

Nanopatterned surface to control bacteria development

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The understanding and control of bacterial biofilms are currently of great interest for the industrial and medical field. Several strategies are investigated to control biofilm formation including antifouling and bactericidal coatings, as well as topographical patterns. In this work, different surface functionalization and nanofabrication techniques have been combined to produce new surfaces to control bacterial organization. Indeed, antifouling hydrophilic polymer brushes were successfully nanopatterned then grafted with bioactive peptides to create innovating chemically nanopatterned surfaces. In order to investigate the influence of the pattern characteristics on the bacterial behavior, two different pattern dimensions (250-pattern and 400 pattern) were prepared. Moreover, three different peptides were grafted on the patterned brushes : a RGD peptide, well-known for its key role in eukariotic cell adhesion and the two antimicrobial peptides (AMPs), LL37 and magainin I known for their antibacterial properties against *Escherichia coli* (*E. coli*), a gram-negative bacteria that was used to investigate the influence of these surfaces on bacterial growth, organization and viability. First, chemical nanopatterns were found to have an indirect influence on bacterial behaviour. Indeed, no clear bacterial alignment nor specific arrangement of dead bacteria was observed on any of the tested surfaces. However, nanopatterned RGD-grafted surfaces clearly induced a higher growth compared to homogeneous RGD-grafted surfaces. Similarly, 400-patterned LL37-grafted surfaces showed a higher killing activity than 250-patterned and homogeneous LL37-grafted surfaces. However, this effect was not observed for MAG-grafted surfaces. Secondly, it was observed that AMP-grafted surfaces were not as effective as expected to kill bacteria : almost no dead bacteria were observed after 1h and about only 20% were dead after 7 h-growth. Further research needs to be done to fully understand the influence of the chemical patterns on the surface proliferation of bacteria. In order to do so, it would be interesting to use different pattern geometries and dimensions as well as different bacterial species. Similarly, further research is most certainly needed to optimize the killing activity of AMP-grafted surfaces.

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LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
AMP	Antimicrobial peptide
ATRP	Atom transfer radical polymerization
ECM	Extracellular matrix
LPS	Lipopolysaccharides
MAG	Magainin I
MIC	Minimum inhibitory concentration
NIL	Nanoimprint lithography
NMRP	Nitroxide-mediated radical polymerization
OD	Optical density
P(HOEGMA)	Hydroxyl-terminated oligo(ethylene glycol)methacrylate)
PCB	Poly(carboxy-betaine)
PCBMA	Poly(carboxybetaine methacrylate)
PDMS	Polydimethylsiloxane
PEG	Poly(ethylene glycol)
PEM	Polyelectrolyte multilayer
PLGA	Poly(lactic-co-glycolic acid)
PMPI	4-Maleimidophenyl isocyanate
POPC	palmitoyloleoylphosphatidylcholine
PSB	Poly(sulfobetaine

PSBMA	Poly(sulfobetaine methacrylate)
PU	Polyurethane
QAC	Quaternary ammonium compound
qPCR	Quantitative polymerase chain reaction
RAFTP	Reversible addition-fragmentation chain transfer polymerization
ROP	Ring opening polymerization
SAM	Self-assembled monolayer
SEM	Scanning electron microscopy
TEG	Triethylene glycol
THF	Tetrahydrofuran
XPS	X-ray photoelectron spectrometry

CHAPTER 1

INTRODUCTION AND OBJECTIVES

Biofilm formation and bacterial adhesion are currently the centre of interest of a number of research. These biofilms consist in structured microbial communities adhering to each other and to surfaces in order to protect themselves.

In most cases, bacterial biofilms are linked to harmful effects. For example, biofilm are of great concern in the medical sector and for industrial processes. Concerning the medical field, they are involved in several human infections such as otitis media, periodontitis, native valve endocarditis and cystic fibrosis.¹ Besides causing these infections, biofilm are also known to induce medical devices failures. Indeed, as bacterial biofilms form dense layers sticking on the surface of materials, they can clog catheters, colonize implants such as prosthetic heart valves or even form on contact lenses.¹ Aside from the medical field, the industrial field is also affected by biofilms and they are of great concern for several processes including oil drilling, paper production and food processing. Extrapolymetric substances secreted by bacteria are not removed by cleaning processes and act as attachment sites for bacteria newly arrived to the cleaned system.^{1,2} As the purpose of biofilms is to protect the bacteria from harmful conditions including antibiotics attacks, bacterial infections are difficult to eradicate once the biofilm is formed.² For implanted devices, the only solution is the removal of the implant, causing discomfort for the patient and leading to additional costs.³

However, it is also possible to take advantage of bacterial biofilm, for example, in the degradation of environmental hazardous chemicals in soil or in bioreactors for waste water treatment.³

Whether it is beneficial or detrimental, bacterial adhesion and biofilm formation need to be fully understood in order to be controlled, namely prevented or promoted depending on the application.

In this work, focus was put on the control of the adhesion and growth of bacteria on the surface to limit their development. For this purpose, two main strategies have been reported : development of antifouling surfaces, preventing proteins and subsequent cell adhesion and bactericidal surfaces, killing bacteria adhered on the surface. How-

ever, in some specific applications such as tissue engineering or prosthesis, eukariotic cells adhesion needs to be promoted while keeping antibacterial properties. Fulfilling these conflicting objectives is still a challenge as eukariotic cells and bacteria are in competition for colonizing the surface. This phenomenon is known as "The race for the surface".³ However, their different properties, such as rigidity, size, shape,... could be used to induce differential behaviour of bacteria and mammalian cells on the surface of biomaterials. For instance, it is believed that nanopatterning could influence bacteria and eukariotic cells differently.⁴

The aim of this master thesis is to investigate the influence of biochemical nanopatterns over bacterial adhesion and biofilm formation. This study will pave the way to novel approach allowing control of bacterial growth. Different approaches have been merged to obtain the final surfaces: antifouling brushes are combined with nanopatterning techniques and biofunctionalization methods. The effect of different types of surfaces will be assessed. The surfaces produced consist in biochemical nanopatterns made from hydroxyl-terminated oligo(ethylene glycol)methacrylate) (P(HOEGMA)) grafted with bioadhesive or antimicrobial peptides and polyethylene glycol (PEG). Three peptides were grafted on the P(HOEGMA) brush. A first type of surface is functionalized with RGD peptides. These are known to be involved in eukariotic cell adhesion events. Hence, RGD functionalized surface could be used to promote eukariotic cells adhesion in some applications. However, the influence of this type of surface over bacterial behaviour need to be investigated. Moreover, an alternation of adhesive and non-adhesive lines could influence bacterial adhesion and growth. If bacteria align according to the nanopatterns rather than forming large 3-dimensional aggregates, they could be less resistant towards antimicrobial treatment.⁵ A second type of surface is functionalized with antimicrobial peptides (AMPs), which show a broad antibacterial activity. Therefore, the goal of these surfaces is to reduce bacterial growth and kill bacteria. The effect of two different AMPs, namely the LL37 and magainin I peptides, will be compared. For each type of surface, the effect of this chemical nanopatterning over the effectiveness of the surface will be assessed by comparing nanopatterned surfaces to homogeneously grafted surfaces. Two different sizes of pattern will be used in order to study pattern dimensions influence. Adhesion, growth and viability of *Escherichia Coli* (*E. coli*), a gram-negative bacteria, will be evaluated on these different surfaces.

This master thesis is thus divided into four main parts. The first part provides an overview of the literature in the field of bacterial adhesion and biofilm formation. More precisely, biofilm formation and bacterial adhesion mechanisms are described. Current strategies to control biofilm formation as well as the influence of the surface patterning over bacterial behaviour are outlined. Finally, the properties of the different peptides used in this work are reported. The second part describes the methodology used to obtain nanopatterned peptide-grafted surfaces by combining nanoimprint lithography, grafting of polymer brush and bioconjugation techniques. The characterization methods are also detailed. Finally, the bacterial experiments performed to analyse the influence of these surfaces on bacterial behaviour are explained. In the third part, the results are presented and discussed. First, surface characterization is achieved by different methods. Then, bacterial behaviour on the different surfaces is described. Finally, the conclusions and perspectives of this work are given in the last part.

2.1 Bacterial adhesion

2.1.1 Characteristics of bacteria

Bacterium definition

Bacteria are relatively simple, unicellular organisms. As their genetic material is not enclosed in a special nuclear membrane, they belong to prokaryotic microorganisms.⁶ Typically, bacteria size is in the range of 1 to 10 μm . Bacteria offer considerable variety in terms of morphology. The two most common shapes are coccus and bacillus. Cocci are spherical or ovoid cells while bacilli are rodlike cells.⁶ Individual bacteria may form pairs, chains, clusters, or other groupings. Such formations are usually characteristic of a particular genus or species of bacteria.⁶

Characteristics of *E. coli*

E. coli is a gram-negative rod-shaped bacterium (see figure 2.1) commonly found in the lower intestine of warm-blooded organisms. Even though most *E. coli* strains are harmless, some types can cause serious food poisoning in humans. The harmless strains are part of the normal intestinal flora, and can serve a useful function by producing vitamins and by preventing the establishment of pathogenic bacteria within the intestine.⁷ Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. Indeed, *E. coli* is a facultative anaerobic, able to make adenosine triphosphate (ATP) by aerobic respiration if oxygen is present, but is also capable of switching to fermentation or anaerobic respiration if oxygen is absent. It can grow in media with glucose as the sole organic constituent.

Optimum growth of *E. coli* occurs at 37 °C and neutral pH.⁸ *E. coli* is frequently used as a model organism in microbiology studies.

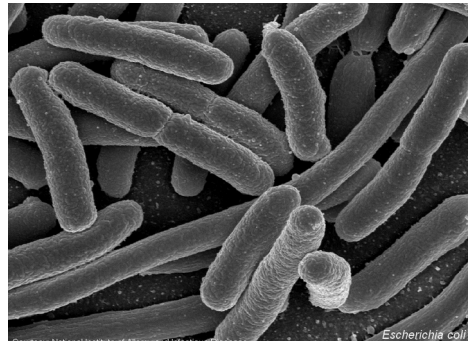


Figure 2.1 – SEM image of *E. coli*⁸

As it was already said, *E. coli* is a gram-negative bacterium. It is thus interesting to describe gram-negative bacteria cell wall. The cell wall is the layer that lies just outside the plasma membrane. This structure is of great importance as it helps maintain cell shape and protect the cell from osmotic lysis or toxic substances and in pathogens, it can contribute to pathogenicity.⁸

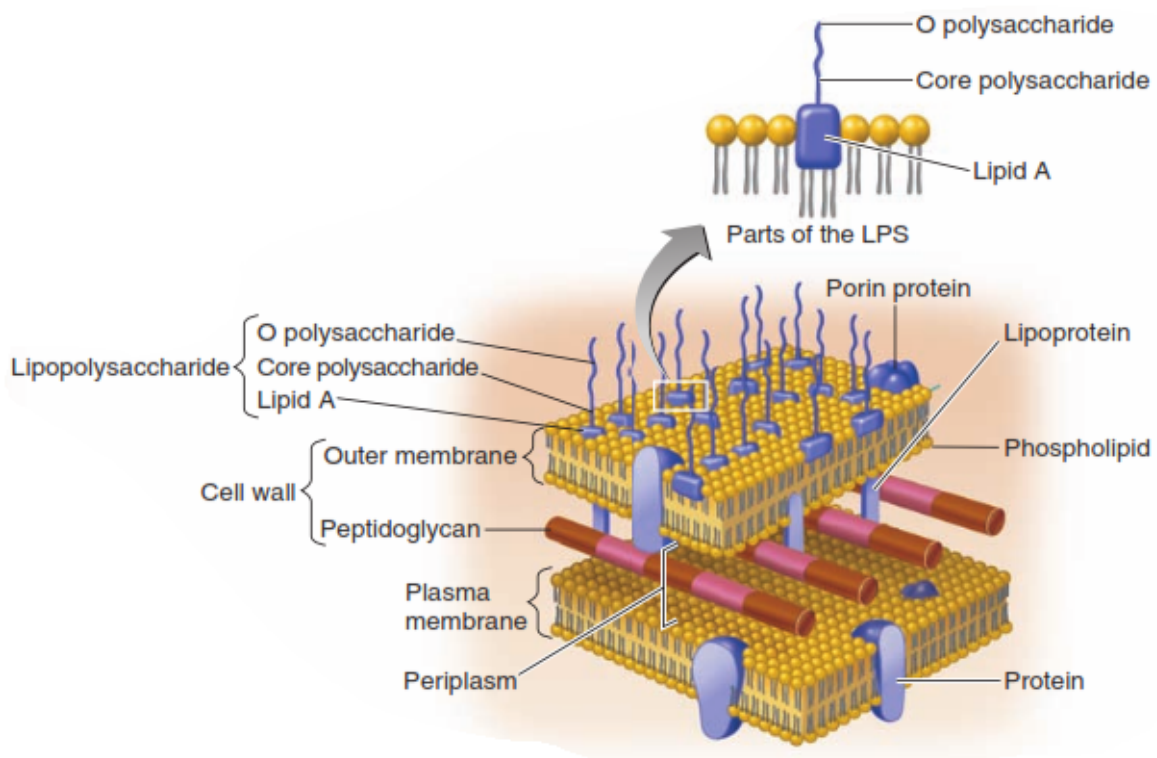


Figure 2.2 – Gram-negative bacterial cell wall⁶

As can be seen from figure 2.2, gram-negative bacteria cell wall is constituted of two distinct layers: a peptidoglycan layer covered by an outer membrane.⁸ The plasma membrane and the outer membrane are separated by a wide (30-70 nm) space called the periplasm in which lies the peptidoglycan layer. Peptidoglycan is a polymer consisting of sugars and amino acids that forms a mesh-like layer, providing structural strength to

the cell. In gram-negative bacteria, this peptidoglycan layer is only 7-8 nm thick while in gram-positive bacteria, its thickness can reach up to 80 nm. The outer membrane consists of lipopolysaccharides (LPS), lipoproteins, and phospholipids and provides a barrier to certain antibiotics, digestive enzymes, detergents,... However, the outer membrane must be permeable to certain substances such as nutrients. Part of this permeability is due to porins, which are proteins forming channels in the outer membrane. They allow the passage of molecules such as nucleotides, disaccharides, peptides, amino acids, vitamins, etc. Lipopolysaccharides (LPS) are large complex molecules that contain lipids and carbohydrates and contribute to the negative charge on the bacterial surface because they usually contain charged sugars and phosphate. They are constituted of three parts (see figure 2.2, upper part) : a lipid A, a core polysaccharide, and an O polysaccharide. Lipid A is the lipid portion of the LPS and is embedded in the top layer of the outer membrane. These lipid A are responsible for much of the toxicity of gram-negative bacteria. When bacterial cells are lysed by the immune system, fragments of membrane containing lipids A are released into the circulation, causing fever, diarrhea, and possible fatal endotoxic shock. The core polysaccharide is attached to lipid A and contains unusual sugars. It has a structural role, as it provides stability. The O polysaccharide extends outward from the core polysaccharide and is composed of sugar molecules but its composition varies from strain to strain. It functions as an antigen : it is a target for recognition by host antibodies.^{6,8}

Role of the Bacterial Cell Wall Properties and Features in the adhesion phenomenon

Bacterial cell surface structure and appendages directly affect bacterial adhesion. Several strains of bacteria possess filamentous appendages of various sizes such as flagella and pili. These appendages have been shown to function as adhesin. Some of these are involved in bacterial adhesion to abiotic surfaces and biofilm formation while others specifically bind to biomolecules on host cells and/or extracellular matrices (ECMs).⁹

Flagella. These cellular appendages are thin, rigid filamentous protein structures of about 20 nm across and up to 20 μm long.^{8,9} Flagella distribution is often different from one species to another. For example, some bacteria present one single flagellum on one end while others present one flagellum at each end. Still others have a cluster of flagella at one or both ends. Flagella can also be spread evenly over the whole cell surface.⁸ They are well known for their role in the locomotion of planktonic bacteria but they have also been found to play a role in adhesion.^{8,9} For example, *Pseudomonas aeruginosa* (*P. aeruginosa*) adhesion on inert surfaces has been reported to decrease in the absence of flagellum.⁹ However, the role of flagellum in adhesion is still not well understood even if it has been suggested that flagella may overcome the repulsive forces between the cell and the surface.⁹

Pili. Pili are non-flagellar filamentous protein appendages anchored in the bacterial cell membrane. These are typically thinner and shorter than flagella. They are usually called pili or fimbriae. A cell may be covered with up to 1,000 pili. They vary from 3 to 10 nm in diameter and up to several micrometers long.⁸ Pili are considered to have a key role in the adhesion to inert surfaces and for the attachment to host tissues.^{8,9}

Lectins. Lectins are carbohydrate-binding proteins that are highly specific for sugar moieties. According to the species and strain, lectins are located in the bacterial cell wall or on the tip of pili. In general, they are an important class of adhesins that are involved in many infections of animal tissues.⁹

Lipopolysaccharides. Aside from proteins, LPS also play an adhesive role in bacterial adhesion. For instance, modifications in the structure of LPS have been shown to significantly affect bacterial adhesion.⁹ Similarly, AFM measurements showed that intact LPS were necessary to obtain strong adhesion between bacteria and surfaces.⁹ However, the mechanisms underlying this phenomenon are not clearly understood but the role of the charge conferred on the whole bacterium by the various LPS structures seems to be involved.⁹

2.1.2 Theory of bacterial adhesion

As it was previously mentioned, there are two opposite goals for the control of bacterial adhesion depending on the context. In some cases, prevention and inhibition of biofilms are required while in others, their enhancement and promotion is necessary. In order to develop specific materials meeting these requirements, the mechanisms governing bacterial adhesion need to be fully understood. Three theoretical approaches have thus been developed to understand the forces that determine adhesion : the DLVO model, the thermodynamic approach and the extended DLVO theory.^{3,10}

Classical DLVO theory (Derjaguin, Landau, Verwey, Overbeek)

Several hypotheses are used in this model. First, given their natural size, bacteria can easily be assimilated to colloidal particles. Bacteria are thus assumed to be perfectly spherical and rigid. Moreover, this model assumes perfectly smooth substrates.^{3,10} According to this theory, the net interaction between a surface and a particle is a balance between van der Waals interactions, which are usually attractive, and electrostatic interactions, which are generally repulsive. Indeed, for a charged particle/surface in an aqueous solution, the surface charge is balanced by an equal but oppositely charged region of counterions, forming what is called an electrical double layer. Since bacteria and natural surfaces in aqueous solution are usually negatively charged, repulsive electrostatic energy is caused by overlap of the electrical double layers of bacterial cells and the substratum.^{3,10} This phenomenon is illustrated in figure 2.3.

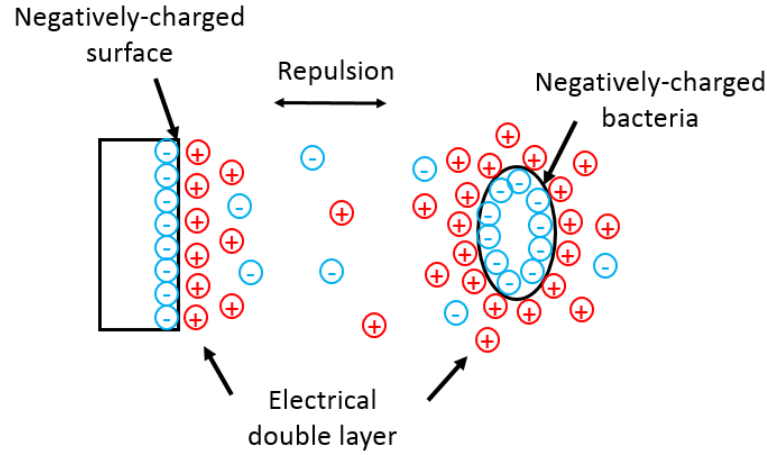


Figure 2.3 – Electrical double layer interaction between negatively-charged bacteria and negatively-charged surface

Electrostatic interactions dominate far from the surface while van der Waals interactions are prevalent in the vicinity of the surface. The balance between these two types of interaction leads to an energy barrier, which is illustrated in figure 2.4.^{3,10}

This energy barrier is influenced by the ionic strength of the medium. Indeed, the electrostatic repulsion decreases as the ionic strength of an aqueous solution increases because of the shielding of the surface charges by the ions in the electrical double layers.^{3,10} This phenomenon is illustrated in figure 2.5.

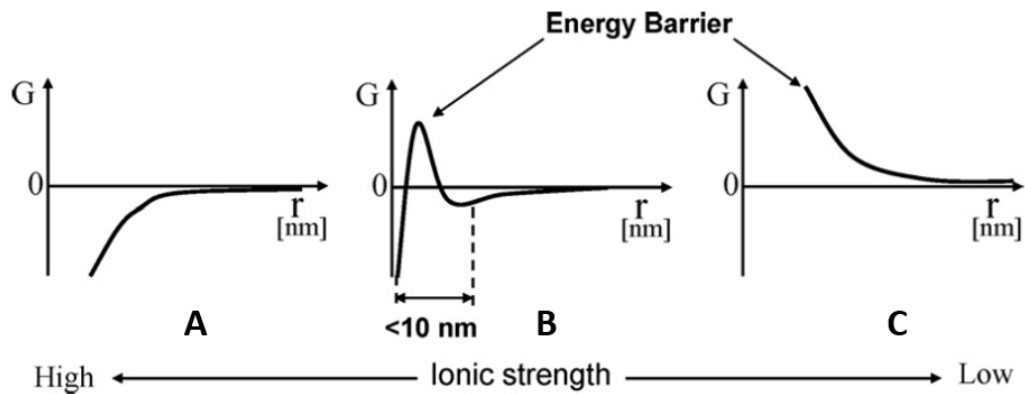


Figure 2.4 – Total interaction energy between a bacterial cell and a surface (A) at high ionic strength (B) at intermediate ionic strength (C) at low ionic strength³

At high ionic strength, the double layer interaction is compressed due to shielding effect and the net interaction may be attractive even if both surface and bacteria bear negative charges. It can be seen from figure 2.4A that the energy barrier disappears resulting in a rapid and irreversible bacterial adhesion.^{3,10}

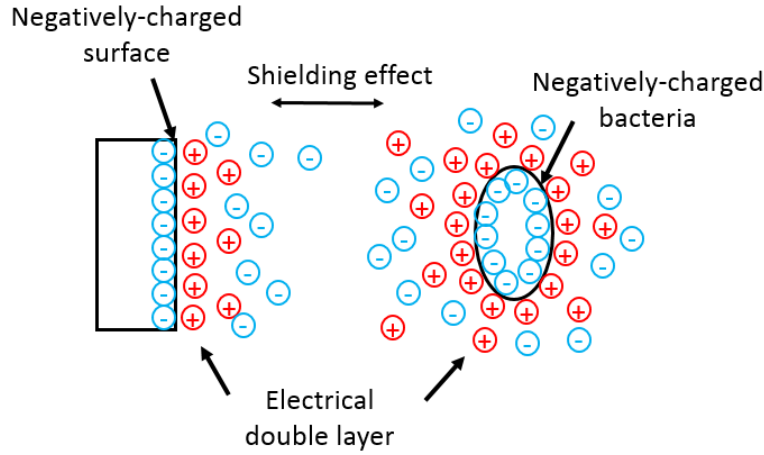


Figure 2.5 – Shielding effect reducing the repulsive interaction between the electrical double layers at high ionic strength

At intermediate ionic strength, illustrated in figure 2.4B, the energy barrier may present a secondary energy minimum at a relatively small distance from the surface. In this case, bacterial adhesion is a two-steps process. In the first step, a bacterium comes to the position corresponding to the secondary energy minimum, either by its own motility or by Brownian motion. As a result, this bacterium can be reversibly adhered to the surface. Then, it can pierce the energy barrier, using surface structures such as pili, flagellum and biopolymers that have a smaller radius and thus less repulsion than bacteria. This is the irreversible adhesion stage. This two-steps process is illustrated in figure 2.6.^{3,10}

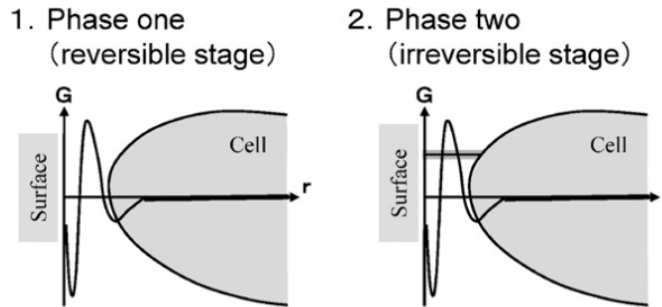


Figure 2.6 – Schematics of two-steps bacterial adhesion process.³

Finally, low ionic strength, illustrated in figure 2.4C, results in repulsion at all cell-substrate distances since bacteria cannot overcome the energy barrier by swimming or by Brownian motion. As bacteria are not sufficiently close to the surface, it becomes difficult for the bacterial surface structures/polymers to reach the substratum, and bacteria are thus unable to adhere.^{3,10}

This model has however some limitations. Bacteria are considered to be rigid solid spherical particles. But, in reality, bacterial membranes are rather elastic and porous.

Secondly, bacteria present a wide diversity of shapes and sizes and usually carry cellular appendages. They are thus far from simple spherical particles. Finally, this model ignores the fact that bacteria are living being able to adapt to their environment.⁹ As a matter of fact, bacteria undergo metabolic changes when passing from a planktonic state to a sessile state. These changes play a key role in bacterial adhesion. For example, cell wall composition can be modified and adhesive appendages and receptors can be expressed, facilitating the irreversible adhesion of bacteria.⁹

Thermodynamic approach

The thermodynamic approach relies on the surface free energy of the interacting surfaces.^{3,10} The adhesion free energy is defined as follows

$$\Delta G_{adh} = \gamma_{sm} - \gamma_{sl} - \gamma_{ml} \quad (2.1)$$

where γ_{sm} , γ_{sl} and γ_{ml} are the solid–microorganism, solid–liquid, and microorganism–liquid interfacial free energies, respectively. Adhesion is favored if the adhesion free energy per unit surface area is negative. In other words, adhesion is spontaneous if γ_{sm} is smaller than the sum of γ_{sl} and γ_{ml} .

This approach assumes that the process is reversible, which however is usually not the case. Moreover, in this case, the model is based on the formation of a new bacteria–substrate interface at the expense of the substrate–medium interface. This means that, in this approach, the adhesion phenomenon requires a contact between the bacteria and the substrate. If the adhesion occurs in the secondary minimum (see figure 2.4B) and no bacteria–substrate interface is formed, this approach is not applicable. As a consequence, the interaction energy is independent of the distance, as opposed to the DLVO model. Despite these limitations, this approach can help understanding a recognized phenomenon : bacteria with a hydrophobic cell surface prefer hydrophobic material surfaces while those with a hydrophilic cell surface prefer hydrophilic surfaces.^{3,10}

Extended DLVO theory

Obviously, the two previously described models cannot completely explain bacterial adhesion. As a consequence, an extended DLVO theory was developed, in which the hydrophobic–hydrophilic interactions and osmotic interactions are included.^{3,10} For bacteria, osmotic interactions were found to be negligible. Accordingly, the total adhesion energy is expressed as follows^{3,10}:

$$\Delta G_{adh} = \Delta G_{vdW} + \Delta G_{dl} + \Delta G_{AB} \quad (2.2)$$

where ΔG_{vdW} is related to the van der Waals interaction, ΔG_{dl} to the electrical double layer interaction and ΔG_{AB} relates to acid–base interactions which account for attractive hydrophobic interactions and repulsive hydration effects. The influence of this last term is believed to greatly exceed the influence of the two other terms. In some cases, the extended DLVO theory seems to qualitatively predict experimental adhesion results better than the classical DLVO theory.

Conclusion

In reality, bacterial adhesion is an extremely complex process which often deviates from the adhesion models described. Some important parameters are not taken into account in these models. First, the presence of various molecules adsorbed on the surface, forming what is called conditioning layers, often modifies the physicochemical properties of the surface, changing the interactions between bacteria and the surface. Secondly, as it was already mentioned, bacteria are far from being simple colloidal particles : they are chemically heterogeneous and present cellular appendages involved in adhesion events. In addition, bacteria are living organisms that are able to adapt and react to their environment. In conclusion, even though these different theories can help to explain some observations, it has not been entirely successful in predicting all the various attachment behaviours observed.

2.1.3 Biofilm formation

In natural environments, there is two distinct bacterial living states : planktonic cells are single-cells that float in a liquid medium while sessile cells are attached to a substrate. The first living state is important for bacteria spreading and the second is necessary to allow bacteria to form biofilms in order to resist environmental conditions.¹¹ In natural environment and within infected tissues, biofilms are the most predominant form.¹²

Bacterial proliferation and colonization following bacteria attachment stage usually lead to biofilm formation. This biofilm is made of dense microbial communities fixed to a substrate and enclosed in a specific matrix. This biofilm possesses a complex structure and is a survival mechanism that protects microorganisms from their environment.⁵ In particular, it provides a high resistance towards mechanical force, UV radiation and antibiotic attack.¹³ Biofilm resistance is believed to have a variety of causes including poor antibiotic penetration, slow growth, adaptive stress responses and the formation of multi-resisting cells.¹¹ The formation of this structure is of great concern for healthcare as well as industrial applications. Actually, biofilms can grow thick enough to obstruct pipelines in various environments. In the medical domain, biofilms can cause several damages by activating human immune response, releasing noxious toxins or obstructing catheters.² Moreover, its protective function makes it difficult to treat infections.

The formation of the biofilm is a multi step process which can take place on any surface conditioned by proteins and other molecules present in the environment (see figure 2.7 step 1). Initially, bacteria are transported to the surface (figure 2.7 step 2) by the effects of physical forces, such as Brownian motion, van der Waals interactions, gravitational forces, etc. Besides this passive transport, bacteria also approach the surface by their own motility using appendages such as flagellae.⁹ The adhesion step is then described as a two-stages process : the reversible adhesion and the irreversible adhesion (see section 2.1.2). During the reversible adhesion step, bacterial cells can adsorb on the surface (figure 2.7 step 3) but they can easily desorb (figure 2.7 step 4). Then, bacterial anchorage on the surface strengthens. In this case, established interactions involve receptor/ligand-like bindings, covalent and hydrogen bondings.⁹ This is the irreversible adhesion step. Once attached firmly to the substratum, bacteria begin cell-to-cell signal communications (figure 2.7 step 5) that induce growth, replication and secretion of

various substances. From this time, bacteria thus begin to proliferate and synthesize a slimy matrix consisting of various polymers (figure 2.7 steps 6-8). The composition of this matrix will depend on the bacteria strains present in the forming biofilm. These polymers are collectively called extracellular polymeric substances, and they include exopolysaccharides, proteins, glycoproteins, glycolipids, and DNA. The matrix allows the bacteria to adhere more stably on the surface. As the biofilm thickens and matures, the bacteria reproduce and secrete additional polymers. Finally, some bacteria detach from the biofilm and spread to colonize other locations (figure 2.7.step 9).⁸ Biofilm are not just bacterial slime layers but biological systems : the bacteria are organized into a coordinated and functional community. Indeed, as it was mentioned, bacteria are able to communicate through molecular signals, a phenomenon known as quorum sensing, allowing them to coordinate their activity to act as a unit.⁶

As it is difficult to eradicate, a better strategy is to avoid the formation of this biofilm.

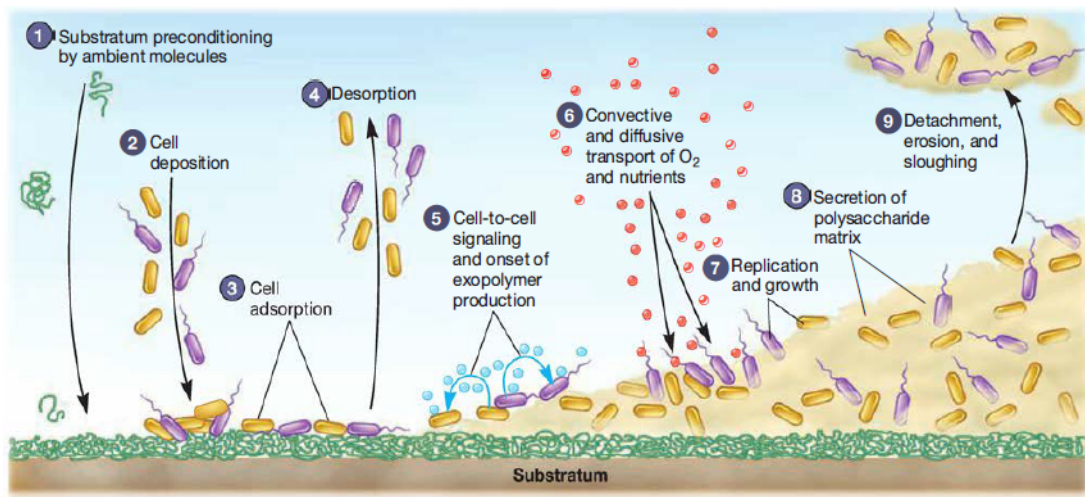


Figure 2.7 – Biofilm formation⁸

2.2 Current strategies to control biofilm formation

Since biofilm formation takes place in several steps, different strategies can be developed to hinder this phenomenon. A first strategy consist in preventing primary bacterial adhesion.^{11,14} Indeed, colonization is impossible if bacteria cannot adhere to the surface. This strategy is based on the development of bacteria repelling surfaces. A second strategy is to reduce the survival of bacteria attached to the surface with antibacterial surfaces.^{11,14} Finally, other strategies¹⁵ require to disrupt early biofilm formation by, for example, interfering with quorum sensing pathway. However, the concepts needed for this strategy are only emerging and will not be discussed here but further information can be found in the literature.^{11,14} It is worth mentioning that physical and chemical patterning techniques also allow controlling bacterial adhesion and viability but these effects will be described in another section (see 2.3).

2.2.1 Bacteria repelling surfaces

As mentioned above, bacteria repelling surfaces aim at reducing the extent of initial bacterial attachment in order to hinder the biofilm formation. In aqueous environment, bacterial adhesion on surfaces is influenced by several parameters including pathogen type, environmental conditions, surface morphology and chemistry, as it can be seen from figure 2.8.¹⁵ More precisely, bacterial behaviour especially depends on material hydrophobicity and electrostatic charges. In fact, effective antifouling surfaces consist in hydrophilic, highly hydrated, non charged surfaces.¹⁵ However, it is worth noting that, even though antifouling surfaces offer promising results in certain application such as catheters, low adhesiveness could also hinder tissue adhesion and integration in other medical applications for which cell adhesion is needed. A potential solution to this issue is the combination of antifouling surfaces with peptides sequences specifically interacting with human cells.¹⁶

Variables controlling bacterial adhesion.	
Variables influencing bacterial adhesion and colonisation on biomaterial surfaces	
Surface morphometry	Macroporosity Microporosity Micro-roughness Nano-roughness
Physico-chemical properties	Surface energy Hydrophilicity/superhydrophilicity Hydrophobicity/superhydrophobicity Hydrophobic functional groups Polar functional groups Charged functional groups Functional groups with specific activities Degree of hydration
Environmental conditions	Electrolytes pH Temperature Host proteins/host adhesins Shear rate/fluid viscosity Fluid flow rate
Pathogen	Gram-positive/Gram-negative Genus/Species Bacterial shape Surface energy Strain type and specific set of expressed adhesins

Figure 2.8 – Variables controlling bacterial adhesion¹⁵

The most commonly used anti-adhesive materials are hydrophilic polymers based on the ethylene glycol repeat unit such as poly(ethylene glycol) (PEG) and its derivatives. The anti-adhesive properties result from a hydration layer maintained by hydrogen bonds. This hydration layer form a physical barrier in aqueous environments, preventing proteins and subsequently cells adhesion.^{13, 16} Several surface-modification methods have been developed to take advantage of the antifouling properties of PEG modified surfaces. Substrates can either be coated or covalently grafted with linear or branched polymers, hydrogels, self-assembled monolayers (SAMs) of oligo(ethylene glycol)-terminated thiol on gold or PEG silanes on oxide substrates, PEG modified polymers and grafted chains of PEG.¹⁶

Polymers bearing zwitterionic groups are an alternative strategy for developing anti-adhesive surfaces. In this case, the hydration layer is more tightly bound through electrostatic interactions.^{13,16} For instance, poly(sulfobetaine) (PSB) and poly(carboxybetaine) (PCB)-based polymers have attracted great interest as they are easy to prepare, chemically stable and low-cost.¹³ Poly(sulfobetaine methacrylate) (PSBMA) and poly(carboxybetaine methacrylate) (PCBMA) have been demonstrated to show an effective resistance toward marine and biomedical bacteria adhesion as well as to prevent biofilm formation.¹⁶

2.2.2 Bacteria killing surfaces

Antibacterial surfaces aim at killing bacterial cells. They can either consist in release or non-release systems.¹⁴ In release systems, bactericidal properties are obtained from the leaching of loaded antibacterial compounds over time, allowing killing of both adhered and floating bacteria. Compared to traditional antibiotic administration techniques, release systems offer the advantage to deliver high concentrations of antibacterial agents locally without exceeding systemic toxicity levels. However, these surfaces are only active for a defined period of time.¹⁴

Non-release systems have thus been developed to circumvent the reservoir exhaustion issue of release-based material. In this case, there should be no loss of functionality over time. These non-released systems consist in contact-killing surfaces.¹⁴

Release systems

In release systems, antimicrobial substances are incorporated in the bulk or on the surface of a material by various methods.¹⁷ Surface can be coated by simple impregnation or by using resorbable carriers materials which are then covalently grafted with antibacterial compounds. These carriers can either be polymer brush made of chitosan, poly(methyl methacrylate) (PMMA), polyurethane (PU),..., self-assembled mono/multilayer films and so on. Several different antimicrobial compounds can be used including antibiotics (e.g: penicillins), antimicrobial peptides (e.g: magainin), elements (e.g: silver), enzymes (e.g: lysosymes), organic cationic compounds (e.g: Quaternary ammonium compounds (QAC)) and so on.¹⁷ The release then occurs by diffusion, erosion of resorbable loaded matrices or hydrolysis of covalent bound and the kinetics of delivery is tailored depending on the bound stability or the degradation rate.^{15,17} For instance, Pfeufer et al. developed titanium surfaces functionalized with the human antimicrobial peptide β -defensin-2. More precisely, titanium samples were functionalized by SAMs forming methoxy silanes and were then coated with human β -defensin-2. This AMP was then continuously eluted from the titanium surface, yielding antimicrobial activity up to several hours.¹⁸

Non-release system

As previously mentioned, non-release systems are contact-based bactericidal surfaces. This type of surface thus requires bacterial adhesion in order to be effective. Non-release systems mainly consist in surfaces coated by bactericidal substances.¹⁷ Antibacterial agents are immobilized on the surface by either covalent conjugation or physical adsorption. Various antibacterial agents can be used including silver nanoparticles, polymer bearing quaternary ammonium, antimicrobial peptides, antimicrobial enzymes, etc.¹⁷

For example, silk and nylon fibers coated with silver-nanoparticles using a layer-by-layer deposition technique were developed by Dubas et al.¹⁹ These fibers exhibited antimicrobial activity and resulted in 80% bacteria reduction for the silk fiber and 50% for the nylon fiber, rendering them useful in applications such as antimicrobial fabrics.¹⁹

2.3 Patterns influence on bacterial behaviour

2.3.1 Topographical patterns

Surface topography has been found to play a crucial role in bacterial adhesion. In order to understand the influence of topography over bacterial adhesion, it is necessary to distinguish the nanotopography from the microtopography.

It is widely accepted that bacteria react to surface topography with size larger than a bacterial cell and that bacterial adhesion and consequently biofilm formation can be modulated by micro-scale patterns.¹³ For example, bacteria preferentially adhere to the bottom of crevices as this location offers a protection against shear forces. This phenomenon was demonstrated by Hou et al.²⁰ by using polydimethylsiloxane (PDMS) surfaces showing 10 μm high square-shaped patterns of varying lateral dimensions and interspacings. *E. coli* was found to preferentially adhere and form biofilm in the valleys between protruding square features regardless of the dimension of the features (2-100 μm tested) and spacing between adjacent features (5-20 μm tested). This phenomenon is illustrated in figure 2.9.²⁰

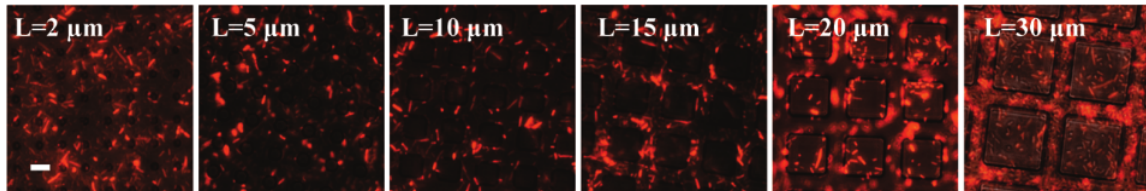


Figure 2.9 – Fluorescences images of *E. coli* on topographically micropatterned PDMS. Features are squares of side L (Scale bar = 10 μm)²⁰

Nanostructured surfaces showing features with dimensions comparable to those of individual bacterial cells are believed to control bacterial arrangement, disrupting the natural organisation of biofilms.¹³ Aizenberg et al.² prepared periodic nanopillars arrays of high aspect ratios with dimensions in the order of bacterial cells. Indeed, these nanopillars were 2 μm tall and with a diameter of 300 nm. Bacteria were found to pattern spontaneously on these nanopillars but different organizations were obtained depending on the spacing between the nanopillars as illustrated in figure 2.10. When the spacing was larger than bacterial cell dimensions, bacterial adhesion was random (figure 2.10A). As the spacing approached bacterial dimensions, bacteria started to adhere only in some specific directions (figure 2.10B). Finally, when the spacing was slightly smaller than bacterial length, bacteria aligned themselves along the nanopillars, oriented normally to the surface (figure 2.10C). It was suggested that bacteria tend to maximize their contact area with the surface by interacting with the nanopillars.² This particular behaviour was observed for both gram-positive and gram-negative bacteria even in the absence of

pili or flagellae. As they interfere with the natural arrangement of bacteria and consequently on the biofilm development, these nanopillar arrays could be used to create novel bacteria-resistant surfaces.² Interestingly, Diaz et al.⁵ reported that a combination of topographical pattern with size comparable to that of bacterial cell, able to promote single cell isolation, and antibiotic treatment act synergistically as an effective strategy to eradicate biofilms. Indeed, the combined strategy involving the use of a microstructured surface, consisting of a grid of 550 nm wide gold rows separated by 750 nm wide and 120 nm deep channels, and streptomycin antibiotic treatment was found to yield a reduction of 1000-fold in the number of surviving *Pseudomonas aeruginosa* (*P. aeruginosa*) bacteria with respect to those obtained with streptomycin treatment or microstructured surface alone.⁵

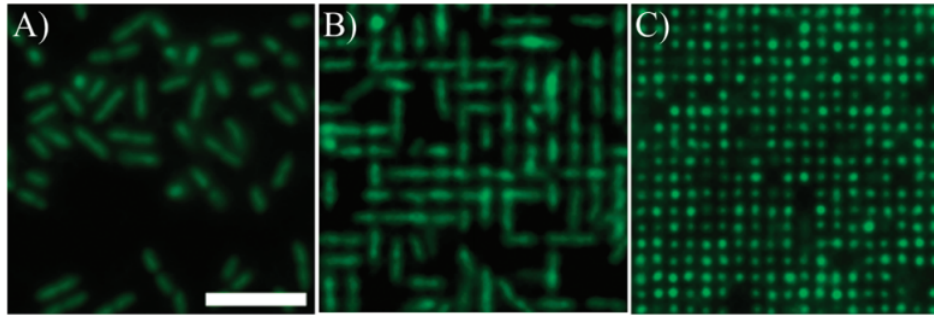


Figure 2.10 – *P. aeruginosa*, a rod-shaped bacteria, adhered on nanopost arrays (Scale bar = $5\mu\text{m}$)²

Surface nanotopographies with patterns considerably smaller than bacterial cells have been found to possess a bactericidal effect regardless of the strain.¹³ The bactericidal effect results from mechanical and structural deformational stresses. Ivanova et al.²¹ reported the rapid and effective bactericidal activity of cicada wings over *P. aeruginosa* that was attributed solely to the wings nanostructure. This nanostructure consists in periodic arrays of nanopillars that are 200 nm tall, 100 nm in diameter at the base, 60 nm in diameter at the top, and spaced 170 nm apart from center to center. Cicada wings showed poor antifouling properties but were instead capable to mechanically disrupt the wall bacterial cells within three minutes.²¹ This phenomenon is illustrated in figure 2.11. Similar results were obtained for other surfaces such as nanostructured black silicon and dragonfly wings.¹³ Similarly, Aurélie Merckx²² investigated the influence of topographical features consisting in 115 nm wide lines separated by 65 nm wide grooves, which are significantly smaller than a bacterial cell. These features were either 7 nm or 22 nm tall. No influence of these topographies was reported on cell adhesion and growth.²² This suggest that features geometry and aspect-ratio are important parameters to induce mechanical stress on bacterial cells.

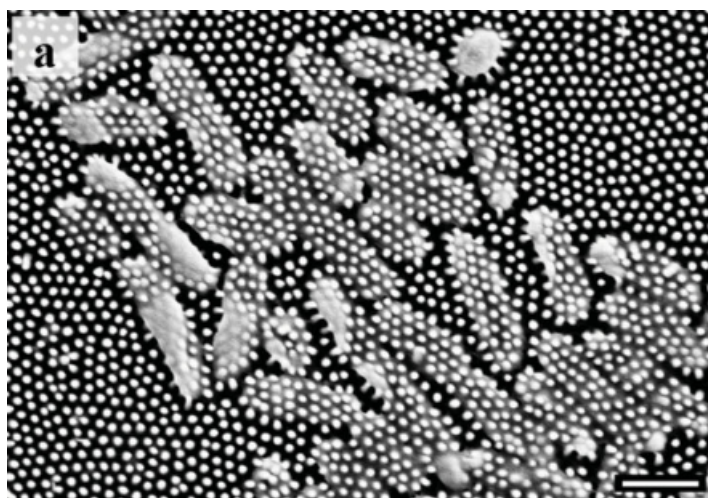


Figure 2.11 – *P. aeruginosa* cells penetrated by the nanopillar structures corresponding to cicada wing surface. (Scale bar = 1 μm)²¹

Up to now, the influence of ordered surface topographies has been described. Indeed, topographical features were based on regularly distributed structural units. Random topographies have also been investigated. For example, enhanced adhesion of spherical *Staphylococcus aureus* (*S. aureus*) was observed by Verran et al. on stainless steel with micron- and submicron-scale roughness.¹³ Similarly, Whitehead et al. showed that more spherical *S. aureus* bacteria were adhered on surfaces of average roughness of about 43 nm compared to smooth surfaces. The opposite behaviour was observed for rod-shaped *P. aeruginosa* bacteria.¹³ As spherical bacteria have a larger contact area on rough surface, which is favourable for bacterial adhesion, they are believed to preferentially adhere on rough surfaces while the opposite is true for rod-shaped bacteria.¹³ On the contrary, Webster et al. observed a decreased adhesion on nanorough titanium compared to smooth titanium regardless of the bacterium shape.¹³ Thus, as conflicting results have been obtained regarding surface roughness, the influence of these random topographic features on bacterial adhesion remains unclear.¹³ However, nanorough random topography has been found to affect bacterial genomics and proteomics. Pompa and coworkers²³ found significant modifications in the genomic and proteomic profiles of *E. coli* adhered on nanorough surfaces compared to those adhered on smooth glass and gold surfaces. More precisely, type-1 fimbriae were observed on smooth glass and gold surfaces with low average roughness (R_a values range from 0.36 to 0.46) while type-1 fimbriae were not present on gold surfaces with higher average roughness. Interestingly, the fimbriae could be recovered by incubating the bacteria on flat glass surfaces, suggesting a topography-induced transient variation in the fimbriae expression. Indeed, a genetic variation in fimbriae regulation was identified by quantitative Polymerase Chain Reaction (qPCR). Moreover, changes in the expression pattern of proteins involved in biosynthesis, peptide transport, metabolic pathway, and DNA repair system were observed when *E. coli* adhered on nanorough surfaces.²³

In conclusion, nanoscale and microscale topographic features have been found to significantly affect bacterial adhesion, viability, organisation and subsequent biofilm formation.¹³ Moreover, topographic patterns are able to induce genes and proteins expression

modifications, suggesting that bacteria actively respond to surface topography at the genetic level.²³

2.3.2 Chemical patterns

Chemical nanopatterns have been much less studied than topographical patterns as it is not straightforward to create chemical patterns without inducing physical topography. Indeed, any topographical difference needs to be avoided in order to ensure that interactions with the surface are purely chemical. Krsko et al.²⁴ created precise patterns of repulsive hydrogels that were able to spatially control the adhesion of *Staphylococcus epidermidis* (*S. epidermis*). Non-adhesive nanohydrogel patches with diameter of 150 nm and interspacing of 2, 1 and 0.2 μm were created on an adhesive glass surface. Bacterial colony size significantly decreased on nanohydrogels with an interspacing of 2 μm (figure 2.12B) compared to the control surface (figure 2.12A). This effect was even more pronounced with the 1 μm interspacing (figure 2.12D), which is comparable to the bacterial cell length. When the nanohydrogels were spaced 0.2 μm apart (figure 2.12C), they formed a continuous layer of PEG hydrogel and bacteria only adhered on the exposed glass surface while the hydrogel completely prevented bacterial adhesion.²⁴

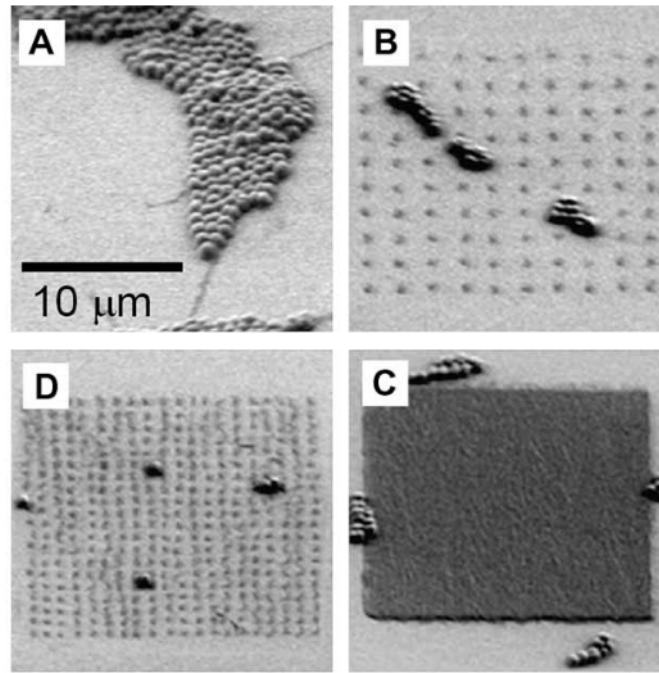


Figure 2.12 – SEM images of *S. epidermidis* after 1 h of culture on: (A) silanized glass (adhesive surface) ; nanopatterned PEG hydrogels showing PEG features with a diameter of 150 nm and an interspacing of (B) 2 μm (C) 0.2 μm and (D) 1 μm .²⁴

The inverse situation, for which circular areas of adhesive glass surface of varying diameter are surrounded by a non adhesive PEG hydrogel, was also investigated. It was shown that adhesive areas small enough to trap single bacterial cells could be fabricated.²⁴

Similarly, Hou et al.²⁵ developed SAMs of alkanethiols presenting different functional groups on gold films. Tri(ethylene glycol) (TEG)-terminated SAM was found to be extremely resistant as it repressed *E. coli* biofilm formation by 99.5% after 1 day compared to bare gold surface. Moreover, patterned surfaces decorated by regions consisting of methyl-terminated SAMs surrounded by TEG-terminated SAMs were elaborated. On these surfaces, *E. coli* was found to form biofilms only on methyl-terminated features.²⁵

Finally, Catherine Philippart²⁶ and Aurélie Merckx²² investigated the influence of chemical nanopatterns consisting in alternated regions of adhesive poly(methyl methacrylate) (PMMA) brush and nonadhesive PEG SAM of different dimensions. Aurélie Merckx observed the behaviour of *E. coli* on three different surfaces²² : alternated 200 nm wide lines of PMMA and PEG (called S400), alternated lines of PMMA and PEG of 65 nm and 115 nm, respectively, (called S180) and circles of PMMA with a diameter of 150 nm and an interspacing of 500 nm in a continuous background of PEG (called S500). *E. coli* was found to align with the pattern direction on the two linearly patterned surfaces. S180 patterns had a greater influence on bacterial alignment compared to S400. On the opposite, no specific bacterial organisation was observed on the S500 nanopattern. Catherine Philippart investigated the behaviour of *Lactococcus lactis* (*L. lactis*) and *Lactobacillus plantarum* (*L. plantarum*) on the same patterned chemical lines composed of PMMA and PEG. *L. lactis* was found to align with the pattern direction only on the S180 surface after 1 h of adhesion. Bacterial alignments were then observed on both surfaces after 2 h- and 4 h-growth and the percentage of surface covered by bacteria increased with time on both surfaces. Interestingly, the S180 pattern induced a higher percentage of surface covered by bacteria after 4 h-growth compared to S400 samples and homogeneous PMMA samples used as control. On the opposite, *L. plantarum* did not exhibit any specific organisation on these surfaces. However, the percentage of surface covered by bacteria was higher on nanopatterned surfaces and the S400 surface had an even more greater impact.

In conclusion, these results suggest that pattern chemistry and dimensions as well as bacterial species are key factors to control bacterial behaviour.

2.4 Surface biofunctionalization with peptides

For this work, three different peptides were used to functionalize polymer brushes. In this section, their characteristics are described.

Compared to proteins, use of peptides for surface biofunctionalization have considerable advantages. Firstly, proteins have to be isolated from other organisms and purified while peptides can be synthesized.²⁷ Purification and characterization of the protein makes the process complicated and expensive. In addition, these proteins may elicit undesirable immune responses and increase infection risks.²⁷ Secondly, proteins are made of several domains and the existence of other domains than the one of interest may lead to undesirable interactions. Finally, because of their smaller size, peptides can be packed with higher density on surfaces.²⁷ Thirdly, proteins are less stable than peptides and need to be refreshed continuously as they undergo degradation. Due to their smaller size, peptides usually do not present a tertiary structure. This structure relies

on non-covalent bonds such as electrostatic interactions and is rather unstable. As a consequence, peptides are rarely denatured but can only be damaged by covalent modification or break of bonds (hydrolysis).²⁷

2.4.1 Adhesion peptide: RGD

Arginylglycylaspartic acid (RGD), shown in figure 2.13 is a tripeptide composed of L-arginine, glycine, and L-aspartic acid also known as aspartate.

It is a signal motif of significant importance for cell adhesion and other vital phenomena linked to adhesion such as cell differentiation and growth. The RGD sequence is found in several proteins that are present in the extra-cellular matrix such as fibronectin, vitronectin, von Willebrand factor, collagen, and laminin.^{27, 28}

This sequence is recognised by specific cell adhesion receptors called integrins. Integrins are transmembrane proteins involved not only in the anchorage of cells but also in other processes such as embryogenesis, cell differentiation, immune response, wound healing and hemostasis.²⁷ They are composed of two subunits, called α and β . There are different types of α and β subunits and the particular combination of these subunits determines the ligand specificity of the integrin. It is worth noting that some integrins can bind to several types of ligands and, in the same manner, some ligands can bind to several types of integrin.²⁷ The process of integrin mediated cell adhesion is composed of four different steps : cell attachment, cell spreading, organization of actin cytoskeleton, and formation of focal adhesions. During the attachment step, the cell comes into a contact with the surface and some ligand links occur that allow the cell to withstand small shear forces. Then, the cell begins to flatten and its plasma membrane spreads over the surface. Thirdly, this leads to actin organization into microfilament bundles. Finally, the formation of focal adhesions occurs, which link the ECM to molecules of the actin cytoskeleton. Focal adhesions consist of clustered integrins and some other molecules. During the four steps of cell adhesion, integrins are employed in physical anchoring processes as well as in signal transduction through the cell membrane.²⁷

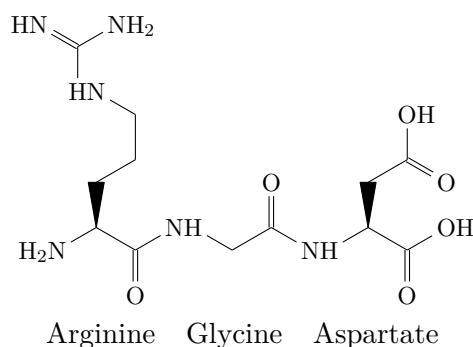


Figure 2.13 – RGD sequence²⁷

Stable linking of RGD peptides on the surface is crucial to induce strong cell adhesion as the formation of focal adhesion is only possible if the ligands withstand the cells contractile forces.

2.4.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) are an important part of the innate immune system and are produced by a wide variety of organisms. Several of these peptides typically show broad and effective antimicrobial activity against bacteria, viruses and fungi.²⁹ Up to now, more than 750 different AMP's have been isolated from various animals, plants, bacteria, fungi and viruses.

Although these AMP's are chemically and structurally different, they present some common characteristics. Firstly, they are composed of a small sequence of amino acids, usually between 10 to 25 amino acids with a molecular weight between 1 and 5 kDa. Then, they present a highly cationic character, though with large variations in the net positive charge. Finally, they form amphipathic molecules that have charged hydrophilic regions separated from the hydrophobic regions.³⁰ This specific organisation leads to strong interactions between the positively charged peptides and negatively charged bacterial membranes, whilst the hydrophobic groups facilitate the penetration of the AMP into the lipidic membrane. As they target negatively charged membranes, they inflict much less damage on the zwitterionic membranes of eukaryotic cells.²⁹

AMPs bactericidal activity results from their structural properties and relies on their ability to permeabilize membranes by creating pores or disrupting the organization of the lipid bilayer.³¹ Not only they show broad bactericidal, fungicidal, viricidal and tumoricidal properties at a very low concentration but they also show a low propensity for the development of bacterial resistance.²⁹ Indeed, as the target of AMPs is the bacterial membrane, bacterial resistance would require bacteria to redesign their membrane, modifying the composition and/or organization of its lipids, which represents an expensive solution for most microbial species.²⁹

All these characteristics make AMPs very interesting candidates for biomedical applications. However, use of some AMPs as drugs has been hindered by their high manufacturing cost, their susceptibility to proteolysis, and their possible toxicity against eukaryotic cells.²⁹ It is therefore necessary to engineer AMPs that are active at low concentrations without harming the host cells.²⁹

Cathelicidins : LL37 peptide

Cathelicidins are specific proteins present in the innate immunity of a wide range of organisms, from fish, amphibians and reptiles to mammals. They act as precursor molecules that can release an antimicrobial peptide after proteolytic cleavage. Members of the cathelicidin family are characterized by a highly conserved region (cathelin domain) and a highly variable cathelicidin peptide domain.^{32,33} More precisely, cathelicidin proteins are characterized by a highly conserved domain of about 100 amino acid residues called cathelin domain flanked by a signal peptide (approximately 30 residues long) on its N-terminus, and by an antimicrobial peptide region on its C-terminus. The sequence formed by the signal peptide, the cathelin domain and the antimicrobial peptide is called a preprotein (figure 2.14A). The signal peptide is used to target the cathelicidin to storage granules or to the exterior of the cell. It is then cleaved off when its purpose is fulfilled leading to the proprotein. The proprotein is a storage form as the peptide

is not active yet (figure 2.14B). The peptide becomes activated only after proteolytic cleavage of the proprotein into the cathelin domain and the cathelicidin-derived AMP (figure 2.14C).^{32,33}

For some species, up to seven cathelicidins have been found, each being the precursor for individual AMPs of very different structural properties.³² On the opposite, only a unique cathelicidin is found in humans. The human cathelicidin protein (hCAP18) contains a 37-amino acid-long peptide with a broad antibacterial activity. This peptide is called LL37, as it starts with two leucines and is constituted of 37 amino acids.^{32,33}

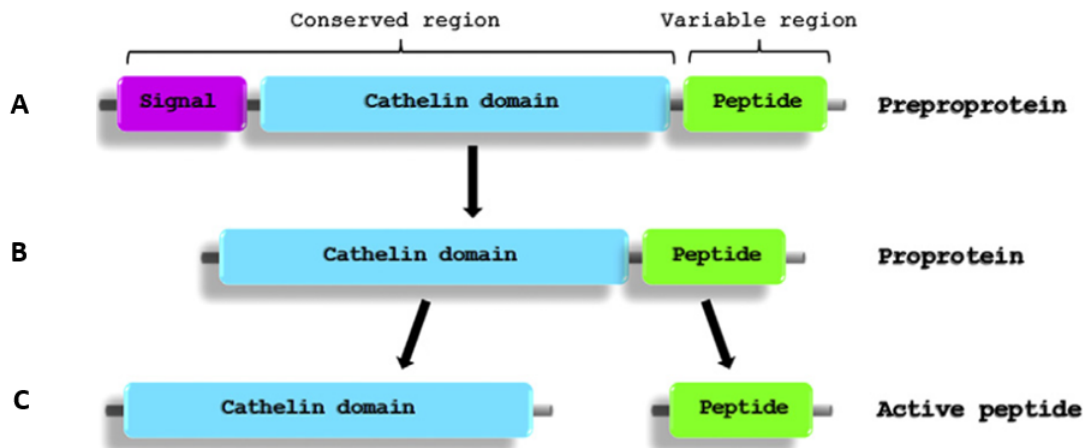


Figure 2.14 – General structure of cathelicidins and their hydrolyzed derivatives³³

LL37 belongs to the class of linear cationic α -helical AMPs. In pure water, LL37 adopts a random coil conformation, but in the presence of membranes or in solutions with millimolar concentrations of salts, it adopts a α -helical structure, consisting of 3 parts: a N-terminal α -helix followed by a C-terminal α -helix, and a C-terminal tail. The two helices are separated by a bend.³³ This structure as well as the amino-acid sequence are illustrated in figure 2.15. An helical wheel projection of LL37 in figure 2.16 illustrates the amphipathic and cationic nature of LL37. Indeed, the hydrophobic residues gather on one side of the helix while the hydrophilic residues (charged and polar residues) populate the opposite face of the helix. At physiological pH, 16 of its 37 residues are charged: 11 residues are positively charged while 5 residues bears negative charges. The resulting net charge at physiological pH is + 6.³²

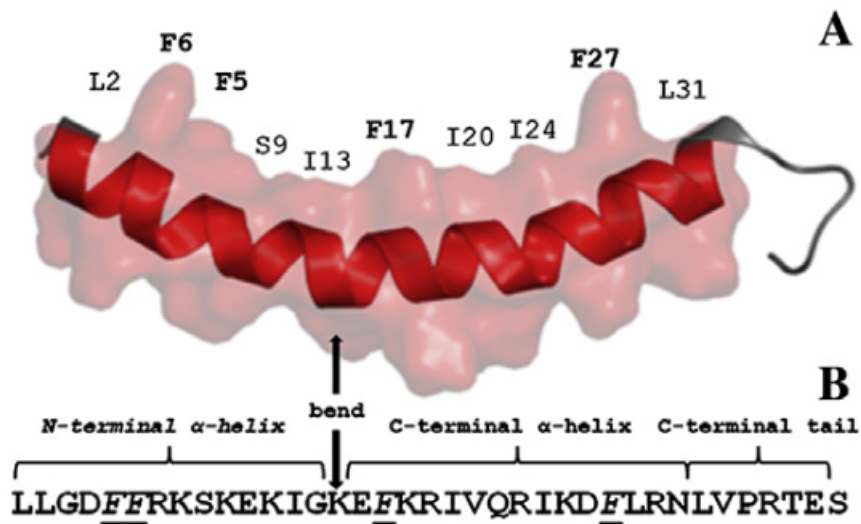


Figure 2.15 – Amino acid sequence and α -helical structure of LL37³³

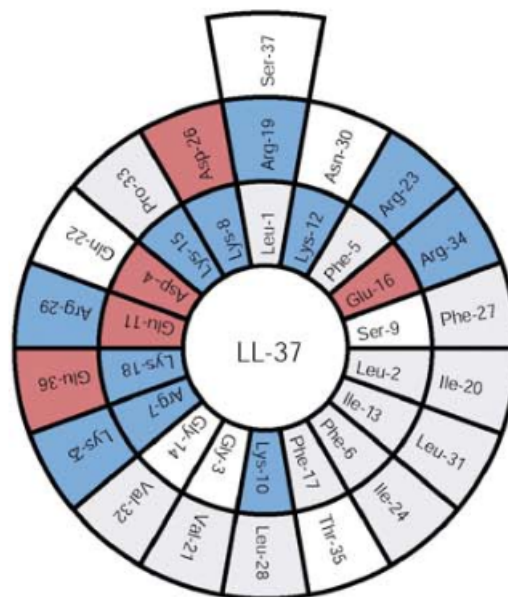


Figure 2.16 – Helical wheel projection of LL37 : positive residues are in blue, negative residues in red, polar residues in white and hydrophobic residues in gray³²

LL37 was found to exhibit several interesting properties:

- **Antibacterial, antifungal and antiviral activity:** LL37 exhibits a broad spectrum of antimicrobial activity against bacteria (both gram-positive and negative), fungi, and viral pathogens.^{33,34} It has also been able to prevent biofilm at low concentration and to destroy already existing biofilms (*P. aeruginosa*) at higher

concentration.³³ The antibacterial activity of LL37 is derived from its secondary structure which allows interactions with pathogens membranes. More precisely, LL37 uses a toroidal pore mechanism also known as worm-hole pore model,¹¹ commonly used by other cationic antimicrobial peptides.^{33,34} This mechanism is illustrated in figure 2.17. Firstly, LL37 monomers or oligomers reach the outer membrane and cover the surface. The peptides bind parallel to the surface, with the positively charged amino acids in contact with the head groups of the cell wall phospholipids. At a certain threshold concentration, the accumulation of peptide molecules starts to induce a bending of the bilayer so that the upper and lower leaflets meet. Small toroidal pores are formed, causing leakage of cellular material.^{30,33} The minimum inhibitory concentration (MIC) of LL37 against *E. coli* has been reported to be 40 μM in solution.^{32,33} It is worth noting that high concentrations of LL37 can also cause leakage of eukariotic cell membranes, resulting in cytotoxicity. However, concentrations associated with cytotoxic activity were always several times higher than the minimum inhibitory concentration.^{33,34}

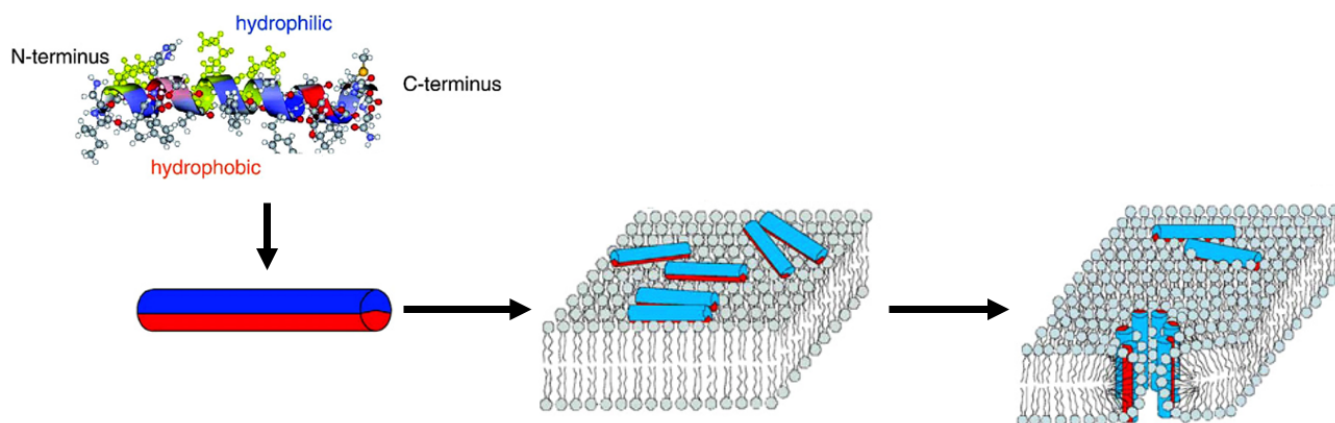


Figure 2.17 – Mode of action of LL37 : toroidal pore model (adapted from¹¹)

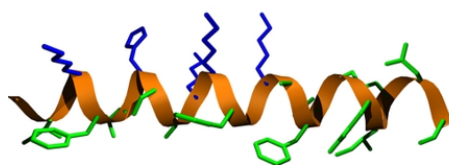
- **Wound healing properties:** When the infection is cured, tissues damaged by pathogens or by the immune system need to be repaired. LL37 plays an important role in this process. It was found to activate the migration and proliferation of fibroblasts, epithelial cells and endothelial cells involved in epithelial wound healing. Similarly, blood monocytes are stimulated by LL37 to migrate to the site of bone fracture and differentiate into bone-forming cells. Finally, another important step of wound healing is the formation of new blood vessels. LL37 is also able to induce angiogenesis.^{32,33}
- **Chemotactic effect:** Besides its bactericidal activity, LL37 also attracts cells capable of dealing with the microbial threat to the infection site. These cells include phagocytic cells such as neutrophils or monocytes and cells that release inflammatory mediators such as mast cells.^{32,33}
- **Immunostimulatory and immunomodulatory effect** After the cells of the immune system have reached the infection site, LL37 is able to stimulate and

modulate their action, leading to a balanced production and release of pro- and anti-inflammatory cytokines. For example, mast cells show degranulation upon stimulation with LL37 leading to the release of pro-inflammatory mediators such as histamine and prostaglandine into the surrounding tissue, thus increasing vascular permeabilization and promoting the infiltration of inflammatory cells. The balance between pro- and anti-inflammatory responses is of great importance as its disruption can either lead to an increased risk of infection and lack of wound healing or to autoimmune diseases or chronic inflammation. Several diseases are associated with an abnormal level of expression of LL37.³³

LL37 coatings were found to be effective in killing bacteria as well as in promoting wound healing. For instance, Gabriel et al.³⁵ developed LL37-grafted titanium surfaces. LL37 peptide molecules were conjugated to long and flexible PEG spacers by a N-terminal coupling mechanism, allowing the parallel alignment of immobilized peptide molecules. These surfaces expressed a clear antimicrobial activity against *E. coli*. Similarly, Pr  at et al. developed poly (lactic-co-glycolic acid) (PLGA) nanoparticles loaded with LL37 peptide molecules for wound healing applications. Indeed, PLGA supplies lactate that accelerates neovascularization and promotes wound healing. As previously mentioned, LL37 was found to play a role in wound healing and angiogenesis. Hence, the administration of LL37 encapsulated in PLGA nanoparticles (PLGA-LL37 NP) was hypothesized to promote wound closure due to the sustained release of both LL37 and lactate. These nanoparticles were found to significantly improve the wound healing activity compared to PLGA or LL37 alone and also showed a bactericidal activity towards *E. coli*.

Magainin

Magainin (MAG) peptides obtained from the African frog *Xenopus laevis* are constituted of 23 amino acid.³⁶ These two peptides, named magainin-I and magainin-II, show similar sequences except at two positions and show similar properties.³⁷ They exhibit antibacterial activity against a broad range of both Gram-positive and Gram-negative bacteria and fungi. Their activity is influenced by their concentration as well as salt concentration in the medium, with higher activity in low salt concentration media.³⁷ These peptides adopt an amphipathic α -helical structure (see figure 2.18) with hydrophobic residues on one side of the helix and hydrophilic residues on the other side as can be seen from figure 2.19. Moreover, they carry a positive net charge (+4) at neutral pH.^{36,37} Magainin-II is currently the most studied peptide but magainin-I was used for this work.



GIGKFLHSAGKFGKAFVGEIMKS

Figure 2.18 – Amino-acid sequence and α -helical structure of Magainin-I : hydrophobic side chains are shown in green, and hydrophilic side chains are in blue. (adapted from³⁸)

Magainin I was proven to show good bactericidal properties even in its immobilized form. Indeed, Glinel et al.³⁹ demonstrated the bactericidal activity of magainin-I functionalized polymer brush and Humblot and Berjeaud proved that magainin-I immobilized on thiols self-assembled monolayers reduced by more than 50% the adhesion of bacteria at the surface and killed the bacteria that nonetheless adhered to the surface.⁴⁰

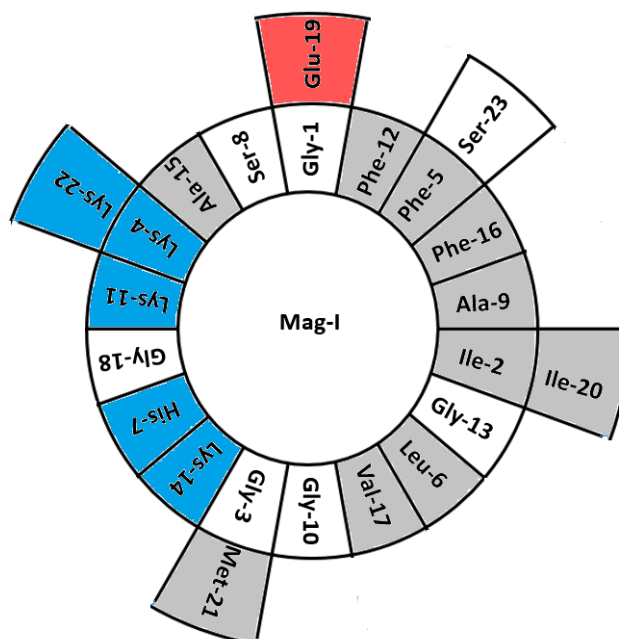


Figure 2.19 – Helical wheel projection of magainin I : positive residues are in blue, negative residues in red, polar residues in white and hydrophobic residues in gray (adapted from⁴¹)

In conclusion, magainin I and LL37 show similar properties. They have the same α -helical structure with hydrophobic residues on one side and hydrophilic residues on the other side, they both show a broad spectrum of antibacterial activity and exert it by the same mechanism, namely the toroidal pore model and finally they both carry a positive net charge. However, the net charge of LL37 is +6 while that of magainin I is only +4.

3.1 Nanopatterned surface elaboration

3.1.1 Overall process

The first part of this master thesis is the realisation of chemically nanopatterned surfaces. These surfaces are obtained following a process of multiple steps. The different steps of the process are summarized in figure 3.1. First, a silicon substrate is cleaned using a standard piranha procedure in order to remove organic residues. The substrate is then spin-coated with a solution of polymethyl methacrylate (PMMA) 4% (w/w) in toluene to obtain a uniform layer. It is then heated at 170°C for 5 minutes to evaporate the solvent (figure 3.1 step 1) and the layer thickness is measured by ellipsometry. Then, the pattern is realized by nanoimprint lithography (NIL) and the bottom of the holes are cleaned thanks to a descum step (figure 3.1 step 2). These different steps are performed in the cleanroom facilities Winfab of UCL. The following steps are performed in the BSMA laboratory. First, we perform a gas phase silanization to graft a ATRP silane initiator on the open parts of the surface (figure 3.1 step 3). The PMMA mask is then removed with acetone (figure 3.1 step 4). A liquid phase silanization is achieved to form a continuous non-adhesive background of PEG surrounding the ATRP initiator (figure 3.1 step 5). The P(HOEGMA) (for poly(hydroxyl-terminated oligo(ethylene glycol)methacrylate)) brushes are then grown from the initiator layer by ATRP (figure 3.1 step 6). ATRP, for atom transfer radical polymerization, provides a controlled architecture and a well-defined thickness and composition of the brush.³⁹ Then, a heterolinker is grafted on the hydroxyl groups present on the lateral chains of P(HOEGMA) brush to allow the grafting of a bioactive peptide in the last step (figure 3.1 step 7). Different peptides are grafted on the linker to obtain functionalized polymer brushes (figure 3.1 step 8).

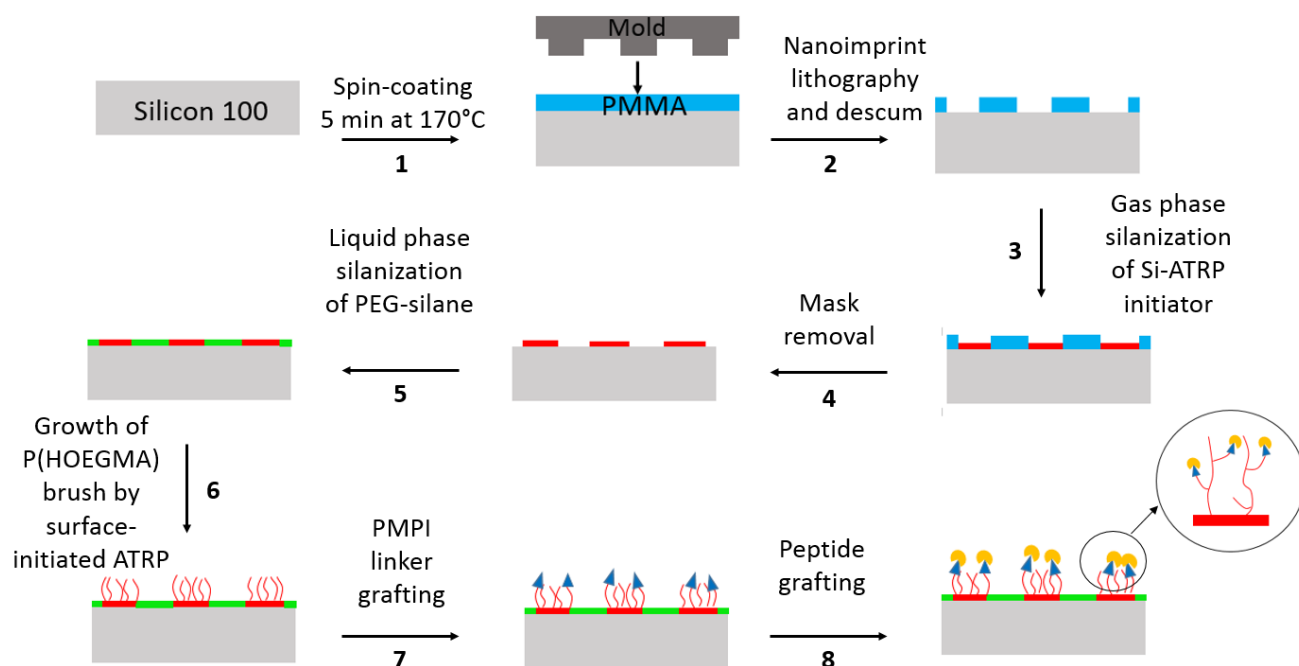


Figure 3.1 – Elaboration of nanopatterned surfaces

3.1.2 Nanoimprint lithography technique

NIL is an advanced patterning method for nanofabrication. NIL consists in the transfer of a pattern by pressing a mold into a polymer. This technique has several advantages. It has demonstrated a sub-10nm resolution. Since there is no need for complex optics or high-energy radiation sources, the process is simple and rapid. Moreover, it allows to rapidly pattern large areas (several cm^2). Also, silicon master molds are rather expensive but they can be used up to a few thousands of imprints. The simplified requirements of the technology lead to its low cost.⁴²

There are two types of NIL processes : thermal NIL and UV NIL. In our case, thermal NIL was used as there is less restrictions on the mold and resist. Indeed, in the case of UV NIL, the resist must be UV curable and the mold needs to be UV transparent, which induces additional costs.⁴³ Thermal NIL process is illustrated in figure 3.2.

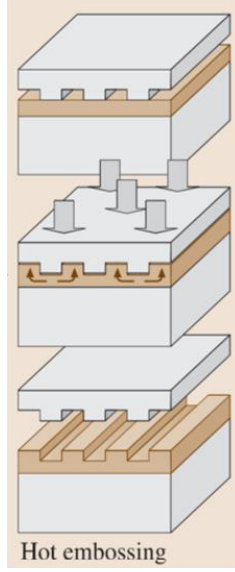


Figure 3.2 – Thermal NIL process

First, a thermoplastic film is coated onto a substrate. The advantage of using a thermoplastic polymer is that the viscosity can easily and reversibly be modified by changing the temperature by just a few tens of degree Celsius. Indeed, a reduction of viscosity is needed to allow the flow of the polymer in the mold. Then a mold is placed and the polymer is heated above its glass transition temperature. During embossing, the film is compressed between the mold and the substrate and the viscous polymer is forced by the pressure and the capillary force to flow into the cavities of the mold. Once the cavities are filled, the polymer is cooled down while maintaining the pressure so that the structure can freeze. After relieving the pressure, the mold can be retrieved and reused. With this technique, a residual layer is left between the features. This residual layer has to be removed in order to allow the surface functionalization.

For this work, PMMA (purchased from Agilent) was used as a resist because it exhibits very good resolution for NIL process.⁴² In the case of PMMA, the substrate is heated to 170 °C, above PMMA glass transition temperature. The mold is then pressed on the substrate using an adequate pressure (60 bars). The substrate is then cooled below the glass transition temperature to let the PMMA solidify and the mold is retrieved. Detailed methodology is presented in appendix ??.

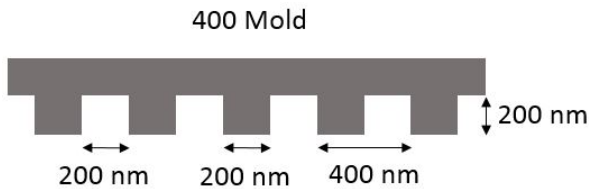


Figure 3.3 – Dimensions of the 400 Mold

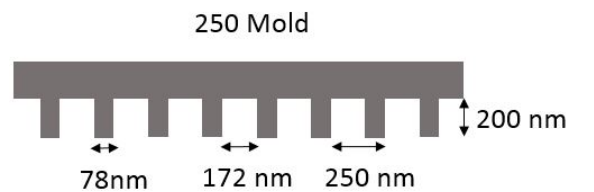


Figure 3.4 – Dimensions of the 250 Mold

Two different linear molds (purchased from AMO GmbH) were used in order to

investigate the pattern dimensions influence. These dimensions are illustrated in figures 3.3 and 3.4. The first mold shows 200 nm wide linear protrusions separated by a gap of 200 nm. The repeating unit is thus 400 nm wide and the mold is referred as the "400 mold". The second mold shows 78 nm wide linear protrusions separated by a gap of 172 nm which gives a repeating unit of 250 nm. This mold is thus called the "250 mold". These molds were coated with a perfluorosilane layer in order to avoid adhesion between the mold and the polymeric layer.

3.1.3 Growth of polymer brush

Theoretical background

A polymer brush refers to polymer chains end-grafted to a supporting surface. More precisely, a polymer brush *"corresponds to an array of macromolecular chains attached to a surface and in sufficient proximity so that the unperturbed solution dimensions (in a good solvent) of the chains are altered. Furthermore, this close proximity causes overlap of adjacent chains and thus significantly alters the conformational dimensions of individual polymer chains such that they extend or alter their normal radius of gyration to avoid unfavourable interactions."*

44

Polymer brushes offer considerable benefits over other surface modification techniques. Indeed, polymer brushes can be easily synthesized from a wide range of monomers and are adaptable to a range of substrates. Moreover, they offer a high mechanical and chemical robustness, ensuring a long-term stability.³⁹

Two approaches can be used to synthesise polymer brush. The first one is a physisorption approach which consists in the selective adsorption of one block of a diblock copolymer to a surface. This approach is rather simple as no chemical reaction is needed. However, the polymer brush can be unstable under certain conditions of solvent, temperature, shear stresses,... or can be displaced by other adsorbents since the physisorption is reversible.

An alternative approach consists in the covalent binding of polymer chains at the surface which leads to a better stability of the tethered polymer chains. This approach is known as chemisorption. These covalently attached polymer chains can be synthesized by two different methods. The Grafting-to approach involves the chemical reaction of preformed, functionalized polymers with surfaces containing complementary functional groups. This approach is technically simple and lead to a more precise characterization of the preformed polymer. On the other hand, it is not possible to produce very dense polymer brushes because the attachment of chains may be impeded by the existence of previously attached chains which cause steric hindrance. Furthermore, the layer thickness is limited by the molecular weight of the polymer in solution. On the opposite, the Grafting-from approach, also called surface-initiated polymerization, involves an in situ polymerization from an initiator functionalized surface. This technique produces polymer brushes of high grafting density. Indeed, since the chains are polymerized from the surface there is less steric hindrance caused by adjacent chains. Moreover, the layer thickness can be tune by adjusting the polymerization time.

The three different methods are illustrated in figure 3.5. In this work, surface-initiated polymerization was preferred over the other techniques for its numerous advantages.

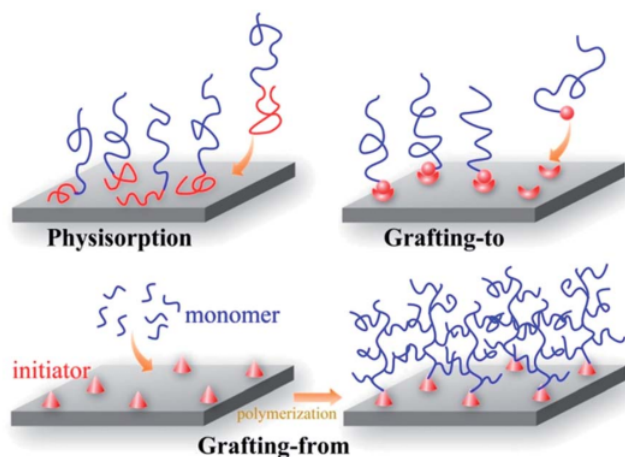


Figure 3.5 – Methods to prepare a polymer brush on a solid surface⁴⁵

More precisely, in order to obtain the best control over grafting density, polydispersity, brush thickness and composition, a controlled or living surface-initiated polymerization is required. Controlled surface-initiated polymerization techniques include living cationic and anionic polymerization, ring opening polymerization (ROP), reversible addition-fragmentation chain transfer polymerization (RAFTP), nitroxide-mediated radical polymerization (NMRP) and atom transfer radical polymerization (ATRP).^{46,47} Among these, cationic, anionic and ring opening polymerization techniques require stringent experimental conditions and sensitive catalysts.⁴⁷ RAFTP, NMRP and ATRP constitute flexible and less constraining techniques allowing the preparation of polymer brushes with controlled structures. All of them are based on the reversible dynamic equilibrium between dormant and active (radical) species. However, NMRP is not applicable to methacrylate-based monomers and chain transfer agents used in RAFTP are not commercially available.⁴⁷ ATRP is thus a good choice to obtain the desired polymer brush. This technique has several advantages including ease of preparation, commercially available and inexpensive catalysts, ligands and initiators. Moreover, it does not require stringent experimental conditions and allows the polymerization and block copolymerization of a wide range of functional monomers.^{47,48}

As mentioned above, hydroxyl-terminated oligo(ethylene glycol)methacrylate) polymer brushes are obtained by ATRP-polymerization. Principle of this synthesis is described below.

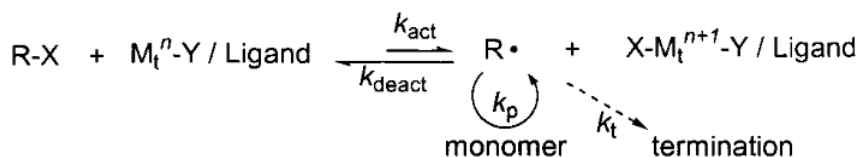


Figure 3.6 – Transition-metal catalyzed ATRP⁴⁸

ATRP process is illustrated in figure 3.6. The dormant species (R-X) is activated through a reversible redox process catalyzed by the transition metal complex ($M_t^n - Y/Ligand$) to generate radicals ($R\cdot$) via one electron transfer process. Simultaneously the transition metal is oxidized to higher oxidation state ($M_t^{n+1} - Y/Ligand$). This process occurs with a constant rate of activation, k_{act} , and deactivation k_{deact} , where the equilibrium is strongly shifted towards the dormant species ($k_{act} \ll k_{deact}$). Polymer chains grow by the addition of the intermediate radicals to monomers with a constant rate of propagation k_p . Radical species can also terminate, mainly through radical coupling and disproportionation, with a constant rate k_t .

Polymer brush elaboration

P(HOEGMA) polymer brushes were thus obtained by ATRP. These brushes contain PEG side chains ended by hydroxyl groups and are expected to be nontoxic, non-immunogenic, non-antigenic, and non-adhesive toward cells.³⁹

1. Gas-phase silanization

The gas-phase silanization allows to graft self-assembled monolayers of ATRP initiator consisting in (5'-dimethyl dichloro silyl propyl) 2-bromo-2-methylpropionate molecules illustrated in figure 3.7. The density of the polymer brush depends on the density of the initiator. The grafting mechanism is illustrated in figure 3.8. Gas-phase silanization allows to easily control moisture level allowing to avoid formation of aggregates. Moreover, the high diffusivity of gas molecules allows to functionalize nano-scale features where the access of liquid is limited by capillary forces. Finally, a liquid-phase silanization could induce undesirable swelling of the PMMA mask, distorting the pattern. The gaz-phase silanization was performed by injecting 20 μl of (5'-dimethyl dichloro silyl propyl) 2-bromo-2-methylpropionate in a degassed schlenk containing the samples. The reaction was run for 2 h at 80 $^{\circ}\text{C}$. Detailed methodology is presented in appendix ??.

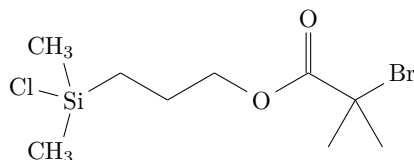


Figure 3.7 – (5'-dimethyl dichloro silyl propyl) 2-bromo-2-methylpropionate

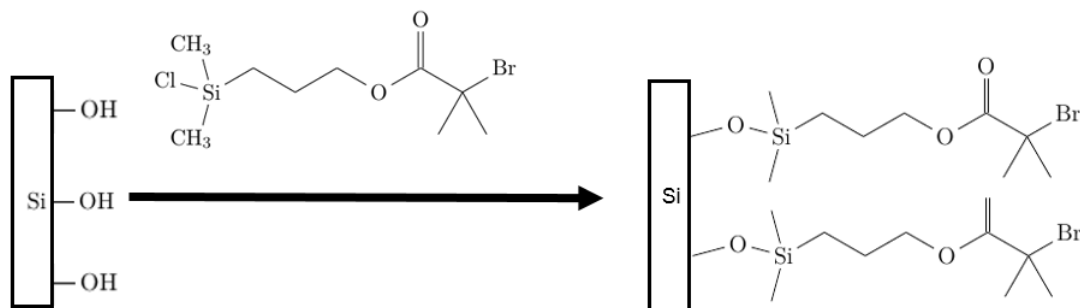


Figure 3.8 – ATRP initiator grafting

2. Liquid phase silanization

When the PMMA mask is removed, a liquid phase silanization is achieved to fill the regions previously covered by the mask and that are now exposed. This step thus allows to form a continuous background of PEG, known for its anti-adhesive properties, around the ATRP initiator. The molecule used for this step, 2-[methoxy(polyethyleneoxy)propyl] trichlorosilane, is illustrated in figure 3.9 while the overall reaction is illustrated in figure 3.10. The samples were immersed in a solution of 2-[methoxy(polyethyleneoxy)propyl] trichlorosilane and triethylamine in toluene. The reaction was run overnight in a glovebox at room temperature. More details on the methodology can be found in appendix ??.

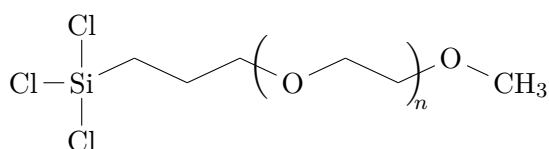


Figure 3.9 – 2-[methoxy(polyethyleneoxy)propyl] trichlorosilane (n is comprised between 6 and 9)

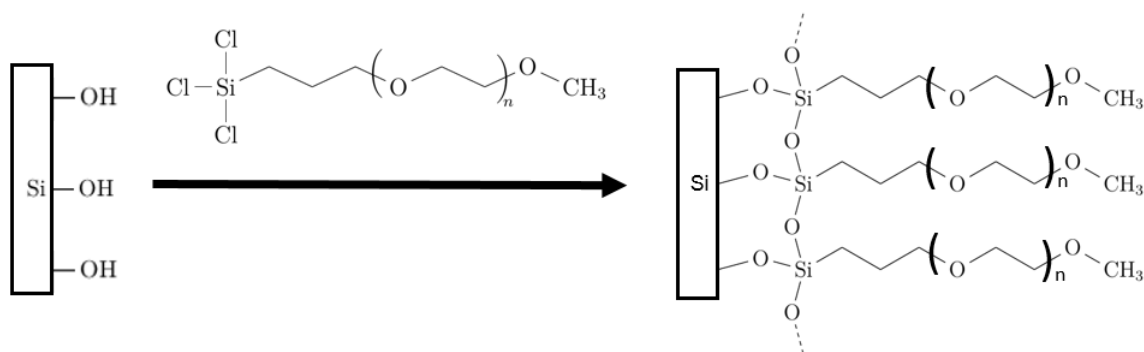


Figure 3.10 – Liquid phase silanization of PEG

3. Polymerization

Figure 3.11 gives the structure of HOEGMA monomer. Based on the structure and molar mass of the monomer, n , the number of ethylene glycol units was estimated to be 6.23. Figure 3.12 illustrates the formation of P(HOEGMA) polymer brush by the ATRP process. As can be seen from figure 3.12, HOEGMA monomers were dissolved in a solution of water and methanol. The catalyst used was based on copper, the two oxidation states being Cu^I and Cu^{II} . Since for copper-mediated ATRP, nitrogen-based ligands work particularly well,⁴⁸ bipyridine was used as a ligand to solubilize the transition metal ion and to adjust the reduction potential of the metal.⁴⁸ The polymerization was performed at ambient temperature during approximately 4 h, resulting in a polymer brush thickness of about 30 nm (measured by ellipsometry). More information about the reaction performed can be found in appendix ??.

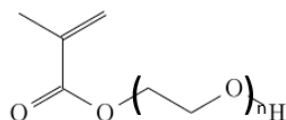


Figure 3.11 – HOEGMA monomer

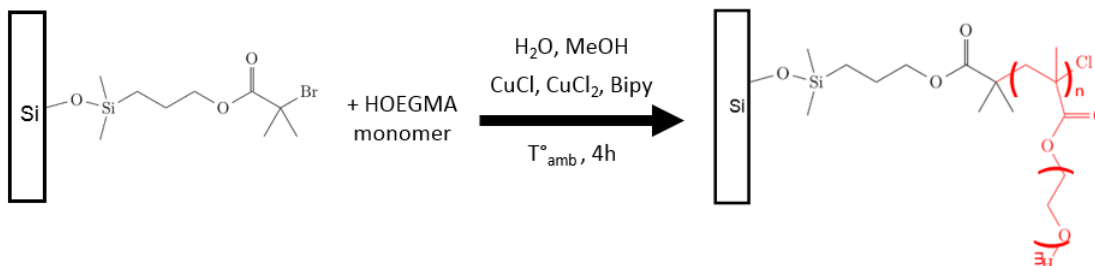


Figure 3.12 – P(HOEGMA) polymer brush growing from the ATRP initiator

3.1.4 Biofunctionalization of the polymer brush

Heterolinker description

The first step to biofunctionalize the polymer brush is to graft a linker which will allow to subsequently graft the peptides by a covalent bond. 4-Maleimidophenyl isocyanate, an heterobifunctional crosslinker, also known as PMPI was used for this purpose. PMPI structure is shown in figure 3.13. Heterobifunctional agents possess two selectively reactive groups that allow coupling to be carried out in a stepwise manner, leading to a better control of the conjugation chemistry. This compound is a maleimide-and-isocyanate crosslinker : the isocyanate group reacts with hydroxyl groups found at the end of lateral chains of the P(HOEGMA) brush to form carbamate linkage while the maleimide group

reacts with sulfhydryl groups present on the cystein residue of the peptide to form stable thioether linkages.⁴⁹

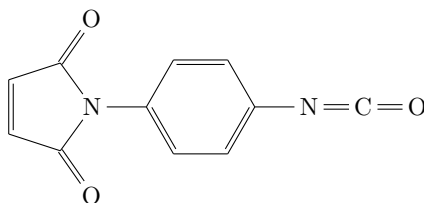


Figure 3.13 – PMPI : 4-Maleimidophenyl isocyanate

Isocyanate group reaction

First, the linker needs to be covalently linked at the end of the lateral chains of the P(HOEGMA) brush. In order to get this, the isocyanate group of the linker is reacted with an hydroxyl group found at the end of the lateral chains of the P(HOEGMA) brush to form carbamate linkage. The grafting of the PMPI linker is illustrated in figure 3.14. PMPI was dissolved in distilled dry THF before being injected in degassed schlenks containing the samples. The reaction was run for 6 h at room temperature.

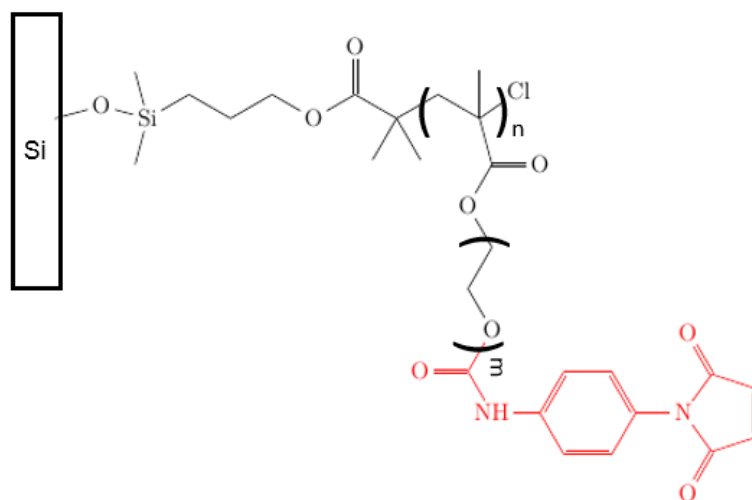


Figure 3.14 – PMPI linker grafted on the lateral hydroxyl groups of the P(HOEGMA) brush

Maleimide group reaction

Then, peptides are grafted at the end of the linker by reacting with the maleimide group. This group reacts with sulfhydryl groups to form stable thioether linkages. Thus, the different peptides must contain a sulfhydryl group to be able to be grafted. Since the amino acid cystein (structure is shown in figure 3.15) is the only one to contain a sulfhydryl group, peptides bearing a cystein were thus used to allow for the grafting (KRDGSPC containing a C-terminal cystein, LL37 with a N-terminal cystein and magainin I with a

Peptide	Sequence
RGD	KRGDSPC
LL37	CLLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES
Magainin I	GIGKFLHSAGKFGKAFVGEIMKSC

Table 3.1 – Sequences of the three different peptides used to biofunctionalize the samples

C-terminal cystein). Peptides sequences are given in table 3.1. The peptide grafting is illustrated in figure 3.16. Peptides were dissolved in 100mM trisbuffer (pH 7.05). The samples were placed in this solution for 2 h 30 min at room temperature. Detailed procedure is given in appendix ??.

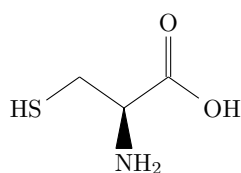


Figure 3.15 – Cysteine amino acid

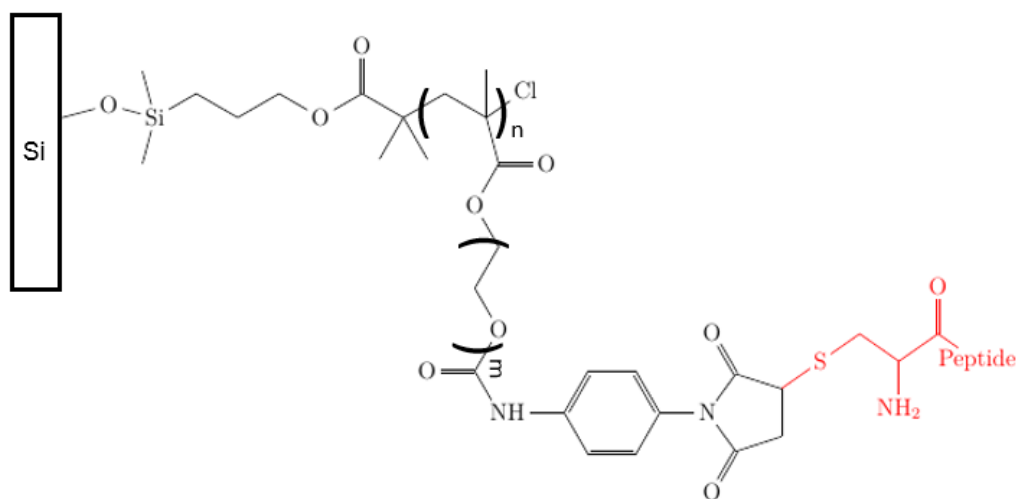


Figure 3.16 – Peptides with N-terminal cystein grafted at the end of the PMPI

Depending on the presence of a pattern and on the peptide grafted on the surface, nine different surfaces have been synthesized and are represented in figure 3.17

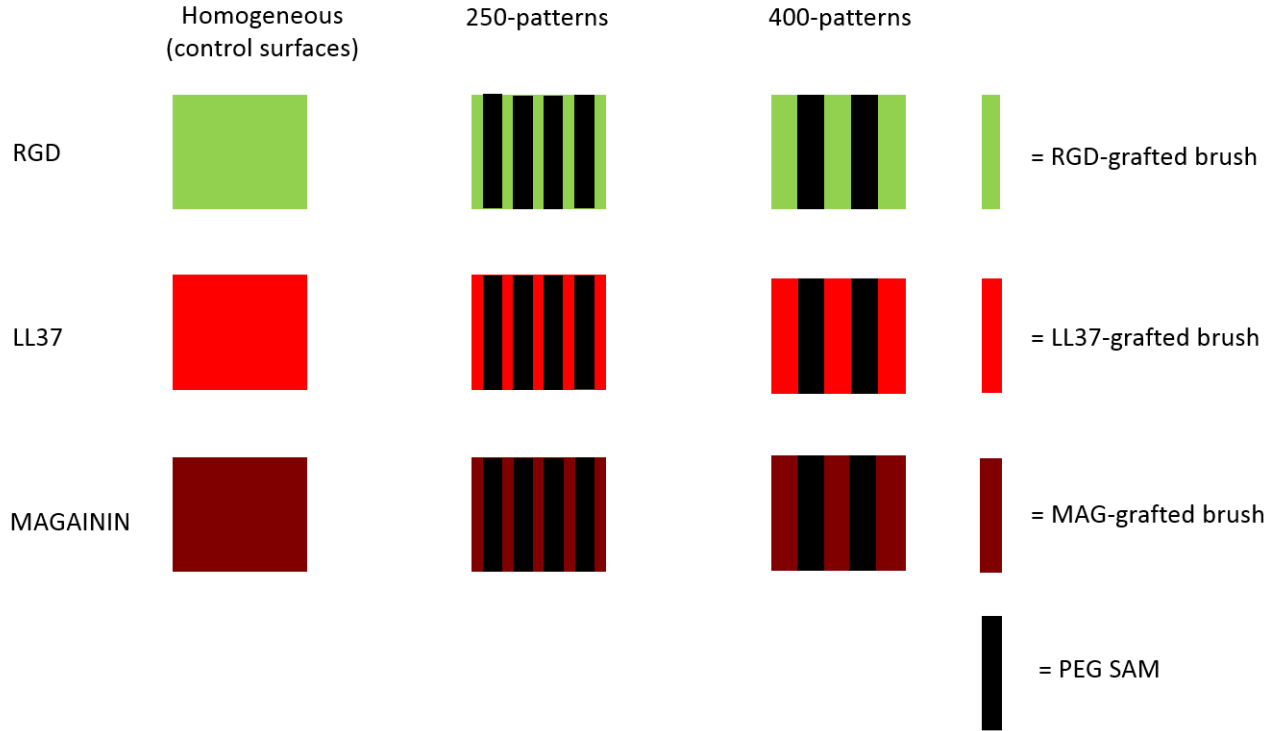


Figure 3.17 – Different types of surfaces synthesized

3.2 Surfaces characterization

3.2.1 Scanning Electron Microscopy

SEM imaging was used to characterize the two different molds used for the nanoimprint lithography. These images were obtained using a JEOL USA JSM-7610F Scanning Electron Microscope at 15keV.

3.2.2 Ellipsometry

Ellipsometry measurements were performed to characterise the layer thickness obtained after the different steps. All measurements were taken on homogeneous surfaces elaborated at the same time as the nanopatterned surfaces and used as control surfaces. Five measurements were taken and averaged for each sample. The first measure was taken after the PMMA coating in order to check if the layer thickness is sufficient to not damage the mold. The second and third measures were taken before and after the etching step to determine the thickness of the removed layer. These three first measures were taken in the cleanroom facilities with a Sentech SE 850 Ellipsometer using SpectraRay software. Another measure was taken after the brush polymerization to quantify the final brush height. Finally, a last measure was taken after the peptide grafting step. These last ellipsometric measurements were carried out with a spectroscopic ellipsometer (RSE from J. A. Woollam Co., Inc.). Details about the models used can be found in appendix ??.

3.2.3 Atomic Force Microscopy

The surface morphology of the nanopatterned surfaces were observed by Atomic Force Microscopy (AFM) with a Nanoscope VIII Multimode AFM (Bruker corporation). AFM observations after the polymer brushes polymerization and after the peptides grafting were carried out in tapping mode with a PPP-NCHR tip with a spring constant of 42 N/m.

3.2.4 X-ray Photoelectron Spectrometry

X-ray Photoelectron Spectrometry (XPS) analysis were performed in order to obtain the elemental composition of the different surfaces. From these, it was possible to quantify the percentage of monomer unit grafted by the different peptides in the polymer brush using the following equations based on the N/C and N/O ratios (developments leading to these formula are detailed in appendix ??).

$$x = \frac{\frac{\%N_{tot}}{\%C_{tot}} * C_{HOEGMA}}{\frac{\%N_{tot}}{\%C_{tot}} (C_{HOEGMA} - C_{HOEGMA_{peptide}}) + N_{HOEGMA_{peptide}}} \quad (3.1)$$

$$x = \frac{\frac{\%N_{tot}}{\%O_{tot}} * O_{HOEGMA}}{\frac{\%N_{tot}}{\%O_{tot}} (O_{HOEGMA} - O_{HOEGMA_{peptide}}) + N_{HOEGMA_{peptide}}} \quad (3.2)$$

where C_{HOEGMA} and O_{HOEGMA} are respectively the number of carbon and oxygen atoms in one monomer of HOEGMA ; $C_{HOEGMA_{peptide}}$, $O_{HOEGMA_{peptide}}$ and $N_{HOEGMA_{peptide}}$ are respectively the number of carbon, oxygen and nitrogen atoms in one peptide-grafted HOEGMA monomer and $\%C_{tot}$, $\%O_{tot}$ and $\%N_{tot}$ are the elemental compositions of the peptide-grafted brush obtained by XPS measurements. From these formula, it can be seen that the number of atoms of each type constituting HOEGMA and peptide-grafted HOEGMA monomers is required. These values are presented in table 3.2. Based on the HOEGMA monomer structure and molar mass, it was possible to estimate the number of ethylene glycol units constituting the side chain of the monomer. For a molar mass of 360g/mol, the number of ethylene glycol units n equals 6.23, explaining the decimal numbers obtained in table 3.2. From this value, it was then possible to determine the number of each atom constituting the HOEGMA monomer. Then, the number of each type of atom constituting the different peptides were added to obtain the final values in table 3.2.

	C	O	N
HOEGMA	16.46	8.23	0
HOEGMA-RGD	56.46	22.23	12
HOEGMA-LL37	235.46	65.23	62
HOEGMA-MAG	139.46	39.23	30

Table 3.2 – Number of atom constituting HOEGMA monomer and peptide-grafted HOEGMA monomer

3.3 Bacterial tests on the surfaces

The next step of this work is to investigate the influence of the chemically nanopatterned surfaces on the behaviour of bacteria. In order to do this, *E. coli*, a gram negative bacteria, is grown on the different nanopatterned surfaces and on homogeneous surfaces. The surfaces are then observed by epifluorescence microscopy after labelling of the cells.

3.3.1 Bacterial culture on the surface

First, Petri dishes are inoculated with *E. coli* MG1655 strain and placed during one night at 37°C (figure 3.18A). Then, some colonies are taken out from the Petri dish and seeded in 20 ml of LB Broth medium which is then placed at 37°C, 180 rpm during one night. This is the overnight culture (figure 3.18B). The next morning, 5 ml are taken from the overnight culture and diluted with 20 ml of fresh LB broth medium. This solution is again placed at 37°C, 180 rpm for 2 h (figure 3.18C). Then, 6 eppendorfs of this culture are retrieved and the bacteria are harvested by centrifugation (3 min, 10000 rpm). The bacteria are then suspended in M9 minimum medium. This procedure is repeated three times. These 6 eppendorfs are then brought together in a tube forming the bacterial suspension (figure 3.18D). The obtained solution is diluted with M9 minimum medium to reach an optical density (OD) between 0.22 and 0.24 at 600 nm, corresponding to about 2.5×10^8 cells/mL. This dilution step is required to avoid having too much bacteria on the surfaces. The nanopatterned and homogeneous surfaces are rinsed with methanol then with filtered Milli-Q water and dried with a stream of nitrogen before being placed in a well plate. Each well was filled with 2 ml of bacteria suspension in M9 minimum. The adhesion step was made without agitation at room temperature during 1 h (figure 3.18E). After the adhesion step, all samples are gently rinsed successively in three tubes filled with filtered 0.15 M NaCl solution ($\text{pH} \simeq 7.25$). The samples for the observation of the adhesion step are then placed 15 min in the viability kit solution in order to stain the cells (figure 3.18F). Afterwards, they are rinsed in one tube filled with M9 minimum and placed in filtered 0.15 M NaCl ($\text{pH} \simeq 7.25$) for observation (figure 3.18G). The samples reserved for the observation of bacterial growth were immersed in 3ml M9 minimum medium and placed at 37°C in static condition. (figure 3.18H). This minimum medium was refreshed after 1 h: 1.5 ml of used medium was changed by 1.5 ml of fresh medium. After 7 h- and 12 h-growth the samples were rinsed in one tube filled with M9 minimum solution and placed in the viability kit solution for 15 min (figure 3.18I). These samples are finally rinsed in one tube filled with M9 minimum and placed in filtered 0.15 M NaCl ($\text{pH} \simeq 7.25$) for observation (figure 3.18J). For each observation time, 2 samples were used. All samples were observed by epifluorescence microscopy and 9 images were taken at random locations on each surface in order to quantify the percentage of surface covered by bacteria. These images were thus analysed with a custom made IGOR PRO routine developed by Pr. Alain Jonas, allowing counting the numbers of green, red and yellow pixels corresponding to lived, dead and dying bacteria, respectively.

The process previously described is the general method for the bacterial tests. For the RGD-grafted surfaces, the surfaces were observed after 1 h (adhesion step), 7 h growth and 12 h-growth. For the AMP-grafted surfaces, the samples were observed after 1 h (adhesion step) and 7 h-growth. These observation times were selected based on the growth curve of *E. coli* in M9 minimum medium. This curve is illustrated in appendix

?? It was found that bacterial exponential phase starts after approximately 450 min ($\approx 7h$). During this phase, microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the environmental conditions.⁸ Since the bacterial population is the most uniform in terms of chemical and physiological properties during this phase, exponential phase cultures are usually used in biochemical and physiological studies.⁸ After more than 12 h in M9 minimum medium, the bacterial suspension presented aggregates and anomalous deposits. Thus, 12 h was selected as the maximal observation time. Materials used as well as media and viability kit preparation are described in appendix ??.

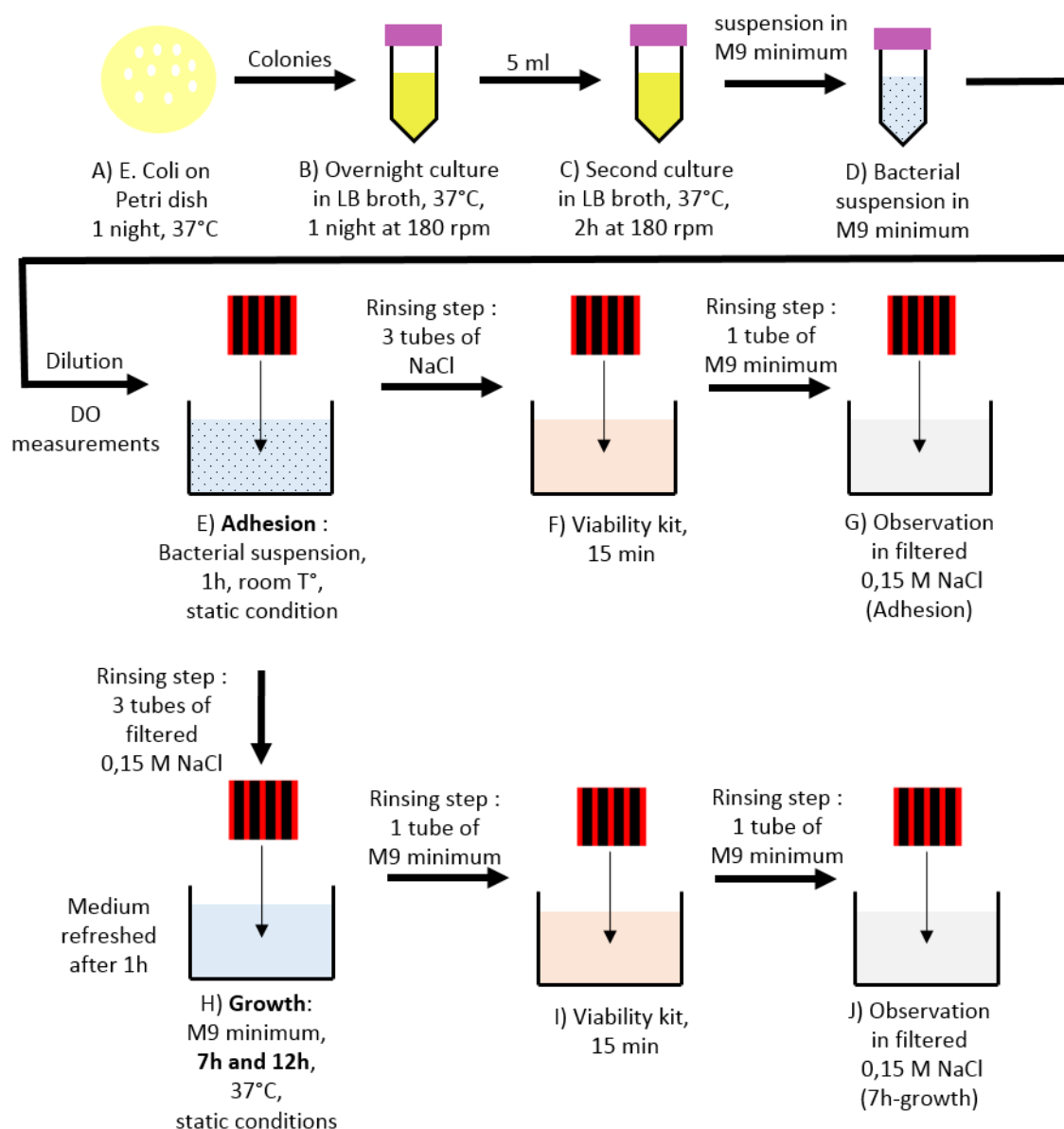


Figure 3.18 – Methodology used to perform bacterial tests on the surfaces

3.3.2 Viability kit

The LIVE/DEAD[®] BacLight[™]Bacterial Viability Kit is used to assess the bacterial membrane integrity. It is based on a mixture of SYTO[®] 9 green-fluorescent nucleic acid dye and the red-fluorescent nucleic acid dye, propidium iodide. These dyes differ both in their spectral characteristics and in their ability to penetrate bacterial cell membrane. The SYTO 9 dye usually labels all bacteria (with intact membrane and damaged membrane as well). In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium iodide dyes, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.⁵⁰

4.1 Mold characterization

The molds used for nanoimprint lithography were characterized by Scanning Electron Microscopy (SEM). As a reminder, the 400 mold theoretically shows 200 nm wide protrusions separated by a gap of 200 nm while the 250 mold shows 78 nm wide protrusions separated by a gap of 172 nm. It can be seen from figures 4.1 and 4.2 that the widths of the lines do not exactly correspond to the theoretical values provided by the company providing the molds. Indeed, for the 400 mold, the protrusions were about 175 nm wide and the gaps were approximately 218 nm wide instead of being 200 nm wide each. Thus, the protrusions were smaller than expected while the gaps were wider. For the 250 mold, the protrusions were about 71 nm wide and the gaps were approximately 171 nm wide instead of being 78 nm and 172 nm wide respectively. The 250 mold dimensions were thus close to the expected values.

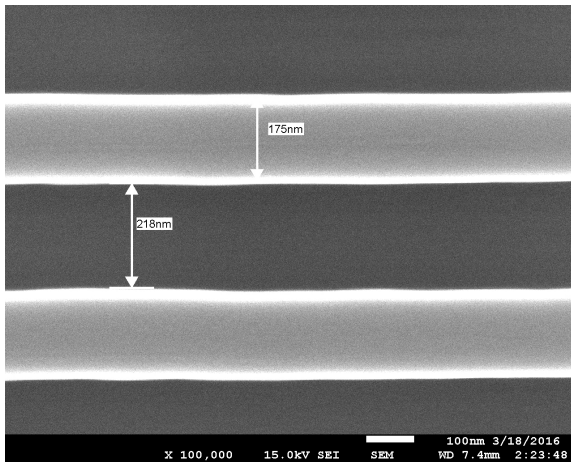


Figure 4.1 – SEM image of the 400 mold

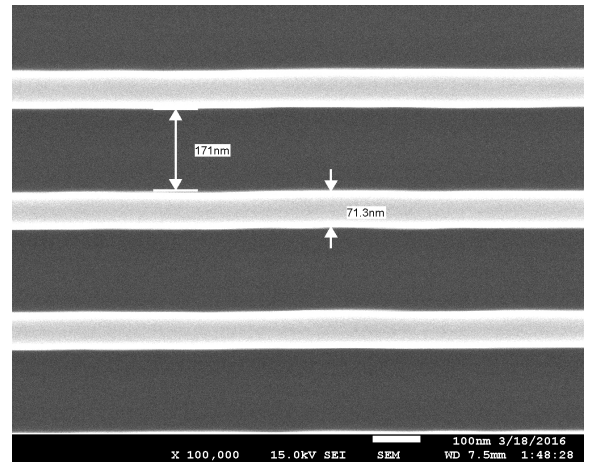


Figure 4.2 – SEM image of the 250 mold

4.2 Surface characterization

4.2.1 Thickness measurements

Ellipsometry measurements were carried out after each step as previously described. The PMMA layer was 150-160nm thick for the 250 pattern and 115-120nm for the 400 pattern. These different thicknesses were required in order to avoid damaging the molds. After the descum, consisting in exposing the PMMA coated substrates to an oxygen plasma during 3 min 40 sec, a 50 ± 4 nm thick layer of PMMA was removed as measured on the control surface. The next measurement is achieved after the brush polymerization. After a polymerization time of 4 h, the brush thickness was of about 28 ± 2 nm. Finally, the last measurement was performed after the peptide grafting step. For RGD-grafted surfaces, the thickness increased by 8 ± 2 nm while for LL37-grafted surfaces and MAG-grafted surfaces the thickness increased by 10 ± 3 nm and 7 ± 1 nm, respectively. This last measurement allows to confirm the successful grafting of the peptides.

4.2.2 Characterization of the surface morphology

The two different nanopatterns were characterized by AFM after the different steps of the elaboration process. As a reminder, these patterns consist in alternating lines of P(HOEGMA) and PEG. These lines of P(HOEGMA) are then grafted with an adhesive RGD peptide or antimicrobial peptides.

Nanopatterned polymer brush

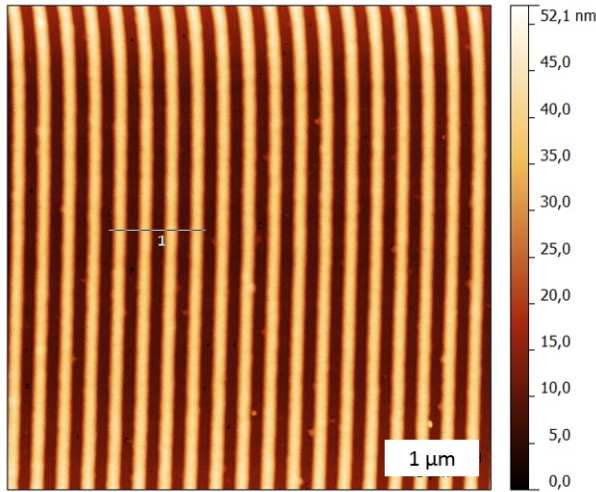


Figure 4.3 – AFM image of P(HOEGMA)-PEG 250-pattern

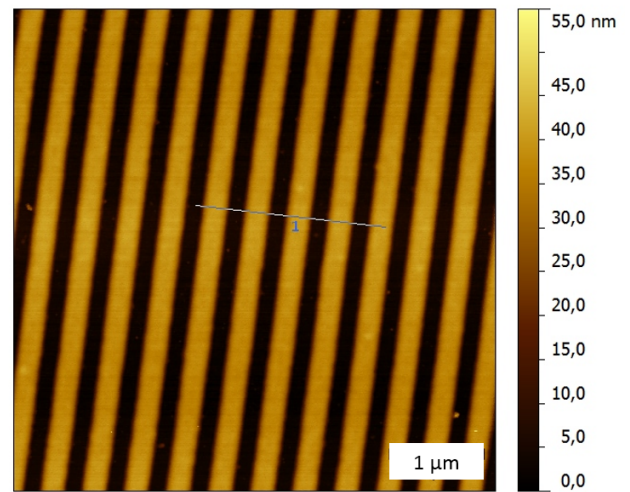


Figure 4.4 – AFM image of P(HOEGMA)-PEG 400-pattern

Figures 4.3 and 4.4 show AFM images of the two nanopatterns before the peptide grafting step, confirming that the surfaces have the expected design.

Figures 4.5 and 4.6 show the height profiles of the two nanopatterns. It can be seen that there is a height difference between the P(HOEGMA) brush and PEG layer of about 32-37 nm depending on the final height of the P(HOEGMA) brush. These values are

coherent with those obtained by ellipsometry.

Moreover, it can be seen from figures 4.5 and 4.6 that the P(HOEGMA) lines width is greater than expected from the molds dimensions. Indeed, 120 ± 2 nm wide P(HOEGMA) brushes are observed for the 250-pattern compared to 78 nm expected and 246 ± 1 nm wide P(HOEGMA) lines are seen for the 400-pattern compared to 200 nm expected. Indeed, these differences are due to the fact that the brush extends beyond the grafting region because of their non-ideal nature.

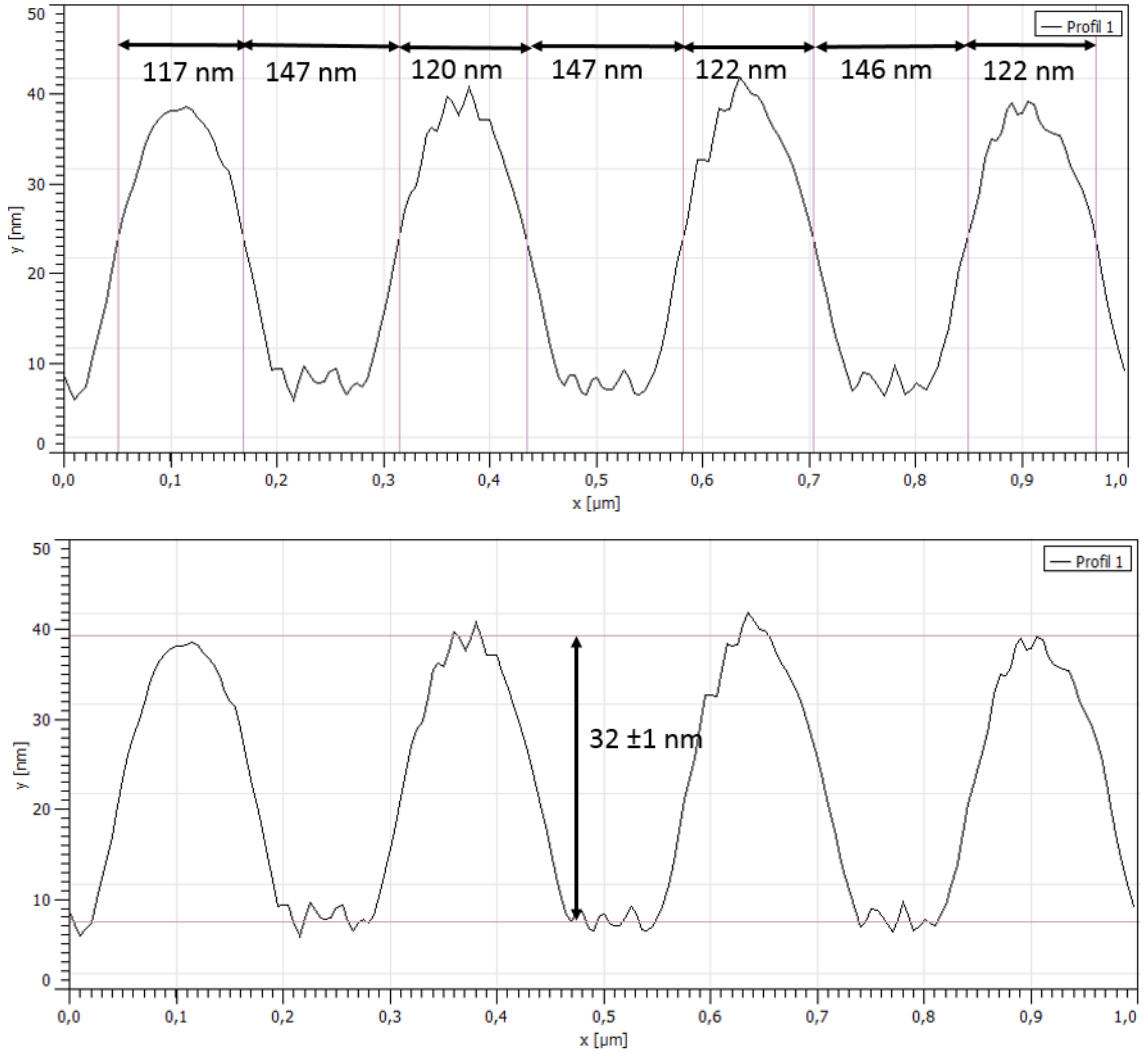


Figure 4.5 – Height profile of P(HOEGMA)-PEG 250-pattern

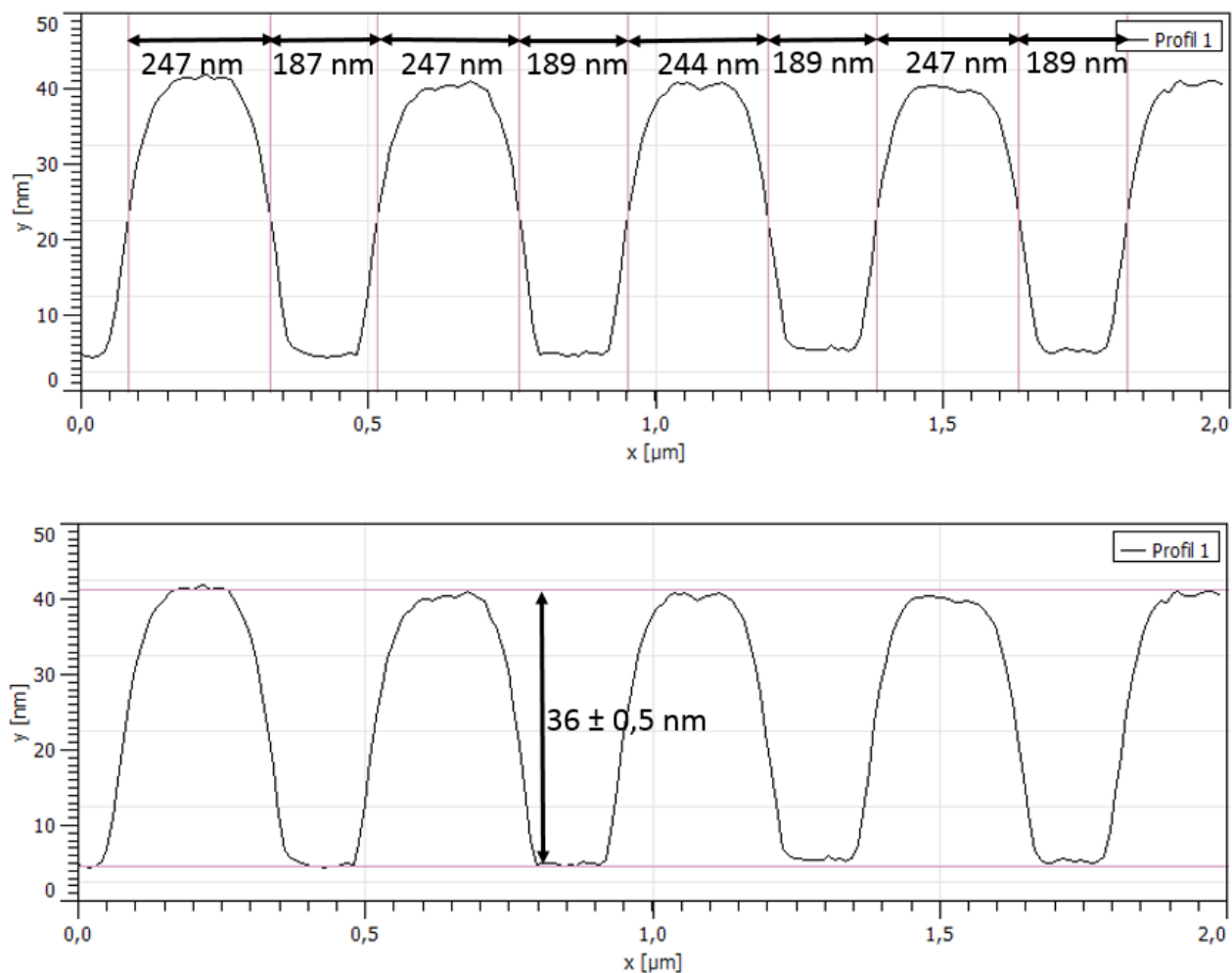


Figure 4.6 – Height profile of P(HOEGMA)-PEG 400-pattern

Nanopatterned grafted polymer brush

Only RGD-grafted surfaces were imaged by AFM at this point. Ellipsometry measurements showed slightly different thickness variations following grafting of the different peptides. However, the surface morphology of the different samples, detailed below, are believed to be similar. AFM imaging of LL37 and MAG-grafted surfaces would be required to confirm this hypothesis.

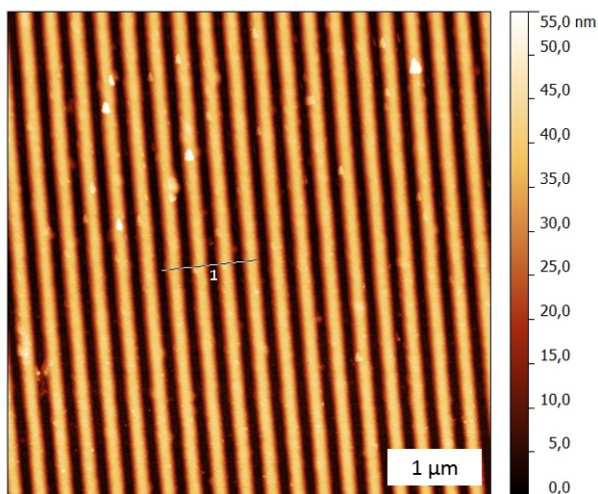


Figure 4.7 – AFM image of RGD-grafted P(HOEGMA)-PEG 250-pattern

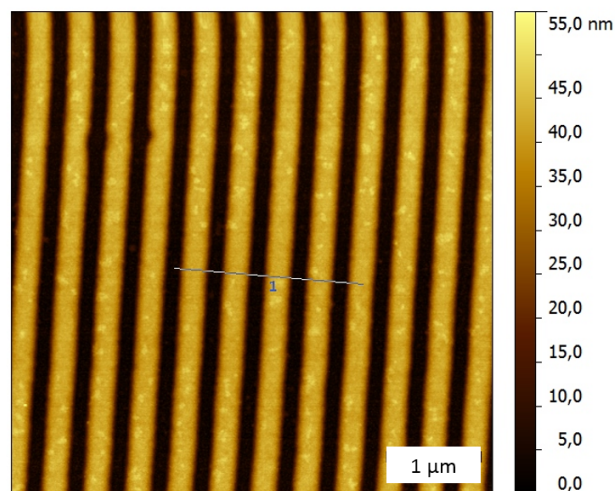


Figure 4.8 – AFM image of RGD-grafted P(HOEGMA)-PEG 400-pattern

Figures 4.7 and 4.8 show AFM images of RGD-grafted nanopatterned surfaces and figures 4.9 and 4.10 show the height profiles for these two surfaces. Compared to non-grafted surfaces, there is an increased in height of about 4-5 nm which is relatively close to the values obtained by ellipsometry (8 nm). At the end, the final height difference between the P(HOEGMA) brush and the PEG SAM is of about 36-40 nm. Since Aurélie Merck found that PMMA-grafted surfaces showing a topographical difference of 22 nm do not influence the bacterial behaviour,²² it has been hypothesized that a height difference of about 40 nm would not influence it either. As a result, any change in bacterial behaviour is attributed solely to the chemical nanopatterning.

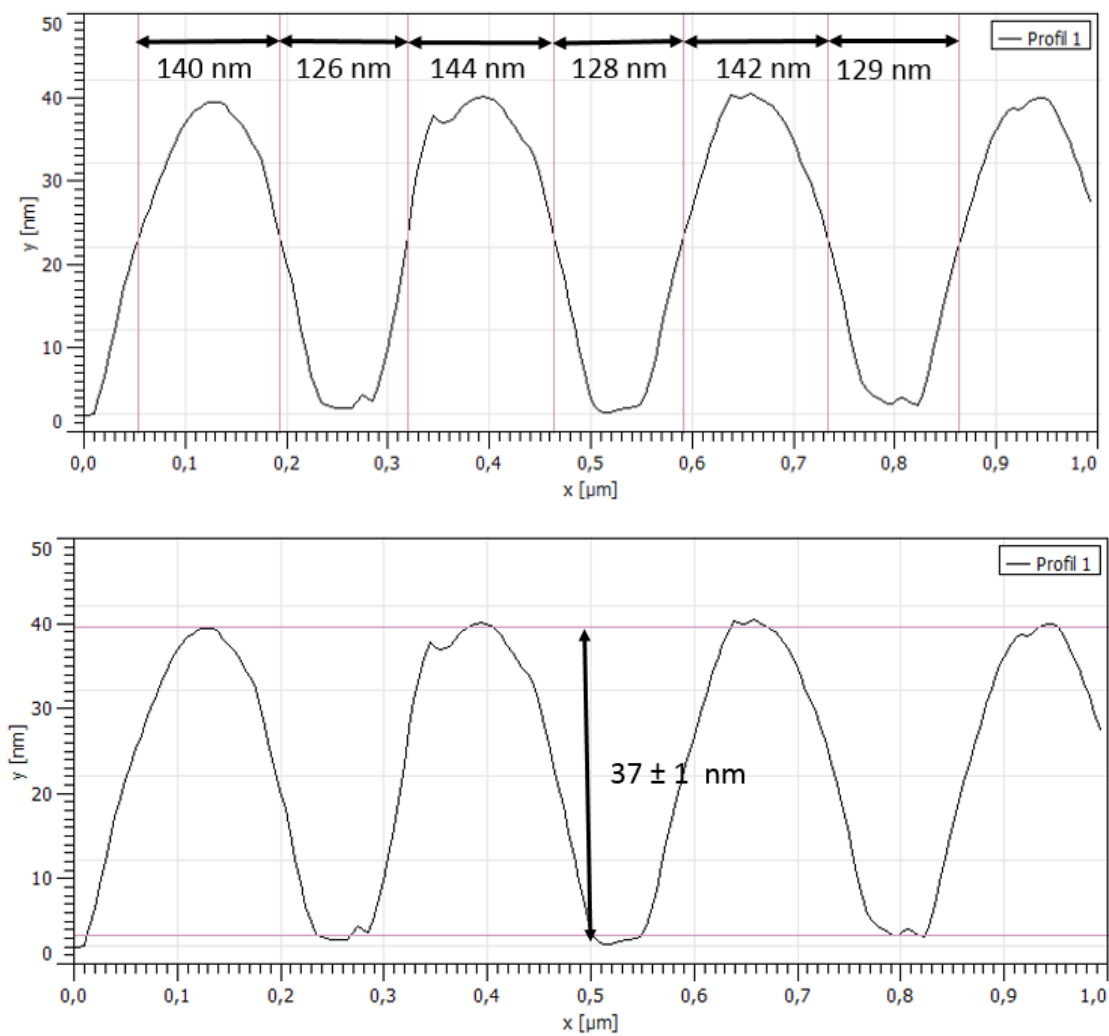


Figure 4.9 – Height profile of RGD-grafted P(HOEGMA)-PEG 250-pattern

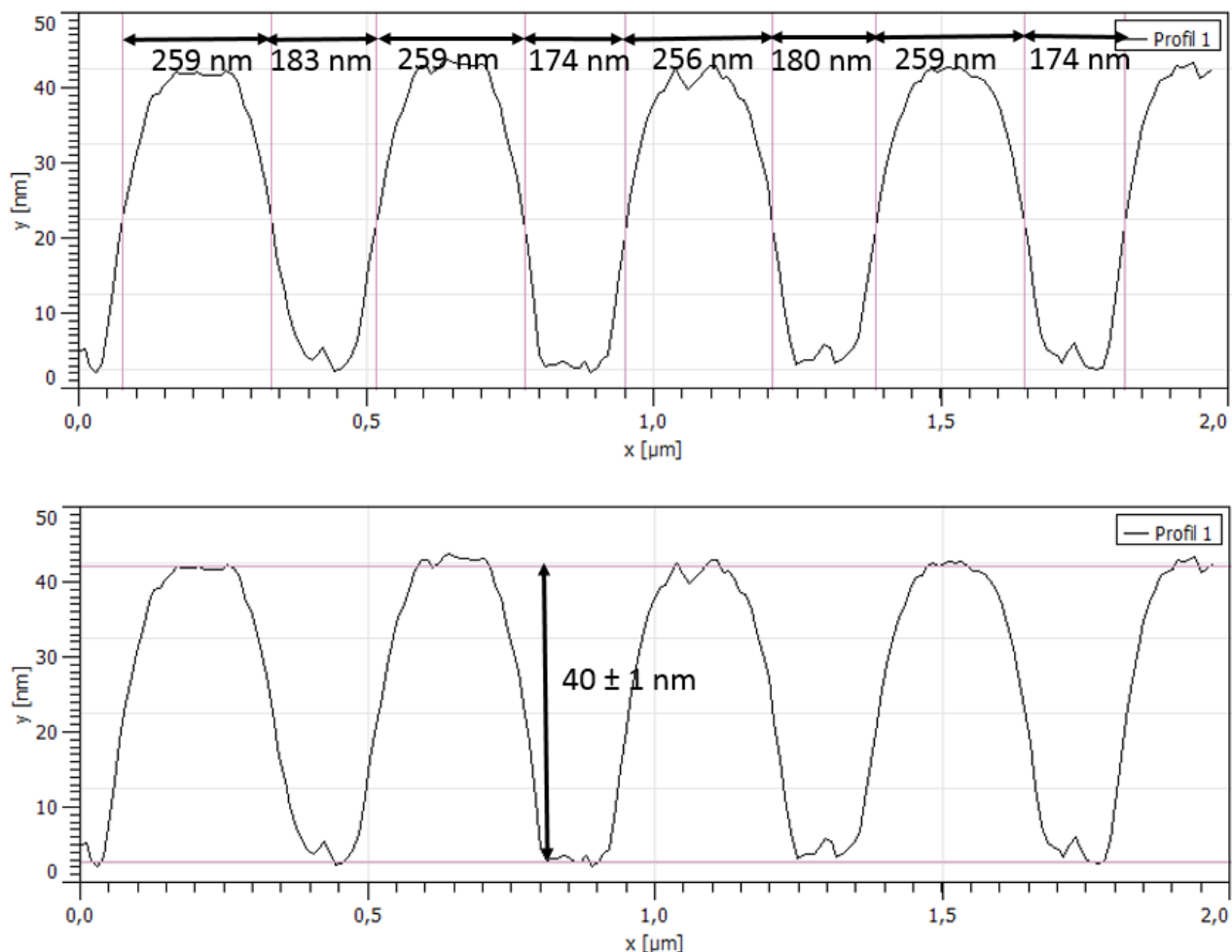


Figure 4.10 – Height profile of RGD-grafted P(HOEGMA)-PEG 400-pattern

4.2.3 Characterization of the chemical composition of the surfaces

XPS spectra obtained for the homogeneous surfaces grafted or not with RGD, LL37 and MAG peptides are presented in figures 4.11, 4.12 and 4.13, respectively. For each type of surface, spectra before and after peptides grafting can be compared. From these figures, it can be observed that an additional peak attributed to nitrogen is present after the grafting step, confirming the presence of peptides on the surfaces. Elemental compositions of the different surfaces were then obtained from these spectra and are presented in figure 4.14. The elemental compositions allowed to quantify the percentage of HOEGMA monomer unit grafted by the different peptides according to equations 3.1 and 3.2 presented page 38. The results are shown in table 4.1. From these values, it can be seen that the percentage of monomer units grafted by the two AMP is quite low as this percentage is approximatively of 5%. However, this value is consistent with previous results obtained on similar brushes grafted with magainin I molecules, which claims that "from 5 to 10% of available hydroxyl moieties are substituted by the magainin peptide".³⁹ This suggests that peptides are mainly grafted at the surface of the

polymer brush due to the size and amphiphilic nature of the peptide.³⁹ The percentage of monomer units grafted by RGD molecule is clearly higher, possibly due to the smaller size of this peptide compared to the two others.

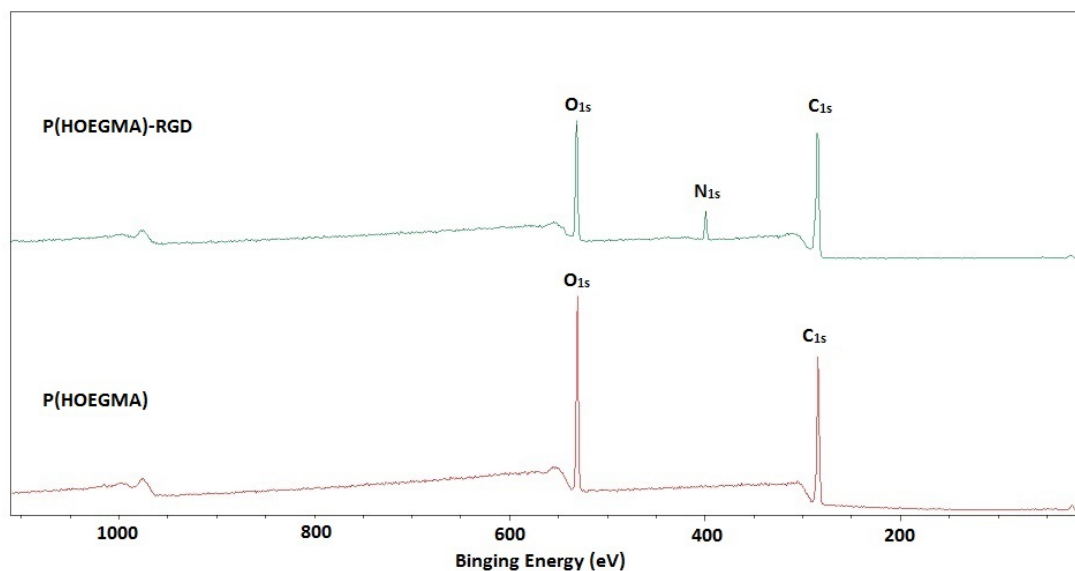


Figure 4.11 – XPS survey spectra of P(HOEGMA) and P(HOEGMA)-RGD brushes

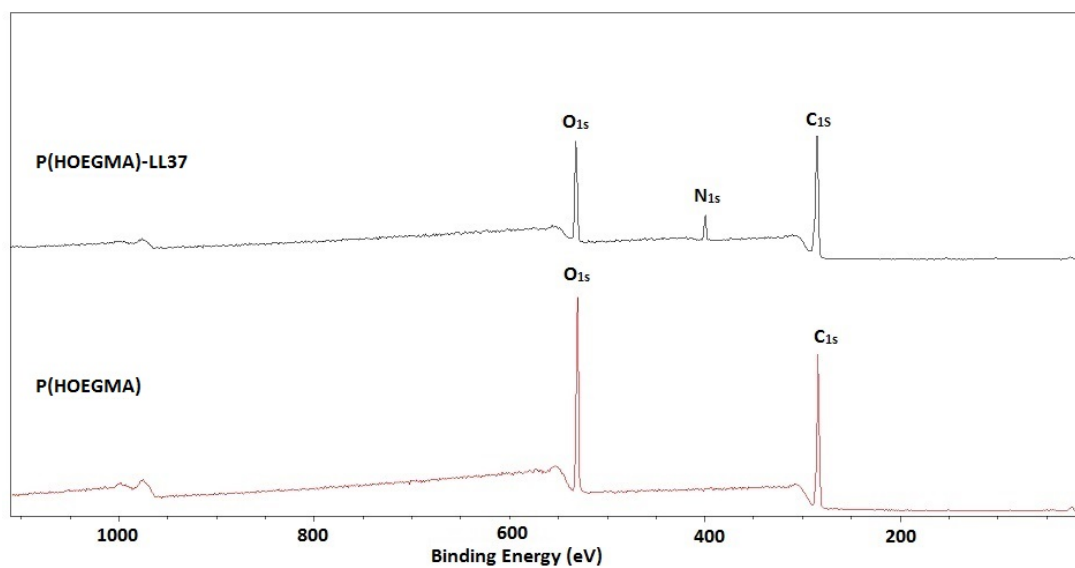


Figure 4.12 – XPS survey spectra of P(HOEGMA) and P(HOEGMA)-LL37 brushes

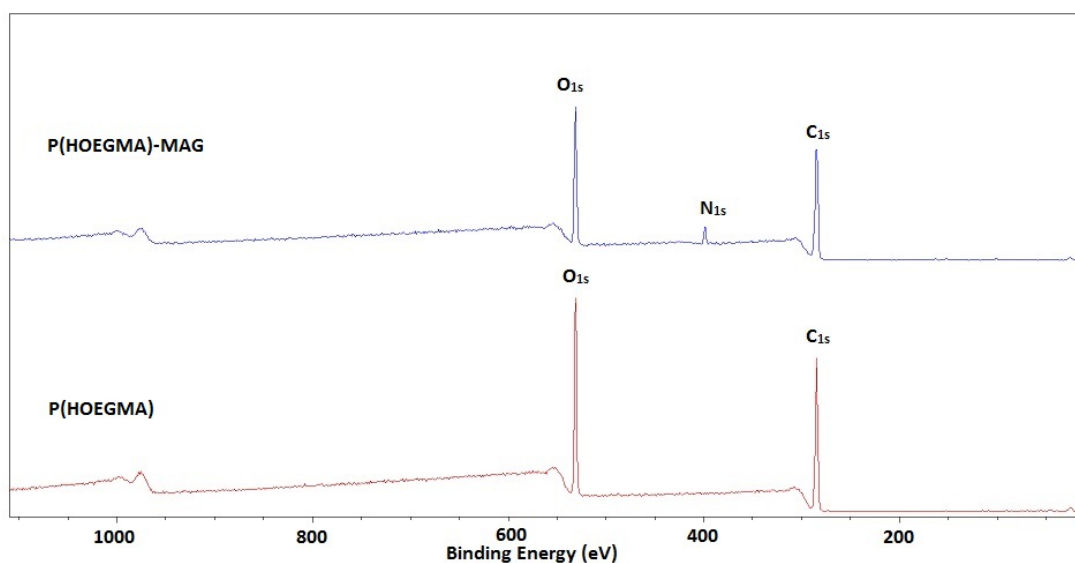


Figure 4.13 – XPS survey spectra of P(HOEGMA) and P(HOEGMA)-MAG brushes

		C				O	N	S	F	Si
		C 1s				O 1s	N 1s	S 2s	F 1s	Si 2p
		C-(C,H)	C-O	O=C-O	C tot					
HOEGMA	Peak Position	284,8	286,24	288,56		532,46				101,96
	FWHM	1,784	1,784	1,784		2,123				1,762
	Atomic Concentration	16	44,78	7,28	68,06	31,76				0,18
HOEGMA-RGD	Peak Position	284,8	286,16	288,51		532,32	400,12			102,22
	FWHM	1,802	1,866	1,803		2,296	2,011			2,016
	Atomic Concentration	33,05	31,43	9,07	73,55	20,49	5,25			0,7
HOEGMA-LL37	Peak Position	284,8	286,18	288,41		532,21	400,11	232,51		102,31
	FWHM	1,813	1,813	1,813		2,392	1,946	0,868		2,128
	Atomic Concentration	35,42	27,62	9,57	72,61	20,33	6,35	0,09		0,62
HOEGMA-MAG	Peak Position	288,36	286,29	284,8		532,61	400,11	227,91		102,31
	FWHM	1,819	1,795	1,819		2,364	1,937	2,647		1,655
	Atomic Concentration	7,718	33,023	27,054	67,795	26,494	4,554	0,36		0,798

Figure 4.14 – XPS analysis results : elemental composition for the different surfaces

	% of HOEGMA units grafted by peptide	
	based on N/C ratio	based on N/O ratio
HOEGMA-RGD	12.84%	25.06%
HOEGMA-LL37	3.35%	5.81%
HOEGMA-MAG	5.08%	5.73%

Table 4.1 – Percentage of monomer units grafted by the different peptides computed from XPS analysis of the polymer brushes

4.3 Bacterial behaviour on surfaces

4.3.1 RGD-grafted surfaces

Microscopy observation

Bacterial adhesion and growth on RGD-grafted surfaces (nanopatterned surfaces and homogeneous surfaces as control) were observed by epifluorescence microscopy. As a reminder, dead and alive bacteria were stained in red or green, respectively. However, as the number of dead bacteria was negligible, images obtained only using the green channel are shown here. The results obtained for the different surfaces are shown in figures 4.15, 4.16 and 4.17 after the adhesion step, figures 4.18, 4.19 and 4.20 after 7 h-growth and 4.21, 4.22 and 4.23 after 12 h-growth. Pattern direction is indicated with white lines. At this point, bacterial surface coverage and aggregates morphology were qualitatively assessed. It is important to mention that all these surfaces were not homogeneous regarding bacterial adhesion. Some regions of these surfaces presented more bacteria than others, leading to large standard deviation values. Regions of interest are presented in this work and it is important to keep in mind that these images are not necessarily representative of the entire surface. It is also important to remember that P(HOEGMA) brushes are non-adhesive toward bacterial cells.

Adhesion

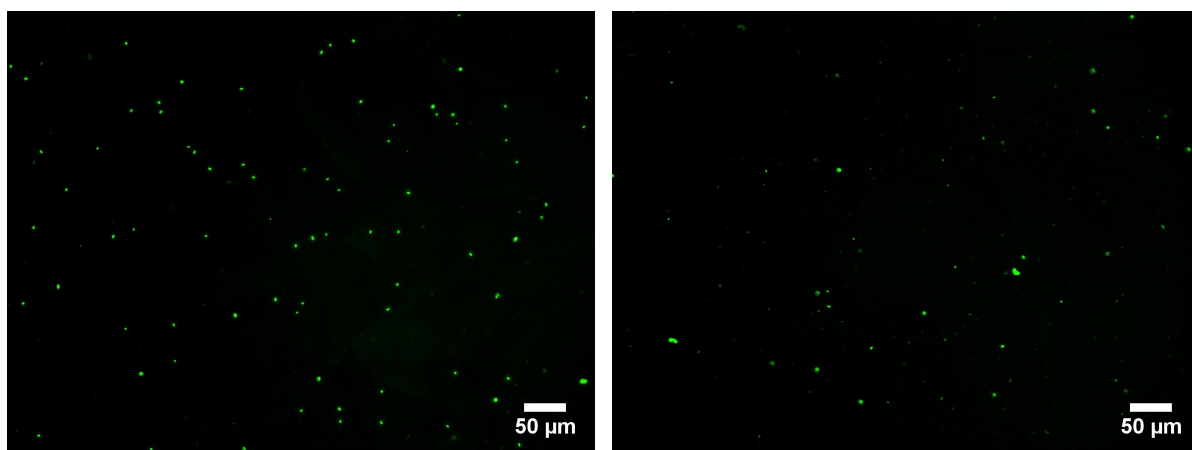


Figure 4.15 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces after adhesion (green channel)

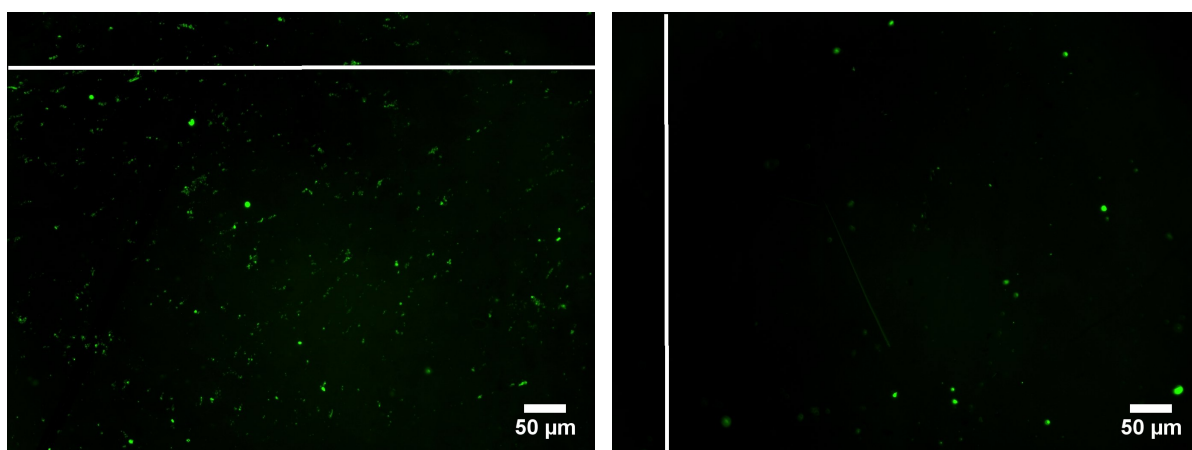


Figure 4.16 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 RGD-grafted surfaces after adhesion (green channel)

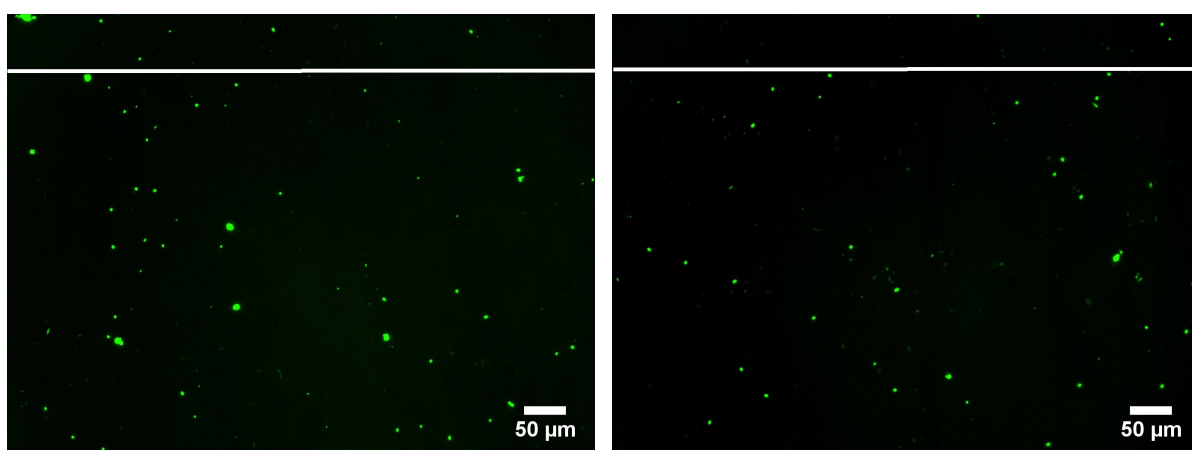


Figure 4.17 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 RGD-grafted surfaces after adhesion (green channel)

7 h-growth

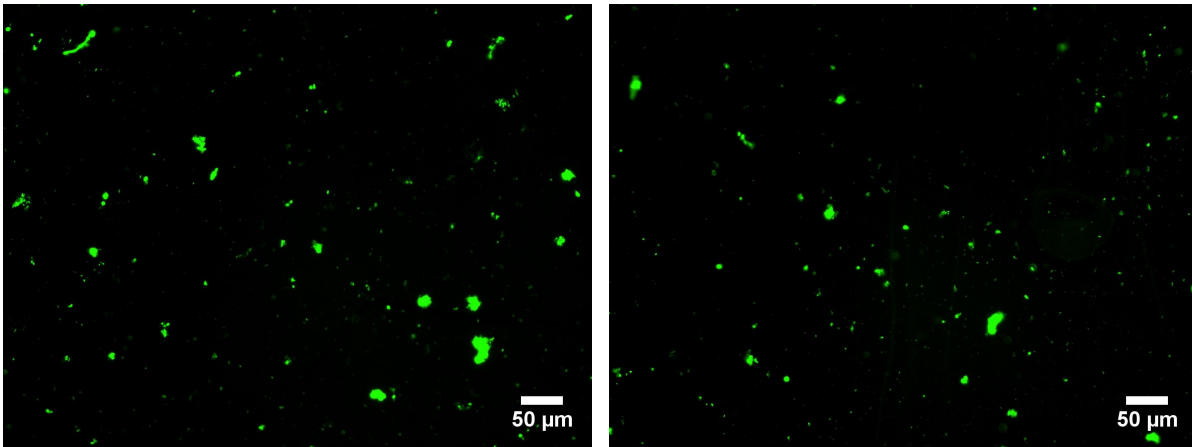


Figure 4.18 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces after 7 h-growth (green channel)

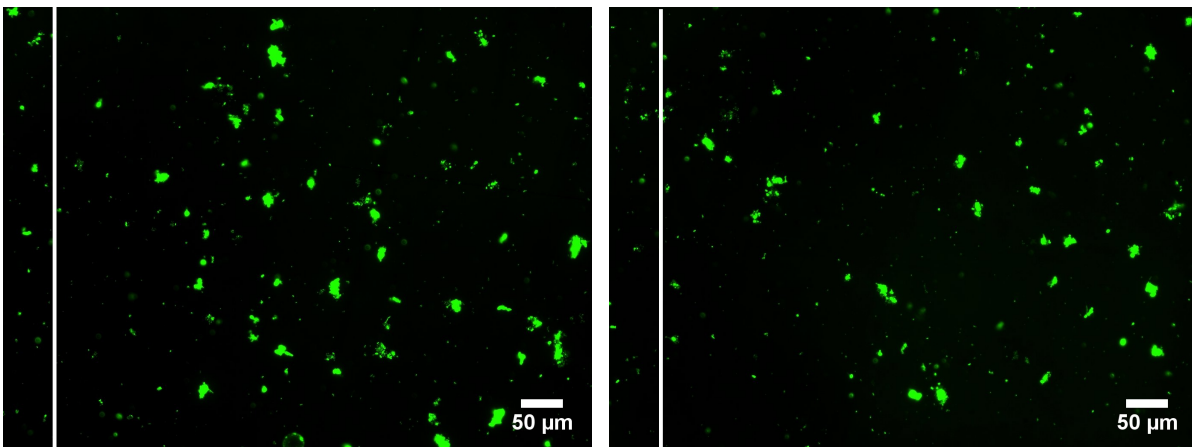


Figure 4.19 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 RGD-grafted surfaces after 7 h-growth (green channel)

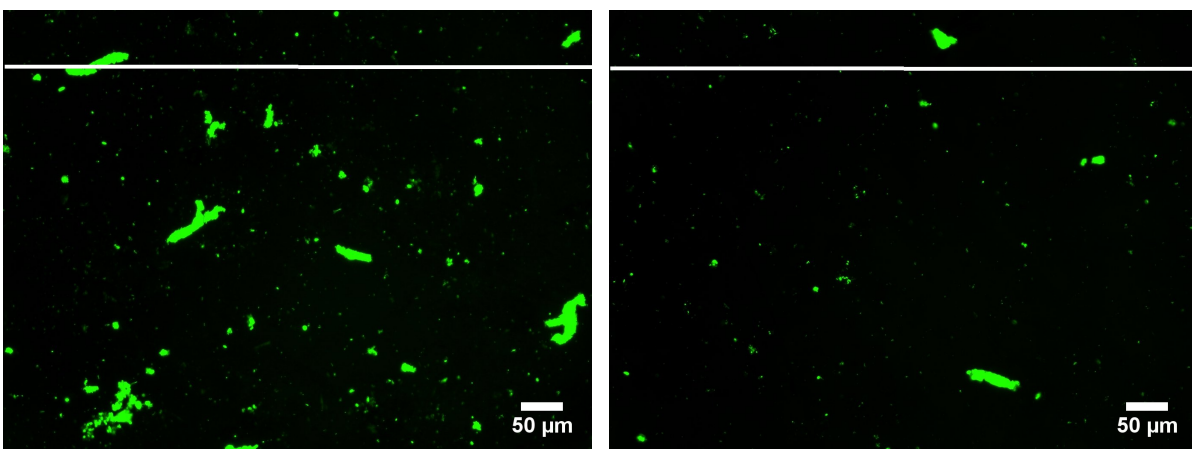


Figure 4.20 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 RGD-grafted surfaces after 7 h-growth (green channel)

12 h-growth

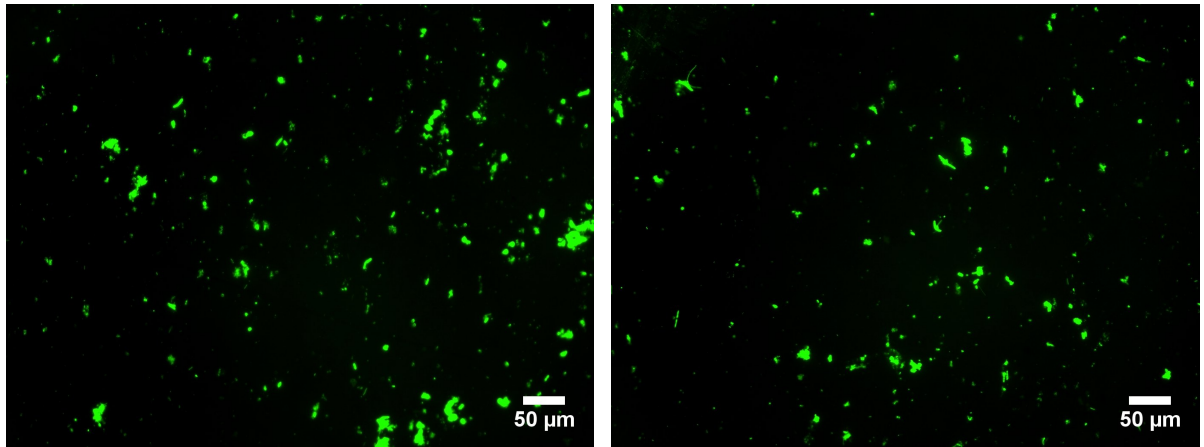


Figure 4.21 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces after 12 h-growth (green channel)

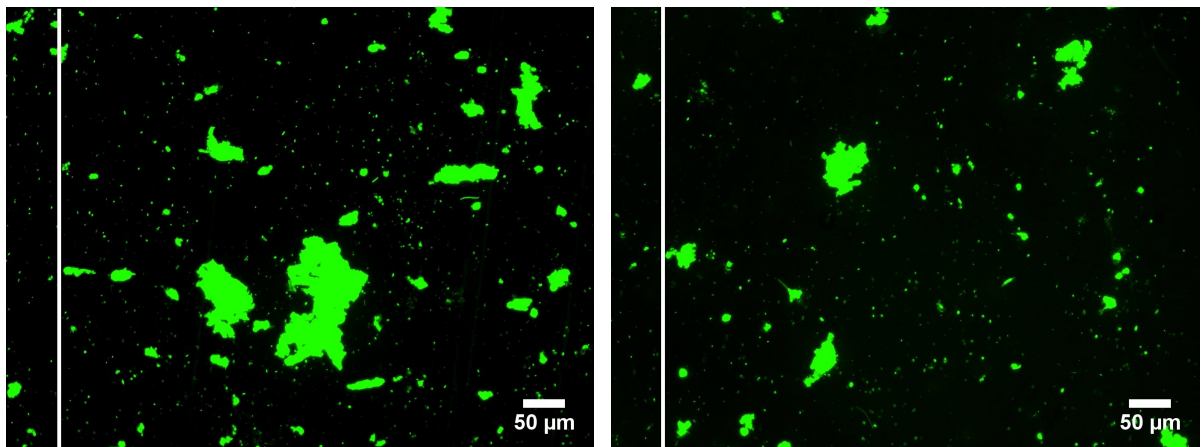


Figure 4.22 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 RGD-grafted surfaces after 12 h-growth (green channel)

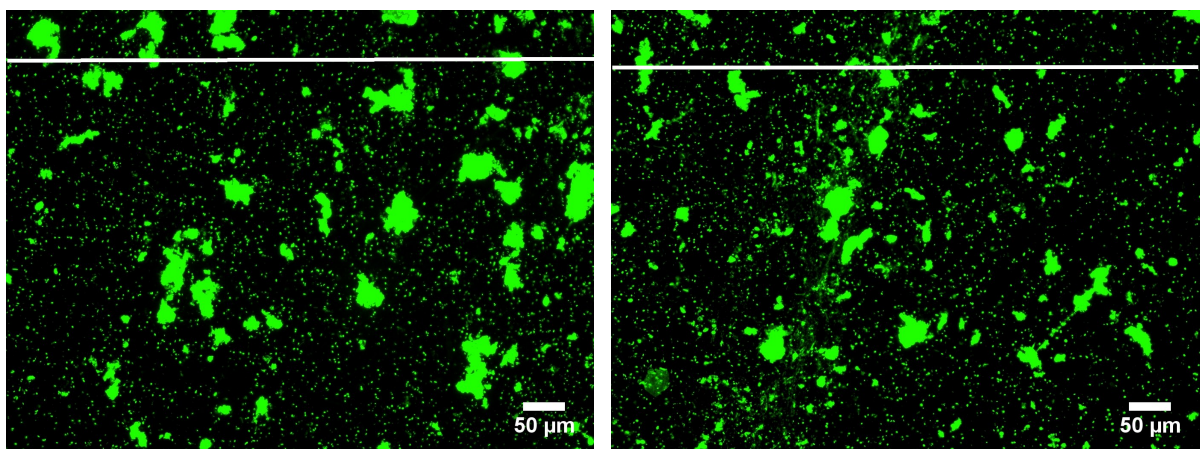


Figure 4.23 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 RGD-grafted surfaces after 12 h-growth (green channel)

A first visual analysis allows concluding that bacterial adhesion and growth was possible on all RGD-modified surfaces. Indeed, bacteria are present on the surface after the adhesion step (figures 4.15 to 4.17) and bacteria surface coverage increased over time on all surfaces (figures 4.18 to 4.23).

After the adhesion step (figures 4.15 to 4.17), there were no distinct differences between the surfaces and no specific bacterial organisation was observed.

After 7 h- and 12h-growth, bacterial aggregates of various sizes and shapes were observed on all surfaces. These aggregates were composed of bacteria forming intertwined chains, arranged on different levels as illustrated in figure 4.24.

More precisely, after 7 h-growth, bacterial coverage seemed to be similar on all surfaces. Aggregates morphology was similar on the homogeneous (figure 4.18) and 250-pattern surfaces (figure 4.19), even though more aggregates were observed on the 250-pattern. On the opposite, 400-pattern surfaces (figure 4.20) showed elongated aggregates more or less oriented in the pattern direction. However, no clear bacterial alignment was observed.

After 12 h-growth, the homogeneous surfaces (figure 4.21) showed rather small aggregates with no specific organization. On the other hand, both patterned surfaces (figures 4.22 and 4.23) clearly showed a greater surface coverage with larger aggregates than the homogeneous surfaces. Similar results were obtained by Diaz et al. on nanostructured surfaces. Gold nanostructured surface consisting in channels of 90 nm height and 900 nm width presented more significant bacterial attachment than the control sample.⁵¹ However, in this case, there was no clear difference in aggregates morphology between the two nanopatterned surfaces.

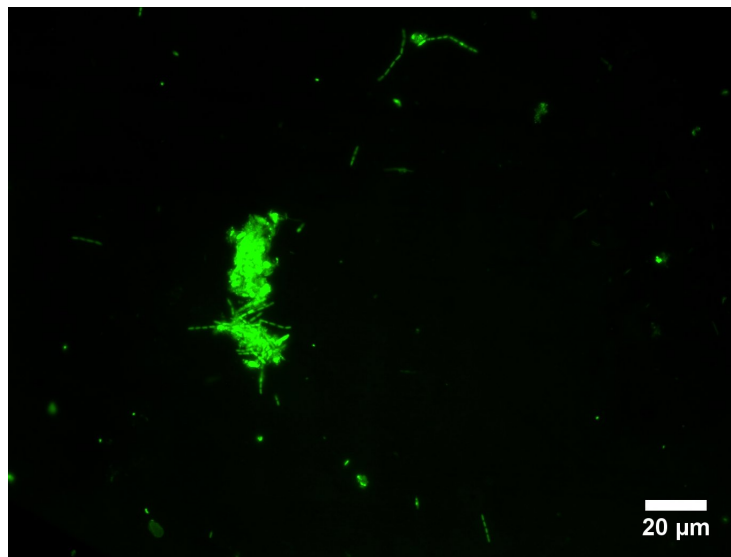


Figure 4.24 – Bacterial aggregate on homogeneous RGD-grafted surface after 12 h-growth in M9 minimum medium

These results suggest that nanopatterned surfaces influence bacterial growth and aggregate morphology but that these effects are not immediate and require a certain time to be visible. Contrary to our expectations, no clear bacterial alignment were observed. This contrasts with the results obtained previously on chemical patterns composed of PMMA nanolines distributed in a PEG background. Indeed, a strong alignment of *E. coli* (rod-shaped, gram-negative) and *L. lactis* (ovoid, gram-positive) was observed along the PMMA patterned lines on these surfaces.^{22,26} Catherine Philippart observed *L. lactis* alignments on S180 PMMA-PEG surfaces after adhesion but more bacteria were adhered to the surfaces (figure 4.25) compared to our surfaces (figures 4.16 and 4.17). Moreover, she observed that *L. lactis* alignment on S400 PMMA-PEG surfaces were only visible after 2h-growth (figure 4.26). However, in our case, clear bacterial alignment did not form at all. On the other hand, *L. Plantarum*, a gram-positive rod-shaped bacterium, showed no specific alignment on the same type of surfaces.²⁶

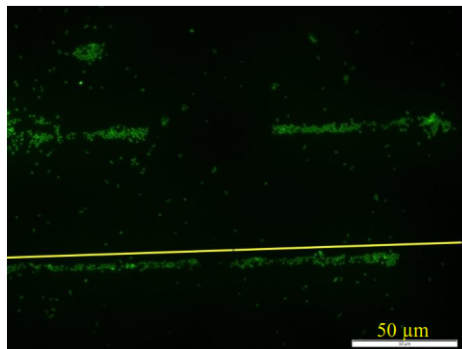


Figure 4.25 – *L. Lactis* alignment on a S180 chemically patterned surface composed of PMMA lines distributed in a PEG background after adhesion²⁶

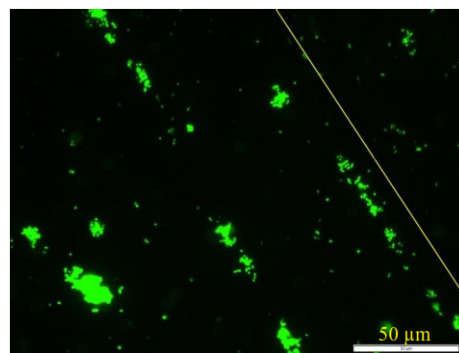


Figure 4.26 – *L. Lactis* alignment on a S400 chemically patterned surface composed of PMMA lines distributed in a PEG background after 2h-growth²⁶

A possible explanation to this phenomenon would be related to the different surface chemistry in these two situations. In previous studies performed by C. Philippart et al., bacteria were directly adhered on adhesive PMMA polymer brushes. Here, bacteria were adhered on RGD-grafted P(HOEGMA) polymer brushes. On one hand, the polymer used here was different and P(HOEGMA) brushes have been proved to be non-adhesive towards bacteria.³⁹ On the other hand, bacteria were supposed to adhere by interacting with RGD-peptides. In this case, if there is not enough peptides, the density of adhesive regions can be too low to induce a bacterial alignment. Moreover, even though these peptides are known to play a key role in eukariotic cells adhesion, no evidence was found of specific interactions between RGD peptides and bacteria. On the opposite, several studies claim that RGD-functionalized titanium surfaces are able to promote cellular adhesion while keeping the non-adhesive/anti-bacterial properties of the underlying surface. These surfaces were effective against several strains of bacteria: *S. aureus*,^{52–55} *E. coli*⁵³ and *S. epidermidis*.⁵⁴ For example, Poh-Hui Chua and coworkers developed titanium surfaces coated with RGD-functionalized polyelectrolyte multilayers (PEM) of hyaluronic acid and chitosan, which is known for its antibacte-

rial properties. The final surface exhibited about 80% reduction in *S. Aureus* adhesion thanks to the PEM while the immobilized RGD was shown to have a marked positive influence on osteoblast adhesion and proliferation.⁵²

In view of these results, RGD-grafted surfaces do not seem to induce any specific interactions with bacteria. However, in our case, bacterial adhesion was actually increased compared to non-grafted surfaces but this phenomenon is thus supposed to result from non-specific interactions.

As a conclusion, it is suggested that bacteria characteristics such as shape and type (Gram-positive and Gram-negative), surface chemistry and pattern dimensions are key parameters to control bacterial alignment on the surface.

Quantitative analysis

A quantitative analysis of the bacteria coverage was then performed. This quantitative analysis required to count the number of green pixels in the fluorescence microscopy images. Since the total number of pixels is known, the percentage of surface covered by bacteria can be computed as the ratio between the number of green pixels and the total number of pixels. It is worth noting that this method only gives an approximation of bacterial coverage as it does not take into account the superposition of bacteria in 3D aggregates. The results are illustrated in figure 4.27. It can be seen that there is no clear difference between the different surfaces (homogeneous and patterned brushes grafted with RGD peptide) after the adhesion step and after the 7 h-growth. However, by looking at the percentage of surface covered by bacteria after adhesion using a different scale (figure 4.28), it can be seen that bacterial coverage seems to be higher on 400-patterns. After 12 h-growth, there is a marked difference in the bacteria coverage between the different surfaces. A higher percentage of coverage was observed for nanopatterned surfaces compared to homogeneous surfaces. Moreover, the 400-pattern seems to have a higher influence on bacterial behaviour since a higher percentage of coverage is observed for these surfaces compared to 250-patterned ones. This could be explained by the greater proportion of non-adhesive PEG regions in the 250-pattern. Indeed, considering the theoretical dimensions of the patterns, 400-patterned surfaces are supposed to present 50% of PEG regions and 50% of RGD-grafted regions while 250-pattern are supposed to present 68.8% of PEG regions and 31.2 % of RGD-grafted regions.

Similar results were obtained by Catherine Philippart on nanopatterned PMMA-PEG surfaces with other bacteria (*L. Plantarum* and *L. Lactis*). Indeed, bacterial growth seemed to be favoured on nanopatterned surfaces compared to homogeneous surfaces and each type of bacteria appeared to have a preferential pattern size that improves its growth.²⁶ This analysis thus supports the observations made during the visual analysis.

Then, Student's t-test were performed in order to confirm these observations. In statistics, this method consists in testing hypotheses about the mean of a small sample drawn from a normally distributed population when the population standard deviation is unknown. This test returns a decision allowing to reject or not the null hypothesis. A t-test may be either two-sided, stating simply that the means of two data sets are not equivalent, or one-sided, specifying whether the observed mean is larger or smaller than an hypothesized mean. In our case, a two-sided t-test is used and the null hypothesis

states that there is no effective difference between the data sets means. Thus, the alternative hypothesis is that these data come from populations with unequal means. In other words, if the null hypothesis is rejected ($H=1$), the data are considered to have different means with a significance level of 5%. As already mentioned, 9 fluorescence images were taken per sample and 2 samples were used per observation time, resulting in 18 values for a given observation. The test results as well as p-values obtained are reported in table 4.2. From these values, it can be observed that, after the adhesion step, the null hypothesis is only rejected for the comparison between homogeneous and 400-pattern RGD-grafted surfaces, which is consistent with figure 4.28. Moreover, the null hypothesis is never rejected after 7 h-growth. This confirms that there is no significant difference between the surfaces after 7 h. It is thus suggested that 400-pattern induced a higher bacterial coverage after adhesion, but this effect was not observed after 7 h-growth. After the 12 h growth, it can be seen that the null hypothesis is rejected when comparing homogenous surfaces and nanopatterned surfaces, supporting our previous observations. Concerning the difference between the nanopatterned surfaces themselves, the null hypothesis is not rejected but the p-value is not really high. Therefore, keeping in mind the visual analysis, it can be assumed that there is a significant difference in the bacteria coverage between the two types of nanopatterned surfaces and that the 400-pattern favours larger bacterial coverage.

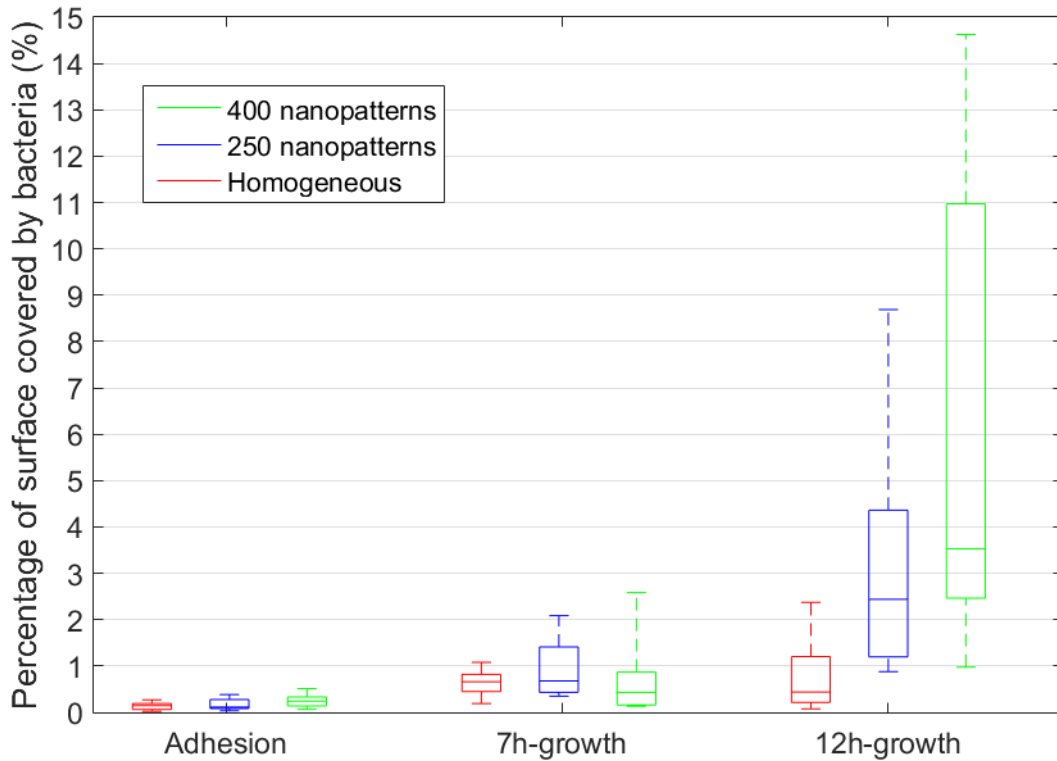


Figure 4.27 – Surface area covered by bacteria on the three types of surfaces after adhesion, 7 h- and 12 h-growth

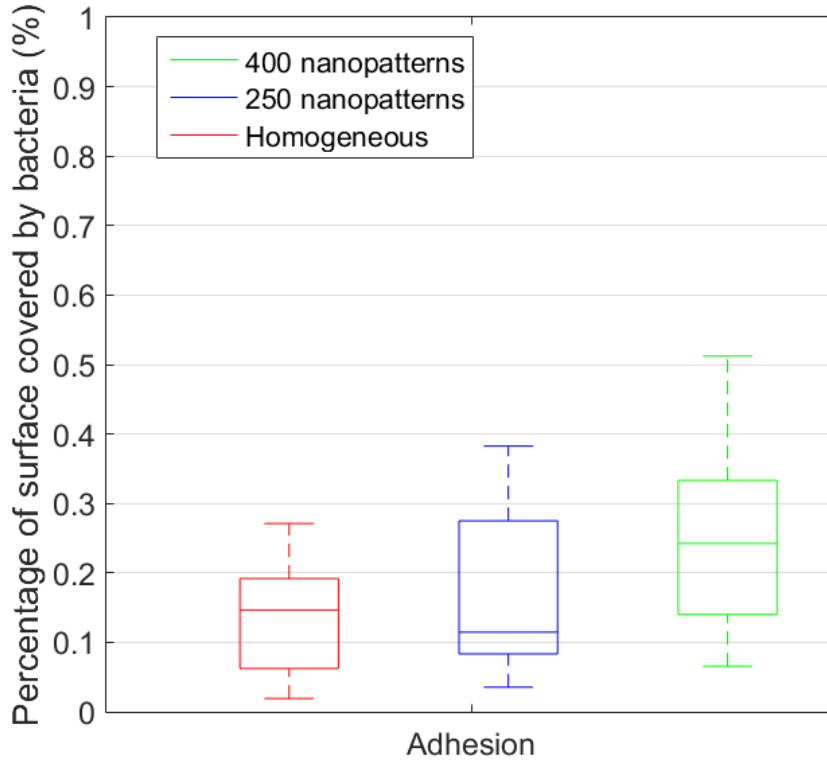


Figure 4.28 – Surface area covered by bacteria on the three types of surfaces after adhesion (different scale from figure 4.27)

Adhesion			7h			12h		
	H	p-value		H	p-value		H	p-value
Hom-250	0	0.6011	Hom-250	0	0.1613	Hom-250	1	0.0097
Hom-400	1	0.0327	Hom-400	0	0.9174	Hom-400	1	0.0045
250-400	0	0.1229	250-400	0	0.3599	250-400	0	0.1829

Table 4.2 – T-test results comparing the average bacterial coverage on the different surfaces after adhesion, 7 h- and 12 h-growth

4.3.2 LL37-grafted surfaces

Microscopy observation

Viability of *E. coli* on LL37-grafted surfaces was investigated by epifluorescence microscopy. Homogeneous RGD-grafted surfaces were used as control. In order to quantify bacterial viability, alive and dead bacteria were stained in green or red, respectively, thanks to a viability kit. Here, fluorescence images are overlay images obtained by combining red and green channels. It is important to remember that yellow regions can be observed, resulting from the superposition of red and green. This regions are considered

to be composed of dead bacteria. The results obtained for the different surfaces are shown in figures 4.30, 4.31, 4.32 and 4.33 for the adhesion step, and figures 4.34, 4.35, 4.36 and 4.37 for 7 h-growth.

By comparing figures 4.30 and 4.34 to 4.15 and 4.18, which show fluorescent images of bacteria on homogeneous RGD-grafted surfaces after adhesion and 7 h-growth for two different experiments (RGD experiment and LL37 experiment for which homogeneous RGD-grafted surfaces were used as control), it can be seen that similar results were obtained for both experiments which were performed in the same conditions.

After the adhesion step (figures 4.30 to 4.33), bacteria were adhered to all the surfaces. Bacterial coverage seemed similar on all surfaces and no evidence of bacterial alignment was found. Moreover, there were practically no dead bacteria regardless of the surface, suggesting that LL37-grafted surfaces were ineffective to kill bacteria at this point.

After 7h of growth in M9 medium, a higher number of bacteria was observed on all surfaces. However, percentage of surface covered by bacteria and aggregate size seemed to be greater on LL37-grafted surfaces (figures 4.35-4.37) compared to RGD-grafted surfaces (figure 4.34). This suggests that LL37-grafted surfaces induce specific interactions with bacteria. Moreover, nanopatterned LL37-grafted surfaces (figures 4.36 and 4.37) showed more elongated bacterial aggregates compared to homogeneous LL37-grafted surfaces (figure 4.35) even though no clear bacterial alignment was observed regarding the pattern direction. Concerning bacterial viability, both homogeneous and patterned LL37-grafted surfaces showed a higher number of dead bacteria, stained in red or yellow, after 7 h-growth compared to RGD-grafted surfaces, suggesting that LL37-grafted surfaces were only effective in killing bacteria after a certain lapse of time. However, the amount of dead bacteria observed on LL37-grafted surfaces was rather low. A bacterial aggregate on homogeneous LL37-grafted surface is illustrated in figure 4.29, showing few dead bacteria.

Another research supports the fact that LL37 killing efficacy is not immediate. Indeed, the activity of LL37 peptide on *E. coli* was investigated in real time by Sochacki et al.⁵⁶ They showed that the interaction of LL37 with bacterial cells can be divided into three stages. The first stage consists in the binding of LL37 peptide molecules to the outer membrane and its lipopolysaccharides. Then, phase two is thought to involve LL37 crossing the outer membrane to gain access to the periplasmic space causing growth interruption. Finally, phase three clearly involves penetration of the cytoplasmic membrane by LL37. The timing of these stages is thought to depend on several parameters. Firstly, cells that are involved in division process are more rapidly damaged than other cells. Then, peptide concentration is a key factor. At four-times the MIC, the three stages took place in less than 1 h. However, at the MIC, phase two was observed to affect less than 1% of the cells after 1 h.⁵⁶

Finally, these experiments were performed by adding LL37 peptide in bacterial suspensions. In our case, the activity of grafted LL37 molecules may be different as peptides are not free to bind all over the bacteria. Moreover, LL37 binding to bacteria requires previous bacterial adhesion events and thus depends on adhesion kinetics. Further in-

vestigations thus need to be performed to evaluate the effect of grafting on the killing efficacy and timing.

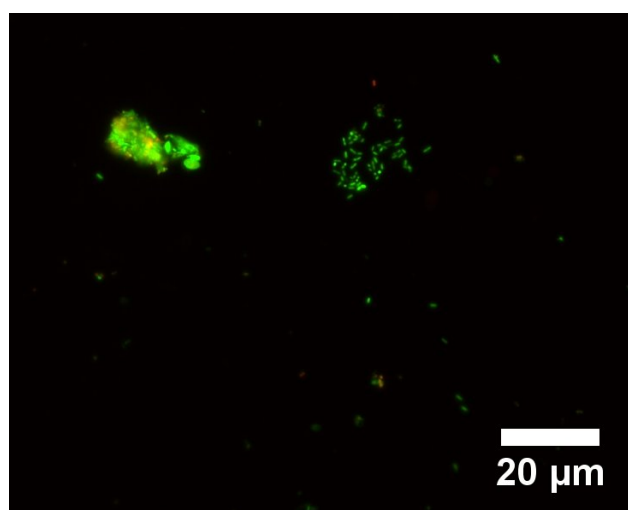


Figure 4.29 – *E. coli* aggregates observed on homogenous LL37-grafted surface after 7 h-growth in M9 medium

Adhesion

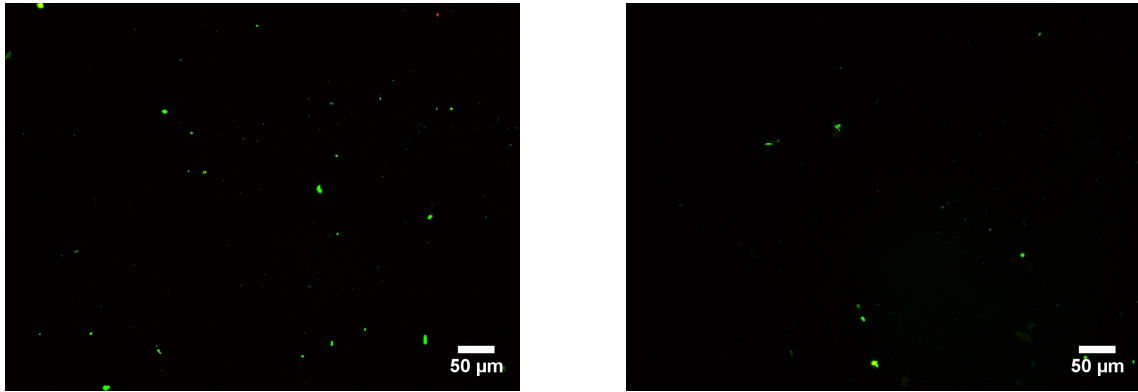


Figure 4.30 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces used as control after adhesion (overlay images)

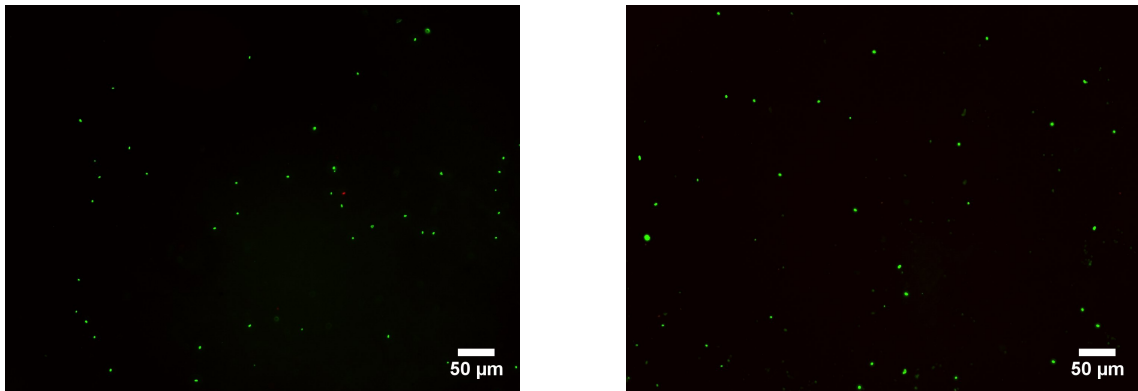


Figure 4.31 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous LL37-grafted surfaces after adhesion (overlay images)

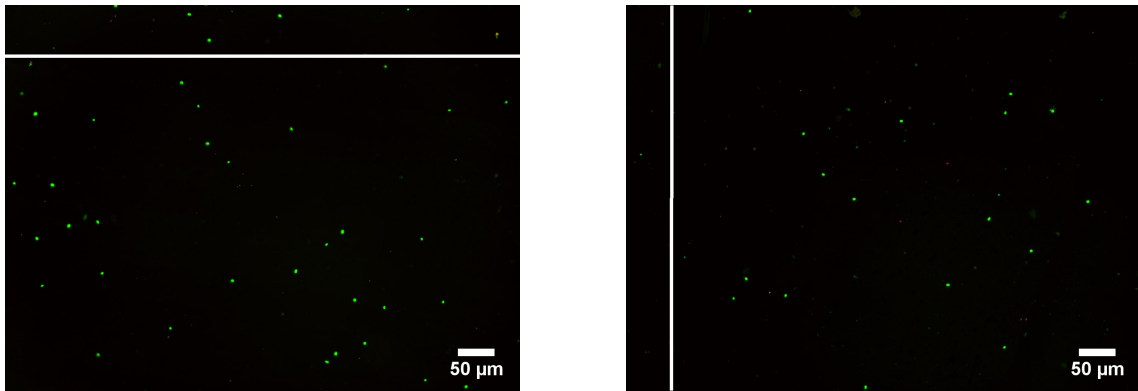


Figure 4.32 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 LL37-grafted surfaces after adhesion (overlay images)

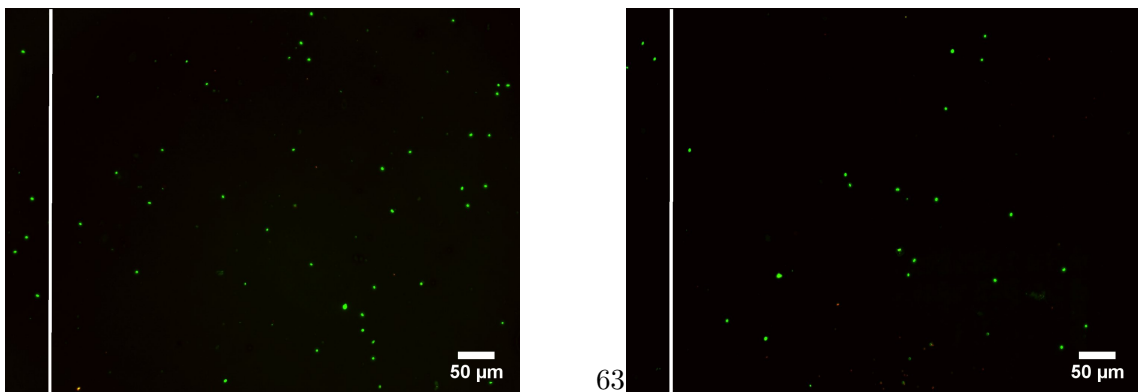


Figure 4.33 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 LL37-grafted surfaces after adhesion (overlay images)

7 h-growth

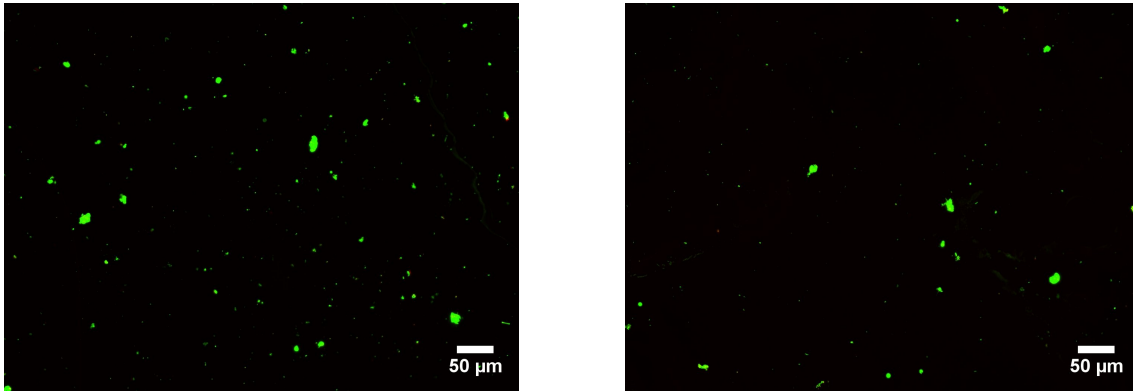


Figure 4.34 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces used as control after 7 h-growth (overlay images)

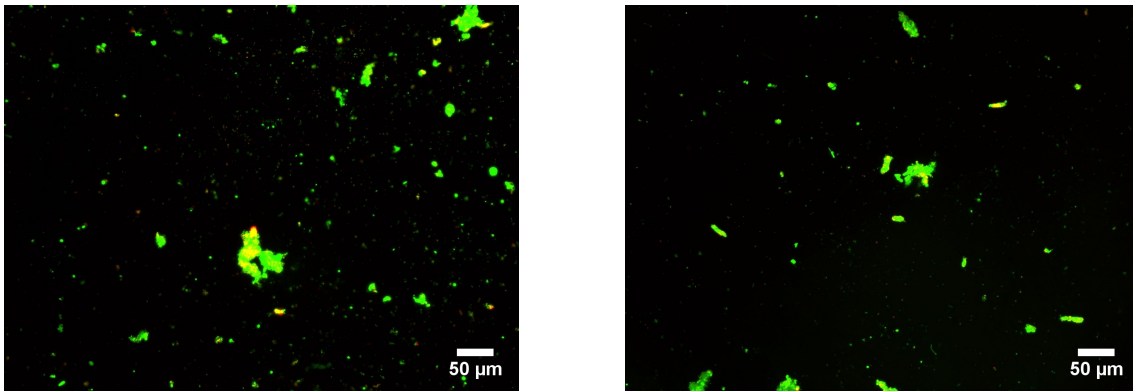


Figure 4.35 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous LL37-grafted surfaces after 7 h-growth (overlay images)

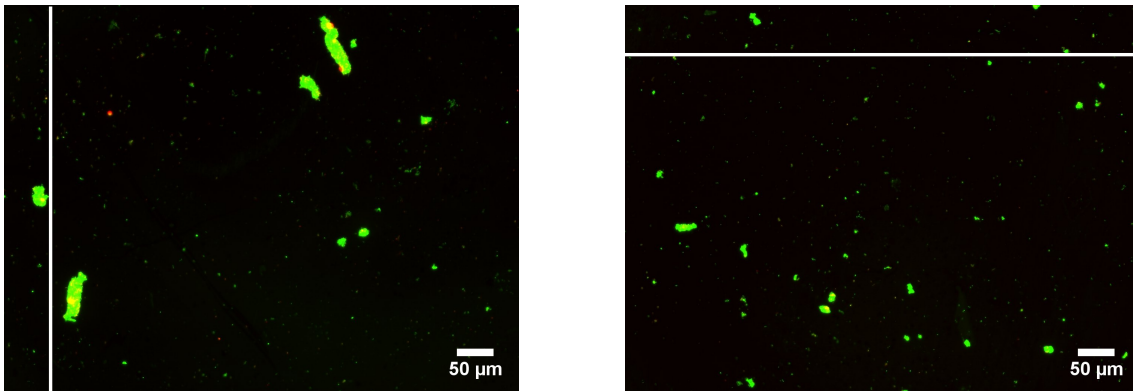


Figure 4.36 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 LL37-grafted surfaces after 7 h-growth (overlay images)

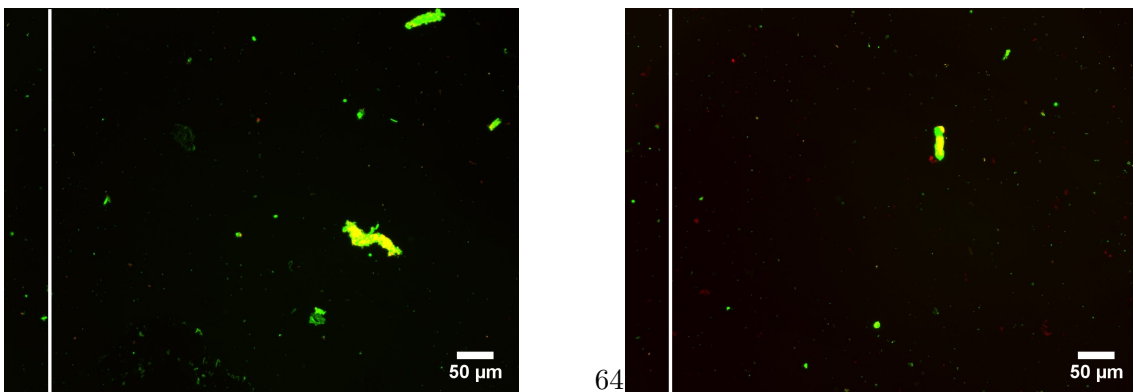


Figure 4.37 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 LL37-grafted surfaces after 7 h-growth (overlay images)

Quantitative analysis

A quantitative analysis of the percentage of surface covered by bacteria was then performed. As shown in figure 4.38, no significant differences of bacterial coverage were found after the adhesion step. Moreover, after 7 h-growth, the percentage of surface covered by bacteria had increased on all surfaces compared to the adhesion step but seemed higher on LL37-grafted surfaces.

Also, the percentage of dead and alive bacteria on each surface were computed. For this, the number of dead bacteria was compared to the total number of adhered bacteria in order to quantify the killing effectiveness of the grafted AMP. The results obtained are displayed in figure 4.39. After the adhesion step, only a really small percentage of bacteria were dead for all the surfaces. Indeed, 93 ± 11 of bacteria were alive on homogeneous RGD-grafted surfaces and $99 \pm 3\%$, $97 \pm 4\%$, $94 \pm 7\%$ of bacteria were alive (green stained) on homogeneous LL37-grafted surfaces, 400-patterned LL37-grafted surfaces and 250-patterned LL37-grafted surfaces, respectively. After 7 h-growth, there was a significant increase in the percentage of dead bacteria for the LL37-grafted surfaces while there was no change observed for the RGD-grafted surface (control). Moreover, the 400-patterned samples induce a lower number of alive bacteria compared to the other LL37-grafted surfaces. The percentages of living bacteria were $94 \pm 4\%$, $81 \pm 8\%$, $73 \pm 13\%$ and $83 \pm 12\%$ on homogeneous RGD grafted-surfaces, homogeneous LL37-grafted surfaces, 400-patterned LL37-grafted surfaces and 250-patterned LL37-grafted surfaces, respectively.

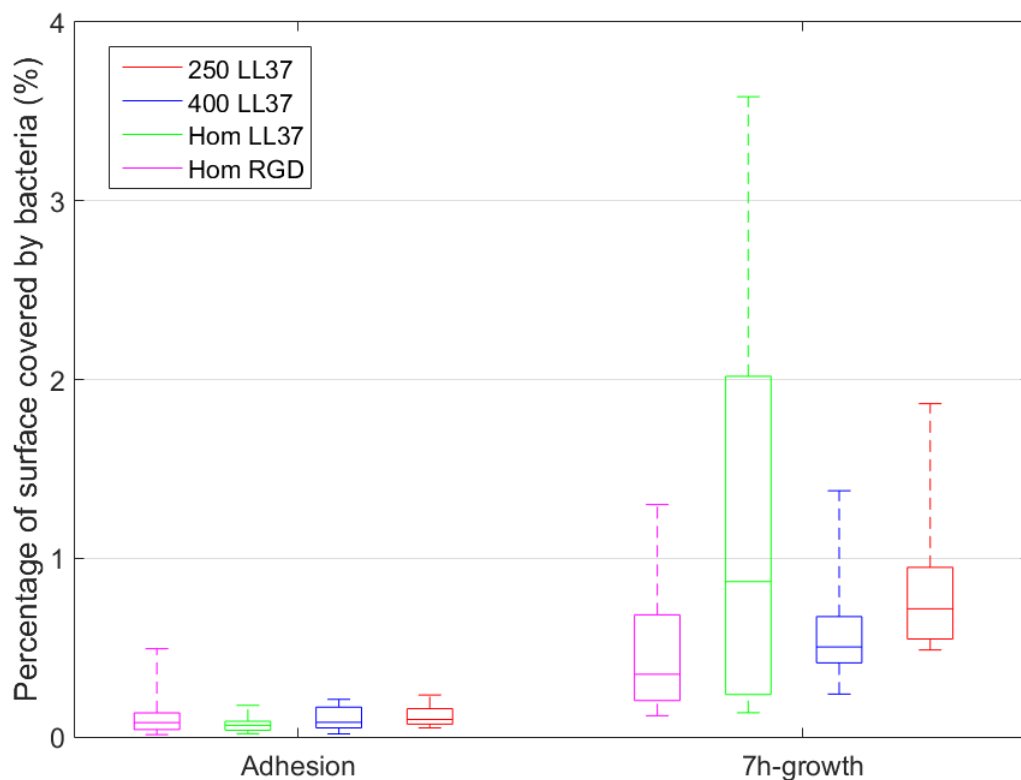


Figure 4.38 – Surface area covered by bacteria after adhesion and 7 h-growth on homogeneous and patterned LL37-grafted surfaces and homogeneous RGD-grafted surfaces used as control

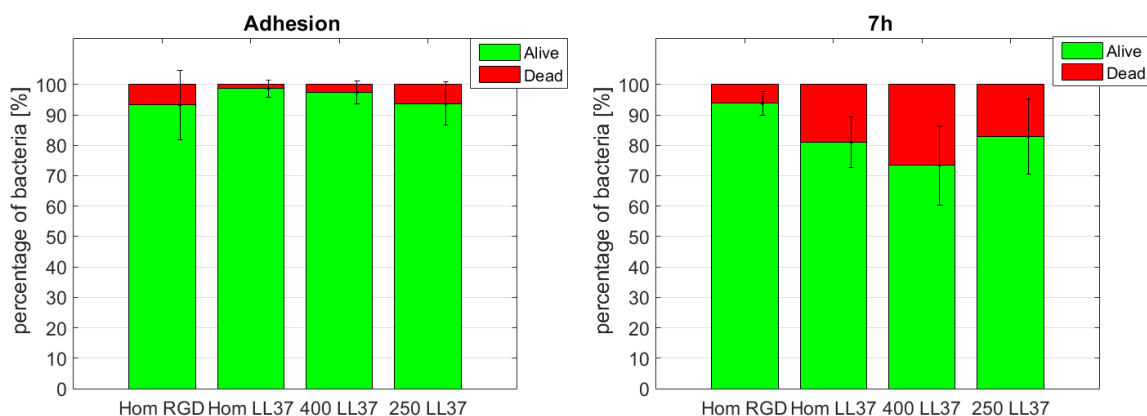


Figure 4.39 – Average percentage of living and dead bacteria after adhesion and 7 h-growth on homogeneous and patterned LL37-grafted surfaces and homogeneous RGD-grafted surfaces used as control.

Again, Student's tests were also performed in order to quantitatively compare the

percentage of dead bacteria on the different surfaces. As a reminder, if the null hypothesis is rejected ($H=1$), the data are considered to have different means with a significance level of 5%. Results are shown in table 4.3.

After the adhesion step (table 4.3 left), the results indicate that there were no significant difference in the percentage of dead bacteria between RGD-grafted surfaces and LL37-grafted surfaces.

After 7 h-growth (table 4.3 right), RGD-grafted surfaces and LL37-grafted surfaces showed significantly different percentages of dead bacteria, with p-values as low as $1.3 * 10^{-10}$. There were no significant difference between homogeneous LL37-grafted surfaces and 250-pattern LL37 surfaces. In addition, the higher effect of the 400-pattern on the percentage of dead bacteria compared to the other LL37-grafted surfaces was further confirmed.

Table 4.4 shows the results obtained by comparing the percentage of dead bacteria after adhesion and after 7 h-growth on a given surface. These results indicate that RGD-grafted surfaces showed similar percentages of dead bacteria after adhesion and 7 h-growth. On the opposite, all LL37-surfaces distinctively showed an evolution of the percentage of dead bacteria with time, confirming our previous observations.

Adhesion			7h		
	H	p-value		H	p-value
Hom RGD - Hom LL37	0	0.0595	Hom RGD - Hom LL37	1	$1.3 * 10^{-10}$
Hom RGD - 400 LL37	0	0.1527	Hom RGD - 400 LL37	1	$2.9 * 10^{-7}$
Hom RGD - 250 LL37	0	0.8926	Hom RGD - 250 LL37	1	0.0012
Hom LL37 - 400 LL37	0	0.2760	Hom LL37 - 400 LL37	1	0.00432
Hom LL37 - 250 LL37	1	0.009	Hom LL37 - 250 LL37	0	0.5966
400 LL37 - 250 LL37	0	0.0585	400 LL37 - 250 LL37	1	0.0312

Table 4.3 – T-test results comparing the percentages of dead bacteria on homogeneous and patterned LL37-grafted surfaces and homogeneous RGD-grafted surfaces used as control after adhesion and 7 h-growth

	H	p-value
Hom RGD	0	0.8627
Hom LL37	1	$6.16 * 10^{-10}$
400 LL37	1	$1 * 10^{-8}$
250 LL37	1	0.003

Table 4.4 – T-test results comparing the evolution of the percentage of dead bacteria of given surfaces (RGD-grafted or LL37 grafted surfaces) with time (adhesion versus 7 h-growth)

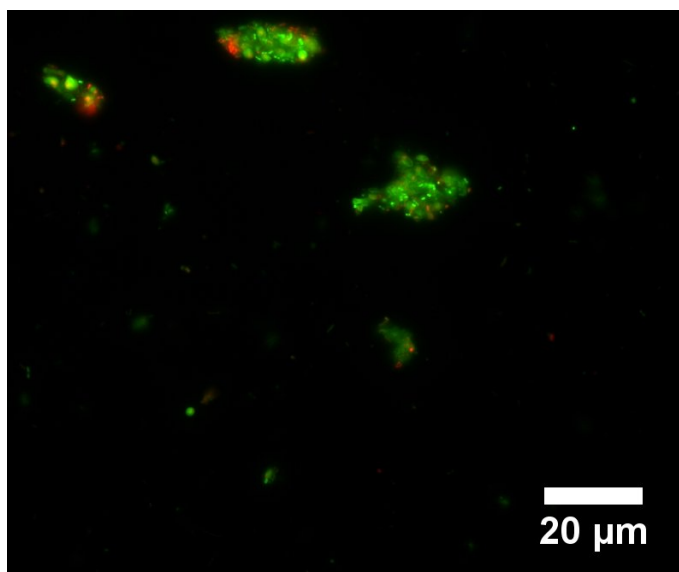


Figure 4.40 – *E. coli* aggregates observed on homogeneous MAG-grafted surface after 7 h-growth in M9 medium

4.3.3 Magainin-grafted surfaces

Microscopy observation

Bacterial viability on Magainin-grafted surfaces was investigated according to the same procedure than the one described for LL37-modified surfaces. It is important to remember that dead bacteria were stained in red while alive bacteria were stained in green. Again, fluorescence images are overlay images resulting from the superposition of images acquired from red and green channels. Homogeneous RGD-grafted surfaces were also used as control. For each surface, observations were made after adhesion and after 7 h-growth. The results obtained for the different surfaces are shown in figures 4.41, 4.42, 4.43 and 4.44 for the adhesion step and 4.45, 4.46, 4.47 and 4.48 after 7 h-growth.

Again, by comparing figures 4.30, 4.34, 4.15 and 4.18 showing fluorescence images of bacteria on homogeneous RGD-grafted surfaces, it can be seen that the results obtained are consistent from one experiment to another (homogeneous surfaces from RGD experiment and homogeneous RGD-grafted surfaces used as control from LL37 and MAG experiments).

After the adhesion step, all substrates presented bacteria on their surfaces. However, RGD-grafted surfaces showed a reduced bacterial coverage compared to MAG-grafted surfaces. Also, no specific influence of the patterns was noticed at this point. Finally, almost no dead bacteria were observed regardless of the surface, indicating that MAG-grafted surface did not yet had a harmful effect on bacteria at this stage.

After 7 h-growth, an increase of the surface covered by bacteria was observed on all the samples. However the degree of surface coverage was lower for RGD-grafted surface. This can be partly attributed to the reduced amount of bacteria observed on

these surfaces after the adhesion step. On the other hand, by comparing these results with the results obtained for LL37-grafted surfaces, and keeping in mind the similarity between LL37 and magainin peptides, it can be assumed that MAG-grafted surfaces have a similar activity on bacteria than LL37-grafted surfaces, causing a higher percentage of surface covered by bacteria compared to RGD-grafted surfaces. A higher percentage of dead bacteria was observed after 7 h-growth on all the MAG-grafted surfaces while no significant difference was noted for the RGD-grafted surfaces. Bacterial aggregates were present on all surfaces. Some elongated aggregates were observed but this tendency was less marked than on LL37-grafted surfaces. Bacterial aggregates showing both dead and alive bacteria can be seen in figure 4.40.

Adhesion

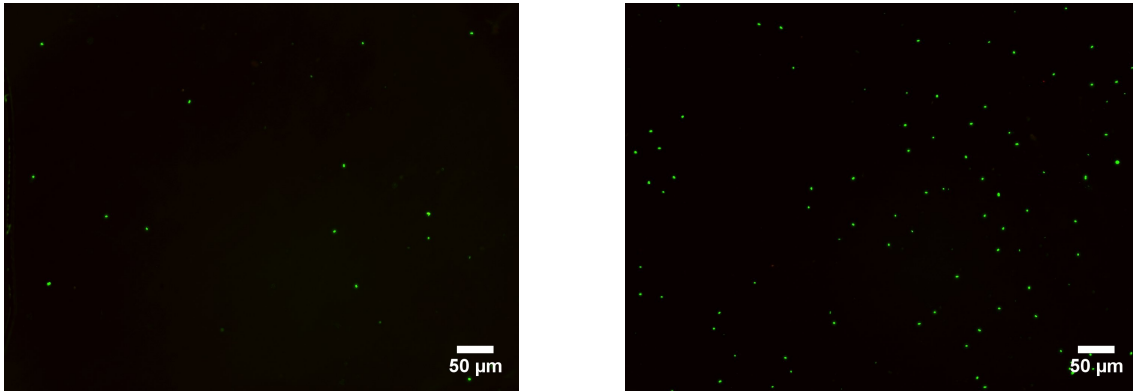


Figure 4.41 – Fluorescence images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces used as control after adhesion (overlay images)

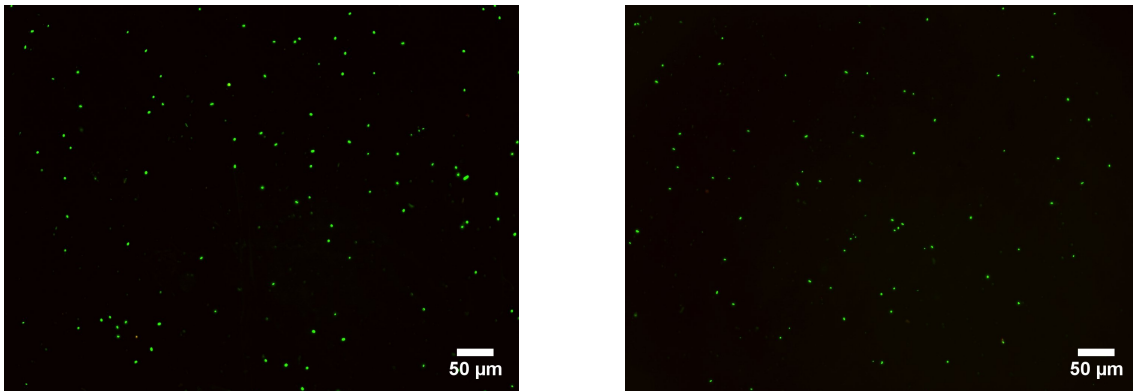


Figure 4.42 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous MAG-grafted surfaces after adhesion (overlay images)

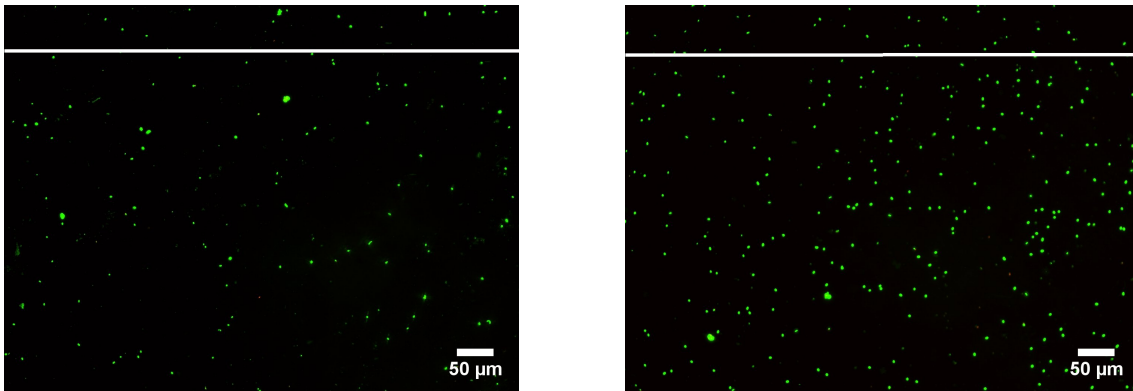


Figure 4.43 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 MAG-grafted surfaces after adhesion (overlay images)

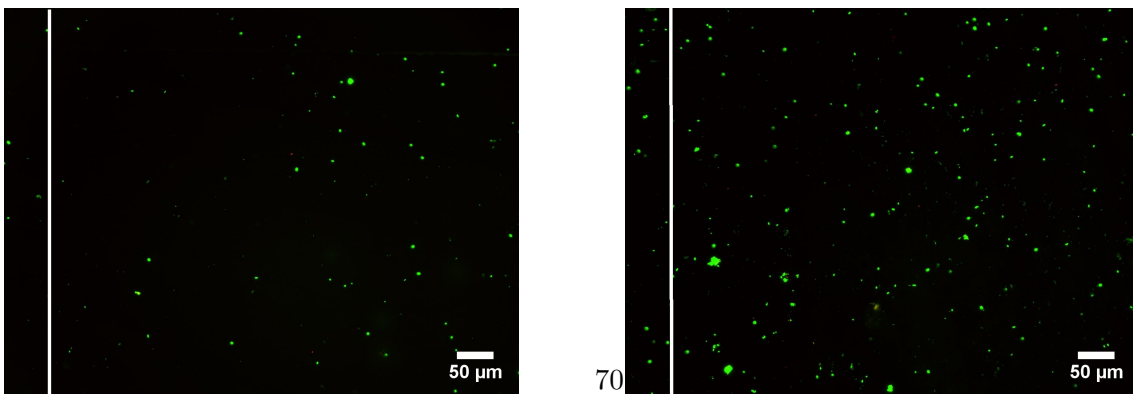


Figure 4.44 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 MAG-grafted surfaces after adhesion (overlay images)

7 h-growth

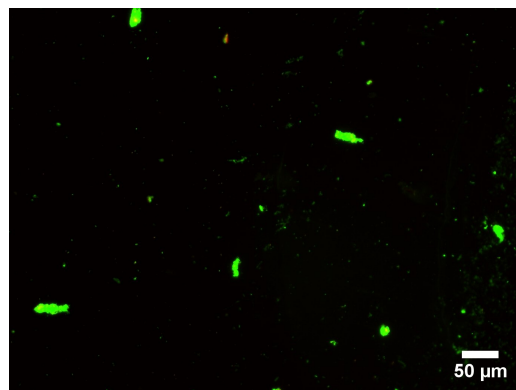
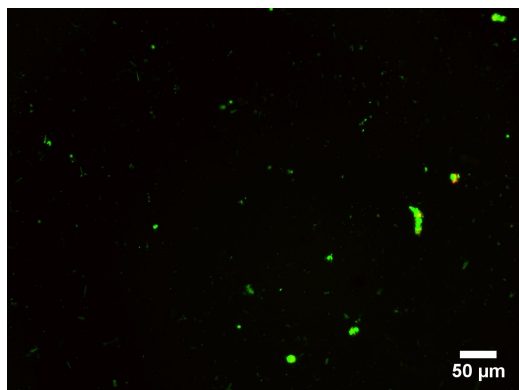


Figure 4.45 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous RGD-grafted surfaces used as control after 7 h-growth (overlay images)

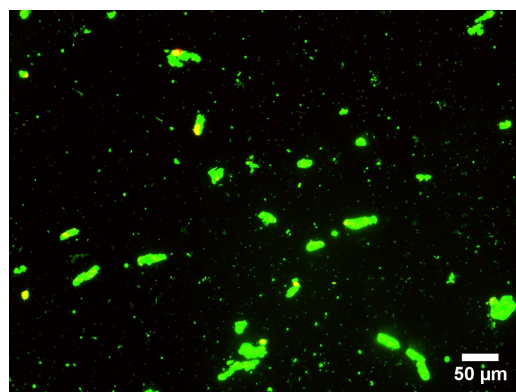
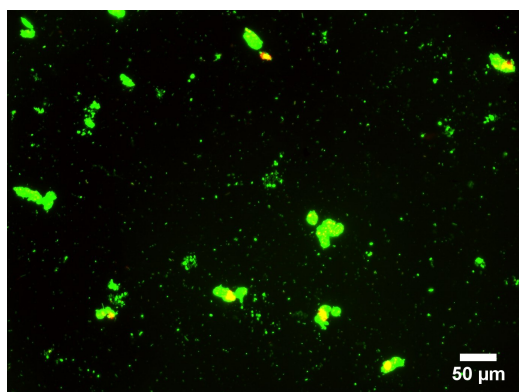


Figure 4.46 – Fluorescence microscopy images of *E. coli* stained with the viability kit on homogeneous MAG-grafted surfaces after 7 h-growth (overlay images)

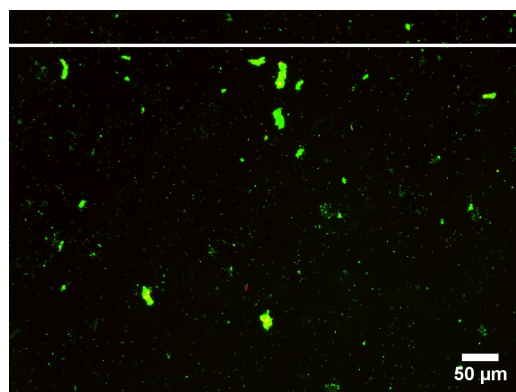
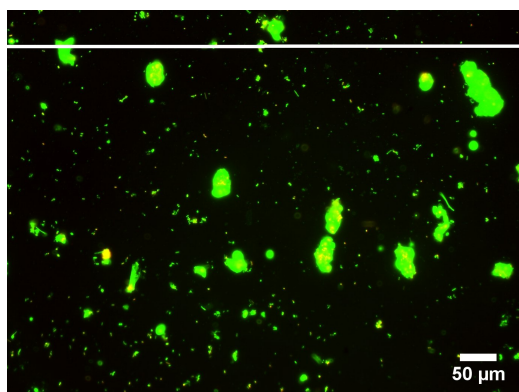
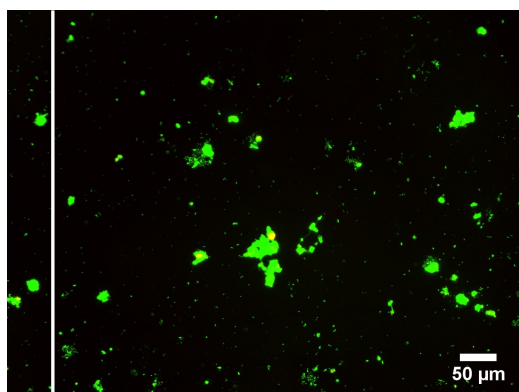


Figure 4.47 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 250 MAG-grafted surfaces after 7 h-growth (overlay images)



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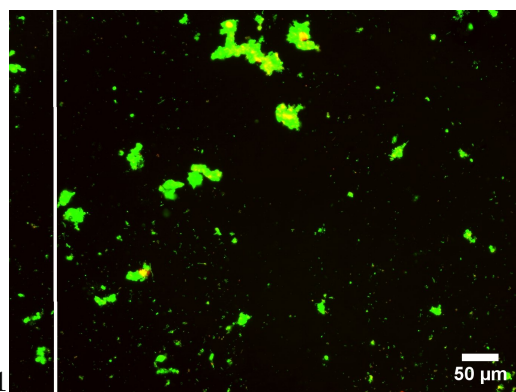


Figure 4.48 – Fluorescence microscopy images of *E. coli* stained with the viability kit on 400 MAG-grafted surfaces after 7 h-growth (overlay images)

Quantitative analysis

Again, a quantitative analysis of the surface covered by bacteria was performed and is illustrated in figure 4.49. From this figure, it can be seen that there was a clear difference between RGD-grafted surfaces and MAG-grafted surfaces after the adhesion step. Two facts can help understanding this observation. Firstly, RGD-grafted surfaces were less recent than MAG-grafted surfaces. Indeed, the RGD-grafted surfaces used had been synthesized almost a month before these bacterial tests while MAG-grafted surfaces had been freshly prepared for the experiment. This may explain the lower bacterial coverage observed on RGD-grafted surfaces. Secondly, some non-adhered bacteria were observed on MAG-grafted surfaces, probably due to an ineffective rinsing. As a consequence, bacterial coverage values on MAG-grafted surfaces are thought to be slightly overestimated. In view of these considerations, it is possible that the difference of bacterial coverage after the adhesion step was not induced by the difference of surface chemistry between RGD- and MAG-grafted surfaces. However, it can also be suggested that MAG-grafted surfaces present a greater affinity for bacteria than RGD-grafted surfaces, inducing a greater bacterial coverage but only further research could confirm this hypothesis. Then, bacterial growth is confirmed on all surfaces as the surface covered by bacteria clearly increased after 7 h-growth. However, the percentage of surface covered by bacteria was much higher for MAG-grafted surfaces compared to RGD-grafted surfaces. This could be attributed to the low bacterial coverage observed after adhesion. However, as previously mentioned, it can also be hypothesized that MAG-grafted surfaces induce a higher bacterial coverage after 7 h-growth due to a higher affinity, which could cause a higher cell spreading and/or growth. Another explanation could be that lysed bacterial cells present on MAG-grafted surfaces reinforce the bioadhesiveness of the surface which promote cell proliferation. Further investigations need to be done in order to verify these hypothesis.

The percentage of living and dead bacteria was computed and is presented in figure 4.50. After the adhesion step (figure 4.50 left), there were indeed really few dead bacteria on all surfaces and there were no significant differences between the surfaces except for the homogeneous MAG-grafted surface which showed an even smaller amount of dead bacteria. After the adhesion step, the percentages of living bacteria were $96 \pm 8\%$, $99 \pm 1\%$, $97 \pm 3\%$ and $96 \pm 4\%$ on homogeneous RGD grafted-surfaces, homogeneous MAG-grafted surfaces, 400-patterned MAG-grafted surfaces and 250-patterned MAG-grafted surfaces, respectively. This observation is further confirmed by the statistical test whose results are presented in table 4.5. These values indicate that there were no difference in the percentage of dead bacteria between RGD-grafted surfaces and MAG-grafted surfaces. There was, however, a significant difference between homogeneous MAG-grafted surfaces and both nanopatterned surfaces. Again, this results from the extremely low percentage of dead bacteria on homogeneous MAG-grafted surfaces. After 7 h-growth (figure 4.50 right), the percentage of dead bacteria had increased on all MAG-grafted surfaces while RGD-grafted surfaces showed no significant difference compared to the adhesion step. This results in a distinct difference of percentage between RGD and MAG-grafted surfaces. These observations are further confirmed by the t-test results (table 4.6) comparing the percentage of dead bacteria on a given surface after adhesion and 7 h-growth, for which p-value as low as 1.15×10^{-9} were obtained, and by the t-test results comparing RGD-grafted surfaces and MAG-grafted surfaces after 7 h-growth

(table 4.5 right). No influence of the patterns on the percentage of dead bacteria was observed even after 7 hours (figure 4.50 right) and this was also confirmed by the t-test results (table 4.5 right). In this case, the percentages of living bacteria were $94 \pm 6\%$, $82 \pm 9\%$, $81 \pm 8\%$ and $80 \pm 11\%$ on homogeneous RGD grafted-surfaces, homogeneous MAG-grafted surfaces, 400-patterned MAG-grafted surfaces and 250-patterned MAG-grafted surfaces, respectively.

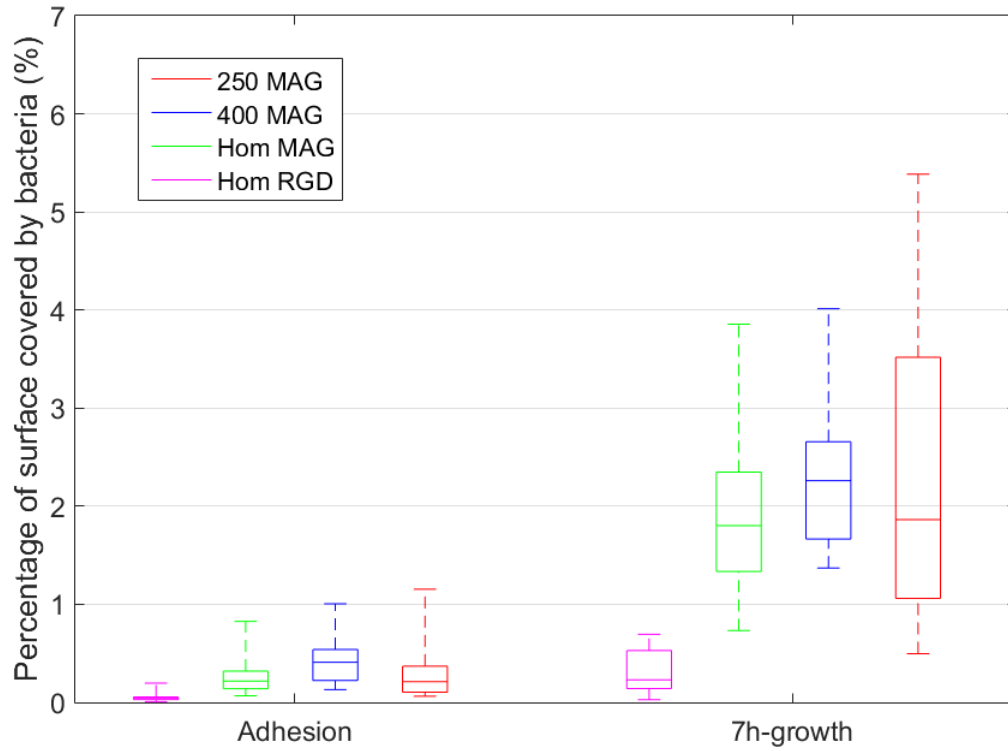


Figure 4.49 – Surface area covered by bacteria after adhesion and 7 h-growth on homogeneous and patterned MAG-grafted surfaces and homogeneous RGD-grafted surfaces used as control.

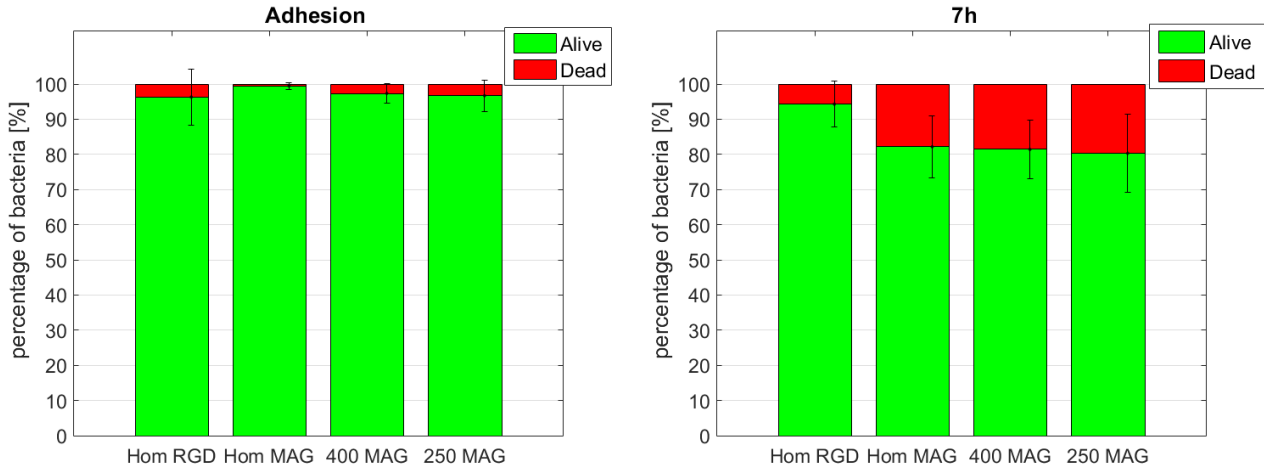


Figure 4.50 – Percentage of living and dead bacteria after adhesion and 7 h-growth on homogeneous and patterned MAG-grafted surfaces and homogeneous RGD-grafted surfaces used as control.

Adhesion			7h		
	H	p-value		H	p-value
Hom RGD - Hom MAG	0	0.1047	Hom RGD - Hom MAG	1	$4.4 * 10^{-5}$
Hom RGD - 400 MAG	0	0.6066	Hom RGD - 400 MAG	1	$1.1 * 10^{-5}$
Hom RGD - 250 MAG	0	0.8606	Hom RGD - 250 MAG	1	$5.02 * 10^{-5}$
Hom MAG - 400 MAG	1	0.0042	Hom MAG - 400 MAG	0	0.7966
Hom MAG - 250 MAG	1	0.0144	Hom MAG - 250 MAG	0	0.5805
400 MAG - 250 MAG	0	0.6014	400 MAG - 250 MAG	0	0.7363

Table 4.5 – T-test results comparing homogeneous and patterned MAG-grafted surfaces and homogeneous RGD-grafted surfaces used as control after adhesion and 7 h-growth

	H	p-value
Hom RGD	0	0.4165
Hom MAG	1	$1.15 * 10^{-9}$
400 MAG	1	$7.61 * 10^{-9}$
250 MAG	1	$1.44 * 10^{-6}$

Table 4.6 – T-test results comparing the evolution of the percentage of dead bacteria of given surfaces (RGD-grafted and MAG-grafted surfaces) with time (adhesion versus 7 h-growth)

4.3.4 Results summary and comparison of the different surfaces

Bacterial behaviour on the different surfaces will now be summed up and compared. RGD-grafted nanopatterned surfaces showed a higher percentage of surface covered by bacteria coverage compared to homogeneous RGD-grafted surfaces. More precisely, the

400-pattern had the greatest influence on bacterial coverage. Moreover, even if bacterial aggregates sometimes showed elongated morphology, bacteria did not clearly align on the pattern. As a result, 3-dimensional aggregates were observed. Since it was hypothesized that aligned bacteria could be less resistant than bacteria in biofilm,⁵ RGD-grafted nanopatterned surface does not seem appropriate to weaken bacteria resistance.

LL37-grafted surfaces and MAG-grafted surfaces presented several similarities. At first, bactericidal activity was not immediate and more than 1 h was required to notice a visible effect. This phenomenon had already been observed for LL37-peptide in solution.⁵⁶ As LL37 and magainin are really similar, it is not surprising to see that they require a certain time to have an appreciable effect.

Secondly, the percentage of dead bacteria after 7 h-growth was similar for these two types of surfaces and were rather low (approximately 20%). The only difference between these two types of surfaces had been observed on 400-pattern after 7 h. Indeed, 400-pattern LL37-grafted surfaces had been found to have a greater impact on bacterial viability compared with homogeneous and 250 pattern LL37-grafted surfaces. This can be further confirmed by looking at the t-test results presented in table 4.7.

Adhesion			7h		
	H	p-value		H	p-value
Hom LL37 - Hom MAG	0	0.2515	Hom LL37 - Hom MAG	0	0.6937
400 LL37 - 400 MAG	0	0.9460	400 LL37 - 400 MAG	1	0.0347
250 LL37 - 250 MAG	0	0.1393	250 LL37 - 250 MAG	0	0.5104

Table 4.7 – T-test results comparing the percentage of dead bacteria on MAG-grafted surfaces and LL37-grafted surfaces at the same time

These findings did not support the findings from previous research.⁵⁷ In this study, cotton-dressings functionalized with antimicrobial peptides magainin I and LL37 were developed. Electrostatic binding of the AMPs was achieved by oxidation of the cotton surface which allowed converting hydroxyl groups of carbon-6 of cellulose into carboxylate groups. This step provided the negative net charge at the surface of cotton enabling the binding of the cationic antimicrobial peptides. Depending on the peptide, different behaviours were observed. The growth reduction rate was evaluated after 18 h of incubation in presence of the functionalized textile. *Klebsiella pneumoniae* (*K. pneumoniae*) reduction was of 15% and 89%, with the cotton functionalized with magainin I and LL37, respectively. On the other hand, *S. aureus* reduction percentage with magainin I and LL37 was 58% and 59%, respectively. Thus, these two peptides clearly displayed different activity against a same bacterial strain and LL37 was found to be more effective. This fact was attributed to the difference of the net charge of both peptides. LL37 has a positive net charge of +6 compared to +4 for magainin I. As a result, higher number of electrostatic interactions can occur between the negatively charged membrane of bacteria and LL37 peptide.⁵⁷

Several hypotheses can be considered to explain the low killing activity of the two

AMPs and their similar behaviour.

Firstly, bactericidal activity was evaluated after 7 h while it was 18 h in this study.⁵⁷ Thus, it cannot be excluded that the bactericidal behaviour would still evolve after 7 h, leading to an increased bactericidal activity and possibly a difference in behaviour for the two AMPs. Hence, it would be interesting to evaluate the bactericidal activity after longer times.

Then, LL37 peptide is immobilized at the surface by a covalent grafting. Previous studies have reported a reduction in the activity of immobilized cationic AMPs with no influence on the activity spectra. For example, Bagheri et al. reported that millimolar concentrations of resin-bound AMP were required to obtain antimicrobial properties toward *E. coli* while micromolar concentrations of the free peptides were sufficient.⁵⁸ Similarly, Haynie et al. indicated that immobilized AMPs presented a 50-fold higher MIC compared to soluble peptides.⁵⁹ Immobilization method, spacer length, peptide concentration and orientation were identified as key parameters regarding bactericidal activity.³⁰ Immobilization methods include random and controlled immobilization. The first technique is carried out through formation of amide bonds between amine from the peptide and surface carboxyl groups and vice versa. Alternatively, controlled immobilization can be obtained by chemoselective formation of a peptide-surface covalent bond. The latter, which was used in this work, is usually preferred as it allows to maintain peptide structural integrity, known to be relevant for activity.³⁰ Concerning spacer length, Gabriel et al. found that directly immobilized LL37 showed no bactericidal activity while LL37 bound to PEG spacers was found to kill *E. coli*.³⁵ However, opposite results were also obtained, claiming that spacer length has no influence on bactericidal activity.³⁰ For instance, Haynie et al.⁵⁹ reported that no difference in bactericidal activity was observed between peptides conjugated to the support with a two-carbon or a six-carbon chain linker.⁵⁹ It was therefore suggested that spacer requirement depends on the mode of action of a specific AMP.³⁰ As already said, bactericidal mechanism of LL37 and magainin peptides involves interaction with the bacterial membrane to create toroidal pores. Thus, long and flexible spacers conferring mobility are thought to be necessary for LL37 and magainin I bactericidal activity. Here, the PEG side-chains of the P(HOEGMA) brush act as spacers. These spacers are relatively long and flexible and were found to be sufficient to ensure peptides accessibility.³⁹ Then, conflicting results have been reported concerning peptide concentration. Some studies indicate that this parameter is not critical for bactericidal activity^{35,39} while others claim that bactericidal activity is clearly concentration dependent.³⁰ Indeed, as a threshold concentration is required for pores to be formed, a too low peptide concentration can cause a reduced bactericidal activity. Finally, peptide orientation is a key parameter. Several studies suggested that only N-terminally attached peptides display antimicrobial activity.³⁰ However, C-terminal immobilization of the peptide is believed to have similar effects.³⁵ Here, an oriented grafting technique was used and peptides were N or C-terminally oriented.

Another parameter that must be taken into account is salt concentration. Several studies suggested that high salt concentration led to a reduction of antibacterial activity.³² Indeed, the effect of salt on the binding of the antimicrobial peptide magainin II to POPC (palmitoyl-oleoyl-phosphatidylcholine) lipid was studied by 40–50 ns molecular

dynamics simulations of a POPC bilayer in presence of different concentrations of NaCl ranging from 0 to 300 mM.⁶⁰ It was found that peptide binding to the lipids is stronger at lower salt concentration. Upon binding, the peptides disorder the lipids in their immediate vicinity, but this effect is diminished as the salt concentration increases.⁶⁰ Similarly, Matsuzaki et al.⁶¹ found that a rise in NaCl concentration decreases the membrane-perturbing activity of magainin I.⁶¹ Thus, the presence of salts in the M9 minimum medium is believed to reduce the AMP killing activity.

Finally, XPS analysis showed that only about 5% of HOEGMA monomers are grafted with MAG or LL37 peptide molecules. It is thus suggested that this low grafting ratio could also explain the low killing activity of the AMP-grafted surfaces.

The aim of this master thesis was to investigate the effect of chemical nanopatterns on bacterial behaviour. The first step was the elaboration of these nanopatterned surfaces. They consisted in alternating lines of P(HOEGMA) brush and PEG SAM obtained by combining nanoimprint lithography, gas phase silanation and ATRP polymerization. Two pattern geometries were used in order to analyse the influence of pattern dimension. Then, the P(HOEGMA) brushes were functionalized with different peptides having different functions. The first peptide is the well-known RGD peptide, recognised for its involvement in eukariotic cells adhesion. This peptide could be used combined with antifouling surfaces to promote cell adhesion while keeping anti-adhesive properties towards bacteria. However, bacterial adhesion on this specific peptide needs further investigations. Then, antimicrobial peptides, namely LL37 and magainin I, were used to functionalize the brush. They were expected to kill adhered bacteria. The successful elaboration of these surfaces was confirmed with different techniques including ellipsometry, AFM and XPS. *E. coli* was then used to assess bacterial adhesion, viability and growth on the different surfaces.

The first important conclusion is that nanopatterns did not directly influence adhesion and growth. In other words, no clear bacterial alignment nor specific arrangement of dead bacteria was observed on any of the tested surfaces. This contrasts with previous results obtained with similar types of patterns, but showing a different surface chemistry.^{22,26} However, nanopatterns had an indirect influence. Nanopatterned RGD surfaces distinctively induced a higher growth of bacteria after 12 h compared to homogeneous surfaces. Similarly, 400-patterned LL37 surfaces showed a higher killing activity compared to homogeneous and 250-patterned LL37 surfaces. However, this effect was not observed for the MAG-grafted surfaces.

The second important conclusion is that nanopatterned AMP-grafted surfaces were not as effective as expected to kill bacteria. Almost no dead bacteria were observed after 1 h and about only 20% were dead after 7 h-growth. It would be interesting to further investigate the cause of this phenomenon in order to optimize the killing properties of the modified surfaces. Moreover, as nanopattern dimensions have a clear influence on

bacterial behaviour, it would be valuable to systematically investigate this phenomenon by using other pattern dimensions and geometries and verifying their influence on other bacterial species.

Also, it would be interesting to investigate the influence of topography on bacterial organization. Indeed, a topographical difference of about 40 nm was observed in our nanopatterned surfaces. Since no influence on bacterial behaviour was found on surfaces presenting similar geometries than our samples and topographical differences of 7 and 22 nm, it has been hypothesized that the surface chemistry of our samples was the only parameter influencing bacterial organization. However, further research needs to be done to confirm this hypothesis.

Finally, it should be a good idea to combine RGD and AMP-grafted brushes on the same surface in order to investigate bacterial and eukariotic cells behaviour. As the MIC of AMP's is usually smaller than their cytotoxic concentration, eukariotic cells would specifically interact with RGD peptides without harm while adhered bacteria would be killed.

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APPENDIX A

EXPERIMENTAL PROTOCOLS

A.1 Bacterial growth in M9 minimum

First, Petri dishes are inoculated with *E. coli* MG1655 strain and placed during one night at 37°C . Then, some colonies are taken out from the Petri dish and seeded in 20 ml of LB Broth medium which is then placed at 37°C , 180 rpm during one night. This is the overnight culture. The next morning, 5 ml are taken from the overnight culture and diluted with 20 ml of fresh LB broth medium. This solution is again placed at 37°C , 180 rpm for 2 h. Then, 6 eppendorfs of this culture are retrieved and the bacteria are harvested by centrifugation (3 min, 10000 rpm). The bacteria are then suspended in M9 minimum medium. This procedure is repeated three times. These 6 eppendorfs are then brought together in a tube forming the bacterial suspension. The obtained solution is diluted with M9 minimum medium to reach an optical density (OD) between 0.22 and 0.24 at 600 nm, corresponding to about 2.5×10^8 cells/mL. This bacterial suspension was distributed in different eppendorf tubes and placed at 37°C . Optical density was evaluated after different times. The results are presented in figure ??.

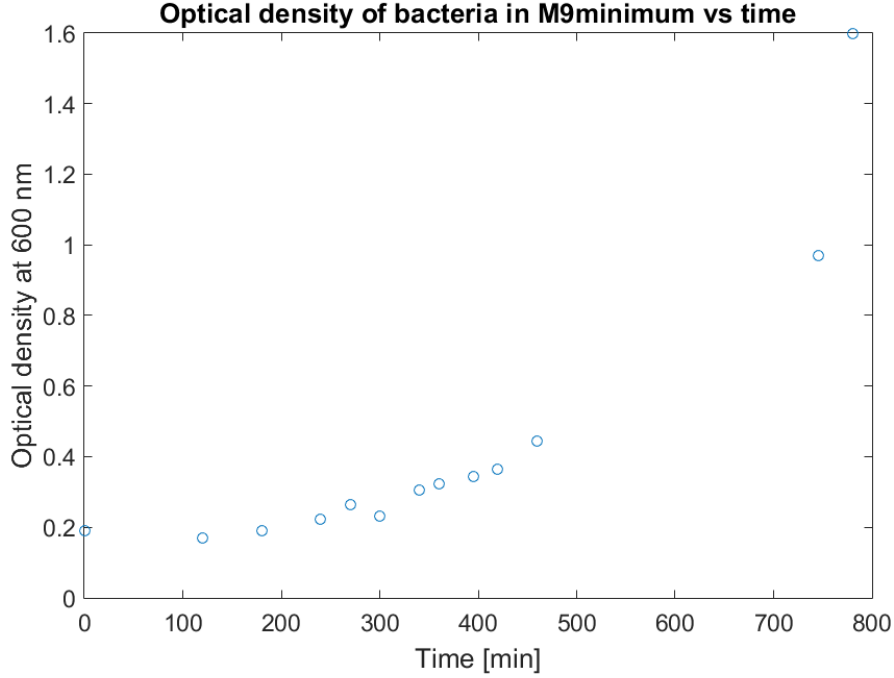


Figure A.1 – Bacterial growth in M9 minimum medium

A.2 Surfaces preparation

A.2.1 Nanoimprint lithography

The nanoimprint lithography process was entirely performed in the WinFab laboratories at Université catholique de Louvain (UCL). Two silicon mold (400 mold and 250 mold as described in section 3.2) with an area of $1 \times 1 \text{ cm}^2$ (purchased from AMO, GmbH) were used. Silicon substrates were first cleaned in a piranha solution made up of a 3:1 mix of H_2O_2 and H_2SO_4 to remove organic residues. The clean substrates were then subjected to a nanoimprint lithography procedure. A 4% (w/w) poly(methyl methacrylate) (PMMA) solution was prepared in toluene and filtered through a $0.45 \mu\text{m}$ membrane. PMMA was spin-coated onto the silicon (Si) samples using a Laurell WS-650MZ-23NPP/LITE spin-coater at a speed of 6400 rpm with an acceleration of 4000 rpm/s for 60 sec for the 400 mold and a speed of 2800 rpm with an acceleration of 2500 rpm/s for 60 sec for the 250 mold. The thickness of the mask was measured by ellipsometry to be approximately 115-120 nm for the 400 mold and 150-160 nm for the 250 mold. To perform the imprint, the mold was pressed onto the polymer mask using an Obducat nanoimprinter. The sample was pre-heated at 170°C for 1 min, then the pressure was increased from 5 bars to 60 bars and left for 3 min to perform the imprint. The system was then cooled down to 60°C and the mold was detached from the sample. Samples were subjected to a descum process for 3 min 40 sec using an Electrotech Plasmafab 310/340 reactive ion etcher, in oxygen plasma, to remove the residual polymer mask left on the patterned regions of the sample, leaving bare silicon with exposed silanol groups for the subsequent functionalization steps. Samples were immediately subjected to the next step to avoid surface contamination.

A.2.2 Growth of polymer brush

Gas-phase silanization

Materials 5'-dimethyl dichloro silyl propyl) 2-bromo-2-methylpropionate molecules were synthesized by Catherine Phillipart in the BSMA laboratories.

Method A Teflon sample holder, a Schlenk reactor and 2 silicon wafers used as control were cleaned by standard piranha procedure, rinsed with milli-Q water and dried in a heat chamber. The septum, valve and micro-syringe were rinsed with methanol and dried with nitrogen. Control surfaces were also cleaned 10 min by UV ozone. Control and descummed samples were placed on a Teflon sample holder in a Schlenk reactor heated in a silicone oil bath at 80 °C. The reactor was filled with argon for 5 min, then vacuum pumped for 10 min, filled again with argon for 15 min and finally vacuum pumped for 90 min before being isolated. 20 μ l of ATRP-silane was withdrawn in a glove box with a micro-syringe, which was previously purged 3 times. It was then injected into the reactor. The reaction was run 2 h at 80 °C. To stop the reaction, the samples were removed from the Schlenk reactor and immediately rinsed with methanol.

Liquid-phase silanization

Materials 2-[Methoxy(polyethyleneoxy)propyl]trichlorosilane (95%) was purchased from FluoroChem. Extra dry Toluene (99.85%) was purchased from Fisher Scientific. Triethylamine (99%) was purchased from Acros.

Method Two crystallizers were cleaned by standard piranha procedure, rinsed with milli-Q water and dried in a heat chamber. The samples were first placed in heated acetone (55°C) for 1 min in order to remove the PMMA mask. They were then placed for 10 sec in acetone in a ultrasound bath, rinsed with methanol and dried with a stream of nitrogen. The 8 samples were placed in 2 crystallizers. Then, 8 ml of toluene, 6 drops of PEG silane and 3 drops of triethylamine were added in each crystallizer. The reaction was run overnight in a glove box with a controlled argon atmosphere to ensure the reaction environment was completely free of water and oxygen. Samples were then rinsed with toluene, placed 20 sec in methanol in an ultrasound bath, rinsed again with methanol and dried with a stream of nitrogen.

P(HOEGMA) polymer brush

Materials Hydroxyl-terminated oligo(ethylene glycol) ether methacrylate (HOEGMA) monomers of molar mass 360 g/mol were obtained from Aldrich and were used without purification. Copper(I) chloride (99.995+%) ($Cu^I Cl$), copper(II) chloride (99.999+%) ($Cu^{II} Cl_2$), and 2,2'-dipyridyl (99+%) (bipy) were from Aldrich. Milli-Q water (resistivity higher than 18.2 M Ω .cm) was obtained from a Millipore Simplicity 185 system.

Method The different schlenks and laboratory glasswork used were cleaned by a standard piranha procedure, rinsed with milli-Q water and dried in a heat chamber. Septums

and valves were rinsed with methanol and dried. For ten substrates, 2 homogeneous surfaces were used as control. The monomer (21ml) was dissolved in a mixture of water (14.7 ml) and methanol (14.7 ml) in a round-bottom flask sealed with a rubber septum. Bipy (521.3 mg) and $CuCl_2$ (14.1 mg) were added to this solution, which was stirred and degassed with a stream of argon for 45 min. $CuCl$ (105.8 mg) was then added quickly to the solution. The solution was stirred and further degassed for 45 min.

Meanwhile, the initiator-grafted Si wafers were sealed into Schlenk tubes (polished face downward) and were degassed (the reactor was vacuum pumped for 5 min and then filled with argon for 15 min, this process was repeated 3 times). Then, 3 ml of polymerization solution was syringed and quickly transferred to each Schlenk tube. The Schlenk tubes containing the samples were kept at room temperature under an overpressure of argon in the absence of stirring, for 2 h. After 2 h, 1 homogeneous sample was removed, washed with water then methanol, dried with a stream of nitrogen, and measured by ellipsometry to assess the remaining polymerization time in order to reach approximatively 30 nm. Finally, the remaining samples were removed, washed with water then methanol and dried with a stream of nitrogen. The last homogeneous surface was measured by ellipsometry to obtain the final brush thickness. All samples were then stored under argon before use. The schlenks were finally cleaned with soap and water.

A.2.3 Biofunctionalization of the polymer brushes

Linker grafting

Materials P-Maleimidophenyl isocyanate (PMPI) was purchased from Apolo Scientific. Tetrahydrofuran (THF) was purchased from Acros.

Methods Glassworks used were cleaned by a standard piranha procedure, rinsed with milli-Q water and dried in a heat chamber. The samples were first rinsed with milli-Q water, methanol and dried with a stream of nitrogen before being placed in schlenk tubes. The reactors were vacuum pumped for 5 min and then filled with argon for 15 min and this process was repeated 3 times. A 6mM PMPI solution prepared in distilled dry THF was placed at 30°C and 700 rpm for 30 min. Then, 2 ml of this solution were added to each Schlenk tube. After a reaction time of 6 h at room temperature, the samples were sonicated in distilled THF for 5 min, and thoroughly washed 3 times with distilled THF before finally being dried in a stream of nitrogen.

Peptides grafting

Materials KRGDSPC (95+%), LL-37 with an additional C-terminal cysteine residue (LL37-Cys) (95+%), Magainin I derivatives with an additional C-terminal cysteine residue (MAG-I-Cys) (95+%) and tris(hydroxymethyl)aminomethane (tris) (99.8+%) were purchased from Sigma Aldrich.

Method Glassworks used were cleaned by a standard piranha procedure, rinsed with milli-Q water and dried in a heat chamber. A 100 mM tris buffer solution was prepared and the pH was adjusted to 7.05. The substrates were then rinsed with filtered trisbuffer and dried with a nitrogen steam. Peptides were withdrawn in glove box and a 280 μ M peptide solution was prepared in trisbuffer. This solution was sonicated for 15 min.

Each sample was immersed in 1 ml of this solution for 2 h 30 min at room temperature. The substrates were then sonicated 5 min in trisbuffer, thoroughly washed 3 times with Milli-Q water, and finally dried in a stream of nitrogen before being stored under argon.

A.3 Surfaces characterization

A.3.1 Ellipsometry

As previously mentioned, the three first measures were taken in the UCL cleanroom facilities with a Sentech SE 850 Ellipsometer using SpectraRay software. The data were fit using a model composed of three layers. The first layer corresponded to the silicon layer characterized by a refractive index of $n=3.6180$ and $k = 0.0015$ (tabulated values). The next layer consisted in a 1.5 nm-thick layer of silicon oxide whose index of refraction was modeled by a Cauchy model with parameters $n_0 = 1.452$ and $n_1 = 36$. The last layer was the PMMA layer also modeled by a Cauchy model with parameters $n_0 = 1.474$ and $n_1 = 47$. Five measurements were taken and averaged for each sample. The next ellipsometric measurements were carried out with a spectroscopic ellipsometer (RSE from J. A. Woollam Co., Inc.). The data were fit using a Cauchy model for the index of refraction of the organic layer, using tabulated values for the index of refraction of silicon. Five measurements were taken and averaged for each sample.

A.3.2 XPS measurements

Measures parameters

The analysis were performed on a SSX 100/206 photoelectron spectrometer from Surface Science Instruments (USA) equipped with a monochromatized micro focused Al Xray source (powered at 20 mA and 10 kV). Samples were stuck onto the aluminium conductive carousel with double sided adhesive tape. The pressure in the analysis chamber was around 10 Pa. The angle between the surface normal and the axis of the analyser lens was 55° . The analysed area was approximately 1.4 mm² and the pass energy was set at 150 eV. In these conditions, the full width at half maximum (FWHM) of the Au 4f peak of a clean gold standard sample was about 1.6 eV. A flood gun set à 8 eV 7/2 and a Ni grid placed 3 mm above the sample surface were used for charge stabilisation. The C(C,H) component of the C1s peak of carbon has been fixed to 284.8 eV to set the binding energy scale. Data treatment was performed with the CasaXPS program (Casa Software Ltd,UK), some spectra were decomposed with the least squares fitting routine provided by the software with a Gaussian/Lorentzian (85/15) product function and after subtraction of a nonlinear baseline. Molar fractions were calculated using peak areas normalized on the basis of acquisition parameters and sensitivity factors provided by the manufacturer.

Percentage of grafted monomers determination

Here, the developments leading to equations 3.1 and 3.2 page 38, allowing to compute the percentage of monomer units grafted by the different peptides, are detailed. By defining

- C_{HOEGMA} , the number of carbon atoms in one HOEGMA monomer,

- $C_{HOEGMA_{peptide}}$ the number of carbon atoms in one peptide-grafted HOEGMA monomer,
- $N_{HOEGMA_{peptide}}$ the number of nitrogen atoms in one peptide-grafted HOEGMA monomer,
- m_{HOEGMA} the number of HOEGMA monomers,
- $m_{HOEGMA_{peptide}}$ the number of peptide-grafted monomers.

From these definitions, it comes :

$$\frac{\%N_{tot}}{\%C_{tot}} = \frac{N_{HOEGMA_{peptide}} * m_{HOEGMA_{peptide}}}{C_{HOEGMA} * m_{HOEGMA} + C_{HOEGMA_{peptide}} * m_{HOEGMA_{peptide}}} * m_{HOEGMA_{peptide}} \quad (A.1)$$

In addition, by defining x the percentage of grafted monomers and n_{tot} the total number of monomers, it comes:

- $m_{HOEGMA} = n_{tot} * (1 - x)$
- $m_{HOEGMA_{peptide}} = n_{tot} * x$

We can thus rewrite equation ?? :

$$\frac{\%N_{tot}}{\%C_{tot}} = \frac{N_{HOEGMA_{peptide}} * x * n_{tot}}{C_{HOEGMA} * (1 - x) * n_{tot} + C_{HOEGMA_{peptide}} * x * n_{tot}} \quad (A.2)$$

$$\frac{\%N_{tot}}{\%C_{tot}} = \frac{N_{HOEGMA_{peptide}} * x}{C_{HOEGMA} * (1 - x) + C_{HOEGMA_{peptide}} * x} \quad (A.3)$$

$$\frac{\%N_{tot}}{\%C_{tot}} = \frac{N_{HOEGMA_{peptide}} * x}{C_{HOEGMA} + (C_{HOEGMA_{peptide}} - C_{HOEGMA}) * x} \quad (A.4)$$

By rearranging equation ?? to isolate x , equation 3.1 is obtained:

$$\frac{\%N_{tot}}{\%C_{tot}} * C_{HOEGMA} + \frac{\%N_{tot}}{\%C_{tot}} * (C_{HOEGMA_{peptide}} - C_{HOEGMA}) * x = N_{HOEGMA_{peptide}} * x \quad (A.5)$$

$$x = \frac{\frac{\%N_{tot}}{\%C_{tot}} * C_{HOEGMA}}{\frac{\%N_{tot}}{\%C_{tot}} (C_{HOEGMA} - C_{HOEGMA_{peptide}}) + N_{HOEGMA_{peptide}}} \quad (A.6)$$

By applying the same reasoning to nitrogen and oxygen atoms, equation 3.2 is obtained:

$$x = \frac{\frac{\%N_{tot}}{\%O_{tot}} * O_{HOEGMA}}{\frac{\%N_{tot}}{\%O_{tot}} (O_{HOEGMA} - O_{HOEGMA_{peptide}}) + N_{HOEGMA_{peptide}}} \quad (A.7)$$

A.4 Bacteria adhesion and growth on biofunctionalized surfaces

A.4.1 Materials

LB Broth with agar (Lennox), LB Broth (Lennox), Ampicillin sodium salt, sodium chloride (99%+) (NaCl), D-(+)-Glucose ($\geq 99.5\%$), ammonium chloride (NH_4Cl) ($>99.5\%$), sodium phosphate monobasic (99%) (KH_2PO_4), sodium phosphate dibasic ($\geq 99.5\%$) (Na_2HPO_4), magnesium sulfate heptahydrate ($>99\%$) ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), calcium chloride dehydrate ($>99\%$) ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) were purchased from Sigma-Aldrich. Pure casein was purchased from Acros. The water used was of Milli-Q grade. LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit L7007 containing SYTO 9 and propidium iodide dyes was purchased from Molecular Probes.

A.4.2 Medium preparation

- Ampicilline solution of 100 $\mu\text{g}/\text{ml}$. Add 10 mg of ampicilline in 10 ml autoclaved water. Dilute 10 times, filter and store in the freezer. Unfreeze before use.
- Petri dishes. Dissolve 7 g of LB Broth with agar in 200 ml of water. Autoclave during 10 min and let it cool then add 200 μl of ampicilline solution. Pour the petri dishes.
- LB Broth medium. Dissolve 4 g of LB Broth in 200 ml water. Autoclave during 10 min and let it cool then add 200 μl of ampicilline solution.
- 0.15M NaCl solution. Dissolve 2.19 g of NaCl in 250 ml of water. Autoclave during 10 min. Adjust pH to 7.25 right before use.
- 20% glucose solution. Dissolve 4 g of glucose in 20 ml of water. Autoclave during 10 min.
- M9 saline solution. Add 0.1 g of NaCl , 0.2 g NH_4Cl , 0.6 g of KH_2PO_4 , 1.1565 g of Na_2HPO_4 in 200 ml of water. Adjust pH to 7.2 and autoclave during 10 min.
- 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution. Dissolve 246 mg of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 ml of autoclaved water. Filter and store in the fridge.
- 10mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution. Dissolve 14.7 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 10 ml of autoclaved water. Filter and store in the fridge.
- Casein solution. Add 1 g of casein in 5 ml autoclaved water. Prepare this solution just before use.
- M9 minimum solution. Add 75 ml of autoclaved water, 25 ml of filtered M9 saline solution, 0.2 ml of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ solution, 1 ml of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solution. Adjust the pH at 7.25. Add 1 ml of glucose solution and 1 ml of casein solution. Agitate the solution until use. Prepare this solution just before use.

A.4.3 Epifluorescence microscopy

Fluorescence images were taken at room temperature with a Olympus IX71 microscope equipped with a FITC filter set and an Epi-Fluorescence module. The samples are placed in filtered 0.15 M NaCl solution ($\text{pH} \simeq 7.25$) in Lab-Tek chambered covered glass. The bacteria were stained with LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit: 30 μl of solution (SYTO 9 dye, 1.67 mM / Propidium iodide, 18.3 mM) was added to 20ml of filtered 0.15 M NaCl solution at neutral pH. Each samples was immersed in 0.8 ml of this solution for 15 min in a container protected from light. After staining, the samples were rinsed in one tube filled with M9 minimum solution and placed in filtered NaCl ($\text{pH} 7.25$) for observation.

