesion, and subsequently biofilm formation and related infections [7].

In order to evaluate these newly designed materials, *in vitro* testing models have been developed [9,70,71]. However, the evaluation is still a challenge as the urinary systems are complex and inconsistent [75] with several difficulties at mimicking listed in Table 1.1. Nevertheless, *in vitro* models are important to test new materials since they can help to avoid ethical issues and higher cost which comes with *in vivo* tests [9]; however, there is still a need for standard and realistic models to be developed in order to evaluate the performance of the new materials in equivalent conditions.

Table 1.1. Limitations of urinary systems used in *in vitro* studies.

Factors	Complexities of urinary systems
Bacteria	▪ The variation in bacterium types in urine [8,9,18] ▪ Strain used with reduced abilities to attach <i>in vitro</i> studies [72] ▪ Use of only cultivable strains for <i>in vitro</i> studies [72] ▪ Variation in bacteria colonisation time [73] ▪ Variation in the number of bacteria [19]
Urinary fluid	▪ The difference in nutrients and nutrient levels [72] ▪ Lack of Host defence system like antimicrobial proteins and peptides for <i>in vitro</i> models [72] ▪ Presence of proteins, cells and crystals in urine [9] ▪ Oxygen amount for bacterial growth [75] ▪ Other parameters like flow, temperature, osmolarity [68,72]
Material	▪ Conditioning film on the material [19,55,63–65] ▪ Crystal formation on the material [8,9,66] ▪ Biocompatibility, stability of the material or modification [72]

Strategies to develop new materials to prevent bacterial adhesion in urinary catheters include mainly (1) incorporating antimicrobial agents and (2) developing antifouling surfaces [33]. The most common commercial products use antimicrobial coatings with silver and antibiotics like nitrofur, sparfloxacin, rifampicin, minocycline, and antifouling coatings by combination with hydrophilic coatings and hydrogels [19,74,75]. However, studies are still being conducted to develop more effective

technologies.

1.4.1. Antimicrobial coatings

Antimicrobial studies involve using antimicrobial coatings comprising antibiotics (e.g., rifampicin, triclosan), silver or nanoparticles (carbon nanotube and graphene oxide), enzymes and peptides [8,9,74,76]. Antibiotic coatings are less effective at preventing biofilm formation since urinary tract infections are mostly caused by antibiotic resistant pathogens [9]. Silver is one of the most used antimicrobial agents [74] and it has provided effective results either alone [77] or in combination with other molecules [78]. The combination of silver with antibiotics (amikacin and nitrofurantoin) also showed a synergistic effect by inhibiting bacterial adhesion more effectively compared to the single use of any of them [78]. Although silver plays an important role in the development of antimicrobial catheters, this type of catheter loses its antimicrobial ability in long term uses. The intermittent use can also cause bacterial resistance [74]. Moreover, silver can cause hypersensitive reactions in patients and there is a growing concern about a possible emergence of resistant bacteria [9]. Another bactericide used is nitric oxide which is an endogenously produced bactericidal gas used in urinary catheter studies [79]. Implementation of nitric oxide has shown some better results compared to silver. However, there can be side effects like decrease in blood pressure, inhibition in platelet aggregation, increased bleeding, skin irritation, skin edema/erythema and uncontrolled erection [80]. Thus, other alternatives like antimicrobial enzymes [81] and peptides [82] are being investigated as they are less likely to cause the development of bacterial resistance and they may be less toxic to tissues compared to silver [74]. Nonetheless, their production is more expensive [74,83] and their use might be limited due to enzymatic degradation [84]. Less toxic surfaces with antibacterial properties can also be achieved using natural substances like phenolic compounds, such as vanillic acid which was found to be effective [85], and chitosan which can offer bactericidal and antifouling properties while being cheap and biocompatible [86].

When designing materials, antimicrobials can be incorporated into the surface by using an antimicrobial release approach which affects the material surface and the surrounding or can be bonded to the surface, affecting only the surface of the materials as shown in Figure 1.3. This incorporation strategy is decided according to the type of antimicrobials incorporated [87]. While some leaching antimicrobials may sensitize patients and cause life-threatening anaphylaxis [87], in other cases surface-bond antimicrobials may lose their efficiency [88]. Surfaces modified with antimicrobials can lose their effectiveness after being implanted since film conditioning and dead bacterial cells can

cover the top of the materials preventing the material surface from functioning [72]. Therefore, it is recommended that antimicrobial strategy is implemented along with another complementary strategy.

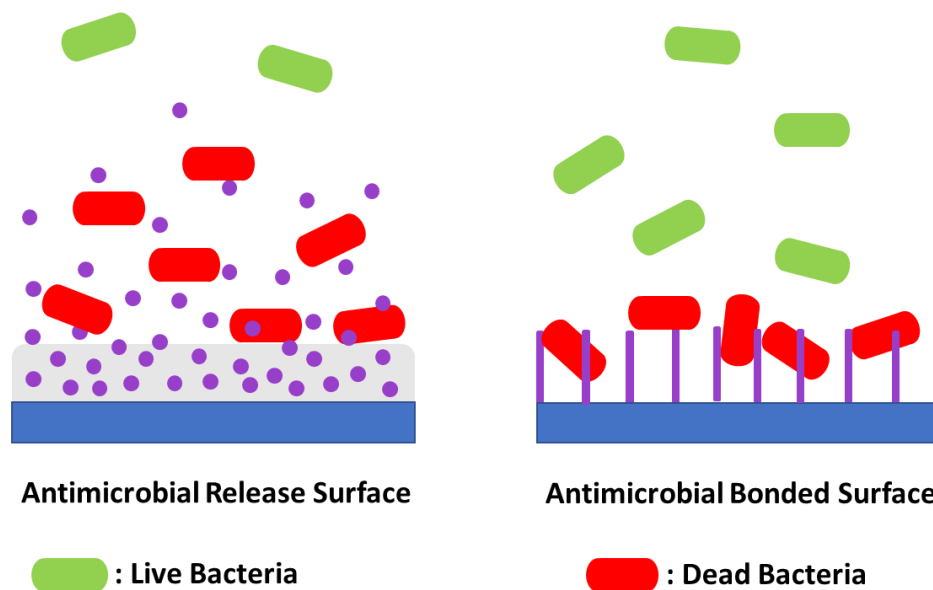


Figure 1.3. Antimicrobial surfaces obtained by the incorporation and release of antimicrobial agent or through binding of an antimicrobial agent onto the surfaces.

1.4.2. Anti-adhesive surfaces

Some other strategies focus on forming anti-adhesive surfaces by modifying the surface physiochemical properties, for example developing hydrophilic or superhydrophobic [74,89] surfaces as shown in Figure 1.4. Among these strategies, catheters with hydrophilic surface properties have already been commercially available [8]. One of the most studied hydrophilic surface modifications has been the formation of polyethylene glycol brushes (PEG) [90], being considered the gold standard; however, these PEG modified surfaces are found to be unstable due to oxidation [91]. Thus, there have been other hydrophilic surface modifications, including poly[N-(2-hydroxypropyl) methacrylamide] (poly(HPMA)) brush [92] or polysaccharides like heparin [93], hyaluronic acid [94] and chitosan [95,96]. Zwitterionic polymers can also be alternatively used to confer hydrophilicity to surfaces. The most common zwitterionic polymers are phosphorylcholine, sulfobetaine and carboxybeteine [74,97,98]. Although they have been extensively studied as the next generation of promising antifouling materials [84], the long-term stability of zwitterionic surface is still a concern [74]. The use of amphiphilic polymeric coatings, by the combination of dodecyl methacrylate, polyethylene glycol methacrylate and acrylic acid, has also proven to prevent bacterial adhesion significantly [56]. An

amphiphilic coating which was effective against both bacteria and protein adhesion was performed by synthesising a polymer from hydrophobic benzyl methacrylate, hydrophilic polyethylene glycol methacrylate and methacrylic acid [99]. Furthermore, surfactants such as surfactin, rhamnolipids and several other surface-active compounds produced by microorganisms were reported in the literature [100]. Biosurfactants are advantageous due to their low toxicity, biodegradability and biocompatibility. They further show both anti-adhesive and antimicrobial properties thus, offer a great potential to be used in urinary catheters [101].

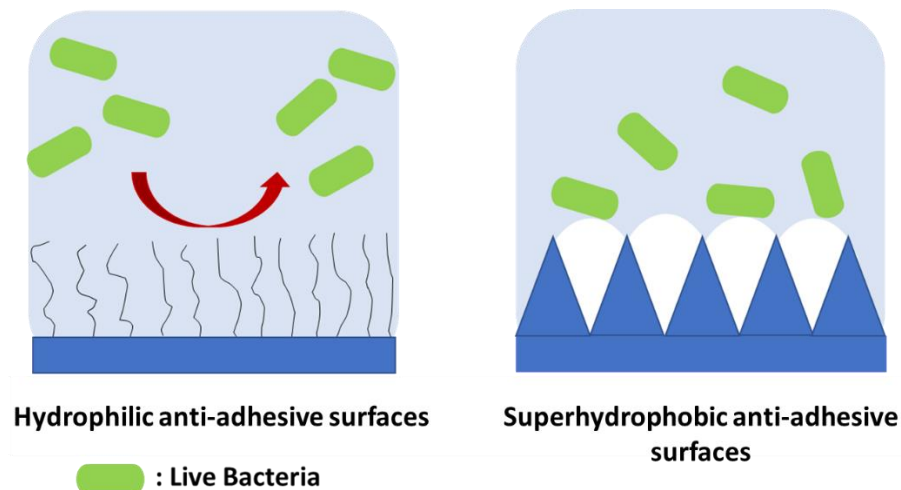


Figure 1.4. Anti-adhesive surfaces via hydrophilic and superhydrophobic approaches.

In general, thin hydrophilic coatings like polymer brushes may not be mechanically stable in the long-term use thus, hydrogel coatings can be promising considering biocompatibility, functional group density and lubricity. However, hydrogel coatings are fragile due to weak interactions between the coating and the substrate. Nevertheless, cross-linking of the coating to the substrate can improve the durability of the hydrogel coating while improving its anti-adhesive performance [102]. Hydrogels can also be used in combination with antimicrobial agents. For instance, Su *et al.* [103] reported a hydrogel from PEG oligomers with the antimicrobial agent polyhexamethylene guanidine.

Although antifouling surfaces are advantageous against antimicrobial resistance, the results obtained using anti-adhesive surface modifications are generally considered to be modest compared to using surfaces modified with antimicrobial agents [56] and combining antifouling with antimicrobial surfaces can increase the life cycle of the surface modification, as dead bacteria cells can be removed from the surface with antifouling functionality [104]. Hence, modifications employing the combination of both strategies are being developed. Polyethylene glycol (PEG) has been a popular choice in studies combining anti-adhesive and antimicrobial functions such as combining PEG for anti-adhesive and

quatarnary ammonium salts for antimicrobial properties [105]. Another study on switchable surface features was also designed by combining PEG as antifouling and poly[2-(dimethyl decyl ammonium)ethyl methacrylate] as bactericidal [106]. Li *et al.* [104] incorporated a coating consisting of copper ions in poly(3-sulfopropyl methacrylate potassium salt) brushes. The study showed that the use of copper ions as antimicrobials increased the efficiency of anti-fouling surfaces. In another study, zwitterionic polymer poly(sulfobetaine methacrylate) used as antifouling and quatarnary ammonium salts as antimicrobial agents were combined and were found to effectively prevent biofilm formation [107]. Moreover, antimicrobial peptides were also combined with zwitterion poly(3-[dimethyl-[2-(2-methylprop-2-enoyloxy)ethyl] azaniumyl]propane-1-sulfonate), polymer brushes used as an anti-fouling lower layer which significantly inhibited bacterial adhesion [108].

Besides hydrophilic surface modifications, the development of superhydrophobic surfaces [109] is another anti-adhesive surface approach that can be explored to prevent and/or reduce bacterial adhesion. The surfaces presenting a water contact angle higher than 150° are called superhydrophobic surfaces. The anti-adhesive principle of superhydrophobic surfaces is the formation of solid-air-liquid, air pockets, interfaces within micro/nano morphologies. Thus, both surface chemical composition and roughness plays a role [89]. Although superhydrophobic surfaces can provide anti-adhesive features through formation of air pockets, the air pockets can be released from the surfaces over time, thus limiting the use of this type of surfaces [33,34]. Nevertheless, superhydrophobic surfaces still attract attention and their effectiveness has been evaluated through different methods and performance tests. In a recent study, trifluoropropyl was used for superhydrophobic surface modification and provided a reduced bacterial adhesion due to cleaning features [110]. Furthermore, both antimicrobial and antifouling strategy of superhydrophobic surfaces can be used to modify the surface. In a study done by Zhang *et al.* [109], silver nanoparticles were modified with 1H,1H,2H,2H-perfluorodecanethiol, which provided anti-adhesive properties. Additionally, it was shown that non-stick polytetrafluoroethylene coating combined with silver inhibited bacterial adhesion [111,112].

1.4.3. Other strategies

In addition to the use of antimicrobial and antifouling surfaces, several other approaches have been explored. There have been studies to reduce bacterial adhesion by forming specific micro and nano patterns on the surface topography. These surfaces reduce the bacterial adhesion by reducing the amount of accessible area for bacteria to adhere onto the surface [8,113]. Further modification via lubricant immersion on nanopatterned or porous surfaces can result in highly effective surfaces [114].

Moreover, those nanopatterns can also be arranged in a way to provide bactericidal properties to the surface [115]. However, it is important to note that nanopattern design parameters like shape, height, diameter and spacing affect the anti-adhesive behaviour of the surface [33,116].

Some other approaches incorporate viral and bacterial treatment which can be alternative to antimicrobial compounds. For example, bacteriophage therapy [117], has provided encouraging results, although the drawback is the strain specific host range and lack of clear regulation [8,118]. Probiotic coatings as a displacement strategy of pathogenic bacteria on urinary catheters have also provided some success [119]. Probiotics work based on the competitive exclusion properties and specific bacterial adhesion ability. For example, lactic acid producing probiotics can have several benefits including antimicrobial, anti-adhesive and anti-QS (quorum-sensing) signalling. They further reduce the pH of the environment and this could also mean anti-encrustation properties [120]. Signal interference is another strategy to hinder bacterial communication within biofilm [74]. A coating with acylase PvdQ (an acylase with an N-terminal nucleophile (Ntn-hydrolase) which is a part of the pyoverdine gene cluster (pvd)) reduces biofilm formation by inactivating long chain N-acyl homoserine lactones (AHL) in QS system [121].

Biodegradable catheter alternatives can be another option to prevent bacterial adhesion and encrustation. Although variable degradation or fragment formation are current problems [122], biodegradable stents with antimicrobials, such as peptides and copper ions, have shown potential in urinary applications [123]. This kind of constantly renewable antimicrobial surfaces could represent the solution to both bacterial adhesion and encrustation, if the problems with un-uniform degradation or fragment formation can be solved.

1.5. CONCLUSION AND OUTLOOK

Urinary catheters are commonly used medical devices for which bacterial adhesion still remains a huge health problem. Some of the most critical challenges that are involved in the bacterial adhesion phenomena include dealing with complex body fluids and molecules; a significant high number of bacterium types; physicochemical interactions with the materials surfaces and problems resulting from encrustation. Understanding bacterial adhesion is key to develop anti-adhesion strategies and to design new anti-adhesive materials for catheters. Several approaches, including hydrophilic coatings, hydrogels, antimicrobial coatings and surface patterning or combinations thereof, seem to offer interesting results. However, despite all the progresses in the materials science field, there is still a great need for durable materials that can efficiently deal with the abovementioned challenges. Achieving

durable and anti-bacterial surfaces with a single approach such as including an antimicrobial or anti-adhesive agent is not an easy task as both exhibit some disadvantages; thus, a proper combination of synergistic approaches can be beneficial at obtaining more resistant surfaces for a longer usage period. Moreover, probiotic coatings seem to be an interesting option for future applications, considering that they gather a combination of several strategies like antimicrobial, anti-adhesive, and anti-QS. However, the stability and shelf-life of such coatings need to be considered. Another recently explored alternative is the use of biodegradable catheters made from hydrogels, being this a promising approach to develop anti-adhesive properties while preventing encrustation due to the constant dissolving state, which warrants further research and development.

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CHAPTER 2

Exploring the potential of polyethylene terephthalate in the design of anti-bacterial surfaces

Polyethylene terephthalate (PET) is one of the most used polymeric materials in the health care sector mainly due to its advantages that include biocompatibility, high uniformity, mechanical strength, and resistance against chemicals and/or abrasion. However, avoiding bacterial contamination on PET is still an unsolved challenge and two main strategies are being explored to overcome this drawback: the anti-adhesive and biocidal modification of PET surface. While bacterial adhesion depends on several surface properties namely surface charge and energy, hydrophilicity and surface roughness, a biocidal effect can be obtained by antimicrobial compounds attached to the surface to inhibit the growth of bacteria (bacteriostatic) or kill bacteria (bactericidal). Therefore, it is well known that granting antibacterial properties to PET surface would be beneficial in the prevention of infectious diseases. Different modification methods have been reported for such purpose. This review addresses some of the strategies that have been attempted to prevent or reduce the bacterial contamination on PET surfaces, including functionalisation, grafting, topographical surface modification and coating. Those strategies, particularly the grafting method seems to be very promising for healthcare applications to prevent infectious diseases and the emergence of bacteria resistance.

Keywords: Polyethylene terephthalate; bacterial adhesion; antibacterial properties; surface functionalisation; grafting; topographical modification; coating; water contact angle

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2.1. INTRODUCTION

Bacterial infections are a big health issue, responsible for high expenditure and death. Among those, nosocomial infections are one of the most life threatening, given that in hospitals and other healthcare facilities the risk of infection is tremendously high. According to the European Centre for Disease Prevention and Control, more than 4 million people suffer from a healthcare associated infection in Europe every year [1].

Most bacteria exist in the form of a biofilm, which are microbial aggregates of diverse species that rely on extracellular products, such as extracellular polymeric substances (EPSs) expressed from the bacteria and a solid material surface. The expression of EPSs renders the attachment irreversible to the solid surface and once the bacteria are settled, synthesis of the bacterial flagellum is inhibited and the bacteria multiply rapidly, resulting in the development of a mature biofilm. At this stage, the bacteria form a resistant barrier to antibiotics, providing a source for systemic chronic infections. Therefore, despite the abundance of antimicrobial drugs and other modern antibacterial agents, bacterial infections remain a threat to humanity, highlighting the urgent need to develop alternative ways to cope with infectious diseases [1].

A significant source of infections are surfaces of both indwelling medical devices or common utilities such as sinks, toilets, door handles, clothes, curtains or computer keyboards [1]. One strategy to prevent infections is to improve the material properties by making them anti-adhesive and/or biocidal. By the analysis of the state of the art, some contradictory results have been observed, mostly due to the experimental conditions applied in the different studies and the joint influences of the different material properties, such as roughness and surface energy, rendering it difficult to extract the exact influence of a material property on bacterial infections [2].

Material surface properties that have been implied in bacterial adhesion are surface charge and energy, hydrophilicity and surface roughness [2]. For instance, a negative surface generally exhibits a reduced bacterial adhesion due to the electric double layer repulsion, since most of bacterial cell surfaces carries a negative charge [3]. On the contrary, materials with positive charge can be used to inhibit bacterial growth, since they can attract and damage the bacterial cell walls, killing bacteria [4-9]. The increase in the surface free energy and the decrease in the contact angle leads to a reduced bacterial adhesion [10]. However, a superhydrophobic surface can also prevent bacterial adhesion due to the reduced protein adsorption and the entrapped air layer between the bacteria cells and the surface [2]. In addition, smooth surfaces exhibit less bacterial adhesion than rough ones, which present an increased area with favorable sites for bacteria to adhere [11]. Nevertheless, microtextured surfaces

have shown less fouling compared to smooth surfaces in the cases where cells are slightly larger than the microtextured gaps. As such, there are a lot of different parameters that interfere with bacterial adhesion (and lately with biofilm formation), highlighting the need to properly modify and tune material surface properties to render them anti-adhesive, which is particularly useful for the health sector. Furthermore, the material surfaces can be modified with biocidal compounds like chitosan, nanoparticles, quaternary ammonium salts (QAS), triclosan and other antibiotics, to inhibit or kill bacteria [12,13]. The simplified illustration of how surface properties can act on the reduction of bacterial contamination is shown in Figure 2.1.

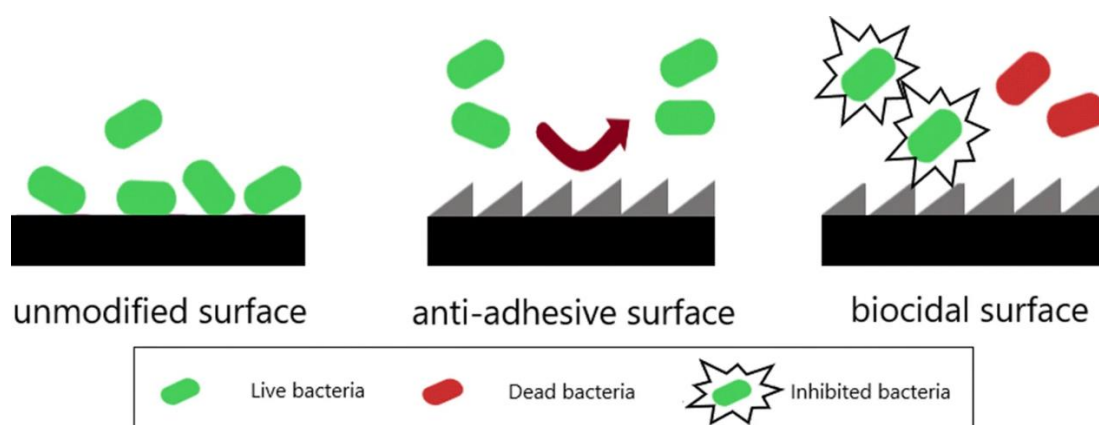


Figure 2.1. Surface action on anti-adhesive and biocidal surfaces.

Currently, implants are made from a variety of synthetic fibres, being the majority made from polyethylene terephthalate (PET), expanded PET (ePET), polytetrafluoroethylene (PTFE), expanded polytetrafluoroethylene (ePTFE) and polyurethane (PU). However, PET, ePET and PTFE are the most used fibres in commercial vascular prosthesis [14]. Other PET applications include sutures, heart valves, surgical meshes, scaffolds, urinary and bloodstream catheters. Its biocompatibility, high uniformity, mechanical strength and resistance against chemicals and/or abrasion make PET a promising material for several biomedical applications. Nevertheless, PET surfaces are prone to bacterial contamination and further modifications are necessary to limit and/or prevent such contamination [15]. This chapter will focus on the most recent strategies and methodologies being explored to confer PET surfaces a permanent antibacterial character.

2.2. SURFACE MODIFICATION TECHNOLOGIES

The recent studies on antibacterial properties of PET have shown that several surface modification technologies have been used to limit and/or prevent bacterial contamination of PET,

namely functionalisation, grafting, surface topography modification and coating. The methodologies are illustrated in Figure 2.2.

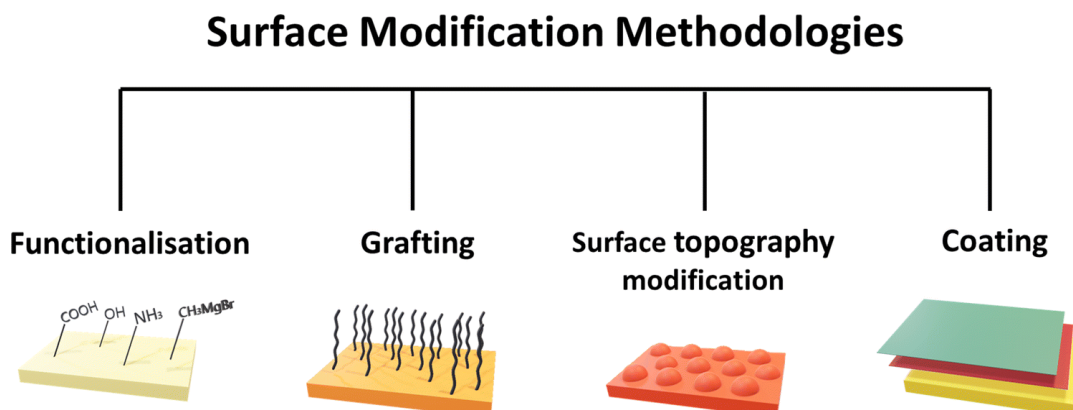


Figure 2.2. Surface modification methodologies used to develop PET anti-bacterial surfaces.

While simple surface topography modification can provide anti-adhesive properties to plastic materials by changing surface properties like roughness, hydrophilicity and surface energy, both anti-adhesive and biocidal modifications can be obtained by the other depicted methodologies, depending on the technologies and chemicals used. Although significant antibacterial activity can be provided by grafting and coating methodologies, further studies need to be performed to obtain PET surfaces that are able to completely avoid biofilm formation (and thus prevent bacterial contamination). When choosing a methodology for PET surface treatment, other two important factors need also to be considered: the durability of the superficial treatment and the cost–benefit ratio of the modified material. Table 2.1 presents a comparative overview of the surface modification technologies for PET, according to these three parameters. It should be noted that the methodologies vary within themselves and thus the generalised table might not give a straightforward comparison.

Table 2.1. Polyethylene terephthalate surface modification methodologies in relation to its antibacterial effectiveness, durability, and cost.

Methods	Anti-bacterial effectiveness	Durability	Cost–benefit
Functionalisation	*	**	**
Grafting	**	**	*
Coating	**	*	***
Surface topography modification	*	***	**

*Low degree; **medium degree; ***high degree

2.2.1. Functionalisation

Several methods can be used for surface functionalisation of PET, including plasma treatment, ozone treatment, radiation, hydrolysis, oxidation and enzymatic modification, among others [15]. Plasma treatment, a commonly used method for surface functionalisation, is effective in creating hydrophilicity and increased roughness at the material surface which ultimately impact bacterial adhesion. Indeed, the surface of polymeric materials can be functionalised with different gas compositions and plasma conditions to enhance their hydrophilic or hydrophobic properties. While functionalisation with fluorine gas can lead to a more hydrophobic surface, functionalisation with air, oxygen, water vapour or carbon dioxide can improve polymer hydrophilicity [16,17]. Junkar and co-workers [16] reported that oxygen plasma treatment of PET foils decreased the contact angle from 74 ° to 22 ° after a very short plasma treatment (3 s). For samples treated directly under the plasma glow, the water contact angle became extremely low (below 5 °) after about 1 min of treatment. However, the authors also showed that the plasma treatment was not stable, since the obtained contact angle for the 3 s treated samples increased to 35 ° after 2 weeks of storage in air and remained constant after 4 weeks. In addition, the average roughness of those samples increased over 10 nm. It is important to notice that the selected functionalisation method can change the microstructure and nanostructure of a polymeric surface, and this can change the surface roughness. The surface roughness by itself could also affect the hydrophilicity of the surface. Ahad and collaborators [18] studied the surface modification of PET films by irradiation with extreme ultraviolet photons to further evaluate the effects on the surface structure and wettability. Results showed that the water contact angle increased with an increased surface roughness. Roughness was found to be 6.6 nm and water contact angle was found to be 81.5 ° for unmodified PET samples, while the modified PET sample exhibited a maximum roughness value of 271 nm and a water contact angle of 98 °.

Rezaei *et al.* [19] also showed that the increased surface energy accomplished by atmospheric plasma treatment can increase hydrophilicity on the material's surface. The authors worked on the effects of different gases like helium, helium/oxygen and helium/nitrogen at different energy levels. It was shown that hydrophilicity, surface energy and surface roughness increased with an increased power. The lowest value for contact angle (25 °) was obtained with the helium/nitrogen mixture, corresponding to the higher surface energy of 67 mN/m. The topographic analysis of the samples indicated a higher value of surface roughness for the samples treated with helium/oxygen plasma, showing that surface energy plays an important role on wettability. In addition, it has been shown that plasma treated samples led to a higher *Staphylococcus epidermidis* inhibition due to an increased

surface free energy and decreased water contact angle. However, the material ageing altered the surface properties (hydrophilicity and surface energy, over time) that limited the bacterial inhibition [10].

Swar *et al.* [15] studied the potential use of Grignard reagents (methyl magnesium bromide (CH_3MgBr) and dodecyl magnesium bromide ($\text{C}_{12}\text{H}_{25}\text{MgBr}$) to modify PET film and fabric surfaces. Modified PET surfaces showed decreased contact angles due to the exposed hydroxyl groups. The use of the methyl derivative Grignard reagent decreased water contact angle for both film and fabric samples, from 82° to 77° and from 111° to 102° , respectively. On the other hand, the water contact angle increased to 87° and 118° when the dodecyl magnesium bromide was used. This was due to the hydrophobicity of longer alkyl groups. The roughness of the film surface also increased from 6.69 to 18.05 nm when $\text{C}_{12}\text{H}_{25}\text{MgBr}$ was used to modify the surface. The modified PET samples were found to be effective against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) growth, as well as against the formation of *Escherichia coli* biofilms.

Another example of PET functionalisation has been performed using benzophenone group terminated cationic quaternary ammonium salts (BP-QAS), which can interact with the cell membrane of bacteria and create reactive oxygen species (ROS) that can cause the death of bacteria. The modification of the PET samples has been done via photochemical hydrogen abstraction. Additional to bactericidal capacity, the modification of the surface has also enhanced hydrophilicity. The results showed that the modified surfaces presented more than 99 % antibacterial efficiency against Gram-negative and Gram-positive bacteria [12].

2.2.2. Grafting

Numerous ways to develop an effective coating that has higher physical and chemical resistance to environmental conditions have been suggested [6,7]; however, they are still limited by the type of the bonds created between the coating and the substrate. A grafting method, where covalent immobilization of the compounds takes place, is an alternative approach to create a resistant film at the polymer surface. The two main grafting methods being currently used are the “grafting to” and “grafting from”. While the “grafting to” method is used to attach polymer chains to the surface; in the “grafting from”, the monomer is attached to the surface and the polymerization occurs at the substrate’s surface [20]. Figure 2.3 shows the chemical structure of some molecules used for surface modification of PET using “grafting to”, “grafting from” and coating methodologies.

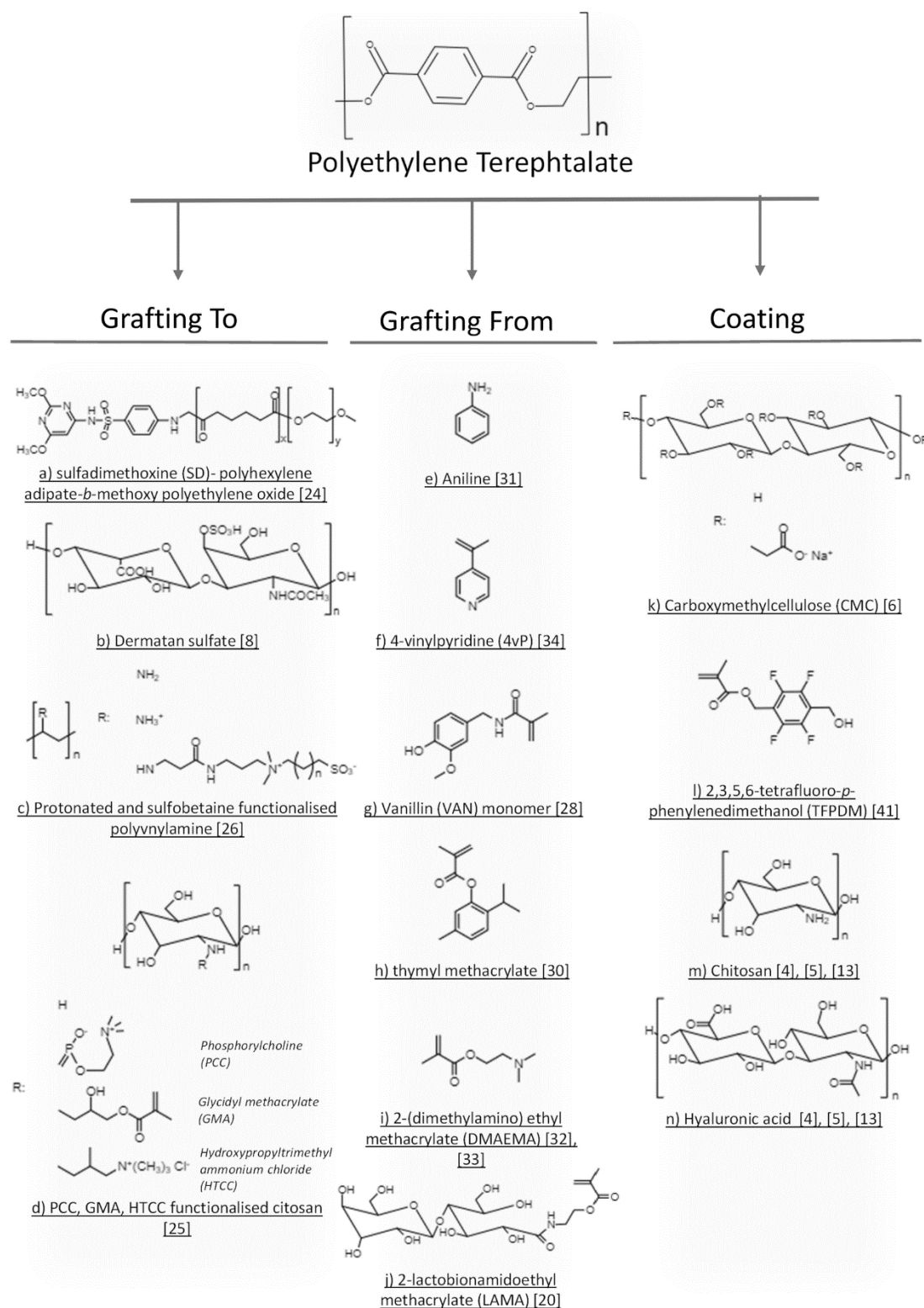


Figure 2.3. Chemical structure of some molecules used for surface modification of PET using “grafting to”, “grafting from” and coating methodologies.

2.2.2.1. “Grafting to” method

In the past years, there have been different attempts to prevent and/or inhibit bacterial contamination using the “grafting to” method. One of the interesting approaches was the immobilization of enzymes. Lysozyme is known for possessing bactericidal activity due to its capacity to hydrolyse the bacterial cell wall peptidoglycan (PG) and highly cationic conventional lysozyme types can kill the bacteria independently of cell wall PG damage [21]. Meslmani *et al.* [22] immobilized lysozyme onto woven and knitted crimped PET surfaces. Although the enzyme activity has been reduced to 55–60 % with the grafting, the resulting samples were able to prevent bacterial adhesion. The anti-adhesive efficiencies of woven and knitted enzyme grafted PET were above 80 % against *S. aureus* and *S. epidermidis*, and above 70 % against *E. coli*, compared to unmodified PET samples.

The effectiveness of chitosan, a widely used natural polysaccharide, was investigated within the “grafting to” approach. Due to its positive charge, it has bacteriostatic and bactericidal effects by damaging the bacterial cell wall. Chitosan is broadly used in layer-by-layer methods to provide multifunctional films due to its partially positive charges. However, the layer-by-layer method does not provide stable coatings, because the layers are pH dependent and not resistant to abrasion [8]. Hayder *et al.* [8] covalently immobilized two separate layers of chitosan and dermatan sulfate (DS), an anionic polysaccharide, on PET using the coupling agent N,N'-dicyclohexylcarbodiimide (DCC). The results showed that chitosan and DS modification increased hydrophilicity. PET-DS-CHI was more hydrophilic exhibiting a contact angle of 71 °, while PET-CHI-DS exhibited a contact angle of 87 °. This was probably due to the higher amount of surface –COOH group on PET-DS-CHI. The tests against bacteria showed that both modified materials were more resistant to biofilm formation compared to the unmodified PET substrate, with PET-DS-CHI coating showing a better inhibition. However, PET-CHI showed a higher reduction of the bacterial adhesion which is thought to be due to the partial positive charges present on chitosan compared to other modified PET samples. The block copolymer of sulfadimehoxine polyhexylene adipate-b-methoxy polyethylene oxide (SD-PHA-b-MPEO) was another multifunctional copolymer studied and it is the combination of hydrophobic PHA to repel bacteria, hydrophilic MPEO to increase the host cell interactions for material integration *in vivo* and negatively charged SD which is a bacteriostatic antibiotic [23]. A porous structure has been obtained after its grafting onto PET by evaporating the solvent from polymer brush solution. Anti-adhesive efficiency of woven and knitted forms of modified PET samples were between 56–62 % against *S. aureus* and *S. epidermidis*, while the efficiency was between 63–64 % against *E. coli* [24].

Zwitterionic polymer brushes is an attractive approach for surface modification in the biomedical

field, since it can provide good biocompatibility and anti-adhesive efficiency due to counteraction of the electrostatic hydration effect [25]. Timma and co-workers [26] developed polyvinylamine polymers that were functionalised with zwitterionic sulfobetaine side chains for PET fabrics. While sulfobetaine provided anti-adhesive properties, protonated amine groups provided bactericidal properties. A high substitution degree of sulfobetaine on the polymer chains might cause pure anti-adhesive properties. Therefore, reducing the substitution degree of sulfobetaine can allow a mixture of anti-adhesive and bactericidal properties due to the existence of uncoupled protonated amine groups. It was shown that the bacterial adhesion decreased with higher amount of substitution degree and 80 % of substitution degree caused the material to lose almost all bactericidal properties. Moreover, the primary action of polymers with 60 % of substitution degree seemed to be dependent of the fibre type. The bactericidal effect against Gram-negative bacteria was noted to be more influenced due to the different structure of the cell wall with the increasing substitution degree.

In another study done by Xv *et al.* [25], a zwitterionic glycidyl methacrylate–phosphorylcholine–chitosan (PCCs–GMA) was photo-immobilized on PET films. Hydroxypropyltrimethyl ammonium chloride chitosan–GMA (HTCC–GMA), cationic chitosan–GMA (Cs–GMA) and pristine PET were used to compare the results. The water contact angle was reduced to 34 °, 36 ° and 47 ° after immobilization of PET–GMA–PCCs, PET–GMA–HTCC, PET–GMA–Cs, respectively. PET–GMA–PCCs has improved surface antibacterial properties and inhibited the adhesion up to 100 % for *E. coli* and 92 % for *S. aureus* compared with pristine PET. Although PET–GMA–HTCC and PET–GMA–Cs improved the antibacterial properties, many bacteria were observed on the surface of the tested materials. The live/dead bacteria assessment showed that no live or dead bacteria were observed on PET–GMA–PCCs surface, while there were some dead bacteria on PET–GMA–HTCC and some live bacteria on both pristine PET and PET–GMA–Cs. Both HTCC and chitosan are positively charged; however, the bacterial growth inhibition and contact killing properties of chitosan are limited in neutral conditions thus HTCC showed attached dead bacteria. Due to strong electrostatic hydration effect of zwitterionic PC, the attachment and killing of bacteria was suppressed [25].

2.2.2.2. “Grafting from” method

The “grafting from” is an alternative surface modification technology, being one of its main advantages the controllable molar mass and grafting density. Lepoittevin *et al.* [20] studied the grafting density by modulating the monomer/free initiator ratio. PET films were pre-treated with polyethylenimine (PEI), followed by the reaction with a surface initiator (bromoisobutyryl bromide).

Finally, the Atom Transfer Radical Polymerization (ATRP) of 2-lactobionamidoethyl methacrylate (LAMA) was carried out and glycopolymer brushes were grown on the surface of the PET films with different grafting degrees. With the higher grafting degree, the authors obtained a water contact angle of 11° and a surface energy of 44.1 mN/m. The study showed the great potential for this type of carbohydrate and further studies should be performed to evaluate its potential for inhibiting bacterial adhesion.

Researchers have been showing an increased interest in the use of natural compounds as alternatives to synthetic active agents. Recent studies on some natural compounds like vanillin monomer which has bacteriostatic effect depending on target (more effective towards Gram-positive bacteria) [27] and thyme, which presents bacteriostatic and bactericidal effect, have been reported. In a study conducted by Mani and co-workers [28], the vanillin derived biobased monomer, N-(4-hydroxy-3-methoxybenzyl)-acrylamide (VAN), was used to modify the PET surface using the photopolymerization technique and N,N-diethylethylenediamine (DEDA) as a crosslinker. The surface modification with VAN reduced the contact angle from 80° to 62° and it was found that VAN grafted PET inhibited the adhesion of the Gram-positive bacteria *Rhodococcus wratislaviensis* and *S. aureus* by 85 % and 97 %, respectively. However, the inhibition of the Gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa* was limited by 50 %. Rhizome *Atractylodes macrocephala* (RAM), is another herbal product which has declared antibacterial properties. Shu *et al.* [29] worked with RAM grafting on PET non-woven substrates. They have found that the grafting of RAM was highly improved when pre-grafted polymerization of acrylic acid or plasma treatment was applied. It was further improved when both acrylic acid and plasma treatments were applied prior to grafting of RAM. The effectiveness against *S. aureus* and *E. coli* increased with the increased grafting percentage. Bedel *et al.* [30] performed ATRP polymerization of thymol monomer. The results showed that the water contact angle increased from 81° to 99° for samples treated with thymyl methacrylate. The total surface energy of PET was reduced from 44.7 to 40.5 mN/m. Furthermore, the thymyl methacrylate treated samples were highly antibacterial exhibiting up to a 99 % decrease in the bacterial attachment against *P. aeruginosa*, *Listeria monocytogenes* and *S. aureus*.

In a recent study, Gallarato *et al.* [31] explored the antibacterial properties of polyaniline (PANI) coating grafted from PET. The authors further microstructured PANI coated surfaces with direct laser interface. They have found that the water contact angle increased from 72° to 84° for PANI coated PET film and it further increased to 101° with additional laser treatment on the PANI coating. PANI film reduced the bacterial adhesion of *P. aeruginosa* by 74 %. Microstructure on PET-PANI film show to reduce the bacterial adhesion by 97 %. The author also showed that the percentage of live bacteria was

lower in modified surfaces and the live bacteria ratio was lower compared to dead bacteria on modified surfaces which proves its bactericidal effect.

A multifunctional coating where zwitterionic polymer brushes of polycarboxybetaine (PC) and polysulfobetaine (PS) formed on PDMAEMA (poly(2-(dimethylamino)ethyl methacrylate)) grafted PET sheets were developed by Jin and collaborators [32]. Both cationic killing behaviour from PDMAEMA and zwitterionic repelling behaviour from PS and PC have been obtained. Water contact angles of 30.4° and 30.6° with a polymerization time of 8 h have been obtained for PS and PC modified samples. A significant reduction of *E. coli* attachment to PC formed PDMAEMA grafted PET sheets was observed [33].

Further improvements in antibacterial properties can be also performed with additional functionalisation of grafted samples. Arslan *et al.* [34] studied the antibacterial effects of amine, chlorine, hydrogen peroxide, and triclosan functionalisation of grafted vinyl monomer on PET fibres. They have found that co-polymerisation of vinyl monomers improved antibacterial properties and further functionalisation with triclosan showed the highest growth inhibition zone in all samples. The most promising vinyl grafting type with the bacterial inhibition was found to be the co-polymerisation of 4-vinylpyridine which also gave higher inhibition zone compared to its oxidized or chlorine forms.

2.2.3. Surface topography modification

As previously mentioned, surface roughness and topography greatly affect bacterial adhesion [2]. In addition, it has been shown that the surface roughness can affect the surface hydrophilicity. Surface wetting can either be homogeneous or heterogeneous, impacting differently the bacterial adhesion. The Wenzel's phenomenon suggests that both hydrophilicity and hydrophobicity are enhanced by an increasing roughness on homogeneously wetted surfaces, meaning that a hydrophilic surface will become more hydrophilic, and a hydrophobic surface will become more hydrophobic [35]. The porous surfaces behave according to another phenomenon so-called the *Cassie-Baxter* phenomenon. In this state, the water droplet heterogeneously wet the surface and affect the wettability [36]. Gillett and collaborators [37] studied the effect of laser modification on surfaces, creating pit structures with $15\ \mu\text{m}$ in diameter and $20\ \mu\text{m}$ gap between each other on PET surfaces. The laser modification increased the roughness more than 30 times, from $R_a = 0.81\ \mu\text{m}$ to $30.1\ \mu\text{m}$. The surface modification has also affected the water contact angle, increasing it from 76.9° to 87.7° . Moreover, the modification was found to affect the *E. coli* distribution on the surface. Although more mature bacteria seem to accumulate around pits, there were no bacteria observed inside the pits. The authors

suggested that this could be due to the presence of air pockets inside the pits following the *Cassie–Baxter* state. It should be noted that Gram-negative bacteria have an extra outer membrane which can ease the interaction with nano-irregularities [38].

Lithography is another method that can be used to modify the surface topography as an alternative to the laser surface modification. It has been reported that the size of the micropatterns can be arranged to prevent microorganisms to adhere and create biofilm onto the materials. Arisoy and co-workers [39] combined nanoimprinted shark skin pattern samples with 1.6 and 3 μm height, 1.3 and 2 μm width, 2.7 and 2 μm spacing by lithography with bactericidal effect of TiO_2 on PET substrates. When shark skin patterned TiO_2 samples were compared to smoother surfaces with the same chemistry, 70 % reduction of the *E. coli* adhesion was observed. In addition, shark skin patterns led to 80 % reduction in the bacterial adhesion as compared to flat PET surfaces. Moreover, it was shown that if the spacing is bigger than the width of the bacteria, then bacteria tend to adhere onto the surface between patterns rather than being repelled by them. Wang *et al.* [40] also evaluated the *E. coli* adhesion on micropatterned PET surfaces obtained by quartz photomask for six different pattern dimensions. The results showed that the shape of the microstructures affect the adhesion of cells and the live/dead cell ratio. In addition, the authors found the minimum adhesion with the smallest micropattern design (i.e., 1 μm).

2.2.4. Coating

In the recent years, the layer-by-layer (LbL) methodology has been widely used. Within this method, the cationic and anionic polyelectrolyte layers can be bond through ionic bonds to form a thin coating film. Alvarez *et al.* [4] showed that the surface of a PET film could be successfully coated by positively charged chitosan and negatively charged hyaluronic acid to create a potentially antifouling surface. Chitosan has contact killing properties, while hyaluronic acid is hydrophilic, and it can repel bacteria due to a steric effect formed by water absorption. This type of coatings can have a nanometre scale thickness. Gallego *et al.* [5] used a similar method to coat the PET surface with chitosan and hyaluronic acid, obtaining coating thicknesses ranging from 45 to 385 nm depending on the number of bilayers (from 5 to 10). The water contact angle of the PET film was 77 ° and decreased to 54 ° after the deposition of the first bilayer. However, the contact angle has not shown any specific trend and varied between 54 ° and 77 ° with the further deposition of bilayers. The authors also observed a reduction of bacterial adhesion against *E. coli* with almost complete bacterial inhibition for ten layers of HA/CHI. However, it is important to bear in mind that the coating degradation is a problem when using

the layer-by-layer surface modification. Indeed, the authors reported that 50 % of the coating was degraded in the first 24 h and 90 % during the first 6 days when exposed to enzymes. This value was 18 % when enzymes were absent and remained stable for more than a month. Further incorporation of triclosan (TRI) and rifampicin (RIF) antibiotics into HA/CHI layer has been done and further antibacterial improvements have been observed. While the reduction in bacterial adhesion was found to be 80 % for 5 bilayers of HA/CHI layers, it was found to be even higher > 99 % for TRI and RIF incorporated HA/CHI layers [13]. An improvement of the material's chemical and physical stability was attempted by Park *et al.* [6] by cross-linking the carboxymethylcellulose (CMC) polysaccharide and chitosan LbL-assembled multilayers on PETG (polyethylene terephthalate glycol modified) samples. The authors used a maximum of 20 bilayers of CMC/CHI to coat PETG and a thickness of 1818 nm was obtained, that was lower than 2 μm , which represents the thickness at which there is a risk of peeling. Moreover, the authors observed that when cross-linking was performed on 20 bilayers of CMC/CHI, the surface roughness increased from 20.3 to 57.7 nm. The cross-linking led to a super-hydrophilic surface exhibiting a reduction of the water contact angle from 35.34 ° to 4.86 ° (10 bilayers of CMC/CHI) and this change of the contact angle was thought to be due to surface roughness. Bacterial adhesion against *Streptococcus mutans* was reduced by 75 % using cross-linked samples as compared to control PETG. Another study showed that an abrasion resistant coating incorporating chitosan can also be developed using a sandblasting method. Wieckiewicz *et al.* [7] formed a chitosan film layer on PET surfaces using the sandblasting method. Prior to chitosan coating, the silica coated sands formed a tribo-chemically hydrophilic adhesive silicate layer by high impact, to improve the stability.

In addition, fluoropolymers have been explored as antibacterial agents. Bao *et al.* [41] studied the use of 2,3,5,6-tetrafluoro-p-phenylenedimethanol (TFPDM) containing acrylate polymer blend (AF) and TFPDM sandwiched epoxy polymer structure (EF) to coat PET. While AF lowered the PET water contact angle from 60 ° to 41 ° and the roughness from 1.6 nm to 1.4 nm; EF lowered the water contact angle to 51 ° and the roughness to 1.3 nm. Both modified materials prevented the initial bacterial adhesion and biofilm formation by *Bacillus subtilis* and *E. coli*. However, AF performed slightly better reducing by 28 % the *B. subtilis* adhesion and by 69 % the biofilm formation, and by 89 % the *E. coli* adhesion and by 94 % its biofilm formation. It was also supported that the use of fluoride damages cell membrane by live/dead bacterial viability study.

The combination of topographical modification and coatings has been reported to increase its effectiveness. For instance, Yamada *et al.* [42] coated a PET film with nanoscale moth-eye cone-shaped protrusions from a hydrophilic resin made of urethane acrylate and polyethylene glycol (PEG) derivatives

[43] with a size of approximately 200 nm in depth and diameter. Bacteria counts were reduced significantly with the use of moth-eye film compared to uncoated PET substrate due to specific structure of moth-eye film. It was also observed that the flat hydrophilic resin coated film reduced bacterial adhesion compared to uncoated PET film. This was attributed to bactericidal characteristics of PEG derivatives.

2.3. CONCLUSION AND OUTLOOK

In this review, recent surface modification approaches for granting antibacterial properties on PET were analysed according to the main methodologies used, namely, functionalisation, grafting, surface topography modification, coating and their combinations. Current developments show that treated PET surface presents a significantly higher antibacterial activity than the pristine form. The most popular methodologies for imparting antibacterial activity to PET surface appear to be the grafting and coating methods. High durability of the surface modification and preservation of PET mechanical properties are particularly important properties for *in vivo* applications, where material experiences excessive wear and thus must always be considered when modifying PET surface.

The studies also showed that coating and grafting methods can be advantageous for providing significantly higher hydrophilic surfaces, being the water contact angle one of the most used characterization methods. Low contact angles were found in literature (around 4 ° with the coating method and around 11 ° with the “grafting from” method) for treated polyethylene terephthalate. As stated by several authors, a surface with a high hydrophilicity behaviour (low contact angle) shows a great potential for developing anti-adhesive PET products for the medical sector. Further topographical surface modifications after grafting methods have been also used to improve the materials antibacterial properties.

Furthermore, combining anti-adhesive properties with bactericidal properties is a popular strategy for achieving high antibacterial efficiencies for PET materials. However, one must consider the potential bacterial resistance, the cost–benefit ratio, the durability of the modification, as well as the possible toxic effects of the antibacterial agents on the environment when choosing the most suitable modification approach for the envisaged PET products. Moreover, it is also important to keep in mind that different types of bacteria react differently to the presence of bactericidal agents due to their different shape (spheres, rods or spirals) and outer membrane structure.

Finally, the use of natural and biological molecules to improve the antibacterial properties of PET materials is a very promising approach to further develop new strategies against infectious diseases

2.4. REFERENCES

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CHAPTER 3

Modification of PET surfaces with gum Arabic

towards its bacterial anti-adhesiveness

using an experimental factorial design

approach

Bacterial adhesion onto hospital material surfaces still represents a big healthcare issue, being preventive measures required to mitigate this problem, such as increasing material surface hydrophilicity. In the present study, gum Arabic, a hydrophilic polysaccharide, was used to modify the surface of polyethylene terephthalate (PET). Initial water contact angle (WCA) and WCA after several washing cycles were studied as response variables by a 2^4 full factorial design. Several reaction parameters, such as contact time between gum Arabic and PET, gum Arabic concentration, curing temperature and curing time for PET modification were investigated. The most significant parameters were found to be the curing temperature and curing time. The optimized parameters led to a WCA reduction from 70° to 27° . The modified PET samples were characterized using several techniques including AFM, colorimetric, ATR-FTIR and contact angle which further confirmed a successful surface modification. Furthermore, bacterial adhesion assays have clearly shown that the treated PET material was highly effective in preventing the bacterial adhesion of *Escherichia coli* expressing YadA, an adhesive protein from *Yersinia* so-called *Yersinia* adhesin. The use of design of experiments techniques allowed for successfully attaining a PET material with a high bacterial anti-adhesiveness, using a simple grafting approach.

Keywords: Grafting; Gum Arabic; Surface modification; Wettability; Hydrophilicity; Design of experiments

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3.1. INTRODUCTION

Bacterial adhesion in the medical context causes several problems including high expenditure and death. Among those, the risk of healthcare associated infections (HAI) is tremendously high. The European Centre for Disease Prevention and Control (ECDC) estimates that 8.9 million HAI occur each year in European Hospitals and long-term facilities combined. Additionally, around 30 % of bacteria responsible HAI were found to be resistant to antibiotics [1] and biofilms are known to be key players in HAI, due to their tolerance and resistance to antimicrobial agents. Once a planktonic bacterium approaches a surface, it can attach irreversibly and grow/produce biofilms in which bacterial cells are colonized within. [2]. Thus, it is clear that the materials surface adhesiveness is a critical factor impacting bacterial adhesion. One preventive approach can be the modification of such surfaces to reduce the risk of bacterial adhesion and ultimately the growth of biofilms, antimicrobial resistant bacteria and bacteria associated infections. Polyethylene terephthalate (PET) is one of the most popular polymers used in the medical context, due to its biocompatibility, hardness, stiffness, and biological, chemical and mechanical stability. PET applications in the medical field include sutures, heart valves, surgical meshes, scaffolds, urinary and bloodstream catheters [3,4]. Nevertheless, PET surfaces are also prone to bacterial contamination and further modifications are necessary to limit and/or prevent such contaminations [4]. An important factor to consider when modifying a surface is its hydrophilicity. It has been found that hydrophilic materials are more resistant to bacterial adhesion compared to hydrophobic ones [5]. Polysaccharides comprise a polymeric group commonly used for surface modification mainly because of their hydrophilic characteristics. Some widely studied examples like hyaluronic acid [6], heparin [7], dextran [8], chitosan [9] have shown potential to decrease the bacterial adhesion and from those, heparin and hyaluronic acid modified materials are being commercially explored [7]. Another polysaccharide, gum Arabic, derived from *Acacia senegal* and *Acacia Seyal* trees [10], is composed of less than 3 % protein and more than 97 % of polysaccharide [11], being the polysaccharide part constructed from neutral sugars (rhamnose, arabinose and galactose) and acids (glucuronic acid, 4-methoxyglucuronic acid) [10]. Gum Arabic has already been used to cover inflamed surfaces, burns, nodular leprosy, as well as treatment of bedsores and wounds traditionally [10]. Moreover, gum Arabic has been evaluated in hydrogel studies [12] and membrane biofouling studies where the membrane was prepared and casted with polymer blends [11]. The membrane blend studies showed that gum Arabic was an effective component at preventing bacterial adhesion due to its antimicrobial properties and hydrophilic nature [13]. Additionally, gum Arabic is negatively charged [14] which is another important factor that impacts bacterial adhesion. Since most bacteria are negatively

charged, they adhere less on negatively charged surfaces due to formation of a negative double layer [15]. Therefore, gum Arabic, a natural polysaccharide, might also provide effective anti-adhesive/antifouling properties to PET, when subjected to a chemical modification process such as grafting.

Chemical reactions are affected by several parameters and statistical design of experiments (DoE) has proven to be a powerful tool to study individual factors and their interactions in a complex, multivariable system. The traditional experimental design, known as OFAT (one-factor-at-a-time), studies one factor/variable at a time, being an inefficient method due to the unnecessarily large number of experimental runs and the inability to study interactions among the factors [16]. Despite its well-known advantages, the use of DoE to evaluate and optimize surface hydrophilicity using contact angle as a response variable is not common. Still, some studies have employed DoE approaches and contact angle as a response variable, when investigating several surface modification technologies, such as atmospheric plasma [17], microwave plasma [18] and surface-initiated polymerization [19].

In this study, we aimed to modify PET surface with gum Arabic using glutaraldehyde as the crosslinker. A full factorial DoE was performed to assess the impact of reaction time, reaction concentration, curing temperature and curing time, as well as the interaction of those parameters in the surface hydrophilicity of PET determined by the water contact angle (WCA) measurement. Using the optimal conditions defined by the models, modified PET surfaces were prepared to be further characterized regarding their ability to prevent bacterial adhesion, as well as other properties including roughness and topography. To the best of our knowledge, the work herein reported is the first study using gum Arabic for PET surface modification by grafting.

3.2. MATERIALS AND METHODS

3.2.1. Materials

PET film (Mylar® A with thickness of 36 μm) was acquired from Isovolta Group. Glutaraldehyde (50 % solution), spray dried gum Arabic (Acacia tree), ethylene diamine ($\geq 99\%$) and diodomethane (99 %) were purchased from Sigma-Aldrich; hydrochloric acid (37 %) from Fisher Chemicals; ethylene alcohol (99.5 %) from Aga; sodium hydroxide from Eka, orange II dye from Acros Organics Luria Broth (LB) from NZYTech; agar powder from VWR chemicals; Isopropyl β -D-1-thiogalactopyranoside (IPTG) from Grisp; anhydrotetracycline hydrochloride from Fluka; kanamycin from Fisher Bioreagents and ampicillin from Applichem Panreac.

3.2.2. Pre-treatment

Thin PET films were cut into 6 X 9 cm rectangles and immersed in ethyl alcohol for 15 min and afterwards rinsed with ethyl alcohol, to clean the surface prior to functionalization. In order to create amine groups on the surface of PET, the films were aminolysed using a protocol reported elsewhere [20] and kept in 50 % aqueous diamine solution at 40 °C for 24 h which enabled the formation of amine groups at the PET ester linkage. Following the aminolysation process, the PET films were rinsed with distilled water and air dried prior to the crosslinking process. The functionalized samples were immersed in 4 % glutaraldehyde aqueous solution with 0.5 % 0.1 M HCl (pH = 2.4). After 3.5 h at 40 °C under continuous agitation at 100 rpm, the samples were removed from the solution, put into the oven and kept for 1 h at 120 °C for curing. Afterwards, the samples were rinsed with distilled water, immersed in water for 3 h and rinsed again with plenty of distilled water.

3.2.3. Grafting of Gum Arabic

Pre-treated PET samples were placed in a distilled water solution with a pre-defined gum Arabic concentration, ranging from 1 % to 5 % for a given period, as defined in Table 3.1. The ranges for the different parameters were defined according to previous preliminary experiments (data not shown). Afterwards, gum Arabic samples were removed from solution and put in the oven to be cured at different temperatures and times, according to Table 3.1. Samples were then rinsed with plenty of distilled water and air dried at room temperature.

Table 3.1. Coded and actual levels for the variables studied in the central composite design.

Independent variables	Factor	Range and levels				
		-1.414	-1	0	+1	+1.414
Curing temperature (°C)	A	71.7	80	100	120	128.3
Curing time (hours)	B	0.08 (-1.280)	0.50	2	3.50	4.12
Reaction Concentration (%)	C	0.2	1	3	5	5.8
Reaction Time (hours)	D	0.08 (-1.131)	1	8	15	17.9

3.2.4. Experimental design and optimization by response surface methodology (RSM)

In a first step, a 2⁴ full factorial design was performed to evaluate the effect of several reaction parameters on PET grafting and later the design was augmented using a central composite design. For

statistical calculation, the variables were coded according to Eq. (1):

$$x_i = (X_i - X_0)/\Delta X_i \quad (\text{Eq. 3.1})$$

where x_i is the independent variable coded value, X_i is the independent variable real value, X_0 is the independent variable real value on the center point and ΔX_i is the step change value. The selected variables and their levels are listed in Table 3.1. Due to constraints in the independent variables curing time (B) and reaction time (D), the minimum level tested in the central composite design had to be adjusted. Minimum curing time and reaction time were set at 5 min (0.08 h), so the coded value changed from $-\sqrt{2}$ (-1.141) to -1.280 for curing time and from $-\sqrt{2}$ (-1.141) to -1.131 for the reaction time.

State-Ease® 'Design Expert' (version 12) software was used for regression and graphical analyses of the data obtained. The statistical significance of the regression coefficients was determined by Student's t-test, the second-order model equation was determined by Fischer's test and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2 . The optimum range of the variables were obtained by the graphical and numerical analysis using the 'Design expert' program, based on the criterion of desirability.

3.2.5. Water contact angle (WCA)

The hydrophilicity of the modified surfaces was evaluated by static WCA measurement using the Optical Tensiometer Attension Model Theta Basic by Biolin Scientific, at room temperature. MiliQ water with a droplet size of 3 μL was used. The measurements were repeated at least 4 times for each sample at different locations. Contact angle measurements were also performed with diiodomethane. Owens-Wendt-Rabel&Kaelble Model (OWRK) equation was used to calculate polar and disperse components of surface energy by using contact angle values from water and diiodomethane.

3.2.6. Acid orange colorimetric method

Acid orange coloration was used for the determination of the presence of amino groups on the substrate's surface. The method used was adapted from Gallego *et al.* [21]. Samples with 2.5 X 2.5 cm were immersed in 6 mL of dye solution in acidic conditions (0.028 g/L, pH=3 adjusted with HCl 37 % in MiliQ water) and agitated at 100 rpm, at 40 °C. Following that, samples were removed and rinsed intensively with aqueous acidic solution (pH = 3) to remove unbound dye. The absorbed dye was

subsequently desorbed by immersing the films in an alkaline NaOH solution, at pH = 12 and then the absorbance of the final solution was measured in a Perkin Elmer Lambda 35 UV-VIS Spectrophotometer using an incident wavelength of 484 nm.

3.2.7. ATR-FTIR

Fourier Transform Infrared Spectrophotometer (FTIR), Perkin Elmer Spectrum 100, with universal attenuated total reflectance (ATR) accessory with diamond crystal was used. The spectra were collected between the region of 4000 – 600 cm⁻¹ wavenumbers with a 4 cm⁻¹ resolution.

3.2.8. SEM

The morphological analyses of the samples were performed using a Scanning Electron Microscope NanoSEM – FEI Nova 200 (FEG/SEM) with a secondary electron detector with 10 keV energy and a working distance between 7 and 8 mm. Before the morphological analysis, the samples were coated with a thin film of Au/Pd (80/20 by weight) 10 nm thick. For each sample, several images were taken by SEM, referring to different magnifications.

3.2.9. AFM

A Keysight Technologies 5500 Atomic Force Microscope was used to perform the AFM measurements. The measurements were performed in air in tapping mode (Mac Mode) at room temperature. The silicone material cantilevers with a spring constant of 13-77 N/m and radius < 10 nm were used. The images were analysed by the Keysight PicoView and Gwyddion software. All images were taken at 5 X 5 µm surface area.

3.2.10. Bacterial adhesion

3.2.10.1. Bacterial strains

E. coli BL21 (DE3) expressing green fluorescent protein (GFP) and an adhesive protein from *Yersinia* so-called *Yersinia* adhesin A (YadA), was used to test bacterial adhesion in the materials. The plasmid pRSFduet_GFP was kindly provided by Maria Sande (unpublished) and was obtained by amplification of GFP from pETduet_GFP(mut3b) [22]. The plasmid pASK_IBA2_YadA was kindly provided by Dirk Linke [23].

3.2.10.2. Preparation of bacteria

E. coli BL21 DE3 (NZYTech, #MB006) harboring pRSFduet_GFP and pASK_IBA2_YadA, was cultivated at 37 °C, 200 rpm in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) with the addition of kanamycin (50 µg/mL) and ampicillin (100 µg/mL). For the adhesion experiments, the cultures were grown at 37 °C and 200 rpm in LB (150 mL) up to an optical density at 600 nm (OD_{600 nm}) of 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) and anhydrotetracycline (aTc) were added (final concentration of 1 mM and 100 ng/mL, respectively) to induce the heterologous protein expression (GFP and YadA). The cultures were then incubated for 5 h at 37 °C and 200 rpm. Next, the cells were harvested by centrifugation at 5000 rpm for 5 min and washed two times with 1 X PBS (phosphate buffer saline). Afterwards, the bacterial concentration was adjusted to an OD_{600 nm} of 0.8 using 1 X PBS.

3.2.10.3. Bacterial adhesion test

Prior to the test, modified and unmodified PET film samples were cut into 1 X 1.5 cm size rectangles. To ensure reproducibility of the results, three replicates of each sample were prepared. The samples were immersed into 1 % detergent solution and placed in an ultrasonic bath for 1 min, rinsed with water to remove contaminants and any possible previously attached microorganism. After that, samples were air dried in the flow chamber and UV treated for 30 min. Each sample was then placed into a falcon tube containing 14 mL of bacterial cells already prepared (OD_{600 nm} of 0.8) and were incubated at 4 °C for 4 h in an orbital shaker at 12 rpm. After incubation, PET samples were removed and washed gently with 1 X PBS buffer to remove unattached bacteria. The bacteria adhered on PET surfaces were visualized using a fluorescence microscope (Olympus BX51), at 60X magnification, coupled with an DP71 digital camera. Fluorescence imaging was done with 470-490 nm excitation and a 520 nm filter in the microscope optical path.

3.3. RESULTS AND DISCUSSION

3.3.1. Water contact angle (WCA) analysis of PET control

WCA gives an indication about the wettability of the surface. However, some environmental conditions like dirt [24], humidity [25] and temperature [26,27] can affect the WCA. Thus, it is normal to expect some variations among each measurement. An average WCA of 70 ° ± 5 ° was found for PET control, in close agreement with other values found in literature for pristine PET (e.g. 72 ° [28] or 77 °

[21]). The values follow a normal distribution, showing that all the individual measurements fall within the control limits.

A surface with a high hydrophilicity behavior (i.e., low contact angle) exhibits a great potential as an anti-adhesive surface thus highlighting the need to further functionalize PET films and one of the commonly used approaches is grafting method to immobilize natural substances including polysaccharides [29].

3.3.2. Pre-treatment of PET substrate

Aminolysis of PET involves a reaction of primary amines with the ester linkage of PET which results in the formation of an amide linkage and hydroxyl group by chain cleavage [20,30]. By using diamine, free primary amine ending groups on the surface of PET were obtained, that can be available for further reaction for gum Arabic attachment (Figure 3.1).

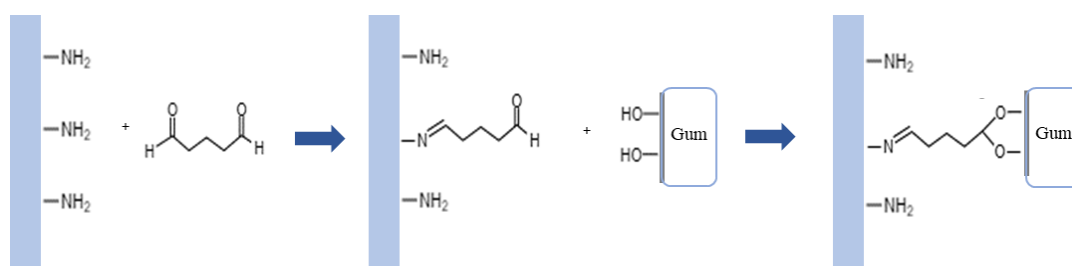


Figure 3.1. Schematic representation of PET modification with gum Arabic.

The aminolysed PET samples were evaluated by ATR-FTIR and the acid orange coloration method to characterize the presence of amine groups on the surface. The ATR-FTIR data in Figure 3.2 shows stretching of the amide groups at 1649 cm^{-1} (amide I, C=O stretching) and 1548 cm^{-1} (amide II, NH deformation) on PET sample [31,32]. Although there were no medium or strong bands representative of the stretching of amine groups between 3000 and 3600 cm^{-1} , the broad weak peak observed between 3000 and 3600 cm^{-1} can be due to presence of exchangeable protons from hydroxyls and amines. However, with a penetration depth as low as $0.6\text{ }\mu\text{m}$ [31], ATR-FTIR lacks high sensitivity for the thin surface modifications. Therefore, it can be assumed that the existence of characteristic peaks in the ATR-FTIR spectrum indicates some degree of bulk material modification, which is also supported by the literature [21,33].

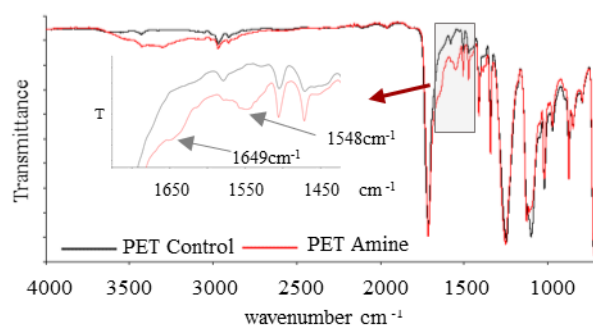


Figure 3.2. FTIR spectrum of unmodified PET (PET control) and diamine treated PET (PET amine).

Additionally, the presence of free amines was also confirmed with a colorimetric method. Acid orange has affinity to primary amine groups in acidic conditions due to electrostatic interactions occurring between the dye and amine groups [34]. The dye treated PET samples can be seen in Figure 3.3. The aminolysed samples showed a higher amount of dye absorption, meaning that the aminolysed samples obtained orange color after the dye application, indicating the presence of primary amine groups, as found also in previous studies [21,34].

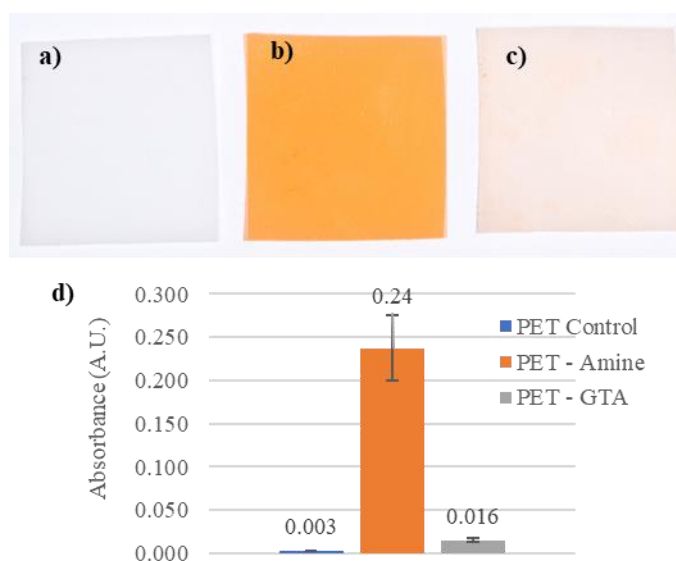


Figure 3.3. Acid orange colorimetric assay; a) unmodified PET sample (PET control), b) aminolysed PET sample (PET-amine), c) glutaraldehyde modified aminolysed PET sample (PET-GTA), d) absorption values from UV-vis spectroscopy for PET control, PET-amine and PET GTA.

Further modification of the aminolysed surfaces was performed with glutaraldehyde. Glutaraldehyde is a well-known crosslinker. The possible crosslinking of glutaraldehyde with amine groups and gum Arabic is shown in Figure 3.1. It is expected that glutaraldehyde forms Schiff bases with the amine groups. Although Schiff bases can be considered unstable and the exact mechanism is still unknown [35], several reports have shown that the bonds created by glutaraldehyde can be stable

due to bulky hydrophobic part near to amine part of Schiff bases [36], or further reaction of the Schiff bases with glutaraldehyde [37]. It is also possible that the monomeric cyclic hemiacetal and multimeric cyclic hemiacetal forms of glutaraldehyde react with the amine groups on the surface since this form is more likely to be stable in acidic conditions [35]. In order to evaluate glutaraldehyde modification, glutaraldehyde treated samples were further characterized with acid orange colorimetric assay, whereas the low absorbance of the orange dye indicates the reduced amount of primary amine groups on glutaraldehyde treated samples as shown in Figure 3.1, thus confirming the bonding of glutaraldehyde on aminolysed PET surfaces. It was also observed that there was a slight yellow interference on the glutaraldehyde treated samples which may be due to the formation of Schiff bases [38-40]. Moreover, further characterization of the surfaces using water contact angle led to values of $64 \pm 1^\circ$ and $75 \pm 6^\circ$ for aminolysed and glutaraldehyde modified samples, respectively.

3.3.3. Statistical analysis of reaction parameters

Organic reactions can be highly affected by the reaction parameters such as reaction time, reagents concentration and curing conditions. In a first step, screening experiments were conducted to identify the factors that influence PET grafting efficiency and to verify if any changes to their settings should be made to improve the process. The effects of different experimental variables on PET grafting process were simultaneously investigated, using a full factorial design experiment (Table 3.2, block 1). Four variables (curing temperature, curing time, gum Arabic concentration and contact time) were considered. The pre-treatment methods (glutaraldehyde modification and aminolysis) were performed for all the samples in the same conditions. The grafting process was then performed in aqueous solutions with 0.5 % 0.1 M HCl (pH values ranged between 3.4 and 4) at 40°C , where the grafting parameters were varied according to the design planning.

Table 3.2. Values for WCA after functionalization (OX), after 5 washing cycles (5X) and after 10 washing cycles (10X), according to the 2⁴ factorial design (block 1) and to the central composite design (block 2), ordered by standard order.

Assay	Block	Variables				Responses		
		Curing temperature (°C)	Curing time (h)	Gum Arabic concentration (%)	Reaction time (h)	Contact angle (OX)	Contact angle (5X)	Contact angle (10X)
1	1	80	0.5	1	1	49±7	49±6	53±6
2	1	120	0.5	1	1	41±4	41±6	44±4
3	1	80	3.5	1	1	37±6	41±5	45±3
4	1	120	3.5	1	1	27±5	34±9	34±6
5	1	80	0.5	5	1	55±2	52±5	55±5
6	1	120	0.5	5	1	49±4	49±5	50±2
7	1	80	3.5	5	1	58±2	50±8	58±3
8	1	120	3.5	5	1	21±3	29±5	37±1
9	1	80	0.5	1	15	39±3	46±7	48±4
10	1	120	0.5	1	15	42±4	39±3	40±3
11	1	80	3.5	1	15	46±8	58±4	53±6
12	1	120	3.5	1	15	25±4	32±3	33±8
13	1	80	0.5	5	15	57±6	52±3	59±4
14	1	120	0.5	5	15	35±5	54±4	51±2
15	1	80	3.5	5	15	45±6	32±3	29±3
16	1	120	3.5	5	15	24±3	42±3	57±3
17	1	100	2	3	8	46±8	45±6	53±5
18	1	100	2	3	8	42±5	49±4	54±3
19	1	100	2	3	8	43±3	57±4	59±3
20	2	71.7	2	3	8	50±1	61±7	55±4
21	2	128.3	2	3	8	24±3	32±2	30±5
22	2	100	0.08	3	8	43±3	55±4	53±4
23	2	100	4.12	3	8	25±5	31±3	46±5
24	2	100	2	0.17	8	50±5	50±6	46±4
25	2	100	2	5.83	8	31±4	46±8	48±4
26	2	100	2	3	0.08	38±3	39±4	47±2
27	2	100	2	3	17.9	38±6	44±2	48±3
28	2	100	2	3	8	37±5	51±5	44±3
29	2	100	2	3	8	48±8	52±8	47±9

The initial WCA and WCA after 5 and 10 washing cycles (one cycle of washing consisted of immersing the sample in distilled water at 40 °C, 100 rpm agitation for 30 min) were chosen as the response variables. At least four measurements were performed in different locations of the sample, being the average values and standard deviation presented as the final response. The experimental matrix and the results for the factorial design are shown at Table 3.2 (block 1).

After the grafting reaction in the conditions set by the planning, the obtained PET grafted films were washed for 5X or 10X successively, to understand the resistance and stability of the produced gum Arabic coatings. After each washing cycle, the samples were removed and placed in a new container for another washing cycle. After 5 or 10 consecutive cycles, the samples were allowed to air dry and WCA was measured in the dried samples.

As can be observed in Table 3.2, the initial WCA after the functionalization process varied considerably (from 21 ° to 58 °), being this variation identical after washing (from 29 ° to 58 ° and to 59 °, after 5 and 10 washing cycles, respectively). However, higher average WCA were observed after washing (the mean WCA increased from 41 ° to 45 ° and 48 °, after 5 and 10 washing cycles, respectively). The increase in the WCA with washing was expected, due to the low stability of the coating in some assay conditions and due to the presence of unreacted components that are being removed during the washing process. However, comparing with PET control samples that have a mean WCA of 70 °, a significant decrease in the WCA was achieved after grafting and washing, being concluded that a stable grafting was achieved.

Table 3.2 also shows that, independently of the other variables, increasing curing temperature from 80 °C to 120 °C resulted in a decrease of the WCA, and consequently, in a higher efficiency in the gum Arabic linkage. Finally, it is also possible to observe that lower WCA occurred when higher curing temperatures and times (more intense curing conditions) were used. Indeed, curing temperature (A) and curing time (B) have played a critical role in PET coating.

According to the Student's t-test results, the most important factors affecting the initial WCA were the curing temperature (A) and curing time (B), thus these factors were considered for building the linear model. Factor C, concentration, the first order interactions AB and AC and the fourth order interaction ABCD were also considered in the linear model, due to their t-test value (above t-value limit).

Using the selected factors for building the linear model, the Fischer's test was used to evaluate the model significance. The ANOVA statistical analysis for the initial WCA is summarized in Table 3.3. A model F-value of 28.15 implies the model is significant ($p\text{-value} < 0.0001$), i.e., there is only a 0.01 % chance that an F-value this large could occur due to noise.

Table 3.3. Analysis of variance (ANOVA) for the linear model obtained for the initial WCA.

Factors	WCA (OX)				
	Sum of Squares	Df	Mean Square	F-value	p-value
Model	1996.0	6	332.67	28.15	< 0.0001
A	930.25	1	930.25	78.73	< 0.0001
B	441.00	1	441.00	37.32	< 0.0001
C	90.25	1	90.25	7.64	0.0172
AB	196.00	1	196.00	16.59	0.0015
AC	156.25	1	156.25	13.22	0.0034
ABCD	182.25	1	182.25	15.42	0.0020
Residual	141.79	12	11.82		
Lack of fit	133.12	10	13.31	3.07	0.2705
Pure error	8.67	2	4.33		
Cor Total	2137.79	18			

The curvature and residual lack of fit were found to be insignificant, and the proportion of variance explained by the model, given by the multiple coefficient of determination, R^2 was found to be 0.9337, highlighting the model adequacy to predict the WCA in the design space. The obtained final model equation for the initial WCA, in terms of coded factors is:

$$\text{Initial WCA} = 41.11 - 7.63A - 5.25B + 2.37C - 3.50AB - 3.12AC + 3.38ABCD \quad (\text{Eq. 3.2})$$

Figure 3.4 shows the 3D surface for the initial WCA, where a clear interaction effect between curing temperature and curing time is observed, thus suggesting lower WCAs values when these 2 factors are simultaneously maximized.

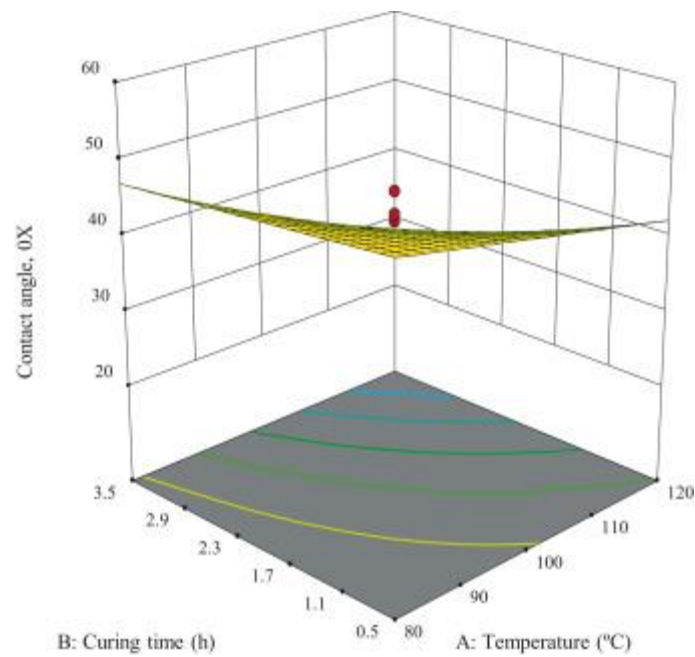


Figure 3.4. 3D graphical visualization of the initial WCA with factors A (curing temperature) and B (curing time). Factors C (gum Arabic concentration) and D (contact time) were kept at the central point.

The same statistical analysis was performed for the WCA after 5 and 10 washing cycles, being the Fischer's test used for evaluating the model significance and the ANOVA statistical analysis summarized in Table 3.4.

Table 3.4. Analysis of variance (ANOVA) for the linear model obtained for WCA after 5 and 10 washing cycles.

Factors	WCA (5X)					Factors	WCA (10X)				
	Sum of Squares	Df	Mean Square	F-value	p-value		Sum of Squares	df	Mean Square	F-value	p-value
Model	997.50	6	166.25	7.31	0.0024	Model	1139.75	7	162.82	6.34	0.0049
A	225.00	1	225.00	9.89	0.0093	A	182.25	1	182.25	7.10	0.0237
B	256.00	1	256.00	11.26	0.0064	B	182.25	1	182.25	7.10	0.0237
AC	81.00	1	81.00	3.56	0.0858	C	132.25	1	132.25	5.15	0.0466
BC	121.00	1	121.00	5.32	0.0415	AC	110.25	1	110.25	4.30	0.0650
ACD	182.25	1	182.25	8.01	0.0163	ABD	110.25	1	110.25	4.30	0.0650
ABCD	132.25	1	132.25	5.82	0.0345	ACD	182.25	1	182.25	7.10	0.0237
Curvature	109.49	1	109.49	4.81	0.0506	ABCD	240.25	1	240.25	9.36	0.0121
						Curvature	191.58	1	191.58	7.46	0.0211
Residual	250.17	11	22.74			Residual	256.67	10	25.67		
Lack of fit	175.50	9	19.50	0.5223	0.7971	Lack of Fit	236.00	8	29.50	2.85	0.2852
Pure error	74.67	2	37.33			Pure Error	20.67	2	10.33		
Cor Total	1357.16	18				Cor Total	1588.00	18			

Although the obtained models were found to be statistically significant with 99.76 % and 99.51 % of probability for WCA 5X and WCA 10X respectively, and lack of fit was insignificant, they both presented curvature and a low coefficient of determination, R^2 . Consequently, the linear model is not adequate to navigate in the design space and an augmented design is recommended.

A Central Composite Design (CCD), with α equal to $\pm\sqrt{2}$ (± 1.414) was chosen to expand the model surface, with the time constraints already described. Table 3.2 block 2 shows the responses obtained for the WCA. When expanding the model, a similar variation of the WCA was found, where initial WCA varied from 21 to 58 °, and WCA 5X and 10X varied from 29 ° and to 59 °, respectively.

The same statistical analysis was performed for the initial WCA and WCA after 5 and 10 washing cycles, being the Fischer's test used to evaluate the model significance. The p-value of obtained models were found to be statistically significant with p-value < 0.0001, p-value = 0.0002 and p-value = 0.0004 for WCA, WCA 5X and WCA 10X respectively. Figure 3.5 shows the contour surfaces obtained for these responses, where it is clearly visible the need to increase curing temperature and curing times, to achieve low values for the WCA.

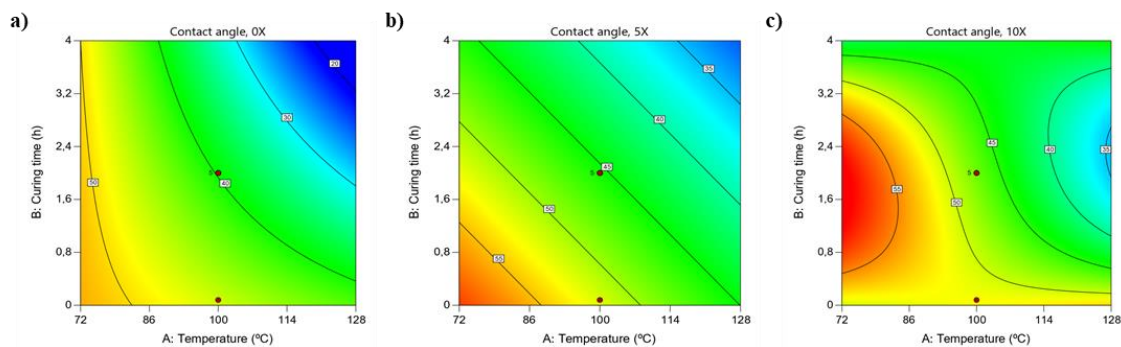


Figure 3.5. Contour plots for factors A (curing temperature) and B (curing time), for the models attained for a) the initial WCA, b) WCA after 5 washing cycles and c) WCA after 10 washing cycles. Factors C (gum Arabic concentration) and D (contact time) were kept at the central point.

The curvature and residual lack of fit were found to be insignificant to all the responses analyzed and the residuals analysis were found to be normally distributed, thus indicating that the statistical studies could be continued with optimization. The predicted model equations for the initial WCA, WCA after 5 washing cycles and WCA after 10 washing cycles, in terms of coded factors, are:

$$\text{Initial WCA} = 39.79 - 7.94A - 5.54B - 6.71C - 3.50AB - 3.12AC + 9.09A^2C + 3.38ABCD \quad (\text{Eq. 3.3})$$

$$\text{WCA 5X} = 45.48 - 5.05A - 4.93B - 2.75BC + 3.37ACD + 2.88ABCD \quad (\text{Eq. 3.4})$$

$$WCA\ 10X = 47.22 - 8.83A - 3.21B + 2.44C + 2.62AC + 2.63ABD + 3.37ACD + 5.46AB^2 + 3.87ABCD \quad (\text{Eq. 3.5})$$

3.3.4. Optimization and characterization of the optimized PET surfaces

To improve the gum Arabic grafting process, the graphical optimization tool from Design-Expert® was used, being the objective, the minimization of the responses found for the WCA. The upper limits (corresponding to the minimum WCA) were set as 35 ° for WCA 0X and 42 ° for both WCA 5X and WCA 10X, since it is recognized that the improved washing resistance of the coatings is an indication of the efficiency of the grafting process. Ideally, after the first 5 consecutive washing cycles, the WCA should stay constant, meaning that the coating is stable, and no more chemicals are being released from the grafted surface.

Figure. 3.6A shows the overlay plot of factor Curing time (B) versus Temperature (A) for the responses fulfilling simultaneously these criteria (yellow area). Observing the yellow shaded area, it is possible to conclude that using combined curing temperatures above 110 °C and curing times higher than 2 h would enable achieving the desired WCA. As such, the study was continued setting the curing conditions at the following parameters: curing temperature of 120 °C and curing time of 3 h. To aid the decision on the best gum Arabic concentration, the Overlay plot of factor C versus factor A (Fig. 3.6B) was analyzed, keeping factor B (curing time) at 3 h.

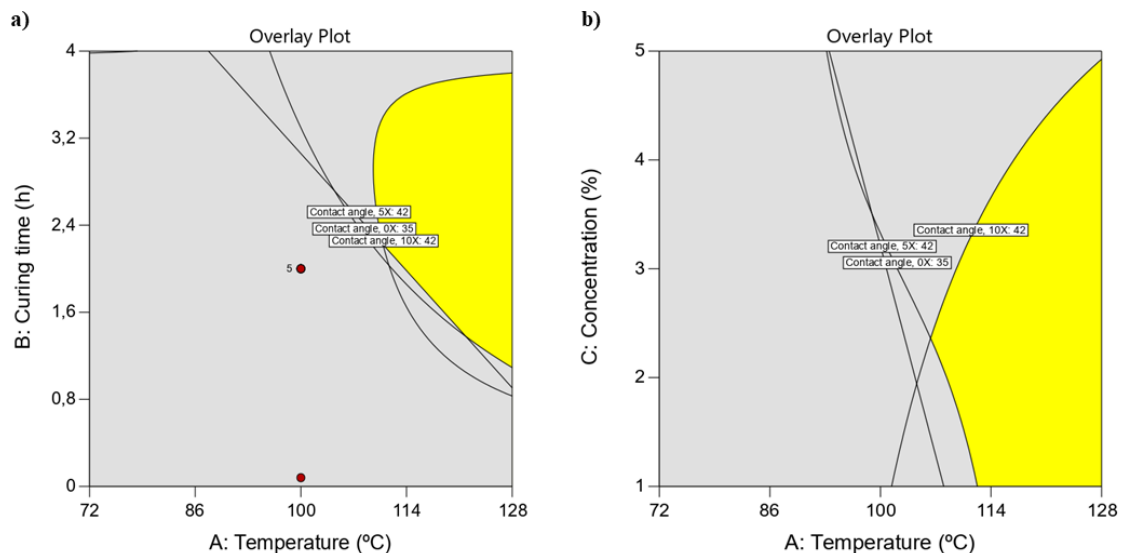


Figure 3.6. Overlay plots highlighting the area where responses fulfill the set criteria (initial WCA of 35 ° and WCA 5X and 10X of 42 °). a) Factor B (curing time) versus A (temperature), keeping C = 3 % and D = 8 h (central point); b) Factor C (gum Arabic concentration) versus A (temperature), keeping B = 3 h and D = 8 h.

Analyzing Figure 3.6B, we can conclude that the range of gum Arabic concentrations to fulfil the set criteria can go from 1 % to approximately 4 %. Since the minimization of consumables is always desired to decrease costs, the validation experiment was performed using 1 % of gum Arabic with 1 h of contact time for the grafting process, whereas the obtained coating was cured at 120 °C for 3 h.

A low WCA ($27^{\circ} \pm 8$) was obtained for this optimized treated sample, and after washing, the developed coating still presented low values for the WCA ($32^{\circ} \pm 8$ for 5X and $30^{\circ} \pm 7$ for 10X), which is correlated with the enhanced grafting performed. Further characterization experiments were conducted on the optimized samples, to assess their efficiency as anti-adhesive materials. Table 3.5 shows the surface energy for PET control and PET treated samples.

Table 3.5. Surface roughness and energy of PET control and PET gum Arabic (optimized gum Arabic treated sample).

Samples	Surface Roughness			Contact Angle		Surface Energy		
	Ra (nm)	Skew	Kurtosis	Water Contact Angle (°)	Diodomethane Contact Angle (°)	SFE Polar (mJ/m ²)	SFE Disperse (mJ/m ²)	SFE Total (mJ/m ²)
PET Control	7.24	1.11	1.4	$70^{\circ} \pm 5$	$34^{\circ} \pm 8$	7 ± 3	42 ± 4	49 ± 4
PET Gum Arabic	21	-0.87	0.016	$27^{\circ} \pm 8$	$38^{\circ} \pm 4$	29 ± 2	41 ± 4	70 ± 4

Besides contact angle characterization, surface energy was calculated since several studies pointed out that the surface energy values of materials can be related with bacterial adhesion [41,42]. According to Owens-Wendt-Rabel&Kaelble Model (OWRK) equation, the polar and disperse surface energy values of treated and untreated samples can be calculated by using water and diiodomethane contact angles. From Table 3.5, it is found that polymers have a relatively high disperse component and low polar component of total surface energy. This is thought to be a characteristic behavior of the polymers [43]. However, after the surface treatment, an increase in the total surface energy from 49 mJ/m² to 70 mJ/m² and an increase in the polar component of surface energy from 7 mJ/m² to 29 mJ/m² has been observed. It is known that the water contact angle and surface energy are important parameters for bacterial adhesion tests, and the negative bacterial adhesion has been correlated with the polar component of surface energy and total surface energy for both gram negative and gram-positive bacteria [41,44], where a material with a high total surface energy and with a high polar component correspond to the requirements towards a decreased bacterial adhesion. This reduced

adhesion can be explained with a strongly hydrated surface providing a physical and free energy barrier [45].

Gram negative bacteria like *E. coli* have a thinner peptidoglycan layer compared to gram positive bacteria and it provides less rigid surface which can promote adhesion on rough surfaces [29,46]. Topographical changes on PET surface is expected due to breakage (aminolysis) and formation of new bonds (attachment of glutaraldehyde and gum Arabic) via chemical reactions. Thus, the gum Arabic treated samples and untreated PET samples were further analyzed by SEM and AFM to evaluate the effect of surface topography and verify the modification (Figure 3.7 and 3.8). SEM images obtained from the surface included surface visualization with lower magnification and small morphological differences were observed (Figure 3.7) and AFM was utilized for further characterization PET surface. As it can be seen from Figure 3.8, PET Control surface is smoother (with $R_a = 7.24$ nm) when compared with the treated sample ($R_a = 21$ nm). In addition to R_a value, other parameters like kurtosis and skewness were further analyzed. While kurtosis can provide information about the sharpness of the peaks (if $R_{ku} > 3$, the peaks are sharp), the skewness value (R_{sk}) can indicate where the distribution is concentrated. The gum Arabic treated samples have a negative skew ($R_{sk} = -0.867$) which indicates that the data collected from the surface were skewed to negative side meaning that they have valleys constructed on the surface and it was also observed $R_{ku} < 3$ for both cases, indicating that the peaks were round [47].

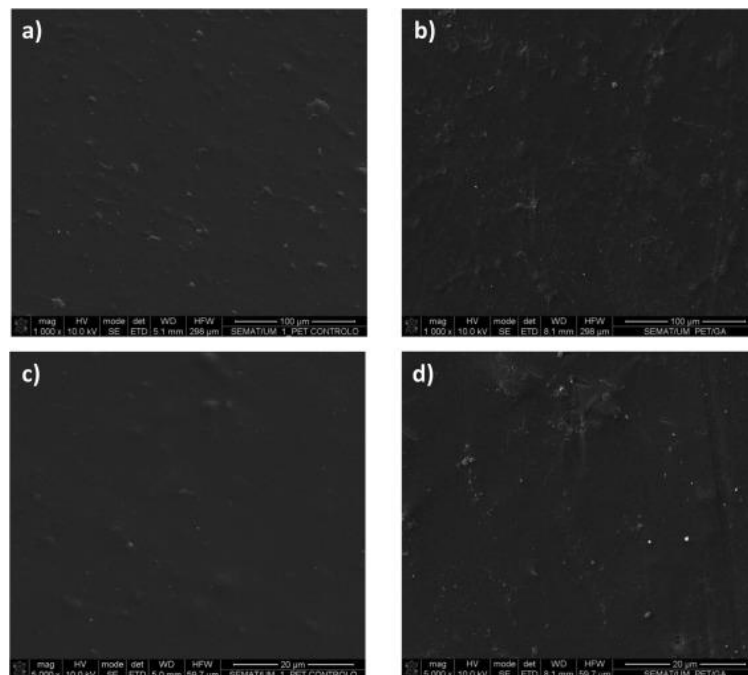


Figure 3.7. SEM images for samples a–c) PET control, b–d) PET gum Arabic treated samples at two different magnifications.

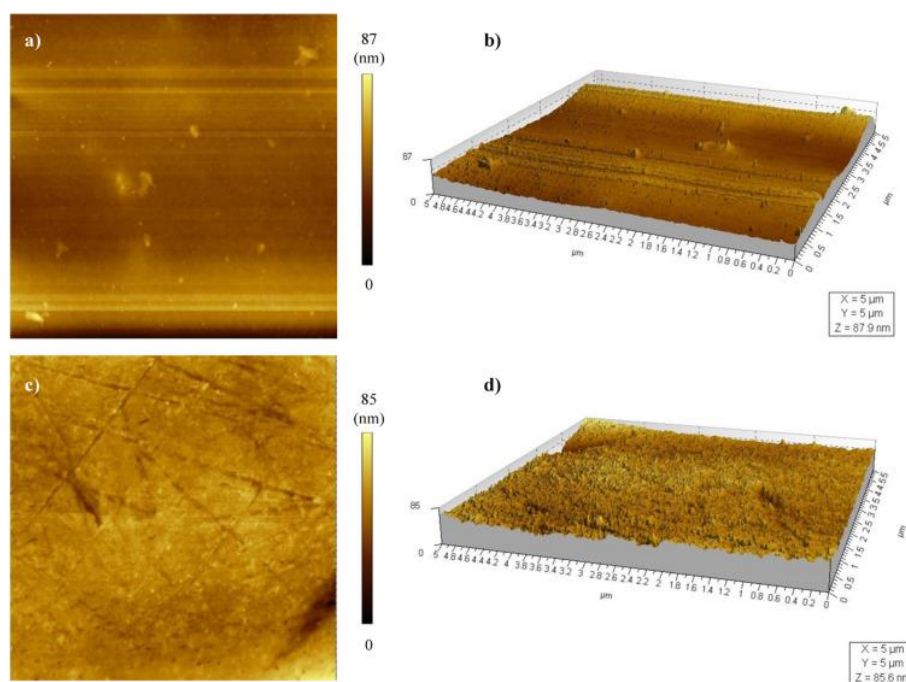


Figure 3.8. AFM images for the samples a, b) PET control, c, d) PET gum Arabic samples.

3.3.5. Bacterial adhesion on PET surfaces

Bacterial adhesion experiments were performed to study the effectiveness of the optimized gum Arabic treated PET surfaces. It is known that bacterial adhesins are involved in bacterial attachment and they further promote biofilm formation [48]. YadA is a *Yersinia* adhesin which promotes adhesion and auto-agglutination, thus suggesting its role in the biofilm formation by *Yersinia enterocolitica* [23]. Although bacterial adhesion mechanism of YadA is unknown, adhesins are known to play a role in maturation of adhesion and irreversible adhesion [48]. Moreover, it is also known to provide resistance to bactericidal activity [49]. In order to assess the adherence mediated by a highly adhesive adhesin like YadA in a low biosafety level laboratory, we assessed the adhesion of *E. coli* BL21 cells harboring pASK_IBA2_YadA and pRSFduet_GFP using fluorescence microscopy where the material was immersed in the culture medium for 4 h incubation time.

It can be seen from Figure 3.9 that PET control sample was fully covered with bacteria after just 4 h of contact, as bacteria can strongly adhere onto surfaces as a key initial step in the formation of biofilms, whereas gum Arabic treated samples showed an extremely low number of adhered bacteria (almost residual). Thus, we can conclude that gum Arabic modified surface clearly exhibits anti-adhesive/antibacterial properties similarly to other polysaccharides reported for the same purpose [7-9]. The anti-adhesive/antibacterial properties of gum Arabic can be due to combination of some properties like hydrophilic nature [12], negative charge [14] and antimicrobial activities. Although the

exact mechanism of antimicrobial action is unknown, there are some studies suggesting that the presence of antimicrobial enzymes like oxidases, peroxidases and pectinases might be the reason for its antimicrobial activity. Some other studies highlight that the presence of the high amounts of salts may be the reason for the antimicrobial properties of gum Arabic [50,51], since glucuronic acid is found as calcium, magnesium and potassium salts in nature [52]. Furthermore, it was also seen that roughness obtained through modification did not promote the adhesion indicating that surface hydrophilicity can be more important at bacterial adhesion.

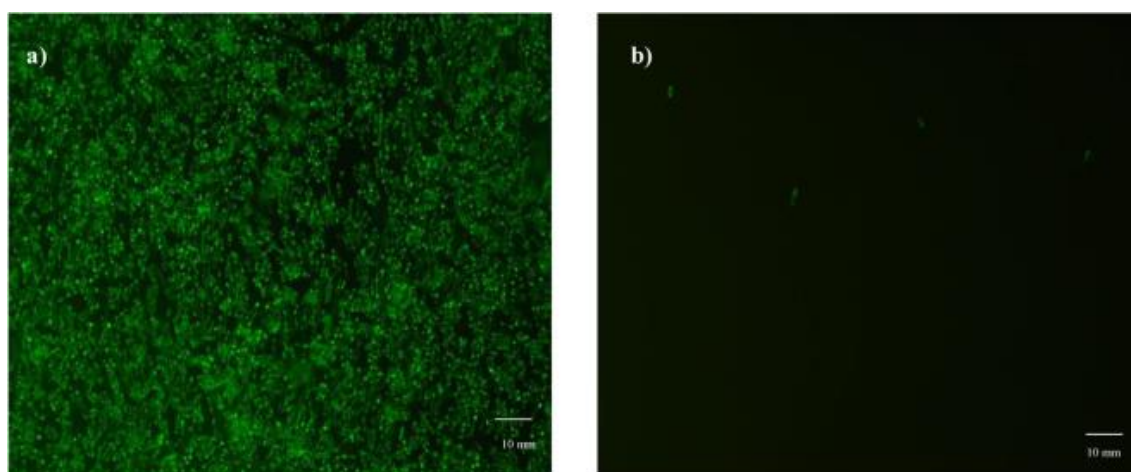


Figure 3.9. Fluorescence microscopy images of *Escherichia coli* BL21 cells harboring pRSFduet_GFP and pASK_IBA2_YadA plasmids a) PET control and b) PET gum Arabic. Scale bar, 10 μ m.

3.4. CONCLUSION AND OUTLOOK

PET surface was modified with gum Arabic through treatments with diamines and glutaraldehyde. The use of WCA as a response for gum Arabic grafting onto PET films was used to find the optimum design parameters within the design of experiment range towards PET bacterial anti-adhesiveness. The parameters chosen were concentration, reaction time, curing temperature and curing time. It was found that curing temperature and curing time were the most significant parameters affecting the WCAs and their simultaneously maximization leads to a decrease of the WCA values. Using optimized conditions (1 % GA concentration, 1 h of reaction time, curing temperature of 120 °C and curing time of 3 h) an initial WCA of 27 ° was found. It was further shown that the treated samples were stable and resistant to several cycles of washings. Furthermore, the total surface energy and polar component of surface energy were found to be higher for treated samples, having increased from 49 mJ/m² to 70 mJ/m² and from 7 mJ/m² to 29 mJ/m², respectively. This corresponds to the requirements for obtaining a low bacterial adhesion on the surface. Finally, the bacterial adhesion susceptibility of treated and untreated

materials was evaluated against *E. coli* expressing YadA, an adhesive protein from *Yersinia* so-called *Yersinia* adhesin A. The adhesion tests clearly showed that the treated materials were highly effective and resistant (numbers of bacteria adhered are residual), which could be explained by the low contact angle and/or bacterial inhibition properties of the gum Arabic and therefore, constitute promising materials to be further used in medical contexts.

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CHAPTER 4

Atmospheric plasma and UV polymerisation for developing sustainable anti-adhesive polyethylene terephthalate surfaces

Enhancing the hydrophilicity of polymeric materials is an important step to achieve anti-adhesiveness. Thus, in this study, atmospheric plasma as a pre-treatment was combined with UV grafting process to obtain durable surface modification on polyethylene terephthalate (PET). The most promising conditions for plasma process were found to be 100 % power and 4 m/s speed, leading to a contact angle reduction from $70 \pm 6^\circ$ to around 30° . However, it was observed that these values increased over time due to ageing and washing, ultimately recovering to its initial value. Therefore, the plasma-pre-treated PET samples were further modified through an UV grafting process, using sodium acrylate (NaAc) and 3-Sulfopropyl Acrylate Potassium Salts (KAc). The grafted acrylate PET samples exhibited contact angles of $8 \pm 3^\circ$ and $28 \pm 13^\circ$ for NaAc and KAc, respectively, while showing durability to ageing and washing tests. Dry film thicknesses for both samples were found to be $28 \pm 2 \mu\text{m}$. Finally, the anti-adhesive properties of NaAc and KAc treated surfaces were evaluated using an *Escherichia coli* expressing YadA, an adhesive protein from *Yersinia*. The modified PET surfaces were highly effective in reducing bacterial adhesion by more than 90 %.

Keywords: Grafting; Hydrophilicity; Surface modification; Wettability; Plasma; UV polymerisation

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4.1. INTRODUCTION

The number of patients with infections increase every year. The European Centre for Disease Prevention and Control estimated that 8.9 million Healthcare-associated Infections (HAI) occur each year in European Hospitals and long-term facilities. Additionally, around 30 % of bacteria responsible for those infections were found to be resistant to antibiotics [1]. More than ever, it is extremely important to reduce the spread of infections. A potential way to reduce the spread of infections and antibiotic resistance is using anti-adhesive materials that can in turn reduce the microbial load on surfaces. A popular approach to obtain such anti-adhesive surfaces is to use hydrophilic surfaces [2], since it has been shown that hydrophilic surfaces can prevent bacterial adhesion by forming a hydration layer [3]. One attractive method to obtain such surfaces is plasma treatment. Plasma surface treatment can be applied on many different materials (metal, wood, paper, glass, polymer, ceramic, nonwoven textile, among others) and the medical sector is one of the main application fields [4]. Plasma systems are well known, and they can be used for a variety of treatments like surface cleaning, etching, functionalisation, activation and polymerisation since they change the chemical and physical properties of the surface [5,6]. The gas type used in the plasma system can determine the hydrophilicity and hydrophobicity of the treated surface [2], and hydrophilic surfaces can be obtained using gases such as air, oxygen, nitrogen, helium and argon [5,7]. Atmospheric air can be a viable option for plasma treatment as it is a natural, ecological, inexpensive and abundant gas mixture [8]. Additionally, since it is a dry surface treatment, using plasma eliminates the need to use harmful chemicals and consequently its effluents, hence leading to more sustainable processes [5]. Furthermore, the use of atmospheric plasma can eliminate the drawbacks from low-pressure plasma systems such as expensive vacuum systems, high maintenance costs, and being limited to batch processes and smaller area treatments. Although atmospheric plasma offers these advantages, it has been seldom studied compared to low-pressure plasma [8].

Moreover, UV-induced photopolymerisation is also an attractive technology for both industry and academy, as it is characterized by having a low environmental impact, a versatile technique, and high efficiency. In most cases, a photoinitiator is needed to create the necessary radicals for the initiation of the polymerisation reaction. However, there are some drawbacks with the use of photoinitiators like storage problems, unwanted odour and colour on final products, migration of remaining photoinitiator molecules, health concerns and cost [9]. Thus, UV can be combined with plasma to generate radicals like peroxy or hydroxyl on the surface [6], increasing the efficiency of the polymerisation reaction and

removing the unnecessary use of photoinitiators.

In this study, we used polyethylene terephthalate (PET) as the base substrate since it is one of the most popular polymers used in the medical context due to its hardness, stiffness, biocompatibility, biological, chemical and mechanical stability [2]. However, PET surfaces need to be modified to prevent/reduce bacterial adhesion. Charged polymer networks exhibit high uptake of water [10], and sodium acrylate (NaAc) and 3-sulfopropyl acrylate potassium salt (KAc) are both used in the preparation of charged polymers commonly used in hydrogel studies [10,11]. Furthermore, poly(sodium acrylate) is known as superabsorbent [12] material, being a good candidate to improve surface hydrophilicity. Thus, a combined process of plasma and UV photopolymerisation for PET modification was employed in this study, in order to render the PET surface hydrophilic. An initial statistical study for the determination of the optimal experimental parameters of the plasma treatment (speed and power) using air as reactive gas was performed. After that, atmospheric plasma treated samples were further subjected to UV polymerisation with NaAc and KAc. Finally, the bacterial anti-adhesive properties of the PET treated surfaces were assessed.

4.2. MATERIALS AND METHODS

4.2.1. Materials

PET film (Mylar® A with thickness of 36 µm) was acquired from Isovolta Group. Sodium Acrylate (97 %) and diodomethane (99 %) were purchased from Sigma-Aldrich; 3-Sulfopropyl Acrylate Potassium Salt (>98 %) was purchased from TCI Chemicals.

4.2.2. Pre-treatment with atmospheric plasma

A Dielectric Barrier Discharge (DBD) atmospheric pressure plasma was used as a sustainable pre-treatment for PET modification. The films were treated using the atmospheric pressure plasma equipment (Sigma Technologies International – Atmospheric Plasma Treatment System), at room temperature and relative humidity around 45 – 55 %. Air was used as reactive gas. The PET film samples passed between two metallic electrodes, at a power that varied from 15 kW (100 % of maximum power) to 7.5 kW (50 % of maximum power) and at a conveyor speed that varied from 4 m/min (1.1 Hz) to 12 m/min (3.3 Hz).

4.2.3. Experimental design and optimization by response surface methodology (RSM)

In a first step, a 2^2 full factorial design was performed to evaluate the effect of treatment power and speed on PET surface modification. Initial water contact angle (WCA), WCA after 2 weeks of ageing, and percentage of oxygen increase were used as response variables.

The selected variables and their levels are listed in Table 4.1. State-Ease® 'Design Expert' (version 12) software was used for regression and graphical analyses of the data obtained. The statistical significance of the regression coefficients was determined by Student's t-test, the second-order model equation was determined by Fischer's test and the proportion of variance explained by the multiple coefficients of determination, R^2 . The optimum range of the variables were obtained by the graphical analysis using the 'Design expert' program.

Table 4.1. Coded and actual levels for the Design of Experiment Variables.

Independent variables	Factor	Range and levels		
		-1	0	+1
Speed (m/min)	A	4	8	12
Power (%)	B	50	75	100

4.2.4. UV polymerisation of PET with acrylates

100 g/L of sodium acrylate and 200 g/L of 3-Sulfopropyl Acrylate Potassium Salt solutions were prepared with MiliQ water and purged with nitrogen for 1 h. Meanwhile, the plasma pre-treated PET samples were placed in a petri dish in nitrogen atmosphere for 30 min before the addition of acrylates solution. After the addition of acrylates solution, they were left in nitrogen atmosphere for 30 min before starting UV polymerisation. For UV polymerisation, an OmniCure S2000, Lumen Dynamics equipped with a 200 W mercury vapor short-arc lamp which emission spectrum is mostly between 300 nm and 450 nm, was used for 35 min. The grafted samples were rinsed and immersed in distilled water for one day to release any adsorbed acrylates from the surface. Finally, the samples were dried under vacuum conditions for 4 h, before any characterization of the modified surfaces was performed.

4.2.5. Water contact angle (WCA)

The hydrophilicity of the modified surfaces was evaluated by static WCA measurement using the Optical Tensiometer Attension Model Theta Basic by Biolin Scientific, at room temperature. MiliQ water

with a droplet size of 3 μL was used. The measurements were repeated at least 3 times for each sample at different locations. Furthermore, contact angle values after 5 cycles of washing (one cycle of washing consisted of immersing the sample in distilled water at 40 °C, 100 rpm agitation for 30 min) and after 2 and 3 weeks of storage were measured to evaluate the stability and durability of the surface treatment under these conditions. Contact angle measurements were also performed with diiodomethane in order to allow the calculation of polar and disperse components of surface energy by the Owens-Wendt-Rabel & Kaelble Model (OWRK) equation [13].

4.2.6. XPS analysis

X-Ray Photoelectron Spectroscopy (XPS) analysis, E557 - X-ray Photoelectron Spectroscopy; Serial Nr. C332844/01; Model Axis Supra; Manufacturer Kratos was used to evaluate the chemical composition of the samples. Monochromatic X-ray source Al K α (1486.6 eV) was used for all samples and experiments with a take-off angle of 90 °. The residual vacuum in the X-Ray analysis chamber was maintained at around 5.6×10^{-9} torr. The measurement was done in a Constant Analyser Energy mode (CAE) with a 10 mA of emission current and 160 eV pass energy for survey spectra and a 15 mA of emission current and 40 eV pass energy for high resolution spectra. Charge referencing was done by setting the lower binding energy C1s photo peak at 285.0 eV C1s hydrocarbon peak.

4.2.7. UV-VIS spectrophotometer

UV-VIS Spectrophotometer, Perkin Elmer Lambda 35, was used for the determination of film thickness of treated materials. The spectra in transmittance were collected in the wavelength range of 250–1100 nm with three repetitions. The Swanepoel method was used for the calculation of film thickness [14].

4.2.8. AFM analysis

A Keysight Technologies 5500 Atomic Force Microscope was used to perform the AFM measurements. The measurements were performed in air in tapping mode (Mac Mode) at room temperature. The silicone material cantilevers with a spring constant of 13-77 N/m and radius < 10 nm were used. The images were analysed by the Keysight PicoView and Gwyddion software. All images were taken at 3 X 3 μm surface area.

4.2.9. Anti-adhesive performance

Bacterial adhesion tests were performed according to the methodology previously described [15]. Briefly, the samples were cut to a size of 1 X 1.5 cm and were first cleaned with 1 % detergent solution in an ultrasonic bath. Afterwards, they were immersed in 1 X PBS (phosphate buffer saline, pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) solution with an *E. coli* expressing YadA (an adhesin from *Y. enterocolitica*) with an OD 600 nm of 0.8 for 4 h [15–17]. After incubation, PET samples were removed and washed gently with 1 X PBS buffer to remove unattached bacteria. A fluorescence microscope (Olympus BX51), at 60 X magnification, coupled with an DP71 digital camera, was used to visualise the adhered bacteria with the fluorescence imaging at 470-490 nm excitation and a 520 nm filter in the microscope optical path. The number of bacteria in each sample was counted using ImageJ Software, and the reduction in bacterial adhesion further calculated.

4.3. RESULTS AND DISCUSSION

4.3.1. Pre-treatment of PET substrate

Plasma medium consists of electrons, ions, radicals, and other charged species which can be utilized for surface applications. Polar groups such as amines, carboxylic acids, alcohols, aldehydes and esters can be formed on the polymer surfaces upon plasma treatment, which eventually affect the wettability of the surfaces [2,18] and can be used for further modification. In this study, the plasma operation parameters - power and speed - were found to significantly affect the surface properties modification process, thus requiring optimization. Therefore, the effect of these parameters on PET surface properties were evaluated using the Design of Experiments methodology. A 2² full factorial design was performed, and the water contact angle (WCA), surface energy (SFE), roughness and chemical composition for PET treated samples were measured as response variables (Table 4.2 and Figure 4.1).

Table 4.2. Water contact angles, surface energy, roughness and chemistry values found for the PET plasma treated samples.

Sample	Speed (m/min)	Power (%)	Water Contact Angle (°)	Surface Energy (mJ/m ²)			Surface Roughness (nm)	Surface chemistry	
			Initial WCA	SFE Disperse	SFE Polar	SFE Total	Ra	O/C ratio	O increase (%)
1	4	50	32±1	49 ± 1	23 ± 1	72.5 ± 1	5 ± 2	0.45	40.62
2	4	100	19±4	49 ± 1	28 ± 1	77 ± 1	3.95 ± 0.03	0.46	43.75
3	12	50	37±10	49 ± 1	21 ± 5	70 ± 5	4 ± 1	0.37	15.62
4	12	100	25±4	49 ± 1	26 ± 2	75 ± 2	4 ± 1	0.41	28.12
5	8	75	28±4	49 ± 1	25 ± 2	74 ± 2	5 ± 2	0.37	15.62
6	8	75	28±3	50 ± 1	29 ± 3	79 ± 4	4± 3	0.36	12.5
7	8	75	30±5	49 ± 0.5	24 ± 2	73 ± 2	7± 1	0.4	25
Untreated PET	0	0	70 ± 6	42 ± 3	7 ± 3	49 ± 4	3 ± 1	0.32	0

From Figure 4.1 and Table 4.2, at an initial stage, it was observed that PET surface hydrophilicity created by plasma treatment was not permanent. Figure 4.1 shows the recovery of WCA after storing the samples at room temperature for up to 3 weeks and after washing. With only 5 cycles of washing, the WCA reached the initial values of untreated PET (around 70 °). Additionally, the hydrophilicity loss due to ageing was lower for the sample treated at 100 % of power and at the lowest speed (4 m/min), presenting a value for WCA around 43 ° after 3 weeks of storage. It has been known that plasma treated samples recover their hydrophobic state over time. This recovery is explained by the rotation and reorientation of the polymer chains and polar groups on the surface to reduce the interfacial surface energy [19]. The ageing and washing tests clearly show the samples hydrophobic state recovery. The recovery to the initial contact angle was significantly faster in water, which is consistent with previously reported literature [20].

Furthermore, Table 4.2 shows that the plasma treatment also changed the surface energy properties, and as expected, an overall increase in total surface energy was observed, from 49 ± 4 mJ/m², on untreated PET, to values between 70 and 79 mJ/m², and with polar component of surface energy changing from 7 ± 3 mJ/m², on PET control sample, to values between 21 and 29 mJ/m² on plasma treated samples. The contribution of the polar component of surface energy was the main responsible for this increase, changing from 7 ± 3 mJ/m², on PET control sample, to values between 21 and 29 mJ/m², for the PET treated samples.

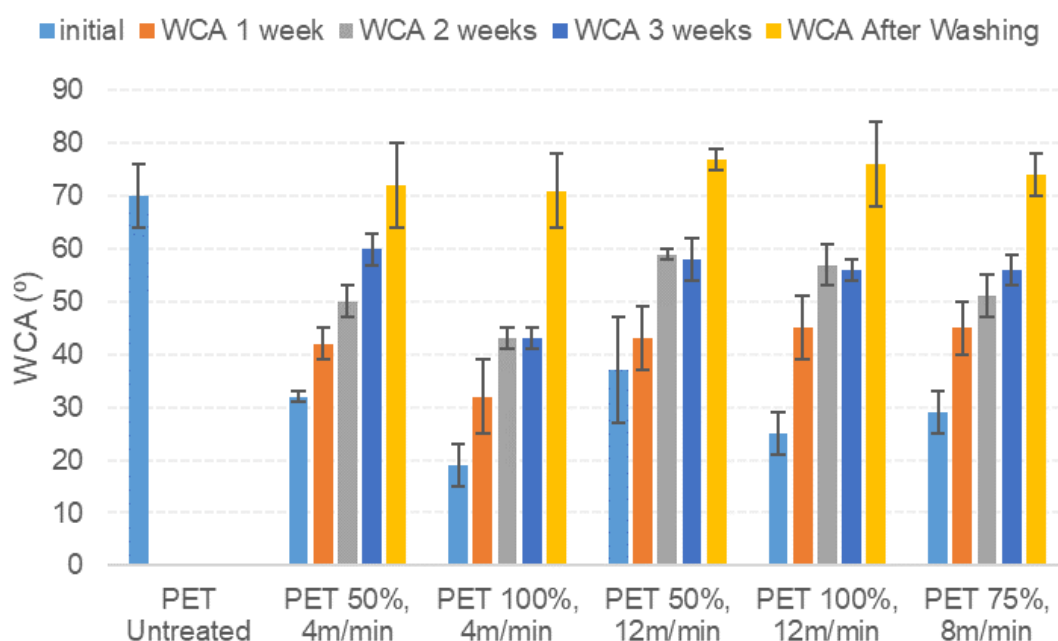


Figure 4.1. Water contact angle values for the PET samples after plasma treatment.

Table 4.3 summarizes the ANOVA statistical analysis for the initial WCA after the treatment, highlighting the significance of both process parameters, power and speed, for this response. The Fischer's test was used to evaluate the model significance. A model F-value of 116.04 implies the model is significant (p-value = 0.0003), i.e., there is only a 0.03 % chance that an F-value this large could occur due to noise.

The analysis also indicates that the main factors speed (A) and power (B) are significant, being the highest impact to the model provided by power with a confidence level of 99.98 % and speed with a confidence level of 99.64%.

Table 4.3. Analysis of Variance (ANOVA) for the linear model obtained for the initial WCA.

Source	Sum of squares	Degree of freedom	Mean Square	F-value	P - value	
Model	186.5	2	93.25	116.04	0.0003	Significant
A-Speed	30.25	1	30.25	37.64	0.0036	
B-Power	156.25	1	156.25	194.44	0.0002	
Residual	3.21	4	0.8036			
Lack of Fit	0.5476	2	0.2738	0.2054	0.8296	not significant
Pure Error	2.67	2	1.33			
Cor Total	189.71	6				

The curvature and residual lack of fit were found to be insignificant, and the proportion of variance explained by the model, given by the multiple coefficients of determination, R^2 was found to be 0.9831, highlighting the model adequacy to predict the WCA in the design space. The obtained final model equation (Eq. 4.1) for the initial WCA after the treatment, in terms of actual factors is:

$$\text{Initial WCA after the treatment} = 41.67857 + 0.6875 * \text{Speed} \left(\frac{\text{m}}{\text{min}}\right) - 0.25 * \text{Power} (\%) \quad (\text{Eq. 4.1})$$

To improve the plasma pre-treatment process, the graphical optimization tool from Design-Expert® was used, aiming at the minimization of the responses found for the WCA (fostering a more hydrophilic surface) and maximisation of the amount of oxygen in comparison to the untreated sample. The upper limits (corresponding to the minimum WCA) were set as 25 ° for the initial WCA after the treatment and 48 ° for the WCA after 2 weeks of ageing and the lower limits (minimum percentage of

oxygen increase) were set as 30 %.

Figure 4.2 shows the overlay plot of factor Power (B) versus Speed (A) for the responses fulfilling these criteria (yellow area), simultaneously. Observing the yellow shaded area, it is possible to conclude that using combined plasma power above 80 % and treatment speed lower than 6 m/min would enable achieving the desired conditions. As such, the study was continued, setting the plasma pre-treatment conditions at the highest power (100 %) and lowest speed (4 m/min), before the UV grafting process.

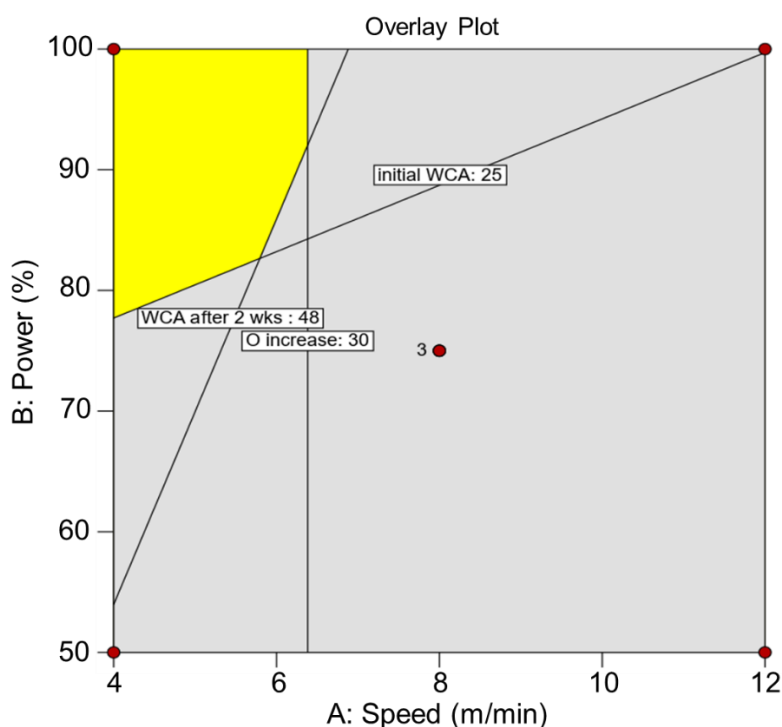


Figure 4.2. Overlay plot of factor Power (B) versus Speed (A).

To reveal the chemical composition of the samples, X-Ray photoemission was used to reveal the chemical composition of the samples. In Table 4.4, the detailed information on carbon and oxygen bonds are listed. The peaks at 285.00, 286.58, 288.99 and 291.53 eV correspond to bonds typical of a benzene ring (C–C, C=C), methylene carbon singly bound to oxygen (–C–O–) and ester carbon atoms (O–C=O), respectively. The peaks at 531.93 and 533.53 eV indicate the carbonyl oxygen (O=C) and singly bonded oxygen atoms in the ester groups (O–C). The chemical changes in treated samples indicated by XPS analysis mainly show a decrease in C=C/C–C and an increase in –C–O–bonds, which is consistent with the expected replacement of hydrogen of aromatic rings by hydroxyl groups [21].

Table 4.4. Relative composition (in percentage) of the different chemical groups detected with the deconvolution of C 1s and O 1s spectra for plasma treated samples.

	C 1s				O 1s		O/C ratio
Peaks deconvolution	C1	C2	C3	C4	O1	O2	
Functional groups	C-C, C=C	-C-O-	O-C=O	-C=C	O=C	O-C	
Binding energy (eV)	285,00	286,58	288,99	291,53	531,93	533,53	
Untreated PET	44.96	15.18	13.12	2.28	11.08	13.03	0.32
PET with 50% power 4m/min speed plasma treatment	37.33	14.25	14.16	3.17	12.74	17.99	0.45
PET with 50% power 12 m/min speed plasma treatment	39.48	15.94	14.18	2.34	10.69	16.12	0.37
PET with 100% power 4 m/min speed plasma treatment	35.29	15.94	14.3	2.27	15.99	15.52	0.46
PET with 100% power 12 m/min speed plasma treatment	38	16.56	14.06	1.95	12.43	16.49	0.41
PET film 75% power 8 m/min speed plasma treatment	43.46	15.09	12.78	1.59	12.93	14.3	0.38

The chemical composition of PET revealed an increase in the O 1s/C 1s ratio from 0.32 to around 0.40 after plasma treatment (Table 4.4), with the highest ratio of 0.46 found for the PET sample treated at 100 % power and with a speed of 4 m/min. The changes in chemical composition with the introduction of these new chemical groups resulted in an increase of hydrophilicity of the polymer surface, as indicated by the results of WCA measurement, which showed a decrease in WCA from $70 \pm 6^\circ$ for PET control to less than 30° for PET-plasma treated samples.

The plasma treatment can also be used to etch away surface molecules and modify the surface topography by increasing roughness, when enough energy is applied [18]. In this way, the adhesion of a coating can be improved by increasing the contact points between the coating and the polymer surface

[6]. The plasma treated, and untreated PET samples were further subjected to AFM analysis to verify if morphological changes occurred in the PET surface topography (Figure 4.3). As can be seen from Figure 4.3, untreated PET surface is slightly smoother (with $R_a = 3$ nm) when compared with the plasma treated samples (R_a values ranging from 4 to 7 nm).

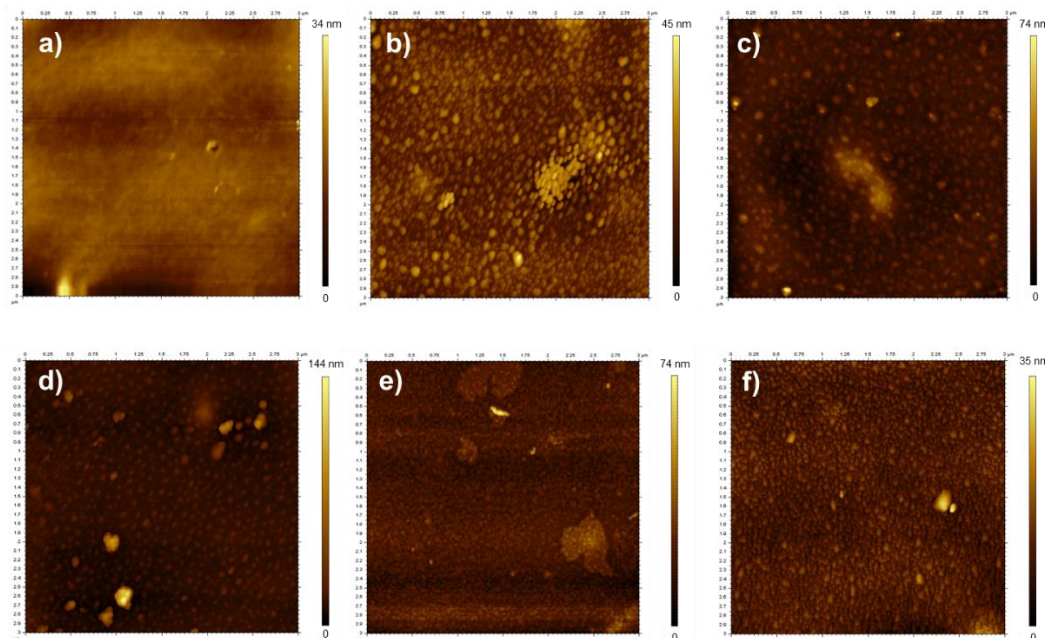


Figure 4.3. Surface topography images from AFM: a) PET Untreated, Plasma treated samples with b) 50 % power and 4 m/min speed, c) 100 % power 4 m/min speed, d) 50 % power 12 m/min speed, e) 100 % power 12 m/min speed, f) 75 % power 8 m/min speed.

4.3.2. Grafting of PET with acrylates

Although plasma treatment was effective at generating local hydrophilicity in PET, the treatment was not permanent and there was a recovery of the surface hydrophobicity over time. To obtain a hydrophilic surface with higher stability, the surfaces can be further modified with acrylates through the formation of active agents' species like peroxides and hydroperoxides to initiate the grafting process [18]. A proposed functionalisation mechanism with plasma treatment and grafting by UV photopolymerisation can be seen in Figure 4.4. From the plasma treatment optimisation experiments, it was seen that the assay performed at the highest power and lowest speed provided the most effective modification. Even though it was not possible to identify the peroxide functional groups generated in this study, several authors have reported an increasing similar trend with increased plasma power and time [18,22]. Thus, the lowest speed (4 m/min) and highest power (100 %) were used to treat the samples with plasma prior to the grafting of sodium acrylate (NaAc) and 3-Sulfopropyl Acrylate Potassium Salt (KAc). It is also important to note according to our experimental study, the plasma modification

procedure with 3 number of passes was shown to be giving stable results for UV photopolymerisation, thus the procedure with 3 number of passes was adopted for further UV polymerisation.

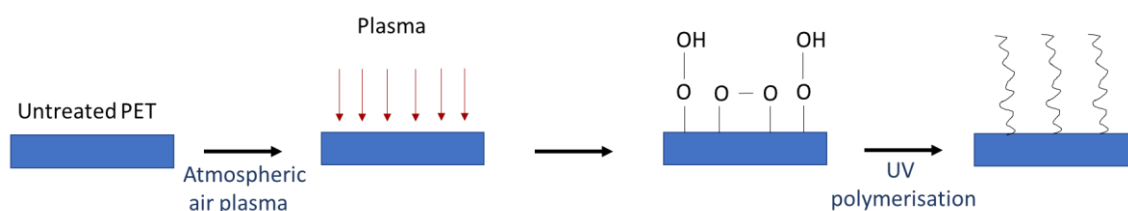


Figure 4.4. Proposed mechanism for atmospheric plasma treatment and UV grafting polymerisation of acrylates on PET surface.

The plasma treated samples were further modified with acrylates under UV light which resulted in a significant WCA reduction, namely to $8 \pm 3^\circ$ (modification with NaAc) making it highly hydrophilic, and to $28 \pm 13^\circ$ (modification with KAc) (Table 4.5). The ageing tests showed that WCA increased to $14 \pm 4^\circ$, $17 \pm 9^\circ$ and $16 \pm 8^\circ$ after 1, 2 and 3 weeks of storage for the NaAc grafted sample, respectively, and it remained around $28\text{--}30^\circ$ for the KAc grafted sample. The washing tests for the NaAc grafted sample suggest that the UV grafting procedure provided stability and durability to surface treatment. However, the hydrophilicity of the KAc treated sample after 10 washing cycles showed a strong variation in the WCA result, as can be seen in Table 4.5, indicating that this acrylate might lead to a lower treatment uniformity. Additionally, total surface energy of the samples increased from $49 \pm 4 \text{ mJ/m}^2$ to $75 \pm 1 \text{ mJ/m}^2$ for NaAc, and to $66 \pm 7 \text{ mJ/m}^2$ for KAc grafted samples, due to high increase in the polar component of surface energy (to $38 \pm 2 \text{ mJ/m}^2$ and $33 \pm 7 \text{ mJ/m}^2$ for NaAc and KAc modified surfaces, respectively).

In order to confirm the effect of plasma pre-treatment in the acrylates grafting enhancement, the same UV grafting process was performed without any prior plasma pre-treatment, No-plasma NaAc grafted PET and No-plasma KAc grafted PET (Table 4.5). Under these conditions, the values found for the WCA were significantly higher for these two samples, thus indicating the positive effect of plasma pre-treatment in the surface modification of PET for preparation to the UV polymerisation procedure with acrylates.

Table 4.5. WCA, surface energy and roughness values of untreated, NaAc treated and KAc treated PET surfaces.

Samples	Initial WCA (°)	WCA After washing 5 cycles (°)	WCA After washing 10 cycles (°)	WCA After 1 week (°)	WCA After 2 weeks (°)	WCA After 3 weeks (°)	Diiodomethane contact angle (°)	SFE Disperse (mJ/m ²)	SFE Polar (mJ/m ²)	SFE Total (mJ/m ²)	Ra (nm)
PET Untreated	70 ± 6	N/A	N/A	N/A	N/A	N/A	34 ± 7	42 ± 3	7 ± 3	49 ± 4	2.7 ± 0.9
NaAc grafted PET	8 ± 3	8 ± 3	14 ± 9	14 ± 4	17 ± 9	16 ± 8	44 ± 5	37 ± 3	38 ± 2	75 ± 1	3 ± 1
KAc grafted PET	28 ± 13	33 ± 10	55 ± 27	30 ± 11	30 ± 8	28 ± 10	52 ± 5	33 ± 3	33 ± 7	66 ± 7	2.4 ± 0.3
No plasma- NaAc grafted PET	39 ± 25	54 ± 12	58 ± 10								
No plasma- KAc grafted PET	23 ± 18	58 ± 16	77 ± 7								

Figure 4.5 shows that the surface topography, as visualized by AFM analysis, of the plasma pre-treated surface has a rougher surface with R_a of 10 ± 2 nm and the structures resembled nanoparticle modified surfaces. Moreover, the surface topography of NaAc and KAc treated surfaces showed a smoother structure, with roughness values 2.62 nm for NaAc and 2.35 nm for KAc like untreated PET sample. The difference in surface nanostructure also corroborates the successful surface modification via photopolymerisation. The unfocused surface appearance of NaAc and KAc treated surfaces indicates that the AFM tip was not effective at displacing polymer brushes and the polymer brushes concealed the surface topography [23].

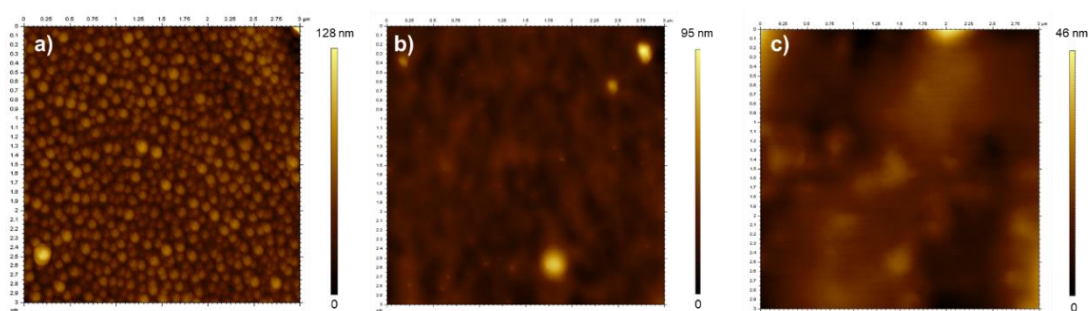


Figure 4.5. Surface topography images obtained by AFM of a) Plasma treated PET with 100 % power, 4 m/min speed, b) NaAc grafted PET, c) KAc grafted PET.

In addition to the structural study, the thickness of the polymeric brushes was measured by UV spectrophotometer using Swanepool method and was found to be 28 ± 2 μm for both NaAc and KAc grafted PET surfaces. This value is thicker than the conventional grafting thickness reported in the literature, and it can expand the uses of this type of polymer brushes [24,25].

4.3.3. PET anti-adhesiveness

Finally, bacterial anti-adhesion studies were performed using *E. coli* expressing YadA which is an adhesin from *Y. enterocolitica* [17]. Bacterial adhesins like YadA are known to promote bacterial adhesion, biofilm formation, and provide resistance against bactericidal compounds [17,26,27]. Adhesins mature the bonding between bacteria and the substrate leading to irreversible adhesion [26] thus, it is important to study bacteria with such highly bonding adhesins. The bacterial adhesion onto the untreated PET and NaAc and KAc treated PET samples can be seen in Figure 4.6. It is evident that the NaAc and KAc treated surfaces successfully prevented bacterial adhesion as compared to untreated PET surface. In order to quantify the effect of these modifications on bacterial adhesion, data gathered in Figure 4.6 was further analysed using the ImageJ® software. The total number of cells attached to

the surfaces were counted and used to calculate the reduction in bacterial adhesion. The bacterial adhesion was reduced by 90.8 % in NaAc grafted PET and more than 99.9 % in KAc graft PET surfaces. The anti-adhesive behaviour of these type of surfaces are thought to be due to higher hydrophilicity and surface energy [7,15]. In this way, a hydration layer is formed on the top of the surface, making it hard for bacteria to approach [28]. According to a recent review, this reduction can be considered highly effective compared to other modifications on PET substrate [2].

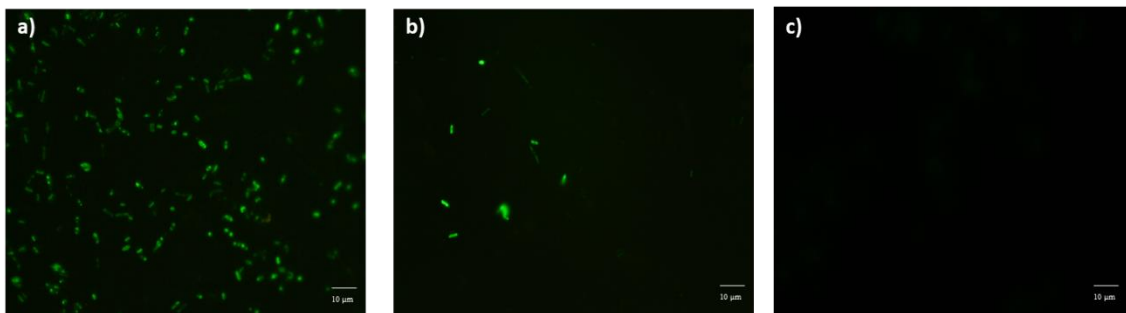


Figure 4.6. Fluorescence microscopy images of *Escherichia coli* BL21 cells harboring pRSFduet_GFP and pASK_IBA2_YadA plasmids a) untreated PET sample b) NaAc treated PET Sample and c) KAc treated PET sample.

4.4. CONCLUSION AND OUTLOOK

Plasma and UV treatments are cost-effective, sustainable and environmentally friendly processes, and the combination of the two can avoid the use of the photoinitiator needed for UV polymerisation. Furthermore, the use of atmospheric plasma can increase the efficiency of an industrial applications. Statistical analysis on plasma treatment parameters showed that power and speed were significant variables influencing the surface WCA, and the highest power and lower speed should be used to increase the plasmatic effect on PET surface.

Using these conditions, plasma pre-treated PET surfaces were successfully modified with NaAc and KAc, creating hydrophilic surfaces with WCA less than 10° for the NaAc grafted samples. The studies on samples washing and ageing showed that the NaAc treated samples hold a durable modification under the tested conditions, while KAc treated samples exhibit some surface heterogeneity.

Finally, the bacterial anti-adhesive performance of treated surfaces was evaluated with *E. coli* expressing YadA and both modified surfaces were able to inhibit the bacterial adhesion by more than 90 %. The combination of these two technologies can bring several benefits to the modification of conventionally hydrophobic substrates, besides being easily industrialised. Furthermore, achieving surfaces with grafting thicknesses at the level of micrometres can have some other potential uses such

as implants, reservoirs to release therapeutic agents, higher membrane selectivity and sensor selectivity and reduced friction.

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CHAPTER 5

Can superhydrophobic PET surfaces prevent bacterial adhesion?

Prevention of bacterial adhesion is a way to reduce and/or avoid biofilm formation, thus hindering its associated infections. The development of repellent anti-adhesive surfaces, such as superhydrophobic surfaces, can be a strategy to avoid bacterial adhesion. In this study, a polyethylene terephthalate (PET) film was modified by *in situ* growth of silica nanoparticles (NPs) to create a rough surface. The surface was further modified with fluorinated carbon chains to increase its hydrophobicity. The modified PET surfaces presented a pronounced superhydrophobic character, showing a water contact angle of 156 ° and a roughness of 104 nm (a considerable increase comparing with the 69 ° and 4.8 nm obtained for the untreated PET). Scanning Electron Microscopy was used to evaluate the modified surfaces morphology, further confirming its successful modification with nanoparticles. Additionally, a bacterial adhesion assay using an *Escherichia coli* expressing YadA, an adhesive protein from *Yersinia* so-called *Yersinia* adhesin A, was used to assess the anti-adhesive potential of the modified PET. Contrarily to what was expected, adhesion of *E. coli* YadA was found to increase on the modified PET surfaces exhibiting a clear preference for the crevices. This study highlights the role of material micro topography as an important attribute when considering bacterial adhesion.

Keywords: Nanoparticles; Surface modification; Polyethylene terephthalate; Superhydrophobic; Bacterial Adhesion

This chapter is based on the following publication:

Caykara T, Silva J, Fernandes S, Braga A, Rodrigues J, Rodrigues LR, Silva C (2022). Can superhydrophobic surfaces prevent bacterial adhesion? (to be submitted)

5.1. INTRODUCTION

Bacterial adhesion and subsequent biofilm formation creates several risks and challenges in both healthcare and industrial applications [1,2]. Some of the approaches to prevent bacterial adhesion include, for instance, leaching biocides from surfaces which kill both the adhered and nearby bacteria. However, there is a growing concern regarding the increased use of biocides as it can cause increased antimicrobial resistance, therefore its use must be limited [1]. On the other hand, superhydrophobic surfaces have been gaining attention due to their self-cleaning and anti-adhesive properties. Superhydrophobic surfaces are defined as surfaces with a water contact angle higher than 150 °. These surfaces combine both low surface energy and nanopatterned structures. Several authors have reported a decreased bacterial adhesion on such surfaces due to the formation of solid-air-liquid interfaces i.e., air pockets between bacteria and the surface [3] that reduce adhesion by preventing bacteria to approach the surface [1] and provide an easy removal of bacteria as the result of a weaker binding at the interface [4].

Since early 2000s after the discovery of hierarchical micro/nanostructure of the Lotus leaf [5,6] and with the development of the technology, interest in superhydrophobic surfaces has increased and this has also boosted the search for other superhydrophobic surfaces in nature, such as those existing in water strider legs, mosquito eyes, rice leaves, butterfly wings and gecko feet [7]. Different methodologies like plasma, etching, electrospinning, sol-gel, self-assembly, layer-by-layer, surface initiated radical polymerization and others can be used to develop superhydrophobic surfaces [7]. Particularly, the sol-gel method offers cheap and versatile fabrication techniques without the need of a special equipment [8] and thus, it has attracted significant attention. This technique includes solution gel transition by hydrolysis and condensation with acidic/basic catalysis [9]. In the study by Zhang *et al.* [10], aminopropyltriethoxysilane was used, via Stöber method, to form micro/nanohierarchical structures with 1H,1H,2H,2H-perfluorooctyltriethoxysilane to achieve low surface energy. The silica sol was then coated on glass slides with silicone resin which resulted in a water contact angle of 168 °. The modified surface further showed a significantly low adhesion to *Navicula pelliculosa*.

The most common low surface energy molecules used to form superhydrophobic surfaces include fluorocarbon chains such as trifluoropropyl [11], polytetrafluoroethylene [12] and 1H, 1H, 2H, 2H-perfluorodecanethiol [13]. In the study by John *et al.* [14], nanoimprint lithography with a low surface energy molecule, 1H,1H,2H,2H-perfluorodecyl acrylate was used to form superhydrophobic surfaces which conferred the surface anti-biofouling properties against *E. coli*. Although a fluorocarbon chain is a common choice, superhydrophobic surfaces have also been formed by other means. For

example, in the study by Zhao *et al.* [15], aminated polydimethyl siloxane was used with epoxy resin cured with isocyanate crosslinker. A bacterial reduction of 79 % was observed with the modified samples. In another study, Han *et al.* [16] studied silicone dioxide (SiO_2) and titanium dioxide (TiO_2) NPs deposited on a surface which was pre-modified with mussels' adhesive protein, and the modified material showed excellent anti-fouling properties. Nanoparticles formed via sol-gel method are generally applied as coating on surfaces via solvent evaporation [17], dip coating/padding [18,19], spray coating [20] and others. However, the superhydrophobic surfaces still lack durability and more studies need to be conducted to improve the longevity of the material properties [1,8].

Several studies on the modification of material surfaces with NPs proved to be effective at minimizing the contact between the liquid and solid surfaces. However, it is still challenging to create stable and durable superhydrophobic surfaces and most of the methods being used do not include chemical attachment of the NPs to the surface, hence making it less durable [1]. An alternative method to increase its durability can be the *in situ* growth of NPs on the surface due to presence of covalent bond between nanoparticles and substrate [21]. Polyethylene terephthalate (PET) has been widely used in biomedical studies. Thus, in this work, polyethylene terephthalate surfaces were modified with *in situ* growth of silica NPs to increase the durability of the coating through an interfacial bonding [9,21], and the obtained surfaces were characterized by contact angle, atomic force microscopy, scanning electron microscopy and dynamic light scattering.

5.2. MATERIALS AND METHODS

5.2.1. Materials

PET film (Melinex 401 CW / 100 μm) was acquired from Putz Folien. Dynasylan F 8261 (FAS13: triethoxy(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-1-octyl)silane) was purchased from Evonik. Nitric acid (70 %) and ammonia (30 %) were from Labkem; tetraethyl ortosilicate (TEOS, 98 %) and diodomethane (99 %) were purchased from Sigma-Aldrich; hydrochloric acid (37 %) was from Fisher Chemicals; ethyl alcohol (99.8 %) from Aga; sodium hydroxide from Eka; sodium chloride from Nzytech; potassium chloride from ChemLab; sodium phosphate from Scharlau; and potassium phosphate monobasic from Panreac.

5.2.2. Pre-treatment

In order to clean the surfaces prior to functionalization, 3 X 6 cm sized PET films were immersed in ethyl alcohol for 15 min. Afterwards, the PET films were rinsed with ethyl alcohol and left to dry at room temperature. For the hydrolysis of PET surface, the alkali method previously reported [22,23] was used. Briefly, PET films were immersed in NaOH (4 M) at 70 °C under constant shaking at 100 rpm for 2.5 h and then were washed with HCl (1 M) until neutral pH.

5.2.3. *In situ* nanoparticle (NP) modification of PET surfaces

PET films were immersed in a solution of 332 mL of ethanol, 90 mL of ultrapure water and 100 μ l nitric acid (as provided, 70%) after being heated to 75 °C. Soon after the PET films were immersed, 5 ml TEOS was poured into the solution under stirring. After 7 min, 32 mL of ammonia and 20 ml TEOS were added with a flow rate of 30 ml/min. After 2.5 h under constant stirring, 3 ml of FAS13 was added to the solution and left to react for 2.5 h. After that time, the sample was removed, rinsed with ethanol, and ultrasonicated for 10 min in ethanol. Finally, the sample was rinsed with distilled water and put in the oven to dry for 30 min at 80 °C. This experimental procedure for the *in situ* NPs formation was adapted from Chen *et al.* [24].

5.2.4. Water contact angle

Static contact angle measurements using the Optical Tensiometer Attension Model Theta Basic by Biolin Scientific were performed at room temperature. For the water contact angle (WCA) measurement, MiliQ water with a droplet size of 3 μ L was used. The measurements were repeated at least 4 times for each sample at different surface locations. Contact angle measurements were also performed with diiodomethane to calculate the polar and disperse components of surface energy by Owens-Wendt-Rabel & Kaelble Model (OWRK) equation [25].

5.2.5. Atomic force microscopy (AFM)

Atomic Force Microscopy (AFM) measurements were performed by a Keysight Technologies 5500 Atomic Force Microscope. The tapping mode (Mac Mode) in air at room temperature was used. The cantilever material was silicone with a spring constant of 13-77 N/m and radius < 10 nm. Keysight PicoView and Gwyddion software were used to analyze the images, with a surface area of 5x5 μ m.

5.2.6. Scanning electron microscopy (SEM) / Energy dispersive spectroscopy (EDS)

The morphological and chemical analyses of the samples were performed at the Materials Characterization Services Laboratory of the University of Minho (SEMAT-UM), using a Scanning Electron Microscopy (SEM) NanoSEM - FEI Nova 200 (FEG/SEM). Before the morphological analysis, the samples were coated with a thin film of Au/Pd (80/20 by weight) 10 nm thick. For the morphological analysis, a secondary electron detector with 10 KeV energy and a working distance between 7 and 8 mm, were used. For the chemical analysis, energy dispersive spectroscopy (EDS) technique was performed by an Integrated System EDAX - Pegasus X4M, using 15 KeV energy, at a working distance of approximately 6.5 mm.

5.2.7. Dynamic light scattering

The size and zeta potential of functionalized NPs were characterized by Dynamic Light Scattering (DLS) using a ZETASIZER NANO-ZS90 (Malvern Parolab) with appropriate software. Dispersions of 3 mg of NPs in 10 ml of ethanol were prepared at room temperature by collecting NPs from three different batches with three replications.

5.2.8. Bacterial adhesion

Bacterial adhesion tests were performed according to the methodology previously described [26]. Briefly, 1 X 1.5 cm sized samples were first cleaned by immersing in 1 % detergent solution in ultrasonic bath. The samples were immersed in 1 X PBS (phosphate buffer saline, pH 7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄) solution with *Escherichia coli* expressing YadA [26,27] with an OD 600 nm of 0.8 for 1 h, 4 h and 6 h. Afterwards, PET samples were removed and washed gently with 1X PBS buffer to remove the unattached bacteria. A fluorescence microscope (Olympus BX51), at 60X magnification, coupled with an DP71 digital camera were used to visualise the adhered bacteria with the fluorescence imaging at 470-490 nm excitation and a 520 nm filter in the microscope optical path.

5.3. RESULTS AND DISCUSSION

5.3.1. Surface modification and reaction mechanism

The modification of polymeric surfaces starts with an initial pre-treatment since most polymer surfaces have good chemical resistance [29]. Like other polymers, PET surface is also quite inert. Nevertheless, different techniques can be applied to introduce functional groups onto the PET surface. Hydrolysis is one of the functionalization methods used [30] to generate hydroxyl groups at the surface [22] for further reaction with the silanol groups present, for instance, on the herein prepared NPs [31]. Furthermore, reaction of organosilanes can be obtained in both acidic and basic reaction conditions, where silanes first go through hydrolysis to form silanol groups and then a covalent bond is established with hydroxyl groups on the surface of the substrate by condensation [31]. In acidic solutions, the rate of hydrolysis increases causing stable silanol groups in the solution, whereas basic conditions can catalyze both hydrolysis and self-condensation [32]. Therefore, acidic conditions were first used to allow the reaction of silanes with the PET surface, followed by an exposure to basic conditions where polycondensation and NPs formation on the surface occurred [24]. Finally, a fluoroalkylsilane was added to the solution in order to functionalize the formed nanoparticles and obtain a surface with low surface energy. The proposed reaction mechanism is illustrated in Figure 5.1.

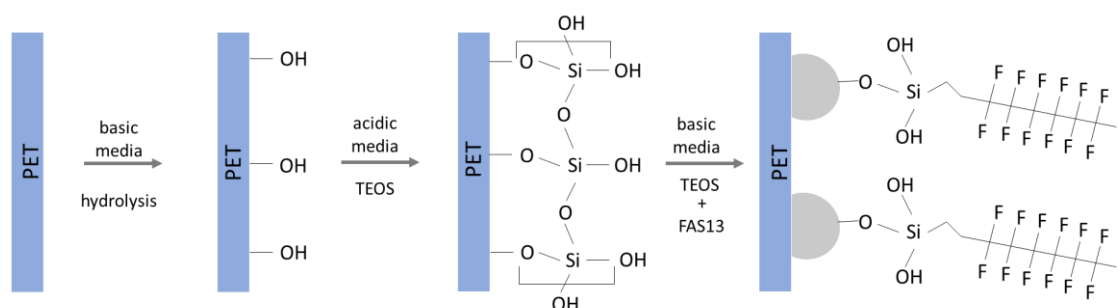


Figure 5.1. Reaction mechanism proposed for the in-situ growth of fluorinated silica NPs on hydrolysed PET.

5.3.2. Water contact angle and surface energy

The surface's hydrophobicity of a material can be increased using low surface energy molecules including methylated and fluorinated carbons. These groups can be ordered by decreasing surface energy as follows: $-\text{CH}_2- > -\text{CH}_3 > -\text{CF}_2- > -\text{CF}_2\text{H} > -\text{CF}_3$. However, the surface modification with the lowest surface energy molecules led to WCA of 119° on a smooth surface [1]. Therefore, it is not possible to create superhydrophobic surfaces using only low surface energy molecules, therefore some surface roughness is also required. Thus, the fluorinated NPs treated surfaces prepared in this work comprise a

promising approach that can be used to achieve superhydrophobic surfaces. The change in the surface's energy due to the chemical modification was assessed by measuring samples WCA with water and diiodomethane. The obtained results showed that untreated PET surfaces exhibited a WCA of $69 \pm 10^\circ$, while hydrolysed surfaces possessed a WCA of $62 \pm 6^\circ$. The WCA value found after hydrolysis agreed with other studies [22,23]. For the NPs treated surfaces (PET NP), the measured WCA was $156 \pm 12^\circ$. In addition, this surface property was maintained after some ageing of the sample, when the WCA was found to be $157 \pm 10^\circ$ after 2 weeks and $151 \pm 14^\circ$ after 3 weeks of storage. Furthermore, the samples were washed with water and ethanol to evaluate the durability of the treatment under these conditions. After 10 cycles of washing with water, where each cycle consisted of immersing the sample in water for 30 min at 40°C , 100 rpm, the WCA was $160 \pm 9^\circ$, and after 10 additional cycles of washing with ethanol the WCA was $157 \pm 10^\circ$, thus showing that the functionalized PET NP samples possessed a resistant superhydrophobic character under the tested conditions.

Table 5.1. Contact angle values determined with water and diiodomethane, and surface free energy (SFE) of PET untreated, PET Hydrolysed and PET NP.

Samples	Water Contact Angle ($^\circ$)	Diiodomethane Contact Angle ($^\circ$)	SFE Disperse mJ/m^2	SFE Polar mJ/m^2	SFE Total mJ/m^2
PET untreated	69 ± 10	35 ± 4	42.0 ± 1.8	7.2 ± 4.0	49.2 ± 4.2
PET hydrolysed	62 ± 6	35 ± 3	41.9 ± 1.5	10.8 ± 3.1	52.7 ± 3.7
PET NP	156 ± 12	114 ± 14	1.3 ± 1.2	3.3 ± 5.7	6.7 ± 4.3

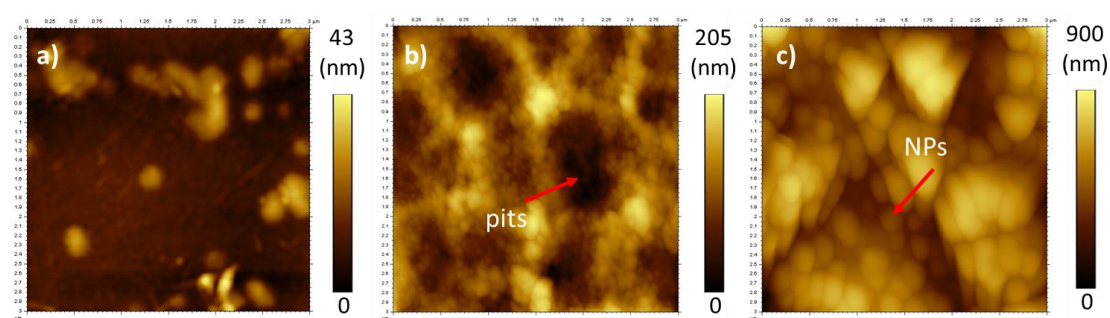
5.3.3. Atomic force microscopy (AFM) and surface roughness

It is well known that surface roughness is necessary to achieve superhydrophobic surfaces [1]. In Table 5.2, surface roughness values for untreated and treated PET determined by AFM technique are summarized. Untreated PET surfaces presented a smoother surface topography with a Ra value of 4.8 ± 1.0 nm when compared to hydrolysed PET (27.8 ± 2.0 nm) and to NPs treated PET (104 ± 20 nm). This last one was considerably rougher, being this an important aspect for the transitioning of the surfaces to the superhydrophobic state [33].

Table 5.2. Surface roughness values (Ra) of treated and untreated PET samples determined by AFM.

Samples	Roughness, Ra (nm)
PET untreated	5 ± 1
PET hydrolysed	28 ± 2
PET NP	104 ± 20

When the surfaces were visualized by AFM, the topography of hydrolysed PET was found to be an irregular granular structure with pits on the film surface, whereas the NPs modified PET presented the typical appearance of NP like structures (Figure 5.2). Interestingly, the hydrolysed surfaces also present small granular like structures on the surface intercalated with pits which are created from the etching process, and these can be better identified by SEM images (Figure 5.3, e)). Therefore, the topography of PET surfaces was further studied by SEM analysis to confirm the presence of these structures.

**Figure 5.2.** AFM images of PET surfaces– a) PET untreated, b) PET hydrolysed, c) PET NP.

5.3.4. Scanning electron microscopy (SEM) / Energy dispersive spectroscopy (EDS) and dynamic light scattering (DLS) measurements

SEM/EDS analysis was performed to characterize the surfaces morphology and chemistry. The morphological changes on both hydrolysed and NPs modified samples at different magnifications can be seen in Figure 5.3. Hydrolysed PET shows signs of etching, which was expected due to breakage of ester groups on the surface of the PET film into hydroxyl and carboxyl groups [22]. It is well known that etching of PET surface depends on the material characteristics like crystallinity and thickness. During hydrolysis, amorphous regions erode faster than crystalline regions leaving a non-uniform surface structure after short hydrolysing times [23]. The formed hydroxyl groups on the PET surface are the probable location where the covalent bonds with TEOS will be established, and that will lead to the subsequent formation of the NPs on the surface, which can be seen for instance in Figure 5.3 i).

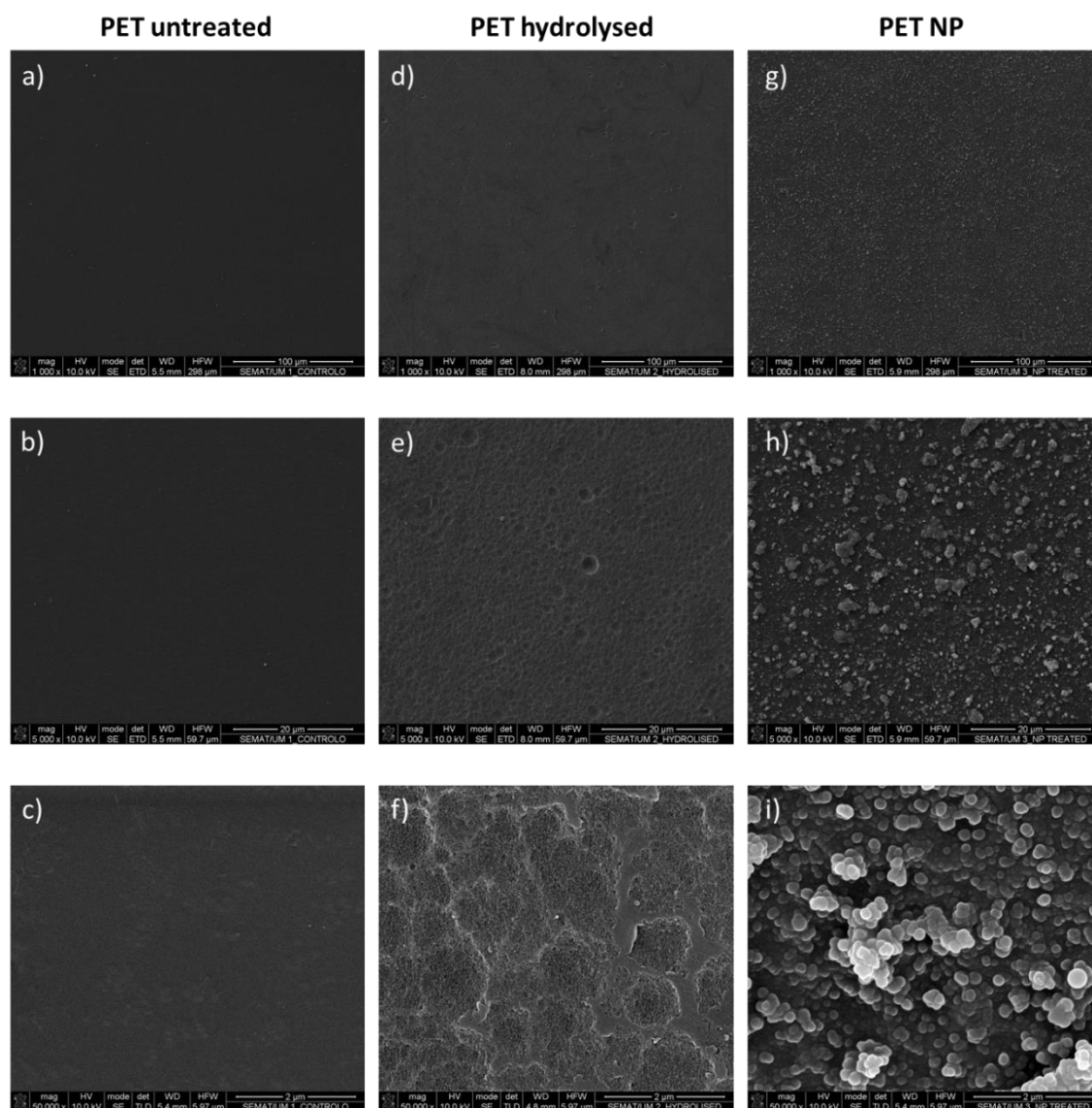


Figure 5.3. SEM images of untreated (a), b), c)), hydrolysed (d), e), f)) and NP modified (g), h), i)) PET samples, at different magnifications (1000 X, 5000 X and 50000 X respectively).

Furthermore, the cross-section of PET NP (Figure 5.4) shows that polycondensation resulted in a clear layer of silica NPs with a thickness around 711 nm on the top of the PET substrate. The growth of the layer thickness is probably due to continuous polycondensation on the surface. In Figure 5.4, the NPs on the surface presented a heterogeneous dimension, with values that vary from 151 nm to 326 nm and with many smaller ones.

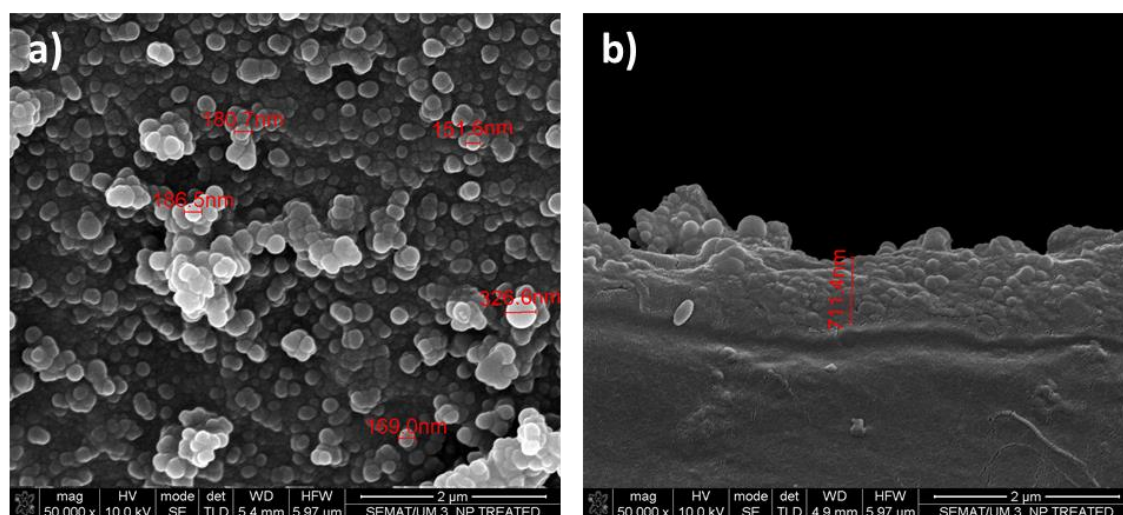


Figure 5.4. SEM images of PET NP modified surface a) top view b) cross-section view at a magnification of 50000 X.

The NPs formed during the surface modification process and that remained in the solution, were also analyzed by DLS to determine their size and zeta potential. The average NP size in the solution was 366 ± 26 nm with a polydispersity index (Pdl) of 0.27 ± 0.13 . Pdl is used to describe the particle size distribution [34] and a value lower than 0.1 is indicative of uniformity [34]. While a Pdl between 0.03 and 0.06 indicates a monodisperse solution [35], a Pdl higher than 0.5 suggests a wide range of particle size distribution [34]. The differences in the NPs size between the surface and the solution herein observed (Figure 5.4) might be due to growth of the layer on the surface and continuous polycondensation which is embedding some nanoparticles while allowing the formation of new ones. Moreover, the DLS analysis provided a zeta potential of -34 ± 2 mV, indicating a stable NPs solution since a zeta potential around -30 mV can be considered a moderately stable solution [36].

In Figure 5.5, the PET surface morphology after 10 cycles of washing with water followed by 10 cycles of washing with ethanol can be observed. Although at lower magnification, washed and unwashed samples seem to have similar features, at higher magnifications it is possible to visualize a NPs free area in the NPs layer on the PET surface (Figure 5.5, b) which indicates that parts of the coating layer were removed from the surface due to washing. Additionally, by observing the sample cross-section, it can be observed that the layer thickness is lower after washing (Figure 5.5, c), which can be explained by the loss of the outermost NPs of the coating layer. It is also important to note that the sizes of the NPs seem to be smaller after washing. Although covalent bonding of the NPs coating layer to the PET surface was expected, it is possible that some NPs were only adhered or were weakly bonded to the surface, or even were only trapped in the network of bonds formed during

polycondensation. Nevertheless, the removal of these fluoroalkylsilane functionalized NPs layer suggests that the treated surfaces have limited durability, even though they continue to provide superhydrophobic surface properties after the washing cycles performed with water and ethanol, as previously shown with WCA measurements. These results strengthen the importance of conducting additional testing such as abrasion when determining the durability of these of surface treatments.

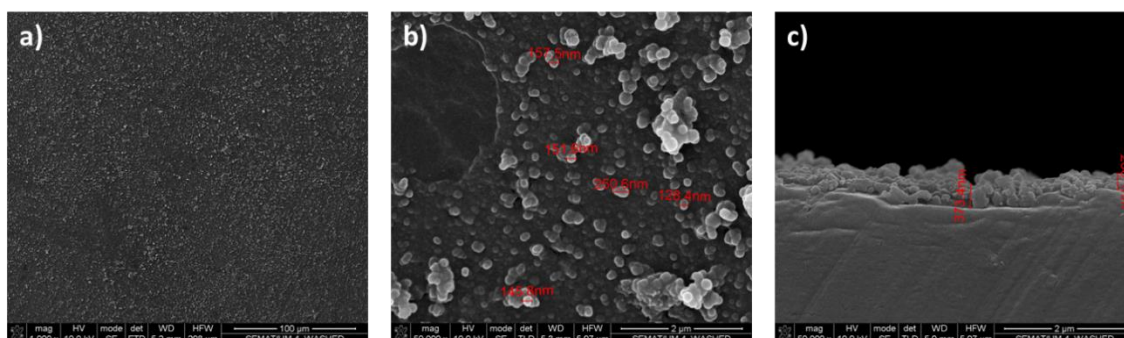


Figure 5.5. SEM images of PET NP modified surface after 10 cycles of water and ethanol washing: a) top view - 1000 X; b) top view - 50000 X and c) cross-section view - 50000 X.

The treated and untreated samples were further analyzed by EDS to determine the chemical composition of the surfaces (Table 5.3). The elemental analysis of samples showed that untreated and hydrolysed surfaces were mainly constituted by carbon and oxygen atoms, while the NPs treated surfaces, including washed samples, also possessed silicon and fluorine in its composition, thus confirming the modification of the surfaces with TEOS and FAS13.

Table 5.3. SEM-EDS results for atomic concentrations of untreated and treated PET samples.

Samples / elements (% atomic)	C	O	Si	F
PET untreated	86	14	n.d.	n.d.
PET hydrolysed	85	15	n.d.	n.d.
PET NP	62	28	8	2
PET NP after 10 cycles of washing with water and ethanol	62	27	7	4

n.d.: not detected

5.3.5. Bacterial adhesion tests

Finally, the performance of the developed modified PET films was evaluated regarding its bacterial anti-adhesion properties. For this purpose, a heterologous *E. coli* expressing the adhesin YadA was used similarly to previous reports [26] and as described in the previous chapters. This protein was cloned into a plasmid containing a reporter protein (GFP) to enable its visualization through fluorescence microscopy. Therefore, it was possible to observe the cells binding to the tested materials. The anti-adhesive mechanism of superhydrophobic surfaces depends on the formation of air pockets on the surface that impair the contact between the bacteria and the surfaces. However, the entrapped air from the air pockets will eventually be replaced over time by the immersion of the solution, potentially leading to an increased adhesion [1,37]. Thus, the duration of the experimental setup to assess adhesion can be an important parameter. For this reason, the untreated and fluorinated-NPs treated samples were incubated with the bacterial solution for 1 h, 4 h and 6 h. Figure 5.6 illustrates the fluorescence microscopy images captured for the different adhesion time periods.

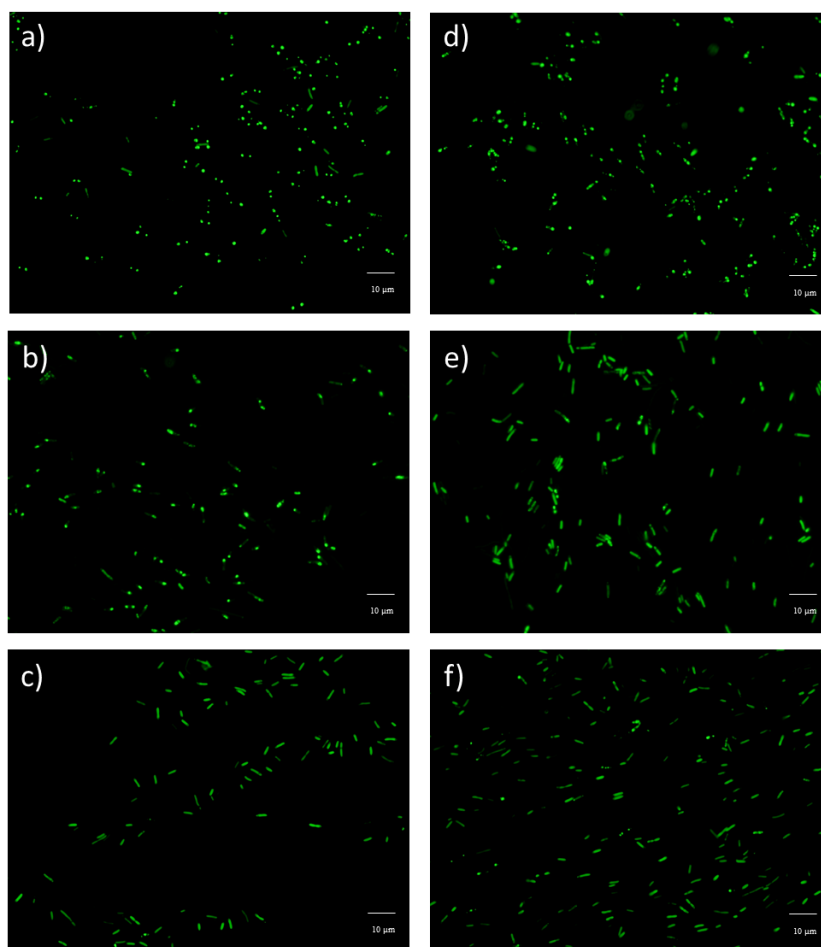


Figure 5.6. Fluorescence microscopy images of PET samples after incubation with bacteria: a) PET untreated after 1 h; d) PET NP after 1 h; b) untreated PET after 4 h; e) PET NP after 4 h; c) untreated PET after 6 h; f) PET NP after 6h. Scale bar, 10 μm .

From Figure 5.6, it seems that after incubation with bacteria for different time periods, the modified PET samples do not seem to show anti-adhesive properties, when compared to the untreated materials using the same incubation times. This result differs from other studies that reported a decreased bacterial adhesion when testing other superhydrophobic materials [38–40]. In our study, the PET modified materials were found to promote the bacterial adhesion over time. In order to better understand these results, the samples immersed in bacterial solution for 4 h were further characterized by SEM, where adhered bacteria on top of the treated surface can be observed (Figure 5.7). In Figure 5.7, c), bacteria seem to position itself in the irregularities where the surface area available for cell adhesion is high. This behavior has also been reported for other superhydrophobic materials where surface irregularities were also found to play a role in promoting bacterial adhesion [41]. In addition, the bacterial cell structure is known to affect the adhesion of *E. coli* that holds a thinner peptidoglycan layer, making the bacterial cell more flexible when adhering on rough surfaces [42]. Besides, in this study the *E. coli* is expressing the YadA adhesin which can be helpful when maturing the adhesion [43]. Furthermore, *E. coli* can explore the local topography through flagella which can improve its adhesion on surfaces with microtopographies [44,45]. Moreover, although it was not evaluated in this study, it is possible that the surface wetting transition from *Cassie-Baxter* to *Wenzel* state have been displacing air pockets and causing an increased adhesion [46]. Thus, altogether these factors that confer the bacterial cell the ability to fit irregularities in the modified surface, can explain the increased adhesion that was herein observed. Also, microtopography and nanotopography can be considered crucial factors at governing bacterial adhesion in this study.

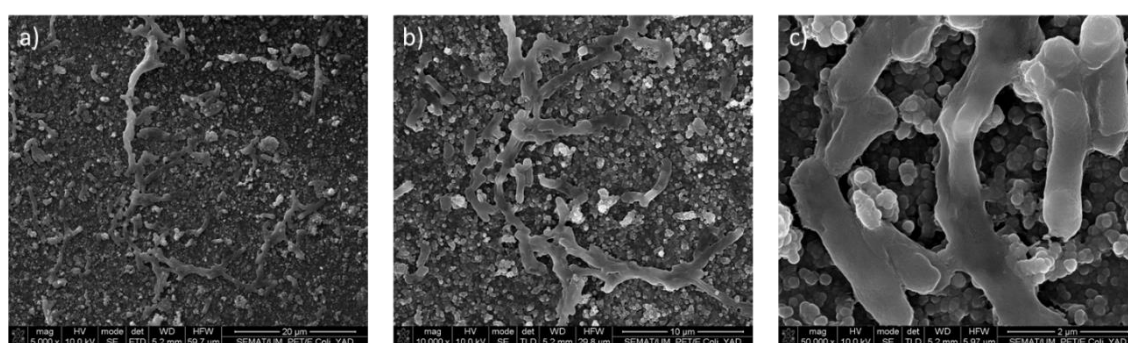


Figure 5.7. SEM visualization of NPs treated samples with different magnifications a) 5 000 X b) 10 000 X and c) 50 000 X, after the bacterial adhesion test (4 h).

5.4. CONCLUSION AND OUTLOOK

Superhydrophobic materials have been shown to possess bacterial repellent properties by several authors. *In situ* growth of NPs on the PET surfaces was studied to create a durable superhydrophobic surface. The WCA of treated surfaces showed a significant increase (226 %) to a value of $156 \pm 12^\circ$, when compared to untreated PET ($69 \pm 10^\circ$). Surface roughness increased to 104 ± 21 nm from the initial 5 ± 1 nm value. This surface modification was further characterised by SEM/EDS showing that the surfaces were properly modified with NPs. Although the samples washing with water and ethanol removed some of the bounded NPs from the material surface, the WCA was still high after the performed washing cycles with water ($160 \pm 9^\circ$) and additional washing cycles with ethanol ($157 \pm 10^\circ$), hence indicating that the superhydrophobicity of the surface was maintained in the tested conditions. These results demonstrated that the functionalized surfaces have some degree of durability. Furthermore, bacterial adhesion onto the treated surfaces was evaluated and it was shown that these surfaces promoted bacterial adhesion, contrarily to other reports. This could be explained by the increased bacterial contact within the surface microtopographies, suggesting that surface topography and roughness are crucial factors at determining the bacterial adhesion outcome of a given material.

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CHAPTER 6

Preliminary Application of Modified PET in Urinary Catheters

Encrustation is a major problem in urinary catheters. Most of the encrustation is caused by the increase in pH due to urease producing bacteria, thus anti-adhesive and anti-bacterial properties of the materials used in urinary catheters play a crucial role in reducing encrustation. Moreover, encrustation on materials causes blockage, leading to damage in kidneys, bladder and ureters, thus highlighting the relevance of the material resistance to the adhesion of such crystals to prevent these damages. In this study, the materials developed in the previous chapters, namely gum Arabic modified PET (Chapter 3), polyacrylate modified PET (Chapter 4) and nanoparticle modified PET (Chapter 5) were evaluated regarding their performance against crystal formation under an artificial urine solution flow in an artificial bladder. The preliminary results showed that all the modified materials promoted crystal formation on their surfaces and no significant differences between hydrophilic and hydrophobic surfaces were found. Although the samples promoted crystal formation, their anti-adhesive properties are still an important characteristic, and they could still be beneficial for the short-term catheters use in real applications.

Keywords: Surface modification; Urinary catheters; Polyethylene terephthalate; Bacterial Adhesion; Anti-adhesive materials

6.1. INTRODUCTION

Catheters offer a simple method for internal drainage of urinary tract. However, they provide an ideal place for bacterial colonization and biofilm formation, leading to infection and even death [1]. Urinary infections account for 30 – 40 % of total hospital associated infections (HAI) worldwide and urinary catheters exhibit the most relevant infection rate among device-related infections with 80 % in urinary infections [2]. Hence, several surface modification strategies have been developed for urinary implants in an attempt to deal with this problem. The use of catheters can be for short term (up to 7 days) or long term (28 days or longer) [3]. Although urinary catheters can be utilized for up to 12 weeks, it is not common due to encrustation and bacterial infection which can block catheters and cause several complications, ranging from mild encrustation and bladder stones to severe septicemia, endotoxic shock and pyelonephritis [4].

Encrustation occurs due to urease producing bacteria like *P. mirabilis*, *P. vulgaris* and *P. rettgeri*. Urease hydrolyses urea in urine into ammonia, which results in increased pH above 8 causing magnesium and calcium phosphate to crystallize on the catheter surface. Encrustation can cause blockage of the catheter leading to bladder, ureter, and kidney damage [4].

The two main problems reported in urinary catheters are the bacterial adhesion and encrustation [4]. Modifying the surface of a material to render it more hydrophobic and hydrophilic are among the strategies to deal with catheter-related problems [3,5,6]. Therefore, in the present study we aimed to investigate the potential usage of the developed hydrophilic and hydrophobic materials for urinary catheters. The performance of the modified PET samples against bacterial adhesion was shown in the previous chapters. In this chapter, the modified PET samples (Chapters 3 -5) were further tested using an artificial bladder model with artificial urine, to evaluate their performance against encrustation and crystal formation.

6.2. MATERIALS AND METHODS

6.2.1. Materials

PET film (Mylar® A with thickness of 36 μ) was acquired from Isovolta Group to be used in gum Arabic and polyacrylate modification. Polyethylene terephthalate (PET) film (Melinex 401 CW / 100 μ m) was acquired from Putz Folien for nanoparticle modification.

Glutaraldehyde (50 % solution), spray dried gum Arabic (Acacia tree), ethylene diamine (≥ 99 %) and diodomethane (99 %) were purchased from Sigma-Aldrich; hydrochloric acid (37 %) from Fisher

Chemicals; ethylene alcohol (99.5 %) from Aga.

Sodium Acrylate (97 %) and diodomethane (99 %) were purchased from Sigma-Aldrich; 3-Sulfopropyl Acrylate Potassium Salt (>98 %) was purchased from TCI Chemicals for polyacrylate modification.

Dynasylan F 8261 (triethoxy(3,3,4,4,5,5,6,6,7,7,8,8,8-tridecafluoro-1-octyl) silane, FAS13, from Evonik, nitric acid (70 %) and ammonia (30 %) from Labkem, tetraethyl ortosilicate (TEOS, 98 %) and diodomethane (99 %) were purchased from Sigma-Aldrich; hydrochloric acid (37 %) from Fisher Chemicals; ethyl alcohol (99.8 %) from Aga; sodium hydroxide from Eka.

Ammonium chloride from Scharlau, Creatinine (anhydrous, ≥98 %) from Sigma Aldrich, di-Sodium Hydrogen Phosphate (anhydrous) from PanReac Applichem, Potassium phosphate monobasic (anhydrous) from Honeywell Fluka, Sodium Chloride from Laborspirit, Sodium Sulfite from Merck, urea from Laborspirit and sodium azide from Laborspirit, were acquired to prepare artificial urine.

6.2.2. Surface modifications

Surface modifications were performed according to the methodologies described in previous chapters: Chapter 3 for Gum Arabic modification, Chapter 4 for polyacrylate modifications and Chapter 5 for nanoparticle modifications. Furthermore, prior to testing, the contact angles of samples were checked to assure that the surfaces were modified.

6.2.3. Artificial urine performance test

Firstly, artificial urine was prepared as follows: 30 g ammonium chloride, 20 g creatinine, 25 g di-sodium hydrogen phosphate, 25 g of potassium phosphate monobasic, 0.15 g sodium azide, 90 g sodium chloride, 26.2 g sodium sulfite and 250 g urea were mixed in 10 L distilled water.

The experimental setup for dynamically assessing the effect of urine on the material was performed according to ASTM F1828 – 97 standard specifications for ureteral stents. The setup consisted in mounting each sample in a silicone-free tube of 3.18 mm of diameter. The tubes were connected to the artificial urine solution reservoir. The artificial urine was circulated through the tubes with a flow rate of 0.25 ml/min. The temperature of the solution was controlled and maintained at 37 °C both at the circulating urinary liquid in the tubes and in the reservoir. The experimental setup for the dynamic urinary assessment is shown in Figure 6.1.



Figure 6.1. Experimental setup for the dynamic urinary assessment.

Finally, the treated samples (5 samples for each different modification) were placed in the tubes and the artificial urine was continuously circulated through the tubes for 30 days to evaluate their performance. The testing was performed in HydruMedicals facilities (Guimaraes, Portugal).

6.2.4. Scanning electron microscopy (SEM)

The morphological and chemical analyses of the samples were performed at the Materials Characterization Services Laboratory of the University of Minho (SEMAT-UM), using a Scanning Electron Microscopy (SEM) NanoSEM - FEI Nova 200 (FEG/SEM). Before the morphological analysis, the samples were coated with a thin film of Au/Pd (80/20 by weight) with 10 nm thick. For the morphological analysis, a secondary electron detector with 10 KeV energy and a working distance between 7 and 8 mm, was used. For each sample, several groups of images were taken by SEM, referring to different magnifications. For the chemical analysis, energy dispersive spectroscopy (EDS) technique was performed by an Integrated System EDAX - Pegasus X4M, using 15 KeV energy, at a working distance of approximately 6.5 mm.

6.3. RESULTS AND DISCUSSION

The urinary catheters can be used in two different ways, (1) short-term usage, that typically last up to 7 days, (2) long-term usage for 28 days or longer [1]. The studies conducted with the modified PET samples were assessed for the long-term usage, as it would be more advantageous to have a more resistant material. It is also important to note that there are two different untreated PET samples used

in this study, as thicker PET films were needed for the nanoparticles modification process (Chapter 5). Figure 6.2 and Figure 6.3 shows the material surface before the urinary solution performance test. The SEM images before urinary tests revealed that no significant differences were found between the untreated PET sample as compared to the hydrophilic treated PET samples shown in Figure 6.2. However, as it can be seen in Figure 6.3, the nanoparticle treated sample has shown to have a different structure which is discussed in Chapter 5. Further SEM images comparisons after artificial urine performance tests are illustrated in Figure 6.4 and Figure 6.5. All surface modifications were found to promote crystal formation compared to the untreated PET samples.

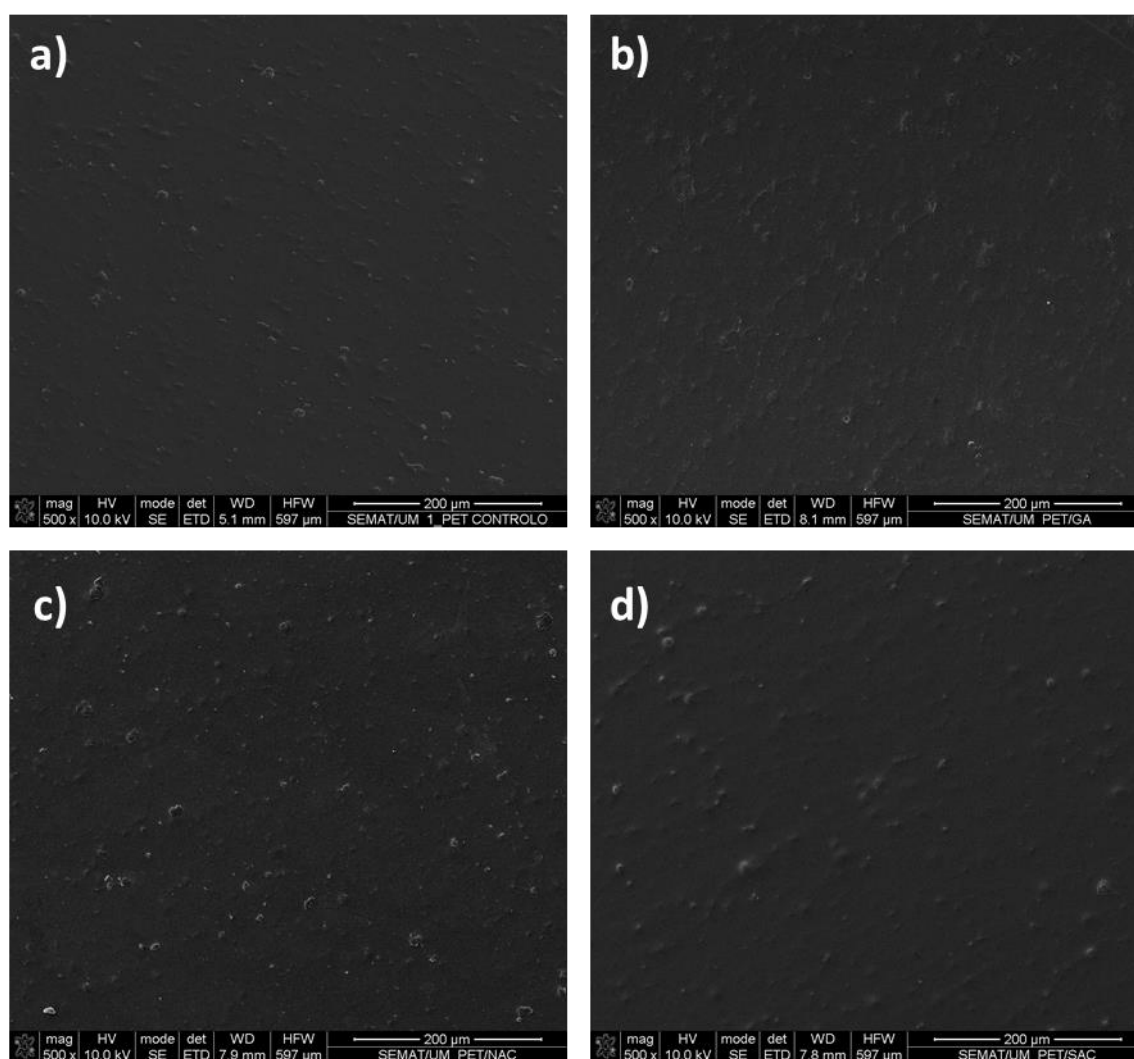


Figure 6.2. SEM analysis of PET samples before exposure to artificial urine using the dynamic urine testing setup; a) untreated PET, b) PET - GA, c) PET- NaAc d) PET- KAc.

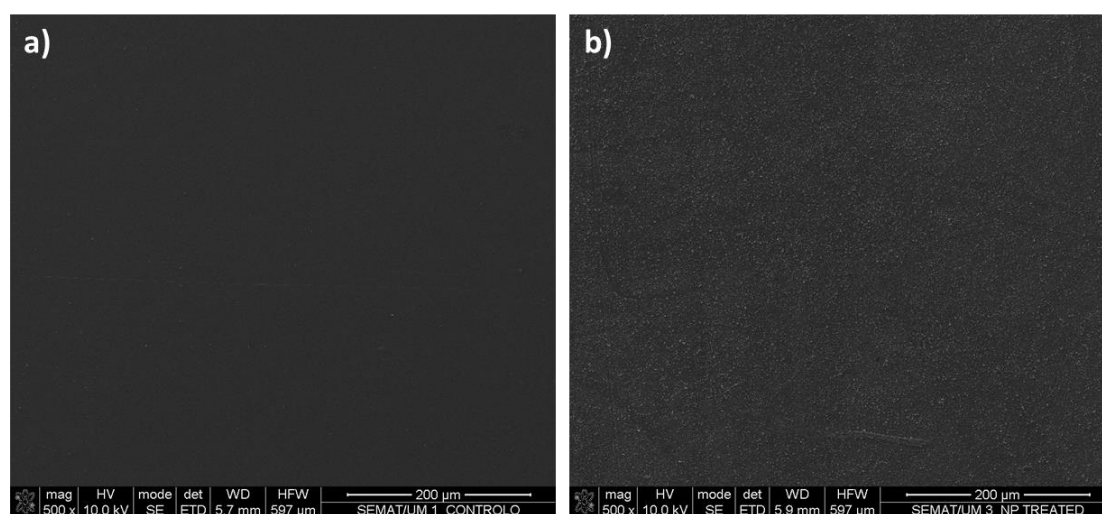


Figure 6.3. SEM analysis of PET samples before exposure to the artificial urine using the dynamic urine testing set up; a) untreated PET and b) nanoparticle treated PET.

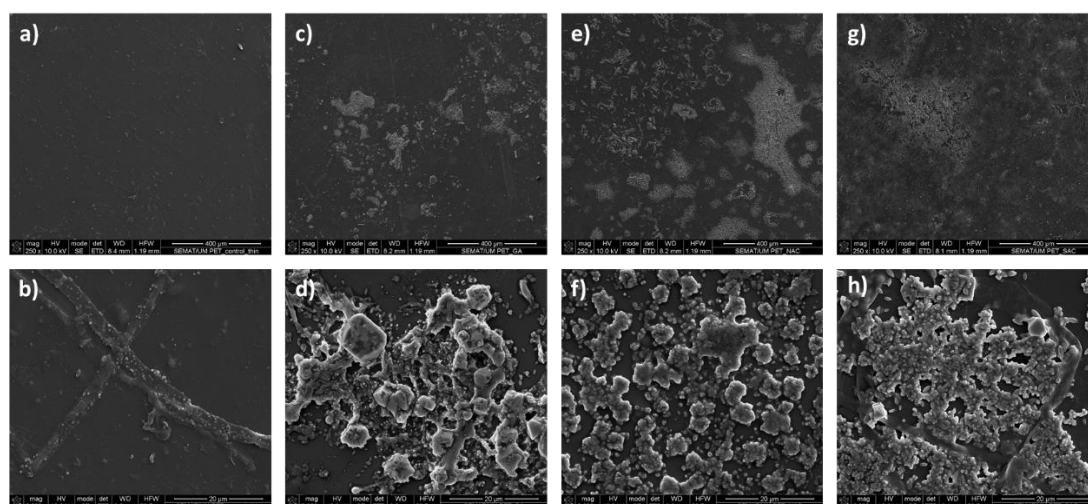


Figure 6.4. SEM analysis of PET samples after 30 days exposure to artificial urine using the dynamic urine testing setup; a) and b) untreated PET, c) and d) gum Arabic treated PET, e) and f) NaAc treated PET and g) and h) KAc treated PET.

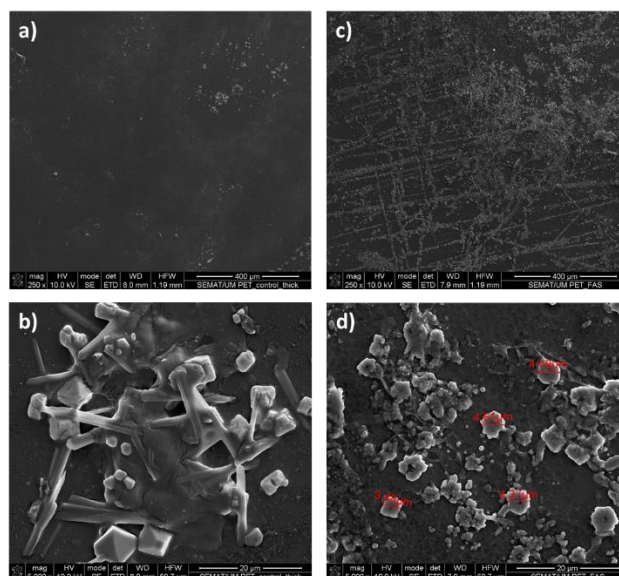


Figure 6.5. SEM analysis of PET samples after 30 days exposure to the artificial urine using the dynamic urine testing set up; a) and b) untreated PET and c) and d) nanoparticle treated PET.

Table 6.1. SEM-EDS analysis of material surfaces after being tested with artificial urine.

Detected Elements (Atomic %)					
	PET - Untreated	PET-NP	PET-GA	PET-NAc	PET-KAc
C	22,8	54,6	49,1	55,9	56,2
N	2,4	11,1	16,9	15,7	13,4
O	12,2	26,8	22,8	20,3	23,9
F	n/a	2,9	n/a	n/a	n/a
Na	25,0	1,8	5,5	4,0	3,5
Si	n/a	1,3	0,3	0,1	0,1
P	4,6	0,1	0,8	0,4	0,3
S	3,7	0,1	0,6	0,3	0,3
Cl	25,7	0,9	3,6	3,0	2,0
K	3,7	0,2	0,4	0,3	0,3

Further elemental analysis on the modified materials after urinary testing were done by SEM-EDS as shown in Table 6.1. Carbon and oxygen are expected elements which are present on all materials. However, the third element with the largest detected amount was found to be nitrogen, N, which is not present in the modified materials and untreated samples except PET-GA, thus it must be obtained through artificial urinary solution. Since, ammonium chloride, urea and creatinine consist of N element in their structure, this element indicate that at least one of them was present on the crystals formed. The other elements that attracted the attention are Na and Cl which are expected to be from sodium

chloride, ammonium chloride, di-Sodium Hydrogen Phosphate, and sodium sulfite. Additionally, sodium is also expected in the PET-NaAc composition. Moreover, a low amount of P, S, K was detected, and these elements are thought to be from artificial urine components including di-Sodium Hydrogen Phosphate, Potassium phosphate monobasic and sodium sulfite. Some of the crystal formations look like the NaCl crystals reported in the literature [11], however in our work, it is hard to determine the crystals through their shape as they are embedded.

Nevertheless, the appearance of the treated surfaces, particularly for hydrophilic ones, were not expected as the hydrophilic properties should have prevented the deposition of crystals [12] and there are currently commercially available catheters with hydrophilic surface modifications [3]. Regarding the superhydrophobic materials, air bubbles might have been the nucleation sites for crystals to develop. Furthermore, surface topography also plays an important role in nucleation, and it was found that rough materials and materials with pores enhance nucleation for a range of crystals [13] which could explain why nanoparticle modified surfaces promoted crystal formation compared to untreated ones.

It is important to note that herein we only used artificial urine and, *in vivo* applications, crystal formation and biofilm formation promote one another [14]. As explained in Chapter 1, the main reason for the encrustation problem is the presence of urease forming bacteria found in catheters. Thus, preventing the adhesion of this type of bacteria is an important step at reducing the crystal formation [4]. Although modified PET materials do not exhibit good dynamic urinary crystal inhibition, they were previously shown to be anti-adhesive (Chapter 3 and Chapter 4), thus they could still provide some degree of protection for short-term applications.

6.4. CONCLUSION AND OUTLOOK

The treated and untreated PET samples were placed under dynamic urinary fluid for 30 days. Samples were observed and analyzed at the final timepoint. The SEM analysis showed that all the modified surfaces promoted crystal formation. Although this is not desired, it is known that adhesion of urease producing bacteria promotes crystal formation. Hence, the strong anti-adhesive properties of the materials herein developed anticipates that these materials can probably reduce the crystal formation by reducing bacterial adhesion. For this purpose, further work is recommended using a mixed bacterial culture (including particularly those that produce urease) with artificial urine.

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CHAPTER 7

General Conclusions & Future Perspectives

The main goal of this thesis was the development of anti-adhesive materials using different surface modification processes such as “grafting to”, “grafting from” and “*in situ* nanoparticles synthesis”. Two different strategies to develop such surfaces were implemented. While the first two strategies included the formation of resistant coatings with hydrophilic characteristics, the third one aimed the formation of repellent superhydrophobic coatings.

In the first strategy, a natural polysaccharide, gum Arabic was used to modify the PET surface using glutaraldehyde as a crosslinker for covalent attachment of gum Arabic. Design of experiments was performed to improve the process parameters like concentration, reaction time, curing temperature and curing time. The curing time and curing temperature were found to be important parameters to reach hydrophilic features in this “grafting to” process. After determining the optimal conditions, a PET sample was subjected to the optimized modification process. Its water contact angle was 27 ° after initial treatment (a significant decrease from untreated PET sample, that presents a WCA of 70 °) and the sample maintained a contact angle of 30 ° after 10 washing cycles, indicating a high degree of durability and stability to usage conditions. Other characterization methods such as UV-VIS FTIR, AFM and SEM were further used to demonstrate the surface modification. Finally, the modified materials proved to be highly resistant (more than 99.9 %) to the adhesion of *E. coli* expressing with YadA.

In the second strategy, the surfaces were modified with sodium acrylate (NaAc) and 3-sulfopropyl acrylate potassium salt (KAc) by combining plasma and UV photopolymerization methods. The water contact angle values for the samples decreased from 70 ° to 8 ° and 28 ° for both NaAc and KAc, respectively. The water contact angle after 10 washing cycles was 14 ° for NaAc, thus showing that the modification was durable and stable. However, with KAc, the contact angle after 10 washing cycles showed variations, indicating a non-uniform treatment. The surfaces were further characterized with XPS, AFM, UV-VIS to further assess the surface modification. As the last step, the bacterial resistance against *E. coli* expressing YadA of the modified materials was evaluated. It was shown that the treated samples possessed more than 90 % reduction for both NaAc and KAc treated samples, proving its potential use to prevent bacterial adhesion in surfaces.

In the third strategy, the modification of the PET surface was implemented by *in situ* growth of nanoparticles using TEOS and fluorocarbon chains (to provide a low surface energy character to the surface). The water contact angle of the modified PET samples was around 156 ° and the contact angle of modified PET samples after 10 cycles of washing with water and 10 cycles of washing with ethanol were 160 ° and 157 °, respectively, proving its durability to usage conditions. However, SEM images showed that some removal of the surface coating occurred after the 10th cycle of water and 10th

cycle of ethanol washing. The modified samples were further evaluated for their anti-adhesive properties against *E. coli* expressing YadA. However, the modified samples were found to promote adhesion instead of inhibiting it as initially expected. This kind of behavior was thought to be due to surface heterogeneity with non-uniform structures that are reported to be an ideal spot for bacteria to adhere.

All the above-mentioned modified PET materials were further evaluated in an artificial bladder model using artificial urine, to assess the modified materials performance as an alternative in the development of urinary catheters. The results showed that the modified PET materials which were immersed in running artificial urine for 30 days, did not show any improvement in the formation of crystals compared to unmodified surfaces. Although this was a preliminary study, it was shown that the modified materials promoted crystallization. This indicates that these type of surface modifications might not be the ideal for materials that are envisaged to be used in the long term in urinary applications. However, it is important to note that most of the crystallization is caused by urease producing bacteria in urinary systems. Therefore, the anti-adhesive nature of the modified PET materials herein developed is as important as determining how resistant a material can be against encrustation. A method combining both the use of bacteria and artificial urine could be better and more realistic at determining how effective a material is. Although this was not tested for the PET materials herein modified, the gum Arabic and acrylate modified samples could still be useful for intermittent catheterization as they can reduce bacterial adhesion very effectively. The results also suggest that the nanoparticle modified PET samples should not be used in urinary catheters as they seem to promote bacterial adhesion instead of preventing it. Instead, other applications where bacterial adhesion is envisaged should be explored for these materials. For instance, the modified nanoparticles may find application in systems where bacteria capture and/or enrichment is envisaged, for example for application in sensors for rapid detection of bacteria.

The hydrophilic modified materials exhibit very interesting anti-adhesive properties, which open several potential applications, particularly for the healthcare sector. Furthermore, these surfaces could be used in diagnosis tools to increase the sensitivity and selectivity of the analysis by preventing non-specific adhesion on sensors and reducing unwanted adhesion on the other parts of diagnostic tools. In this way, they can reduce misdiagnosis by providing faster and more accurate results thus, preventing wrong use of drugs and reducing the cost and effect on antimicrobial resistance.

While surfaces that work with antimicrobial agents can be highly effective against initial stages of bacterial adhesion, accumulation of dead bacteria on the surface will change the surface properties, thus once the surface is covered, the expected antimicrobial effect might be highly inhibited. Moreover,

given the current concerns with the growth of resistance to antimicrobials, it is important to consider and reduce the use of these kind of agents. Thus, the alternative materials with anti-adhesive properties can be highly effective at preventing bacterial adhesion without contributing to the antimicrobial resistance. They can also lead to a reduction in the use of antimicrobial agents, which in turn has a strong effect of fighting antibacterial resistance. However, this kind of surface modification does not prevent bacterial adhesion at 100 %. Thus, it could be used as a combination with other methods to prevent bacterial adhesion. For example, antimicrobial peptides are gaining attention and they are less likely to cause antimicrobial resistance therefore, the combination of anti-adhesive materials with antimicrobial surface structures or agents like peptides can be very useful.

Furthermore, in these applications, it would be important to understand the material behaviour regarding the stability and durability of the anti-adhesiveness. There is still a need to understand the long-term durability of these anti-adhesive materials since there is lack of testing long term durability of these materials. The further characterization tests (e.g., abrasion) would be needed to verify the material resistance to use since those applications require longer term use and it would not be feasible to change those materials often. However, durability tests are also particularly hard considering that there is no standard testing comparing the materials.

Finally, the outcomes obtained in this thesis reinforce the potential use of hydrophilic materials to prevent bacterial adhesion. Furthermore, it also highlights the need for further durability tests to understand the bacterial the involvement in urinary applications.

