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PREVENTION OF BACTERIAL COLONIZATION IN HOSPITAL-ACQUIRED INFECTIONS USING ELECTRICALLY CONDUCTING POLYMERS

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Prevention of bacterial colonization in hospital-acquired infections using electrically conducting polymers

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Biofilms are bacterial assemblies developed as response to adverse environmental conditions and external threats. Within a biofilm, a complex and highly regulated internal architecture is developed, resulting in a network of interconnected microniches. This leads to the formation of an intricate internal electrochemical balance, key to aspects such as metabolism and inter-cell communication. Due to their highly optimized physiology, biofilms heavily influence a wide variety of aspects of the human life. In a medical context, biofilms constitute a serious health threat due to their low susceptibility to antibiotics and other biocides. In particular grave risk are patients treated with indwelling devices, as device-associated infections often result in the biofilm contamination of the implant. This requires the development of novel materials and strategies, so biofilm colonization of the device surface can be prevented.

Electrically conducting polymers have recently emerged as an interesting group of materials with properties from organic polymer, metals and semiconductors. With their dual organic-conductive nature, these materials can be used to synthesize versatile electrochemical systems with which monitor and influence biological systems. In this thesis, the use of electrically conducting polymers is explored with the aim of modulating biofilm formation.

First, composites of the conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT) complexed with either chlorine (Cl), heparin (Hep) or dodecylbenzenesulfonate (DBS) were studied. In all three cases, PEDOT acted as an electron mediator for bacterial metabolism, modulating *Salmonella* biofilm growth with the polymer electrochemical state. Furthermore, bacteria induced an electrochromic response on PEDOT. This allowed the use of the polymer composites as visual indicators of bacterial colonization, with applications in sterility assurance of medical devices and in food packing for contamination control.

To gain a deeper understanding of the effects of the PEDOT composites on biofilm growth, a fluorescence confocal microscopy study was performed. Using a custom-made image processing software tool, differences were found in the architecture of *Salmonella* biofilms that depended on the electrochemical state and composition of the composite. This revealed the suitability of conducting polymers as a platform for both fundamental microbiologic studies and biofilm engineering applications.

Next, we investigated whether a more refined control of *Salmonella* biofilm formation could be obtained with a more elaborated electrochemical device. Different electrochemical gradients were established along the channel of a PEDOT:Cl-based organic electrochemical transistor (OECT) using different voltage inputs in the source, drain and gate terminals. A fluorescence confocal microscopy study with the developed custom-made software tool revealed biofilm gradients mimicking the imposed electrochemical gradients. This illustrated

the potential of conducting polymers to modulate biofilms formation in complex patterns, which has applications in areas like design of antifouling surfaces, biocatalysis, and the study of bacterial colonization.

Finally, we explored the functionalization of conducting polymers with biocide agents. Surfaces based on poly(hydroxymethyl 3,4-ethylenedioxythiophene):polystyrene sulfonate (PEDOT-MeOH:PSS) were functionalized with silver nanoparticles (AgNPs) by means of an aminosilane linker. A nearly complete prevention of *S. aureus* biofilm growth was obtained when a voltage input was applied. This was not explained by the individual effects of either the AgNPs or the electrical input, indicating the presence of a synergistic effect. Moreover, it was also observed that bacterial colonization affected the electrical properties of PEDOT-MeOH:PSS, indicating a possible use of our system as real-time bacterial sensor. This opens the door to use the material as dual sensor-effector system, detecting bacterial colonization and acting when necessary.

In conclusion, the work performed in this thesis shows the potential of conducting polymers as biotransducers to both monitor and influence biofilm growth. This can be applied to the synthesis of smart coatings to effectively prevent the bacterial colonization of indwelling devices as well as to many other applications.

LIST OF SCIENTIFIC PAPERS

- I. S. Gomez-Carretero, B. Libberton, M. Rhen, and A. Richter-Dahlfors. Redox-active conducting polymers modulate *Salmonella* biofilm formation by controlling availability of electron acceptors. *npj Biofilms and Microbiomes*, 3(19):1–10, 2017.
- II. S. Gomez-Carretero, B. Libberton, M. Rhen, and A. Richter-Dahlfors. Image processing algorithm for the discovery and quantification of phenotypic patterns in biofilm microstructure. Manuscript.
- III. S. Gomez-Carretero, M. Rhen, and A. Richter-Dahlfors. Electrochemical patterning of biofilm growth along the channel of an organic electrochemical transistor. Manuscript.
- IV. S. Gomez-Carretero, R. Nybom, and A. Richter-Dahlfors. Electroenhanced antimicrobial coating based on conjugated polymers with covalently coupled silver nanoparticles prevents *Staphylococcus aureus* biofilm formation. *Advanced Healthcare Materials*, 6(20):1–10, 2017.

Work not included in this thesis:

- S. Gomez-Carretero and P. Kjäll. Medical Applications of Organic Bioelectronics. In F. Cicoira and C. Santato, editors, *Organic Electronics: Emerging Concepts and Technologies*, chapter 3, pages 69–89. Wiley Online Library, 2013.

CONTENTS

1	Introduction	1
1.1	Biofilms: a threat and an opportunity	1
1.2	Biofilm regulation	2
1.2.1	<i>Salmonella enterica</i> serovar Typhimurium	2
1.2.2	<i>Staphylococcus aureus</i>	3
1.3	Electron transport chain in bacteria	3
1.4	Strategies to control biofilm formation	5
1.4.1	Control of surface properties to prevent bacterial attachment	5
1.4.2	Surfaces with bactericidal compounds	6
1.4.2.1	Attached antimicrobials	6
1.4.2.2	Releasable antimicrobials	6
1.4.2.3	Silver nanoparticles	7
1.4.3	Electrochemical control of biofilm formation	8
1.4.4	Bacterial sensing	8
1.5	Introduction to the theory of solids	9
1.5.1	Solid formation and energy bands	9
1.5.2	Conducting, insulating and semiconducting solids	11
1.6	Fundamentals of conducting polymers	13
1.6.1	Description of selected conducting polymers	13
1.6.2	History of conducting polymers	16
1.6.3	Structure of conducting polymers	17
1.6.4	Charge carriers in conducting polymers	18
1.6.5	Synthesis of conducting polymers	21
1.6.5.1	Chemical polymerization	21
1.6.5.2	Electrochemical polymerization	22
1.6.5.3	Other polymerization methods	23
1.6.6	Functionalized conducting polymers	24
1.6.7	Conducting polymer-based electrochemical devices	26
1.6.7.1	The two-electrode architecture	26
1.6.7.2	The single electrode architecture	27
1.6.7.3	The organic electrochemical transistor	28
2	Aims	29
3	Results and discussion	30
3.1	Paper I. Electrically conducting polymers modulate biofilm formation	30
3.2	Paper II. Image processing algorithm to discover consistent phenotypic patterns in the biofilm architecture	32
3.3	Paper III. Biofilm gradients along the channel of an organic electrochemical transistor	34

3.4	Paper IV. Electroenhanced antibacterial activity of silver nanoparticles	35
4	Conclusions and future perspectives	38
5	My scientific contribution	40
6	Popular science summary	41
7	Acknowledgements	42
8	References	43

LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
AgNP	silver nanoparticle
APTES	(3-aminopropyl)triethoxysilane
ATP	adenosine triphosphate
Cl	chlorine
DBS	dodecylbenzenesulfonate
DNA	deoxyribonucleic acid
EDOT	3,4-ethylenedioxythiophene
EDOT-MeOH	hydroxymethyl 3,4-ethylenedioxythiophene
Hep	heparin
ITO	indium tin oxide
LB	lysogeny broth
OECT	organic electrochemical transistor
PEDOT	poly(3,4-ethylenedioxythiophene)
PEDOT-MeOH	hydroxymethyl poly(3,4-ethylenedioxythiophene)
PSS	poly(styrenesulfonate)
RNA	ribonucleic acid
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SEM	scanning electron microscopy
SPR	surface plasmon resonance
<i>S. Typhimurium</i>	<i>Salmonella enterica</i> serovar Typhimurium
TSB	tryptic soy broth

1 INTRODUCTION

1.1 BIOFILMS: A THREAT AND AN OPPORTUNITY

Bacterial biofilms are sessile microbial communities anchored to a surface and covered with a matrix of extracellular polymeric substances [1–5]. Bacterial biofilms are ubiquitous, being normally formed as a response to any form of environmental stress, including adverse temperature and pH levels and, the shear forces or running water, lack of nutrients and competing pathogens [5]. Against these external threats, biofilms confer higher chances of survival due to, among others, the formed extracellular matrix, a high interbacterial coordination, a slow metabolism and the development of a variety of phenotypes within the biofilm.

Developed biofilms constitute complex optimized ecosystems with an elaborate internal organization. For example, several strains show specific patterns in the location of dead cells in order to withstand the mechanical stress affecting the biofilm [6]. In addition, localized patterns are also observed in the expression of phenotypes related to biofilm synthesis, such as motility and synthesis of cellulose and curli, typical biofilm structural materials [7–10]. Interestingly, different parts of the biofilm also seem to show differentiated growth rate and metabolism [11, 12], which can create interdependence relationships and increase inter-cell coordination [13]. In relation to these differentiated phenotypes, different local microenvironments are also found within a biofilm, with local pH levels, oxygen concentrations, redox potentials and local concentrations of various metabolites [14–22]. To support cell survival and the coordination between the niches within the biofilm, an extensive network of water channels is deployed [1–4, 23], which, combined with diffusion [24], allows the distribution of nutrients, oxygen and chemical signals. In addition, a recent study indicates the use of ion channels and potassium waves for long-range electrical inter-bacterial communication within the biofilm [25].

The optimized phenotype of biofilms has severe consequences in many contexts of human activity. A main implication is the low susceptibility of biofilms to antibiotics and other antimicrobials, which poses an important hazard in clinical contexts [23, 26, 27]. Biofilms severely affect people with cystic fibrosis, causing chronic infections [28]. Biofilms are also typically present in chronic wounds [29] burns [30, 31], and in dental plaque [32, 33]. However, the medical context where biofilms are most often a problem is in indwelling devices such as catheters and respiratory tubes, since their abiotic surface constitute an ideal substrate for pathogens to colonize [34–36]. This frequently leads to device-associated infections, which cause elevated mortality, morbidity and economic costs every year [36–41]. But biofilms are undesirable also in other context of human activity. One is food industry, where biofilms, with low susceptibility to sanitizers, can contaminate food-contact surfaces and reach the consumer [42–44]. Other examples include water contamination [45] and corrosion in metal

pipes [46, 47].

Biofilms can, however, be also beneficial. Examples include corrosion protection by non-corroding bacteria,[47, 48] processing of waste-water [49–51], generation of electricity in microbial fuel cells [52] and the biocatalyzed production of chemicals [53]. Due to the ubiquitous presence of biofilms, it is important that we deepen into the study of biofilm formation, understanding how biofilm growth can be prevented or promoted depending on the context of application.

1.2 BIOFILM REGULATION

1.2.1 *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a Gram negative, rod-shaped, motile pathogen. It causes gastroenteritis in humans and other animals, being a common cause of food poisoning and constituting a cause of concern in food industry [43, 44]. *S. Typhimurium* binds to biotic and abiotic surfaces through diverse structures. This includes several types of fimbriae, secreted substances such as SiiE (*Salmonella* intestinal infection E) and BapA (biofilm associated protein A) adhesins and structures with alternative functions like flagella [54–56]. *S. Typhimurium* biofilm matrix is mainly constituted of proteins such as curli fimbriae (encoded by the *csg* operons), the BapA protein, flagella and extracellular polysaccharides such as cellulose, colanic acid and the O-antigen capsule [54, 57–60].

CsgD is the master regulator of biofilm formation, regulating transcription of the *csgDEFG-csgBAC* operons, involved in curli synthesis. CsgD also indirectly activates cellulose production via the positive regulation of *adrA* transcription. *adrA* positively regulates cellulose synthesis through the production of the secondary messenger (3'-5')-cyclic diguanosine monophosphate (c-di-GMP), which acts as an activator of the cellulose synthase BcsA [58]. In addition, c-di-GMP can also directly control biofilm growth through other mechanisms, such as curli synthesis through activation of *csgD* expression, and inhibition of motility [57–59]. CsgD was also found to induce expression of BapA and the O-antigen capsule on *Salmonella enterica* serovar Enteritidis [58].

Several factors influence biofilm formation through the influence on *csgD*. A major role is played by external environmental conditions such as temperature, osmolarity, oxygen tension and nutrient availability.[54, 57–60] These influence *csgD* synthesis through several global regulators, like the osmolarity response regulator OmpR, the integration host factor (IHF), the histone-like nucleoid structuring protein (H-NS) and the stress/stationary sigma factor RpoS, among others [54,57–60]. Small regulatory RNA (sRNA) can also regulate biofilm formation. ArcZ is a sRNA that, coupled to Hfq, regulates *csgD* both dependent and independent of RpoS [58,60–64]. ArcZ is regulated by the ArcB/A two-component system, activated in conditions

of oxygen deprivation [61–63]. In addition, RpoS also seems to be directly controlled by ArcB/A [63]. These results highlight the relationship between biofilm formation and bacterial respiration and metabolism.

1.2.2 *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive, round-shaped, non-motile bacteria. It is commonly implicated in device-associated infections, normally of nosocomial origin. Typical examples include infections associated with the use of urinary and ventricular catheters [35,41]. A major biofilm component in staphylococci is the polysaccharide intercellular adhesin (PIA), also called poly-N-acetylglucosamine (PNAG), together with teichoic acids and several different proteins [57, 65–68]. PIA is synthesized from the products of the *icaADBC* locus, which is regulated by many factors. It is repressed by TcaR and IcaR. In addition, *icaR* expression is negatively regulated by the protein regulator of biofilm formation, Rbf, therefore promoting biofilm formation. Conversely, Spx, a global regulator of the stress response, positively regulates *icaR* expression and prevents biofilm formation [57, 67].

Biofilm formation in staphylococci also seems to be controlled by several other global regulators, possibly both dependent and independent of the *ica* operon [57, 65–68]. The staphylococcal accessory regulator (*sarA*) and the sigma factor *sigB* positively regulate biofilm formation. Conversely, the accessory gene regulator (*agr*), heavily involved in the *S. aureus* quorum sensing system, downregulates biofilm formation. The *agr* system negatively regulates microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) adhesin proteins, involved in adhesion to host tissue, through the action of the RNAIII sRNA [57, 65–68]. In addition, *agr* affects and is affected by SarA [57, 67, 68]. Biofilm formation is also affected by environmental factors like oxygen concentration [57, 67]. The staphylococcal respiratory response regulator, SrrAB, induces PIA expression via positive regulation of *icaADBC* under anaerobic environments in *S. aureus* [57, 67, 69]. Conversely, in *Staphylococcus epidermidis* the oxygen-dependent control of biofilm seems to be performed by *sigB* [70]. These results highlight again the relationship between biofilm formation and bacterial respiration and metabolism.

1.3 ELECTRON TRANSPORT CHAIN IN BACTERIA

Bacterial respiration is a fundamental part of metabolism in which electrons are transferred from electron donors to electron acceptors through a series of redox reactions. This electron transport chain generates energy used to actively pump protons out of the cytoplasm, so they can re-enter through the ATP-synthases and synthesize ATP by oxidative phosphorylation [71–73]. We will now summarize the main components and processes of the electron transport chain.

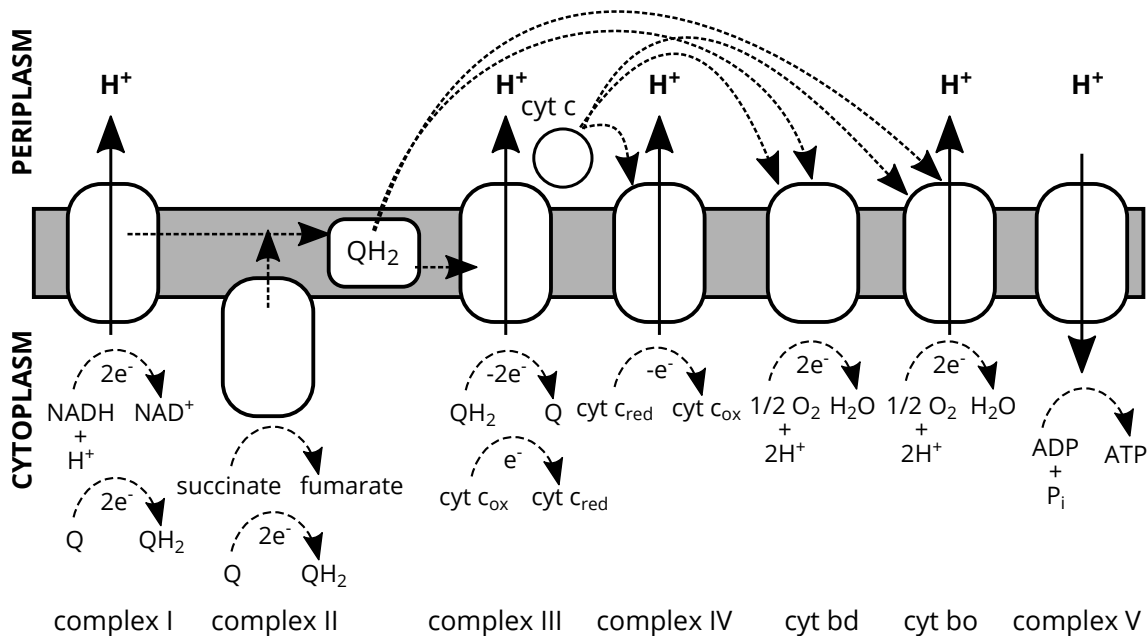


Figure 1. Bacterial electron transport chain. Differences might exist among different bacterial strains.

The electron transport chain in bacteria is illustrated schematically in figure 1. Generally speaking, the bacterial respiratory chain consists of a series of dehydrogenases and terminal oxidoreductases connected by mobile electron carriers such as quinones and the cytochrome c. The main electron donor in the bacterial electron transport chain is nicotinamide adenine dinucleotide (NADH). NADH is oxidized to NAD^+ in the respiratory complex I (also called NADH:ubiquinone oxidoreductase or NADH dehydrogenase). This in turn reduces ubiquinone (Q) to ubiquinol (QH_2), and uses the obtained energy for active proton pumping. In addition, there are many secondary dehydrogenase systems that oxidize a variety of electron donors, which highlights the versatility of bacteria [72–75]. One case of particular importance is the oxidation of succinate to fumarate, which, similarly than before, reduces ubiquinone to ubiquinol (succinate dehydrogenase or respiratory complex II). Ubiquinol can freely diffuse within the cytoplasmic membrane, carrying electrons to terminal sites to transfer electrons to final acceptors. A wide range of terminal oxidoreductases exists, making bacterial respiration very versatile. Among them, we can cite several ubiquinol oxidases and cytochromes, used in aerobic respiration, and nitrate and fumarate reductases, used in anaerobic respiration. One common electron acceptor of ubiquinol is the cytochrome bc1 complex (also called respiratory complex III), which transfer electrons from the low reduction potential compound ubiquinol to the high reduction potential compound cytochrome c, an electron carrier that can freely diffuse into the periplasmic space. Cytochrome c then delivers electrons to terminal oxidoreductases such as cytochrome c oxidase (respiratory complex IV), cytochrome bd, that uses oxygen as final electron acceptor, and cytochrome bo, able to oxygen but also use copper as final electron acceptor. Some of these terminal oxidoreductases, like cytochrome bd and cytochrome bo, can be used as quinol oxidases, so electrons are transferred directly from ubiquinol. This is the case of *E. coli*, where the cytochrome bc1 complex, cytochrome c and cytochrome c oxidase are missing, therefore relying on the Q/ QH_2 system as the sole electron carrier

[72–75]. Finally, the potential energy accumulated in the form of proton gradients is stored in the form of ATP from ADP and inorganic phosphate (P_i) (respiratory complex V).

The capability of bacteria to transfer electrons during the metabolism of organic substrates constitutes an interesting method to generate electricity. This was recognized already in the beginning of the twentieth century, giving rise to the technology of microbial fuel cells [52]. One main obstacle in the construction of efficient microbial fuel cells is, however, the low efficiency of electron transfer to solid state electrodes. Several solutions have been proposed. One is the use of electron mediators either dissolved in the culture medium or chemically coupled to the electrode [76–78]. Another solution is the use of exoelectrogenic bacteria such as *Shewanella oneidensis* and *Geobacter sulfurreducens*, which perform direct electron transfer to solid metal electrodes with high efficiency [71, 79–81]. Interestingly, *E. coli* has been shown to evolve in mediator-less microbial fuel cells to perform direct electron transfer to carbon-based electrodes [82, 83].

1.4 STRATEGIES TO CONTROL BIOFILM FORMATION

1.4.1 Control of surface properties to prevent bacterial attachment

Bacterial attachment to a surface is commonly regarded as the first step of biofilm colonization [84, 85]. While bacterial attachment to biotic surfaces is mainly driven by the specific binding of bacterial adhesins to receptors in the host tissue [86, 87], binding to abiotic surfaces is considered to be governed by unspecific physicochemical interactions. This includes the Lifshitz–van der Waals forces (normally attractive), the electrostatic interaction between the bacterium and substrate double layers (normally repulsive), and the Lewis acid-base interactions (the main responsible of the “hydrophobic interaction”, attractive or repulsive depending on the specific case). In addition, surface geometry of both the bacterium and the surface will severely affect the balance between these forces. Several attempts have been made to predict bacterial attachment from the physicochemical properties of the bacterial membrane and the physical substrate. A common method has been the calculation of the energy barrier between the interacting objects via the application of the extended DLVO (Derjaguin, Landau, Verwey, and Overbeek) (XDLVO) theory, commonly used in colloids science. These methods have, however, generally provided predictions of poor accuracy due to an oversimplification of the complexity of the bacterial membrane, although they have been useful in providing general trends for the design of antifouling surfaces [88–92].

One main surface property used to prevent bacterial adhesion is the surface charge [89–94]. The bacterial membrane is generally considered negatively charged in average, so negatively charged surfaces have often been employed to prevent bacterial attachment. Hydrophobicity is another factor often employed to prevent biofouling [90–93]. Bacterial membranes are generally considered hydrophobic, so hydrophilic surfaces are often employed to prevent

bacterial attachment. Another strategy is to employ particular surface nanopatterns [95–97]. These patterns can severely affect the correlation of physicochemical forces and increase the energy barrier to bacterial adhesion. In addition, certain nanopatterns have been shown to affect the integrity of the bacterial membrane, leading to cell death. Taken together, these methods constitute useful strategies to prevent bacterial attachment, although they also present several drawbacks. They are prone to heavily depend on the characteristics of the membrane of the bacterial strain tested, which hinders their general applicability. Besides, they are also heavily affected by surface fouling from the components of the bacterial medium.

1.4.2 Surfaces with bactericidal compounds

Another option to prevent bacterial attachment and biofilm formation is the use of biocidal compounds. Two strategies can be used: to attach the antimicrobial compound to the surface exposed to bacterial colonization or to release the biocide from the surface.

1.4.2.1 Attached antimicrobials

Attached antimicrobials have the advantage of presenting a high local concentration on the prepared surface and preventing the removal of the compounds in the presence of liquid flow. Their efficacy might, however, be compromised by fouling from the components of the bacterial medium. A wide variety of attached antimicrobials has been tested. One typical example is the use of antibiotics grafted to the surface [93, 96–99]. Another example is the use of antimicrobial peptides. Generally with an overall positive charge and abundant hydrophobic residues, antimicrobial peptides attract the negatively charged, hydrophobic bacterial surface and disrupt the cell membrane [96, 97, 99]. However, their high price and low stability have encourage research on synthetic polymers with equivalent properties, like cationic charge and equivalent functional moieties [100, 101]. Polymer coatings also provide high flexibility in their composition, for example through the use of polyelectrolyte multilayers [102]. Besides, the architecture of the polymer coating can also be finely controlled. One example is the use of polymer brushes, which provide an additional factor to prevent bacterial attachment by means of the steric repulsion driven by the surrounding osmotic pressure [99].

1.4.2.2 Releasable antimicrobials

On the other hand, surfaces with releasable antimicrobials does not generally suffer from fouling from the components of the liquid medium. Their efficacy is, however, critically dependent on parameters such as the concentration of the released compound, the kinetics of the release and the duration of the release. This is particularly important in contexts where liquid flow is present. The release mechanism is often based on degradation or swelling of polymer scaffolds, while the employed antimicrobial compound is typically an

antibiotic, an antimicrobial peptide or an antimicrobial synthetic polymer [98–100, 103, 104]. Polyelectrolyte multilayers are also here a typical option for the release of one or several antimicrobials [102]. Also of interest is the novel generation of polymer scaffolds of “smart materials” able to initialize antimicrobial release when stimulated by changes in pH, ionic strength or temperature [105–107]. New approaches are also being explored in terms of the released compound. One interesting strategy is the use of quorum sensing signaling compounds to prevent biofilm formation [44, 93].

1.4.2.3 Silver nanoparticles

Silver nanoparticles (AgNPs) constitute an interesting biocidal compound to create antibacterial surfaces [108]. Their mechanism of action seems to be originated in the slow release of silver ions as the AgNPs become oxidized due to the action of oxygen and other elements present in the bacterial medium [109, 110]. Silver ions affect bacteria in a number of ways. Silver ions interact with the peptidoglycan cell wall and the plasma membrane, causing their disruption. They also interact with the bacterial DNA, forcing it into its condensed form and preventing DNA replication. In addition, silver ions bind to thiol and amino groups of proteins, affecting, among others, proteins involved in cell division and bacterial respiration. Reactive oxygen species also seem to be generated in some bacterial species due to the malfunction of the bacterial respiratory chain, further affecting the cell [109–112]. Compared to bulk silver coatings, AgNPs offer increased antibacterial activity [111, 112]. This is likely originated in the increased amount of released ions due to the larger exposed surface of AgNPs and their easier oxidation, as observed by the smaller oxidation potential of AgNPs compared to bulk silver [113]. Interestingly, AgNPs have also shown increased antibacterial activity respect to a similar molar concentration of silver in ionic form, like in the case of the salt silver nitrate (AgNO_3) [111, 112]. Moreover, several studies indicate the presence of a biocidal effect caused by AgNPs in the absence of silver ions release [112]. Taken together, this indicates particular effects ligated to silver in nanoparticle form. Possible explanations include enhanced penetration into the bacterium, a more optimal silver ions release kinetics and the catalytic generation of free radicals [111].

Several reports account for the existence of silver ions-resistant bacteria due to mechanisms such as the presence of efflux pumps and metal-binding proteins. Silver ions resistance has been found encoded in plasmids, therefore allowing transfer of resistance, and sometimes in the chromosome [109–112, 114]. Silver nanoparticles have been found effective against multidrug resistant bacteria [115], and it is thought that the broad antibacterial mechanisms of AgNPs would hinder resistance development [109, 114]. However, resistance to AgNPs has been reported [112]. Special AgNPs coatings or the combination of AgNPs with antibiotics might further help to overcome resistance [115, 116].

Also of concern is the toxicity of AgNPs [108–112]. Toxicity in AgNPs, however, seems to be directly related to the presence of released silver ions [110, 111], so toxicity might be prevented with a sufficiently low concentration of AgNPs or with more stable AgNPs produced using special preparation routes or coatings [110, 111]. An interesting example is the lack of appreciable toxicity in commercially available AgNPs-coated catheters [108], which points to the safety of AgNPs when used at limited concentrations [108–112, 117].

1.4.3 Electrochemical control of biofilm formation

The finely tuned electrochemical environment conformed within a bacterial biofilm suggests the use of electrical signals as a possible strategy to prevent biofilm formation or remove an already formed biofilm. This is usually termed the “electricidal effect” [118–120]. Several studies have reported the successful electrochemical control of biofilm formation, using constant voltages and currents as well as time-varying signals with frequencies up to several megahertz. Although the causes are yet not completely clear, some explanations have been suggested. These include biofilm disruption by the flow of hydrated ions, the electrochemical generation of potentially biocidal compounds such as hydrogen peroxide and other oxygen reactive species, the generation of electrostatic charges, causing the prevention or delay of the bacterial attachment, and electrochemically-driven changes in pH in the proximities of the electrodes [118–120].

Another interesting mechanism is termed the “bioelectric effect”, which consists in the enhancement of the biocidal activity of certain compounds when they are employed together with an electrical signal. Although the causes are still unknown, some explanations have been proposed. One is the electrophoretic movement of antibiotics, which would help them to cross over the matrix of extracellular polymeric substances of the biofilm and penetrate the bacterial membrane. Another is the electrochemical modification of antibiotics and other bactericidal compounds, conferring them new functionalities and constituting a possible way to elude bacterial resistance. An interesting proposed explanation is the electrochemical increase of metabolic activity in bacteria, either directly with the applied electrical input signal or indirectly by, for example, the electrochemical increase of oxygen concentration. This would stimulate bacterial metabolism, increasing the susceptibility of bacteria to biocidal compounds such as antibiotics [118–123].

1.4.4 Bacterial sensing

Finally, another way to prevent bacterial colonization can be, in certain situations, an early bacterial detection. One example would be the detection of catheter colonization, so antimicrobials can be applied or the catheter removed and cleaned or replaced. Another example would be the detection of colonization of food-contact surfaces, protecting the customers’ health and preventing economic losses.

Bacterial sensing is based on the bacteria-triggered alteration of one or several of the physical magnitudes monitored by the sensing device. Typical examples include detecting the bacterial mass, like in quartz crystal microbalance (QCM), the bacteria-trigger modification of the response to an incident light, like in UV-Vis absorbance, Raman spectroscopy, optical fiber-based sensors and surface plasmon resonance (SPR)-based sensors, and the bacteria-triggered changes of the measured electrical response [124]. Electrical sensors (also termed electrochemical sensors) in particular constitute a very powerful method of bacterial sensing. They are generally robust, precise and accurate, with several electrical properties serving as sensing candidates. In addition, they are generally easy to implement, requiring only the use of electrically conducting electrodes in some point of the biological system to monitor, and need equipment that is commonly inexpensive and portable [125, 126]. Besides, the sensing electrodes can be functionalized for enhanced performance [124]. The main classes of electrical sensors are potentiometric, where no electrical current circulates and only the open circuit potential is measured, amperometric, where a voltage is applied and the resultant electrical current is measured, and impedimetric [126–128]. In impedimetric sensors the ratio between voltage and current is calculated. The most common form of impedimetric sensing is the technique known as electrochemical impedance spectroscopy (EIS), where the input signal (voltage in potentiostatic EIS and current in galvanostatic EIS) is a sinusoidal waveform of varying frequency [127, 128]. The monitoring of the electrical response of the system in the range of frequencies of interest generates an impedance spectrum that can be adequately interpreted with the right electrical circuit-based theoretical model. This allows a very precise characterization of any biological event occurring in the system, like bacterial colonization [127]. In addition, the versatility of electrical sensing has allowed the use of many other sensing techniques employing a variety of input waveforms to record a variety of phenomena. This includes techniques such as cyclic voltammetry (CV), square-wave voltammetry (SWV), differential pulse voltammetry (DPV) or the different forms of stripping voltammetry [128, 129].

1.5 INTRODUCTION TO THE THEORY OF SOLIDS

To develop technologies to successfully control bacterial attachment and biofilm formation, it is important to understand the different types of solid materials and how they are formed. To this end, some fundamental concepts will be introduced.

1.5.1 Solid formation and energy bands

Solids are formed by the tight bonding of a large collection of atoms, which results in properties and phenomena not present in the individual atoms when they are considered alone. When atoms are brought together to form a solid, their atomic orbitals split, so the Pauli exclusion principle is fulfilled. This process continues as more atoms interact and the inter-atomic distance is reduced, leading to a situation where the energy levels are so numerous

and tightly packed that they can be considered energy bands. Several energy bands are then formed from the combination of the different orbitals, with the electrons filling the bands from the lowest available energy state. Consecutive bands are separated by band gaps where no electrons can be placed. Also termed “forbidden bands”, they are originated by the energy states not covered by the combined atomic orbitals of the formed solid and constitute an energy barrier that electrons must overcome in order to travel to a band with higher energy.

In terms of electrical conductivity, only the outermost orbitals, the valence orbitals, are of interest due to their involvement in electron transport. This encouraged the particular study of the corresponding frontier energy bands, termed the valence band and the conduction band. The valence band comprises the energy states that the electrons of the outermost orbitals would normally fill, while the conduction band comprises the collection of electronic states located immediately above, in the sense of higher energy, available for electrons to occupy. To fully understand the concepts of valence band and conduction band and the behavior of electrons within them we need, however, to look a bit deeper into the theory of solids.

The occupancy of a particular energy state of a solid by an electron depends on whether this state is available and on the probability of an electron to occupy it. The distribution of available states in the solid is termed the “density of states” of the particular solid. It depends on the structure of the solid and, as previously discussed, should be zero at the forbidden band. Meanwhile, the probability of occupancy is provided by the probability distribution of the energy of the electron. Electrons belong to a class of particles called fermions. As such, their probability distribution $f(E)$, with E being the energy level, is described by Fermi-Dirac statistics:

$$f(E) = \frac{1}{e^{(E-E_F)/kT} + 1} \quad (1)$$

where E_F is called the Fermi level, k is a constant value called the Boltzmann constant and T the temperature in kelvins. The Fermi level is an important parameter dependent on the structure of the solid that indicates the hypothetical energy level (without considering whether it is a forbidden state or not) where the probability of occupancy is $1/2$.

To understand the behavior of electrons inside the solid we will start by analyzing the situation at absolute-zero. This is depicted in the band diagram of figure 2.a, where the vertical axis corresponds to the energy levels and the horizontal axis to a physical dimension of the solid. At 0 K, according to equation 1, all the states below E_F have probability of occupancy $f(E) = 1$, while all the states above E_F have probability of occupancy $f(E) = 0$. We can therefore see that the Fermi level indicates the highest energy level that electrons have a larger than zero probability to occupy at 0 K. However, as can be seen in figure 2.a, the Fermi level is in the forbidden band so no available state exist at the Fermi level according to the density of states of the material. Electrons therefore occupy up to the lower edge of the band gap, which defines the valence band. In turn, the upper edge of the band gap defines the conduction

band. At 0 K no electrons can occupy the conduction band since, although there are available states, the probability distribution $f(E)$ dictates a zero probability of occupancy. When the temperature is raised, as shown in figure 2.b, some electrons are “excited” and acquire more energy so they are able to “jump” to the conduction band. This is indicated by the new shape of $f(E)$ in figure 2.b, which becomes larger than zero on the lower part of the conduction band.

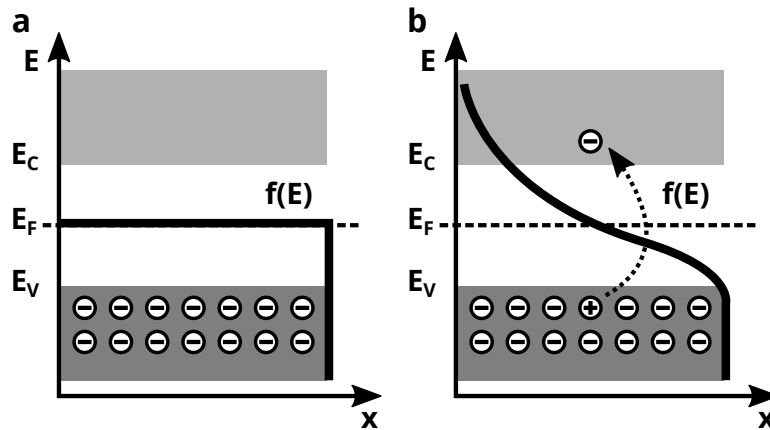


Figure 2. Band diagram of a semiconductor with the probability distribution at 0 K (a) and at high temperature (b). The vertical axis corresponds to the energy values. E_V is the energy of the edge of the valence band, E_C is the energy of the edge of the conduction band and E_F is the Fermi level. The horizontal axis corresponds to a physical dimension of the solid. Electrons are represented with the minus sign and holes with the plus sign. An electrical current would correspond to electrons and holes moving in the horizontal direction. An external voltage is, however, needed to move the charge carriers in the horizontal direction.

The excitation of electrons to the conduction band is of key importance for electrical conduction. No net current flow is possible in a completely full valence band since there are no available states. No current flow is possible in a completely empty conduction band since there are no electrons to move. By exciting an electron from the valence band to the conduction band a double effect occurs. First, an electron is placed on the conduction band, making possible the existence of a current in the conduction band. Second, an empty space or “hole” is created in the valence band, making possible the existence of a current in the valence band. It should be noted, however, that the application of an external voltage is necessary to effectively create an electrical current. This would correspond in the band diagrams of figure 2 to the movement of electrons and holes, generically termed “charge carriers”, towards the right or the left. Further information about electrical conduction in solids can be found in specialized publications [130, 131].

1.5.2 Conducting, insulating and semiconducting solids

With the definitions of the previous section it is now possible to classify the different solids according to their electrical conductivity. Three types of solids are typically defined: conductors, insulators and semiconductors.

Electrical conductors, depicted in figure 3.a, possess an elevated electrical conductivity. They are characterized by the overlapping of their valence and conduction bands, which allows electrons to freely travel to the conduction band and, upon the application of an external voltage, originate an electrical current. Examples include solids made of transition metals, like gold or silver solids.

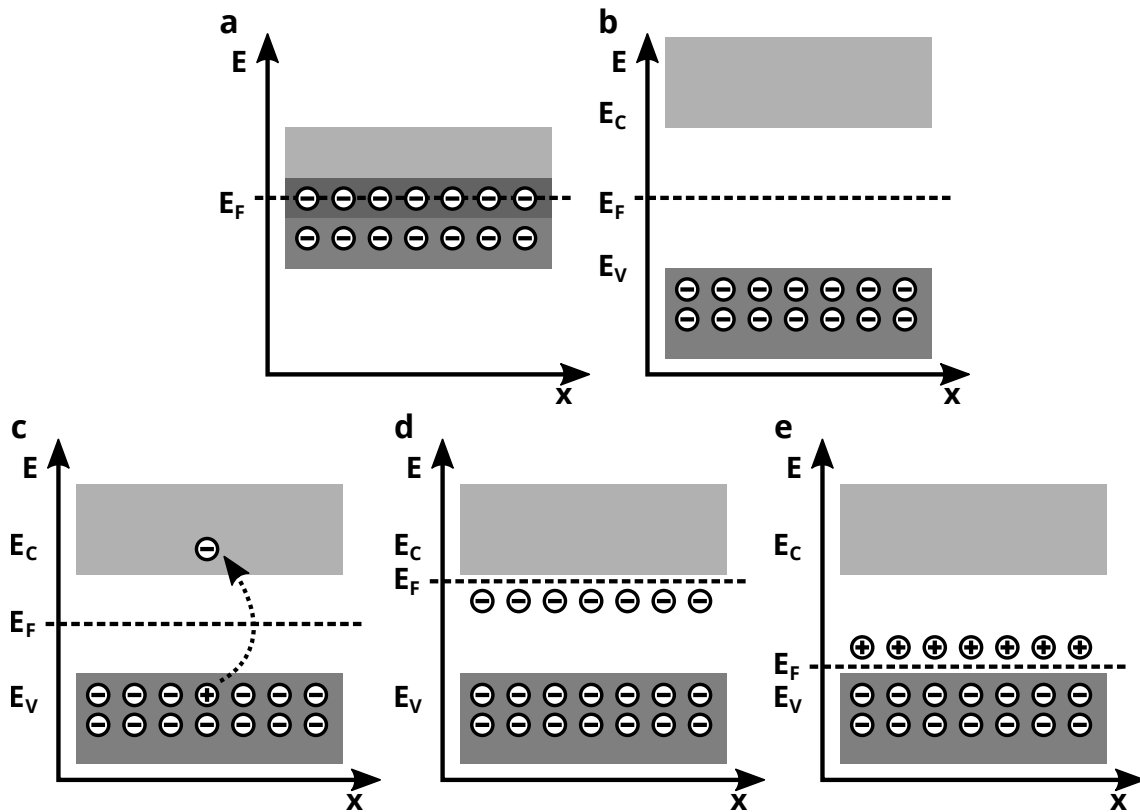


Figure 3. Types of solids according to their electrical conductivity: conductors (a), insulators (b), intrinsic inorganic semiconductors (c), n-doped inorganic semiconductors (d) and p-doped inorganic semiconductors (e).

Insulators, depicted in figure 3.b, possess an extremely low electrical conductivity. They are characterized by a very large band gap, with the Fermi level lying in the gap region. This large band gap prevents electrons from travelling from the valence band to the conduction band unless they acquire an extremely large amount of energy. This, as seen in the previous section, results in a poor electrical conductivity. Glass is a typical example of an insulator.

Intrinsic inorganic semiconductors, depicted in figure 3.c, possess an electrical conductivity between that of metals and insulators. Their band structure is similar to that of insulators, possessing a certain band gap with the Fermi level lying within it. However, the band gap is much smaller than in insulators, which allows a certain amount of electrons to cross to the conduction band under normal ambient temperatures. This leads to an intermediate electrical conductivity. Silicon and germanium are typical examples of semiconductors.

An interesting characteristic of semiconductors is the possibility to greatly increase their electrical conductivity by a process called doping. Two classes of doping processes exist:

n-doping, presented in figure 3.d, and p-doping, presented in figure 3.e. Taking silicon as example, n-doping is typically produced by the addition of phosphorous atoms, while boron atoms are used for p-doping. A phosphorous atom has five valence electrons but only four are used when bonding with four neighboring silicon atoms in the silicon crystalline lattice. This results in an additional electron per phosphorus atom added. As a consequence, the band structure of the solid radically changes due to new energy levels occupied by electrons. These additional levels lie very close to the conduction band so electrons can readily jump into them, which increases electrical conductivity. The introduction of these new energy states also causes the Fermi level to shift towards the conduction band, reflecting the increased probability of electrons occupying energy states closer to the conduction band respect to the undoped situation. When boron is used the opposite effect occurs. Boron has three valence electrons, therefore leaving one of the four bonds with neighboring silicon atoms without an electron. This can be interpreted as a “hole” in the crystalline structure of silicon, resulting in one hole per boron atom inserted. As a result, additional energy levels with “holes” appear close to the valence band, so valence band electrons can readily jump into them. This leaves available electronic states in the valence band, increasing electrical conduction. The presence of these new empty energy states in the band gap causes here a shift of the Fermi level towards the valence band, reflecting the new increased probability of electrons occupying energy states closer to the valence band in comparison to the undoped case. More information about the effects the doping process can be found in specialized publications [130, 131].

1.6 FUNDAMENTALS OF CONDUCTING POLYMERS

Polymers are macromolecules formed by a large repetition of a reduced number of different units called monomers. From DNA to proteins and carbohydrates, polymers are fundamental to form and maintain life. They are also a fundamental part of modern technology, with synthetic polymers like poly(vinyl alcohol) (PVC), poly(ethylene terephthalate) (PET), poly(styrene) (PS), poly(propylene) (PP) and poly(urethane) (PU or PUR), among others, ubiquitously found in almost any consumer product. An important limitation of the traditional synthetic polymer technology, however, is the lack of electrically conductive materials, which prevents their applicability in areas like flexible electronics, wearable sensors and disposable diagnostics. This obstacle has, nevertheless, recently been solved with the synthesis of organic electrically conducting polymers, which has given rise to the new field of “plastic electronics”. To evaluate the possible use of organic electrically conducting polymers to successfully control bacterial attachment and biofilm formation, some fundamental aspects of these materials will be analyzed.

1.6.1 Description of selected conducting polymers

A wide variety of conducting polymers exists. Among the most common are: trans-polyacetylene, cis-polyacetylene, poly(p-phenylene vinylene), polyaniline, polypyrrole,

polythiophene and poly(3,4-ethylenedioxythiophene) (PEDOT). Also of interest are PEDOT derivatives such as hydroxymethyl poly(3,4-ethylenedioxythiophene) (PEDOT-MeOH). Their chemical structures are shown in figures 4.a-h.

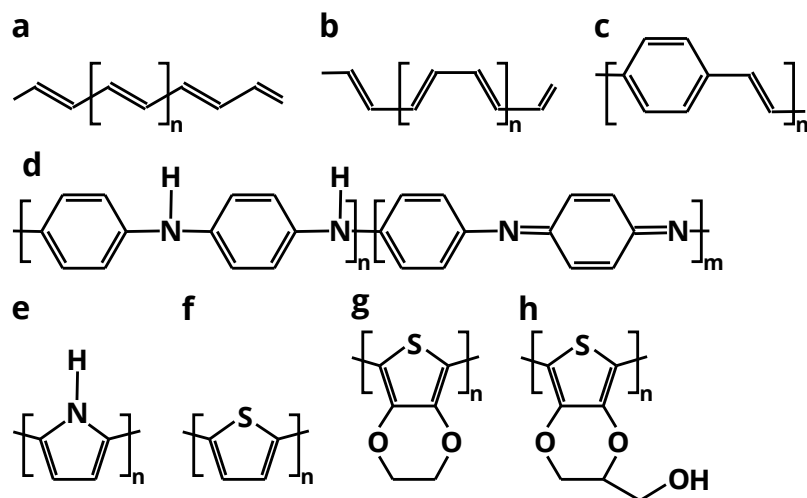


Figure 4. Chemical structure of trans-polyacetylene (a), cis-polyacetylene (b), poly(p-phenylene vinylene) (c), polyaniline (d), polypyrrole (e), polythiophene (f), PEDOT (g) and PEDOT-MeOH (h).

Polyacetylene is the simplest conducting polymer. Although it has a high conductivity upon doping, it is unstable in air, difficult to synthesize and not processable, so its use is restricted to pure fundamental research [132–134].

The polymer poly(p-phenylene vinylene) was the first conjugated polymer where electroluminescence was described. It is mainly used in light emitting diodes (LED) production [135]. It is not processable after synthesis, although several soluble variants have been synthesized [135]. Intrinsic poly(p-phenylene vinylene) possesses a good ambient stability, a feature improved even further in several developed poly(p-phenylene vinylene) variants [132–137]. However, its intrinsic conductivity is very low, in the range of $10^{-13} \text{ S cm}^{-1}$, and although its doped state presents highly increased conductivity, the material becomes then usually unstable in air [132, 136, 137].

Polyaniline is one of the most currently used conducting polymers. This conducting polymer can exist in one of three possible forms depending on the oxidation state: pernigraniline (fully oxidized), emeraldine (half-oxidized) and leucoemeraldine (fully reduced). Only the emeraldine form is conductive, with the more oxidized emeraldine salt having a much higher conductivity than the emeraldine base.[138] Polyaniline can be easily synthesized by chemical or electrochemical polymerization at low pH [138], which is required to solubilize the monomer and generate the emeraldine salt form [138]. After synthesis polyaniline is typically insoluble in water and organic solvents, although several monomer variants offer improved processability. Besides, electrochemical polymerization can be employed to obtain polyaniline films deposited over electrodes [138]. Polyaniline possesses a moderately high conductivity, with typical values of 7 S cm^{-1} [138], and good ambient stability [133, 138].

However, several studies question its biocompatibility, which could limit its use in biological applications [139–141].

Polypyrrole is also a very commonly used conducting polymer. Its unsubstituted form is generally insoluble, with only moderate solubilities achieved in certain organic solvents upon the inclusion of surfactants like dodecylbenzene sulfonate (DBS). This makes polypyrrole hard to process [133, 134, 138]. However, its low oxidation potential allows easy electrochemical polymerization on electrodes, typically in aqueous solutions with sulfonate salts like sodium dodecyl benzene sulfonate (NaDBS) [133, 134, 138]. Due to its low oxidation potential, the undoped state of polypyrrole is unstable in ambient oxygen, as it progressively turns into the doped state. Conversely, the doped state of polypyrrole is stable at ambient temperature, although some decrease in conductivity can appear, depending on the counter ion used, at moderately high temperatures [132–134]. Polypyrrole possesses a high conductivity, with typical values around 100 S cm^{-1} , although higher values can be obtained with special polymerization conditions [132–134, 138]. Besides, it is generally regarded as biocompatible [141]. In addition, polypyrrole presents a characteristic anisotropic volume change pattern, with a larger perpendicular volume change upon doping and dedoping [142]. This feature has made it a very promising candidate for the manufacture of microactuators [142].

Polythiophene is one of the most employed conducting polymers. Unsubstituted polythiophenes are insoluble and therefore not processable [138, 143]. Besides, their high oxidation potential complicates their electrochemical polymerization [138, 143]. However, several 3-substituted thiophenes have been developed, with the added side chain conferring solubility in several organic solvents as well as water, although their high oxidation potential remains a problem for their electrochemical polymerization in many cases.[143] Generally speaking, polythiophenes are very stable at ambient conditions [133, 143, 144] and have high electrical conductivity, with typical values of $100\text{--}1000 \text{ S cm}^{-1}$ and reports of conductivities up to 7500 S cm^{-1} [138, 143–145]. Several 3-substituted thiophenes are regularly used in many applications. A popular example is poly(3-hexylthiophene) (P3HT), used in photovoltaics but also in biological systems, where it shows great biocompatibility [146]. However, the most used polythiophene is the 3,4-substituted polythiophene poly(3,4-ethylenedioxythiophene) (PEDOT), synthesized by first time in the late 1980s [144]. PEDOT possesses a very good ambient stability while showing a diminished oxidation potential, which facilitates its electrochemical synthesis and improves its electrochemical switching characteristics [145]. Although PEDOT remains a fairly insoluble polymer, this problem is circumvented with the use of dopants like poly(styrene sulfonic acid) (PSS), which can be used to obtain a processable PEDOT:PSS water dispersion [144]. Furthermore, PEDOT is notable for its high electrical conductivity [132, 141, 145], reaching values up to 1000 S cm^{-1} with the use of secondary doping strategies [144], its transparency [144, 145], its stability in water in a wide range of pH values [147, 148] and its biocompatibility

[141]. Besides, several functional PEDOT derivatives have been prepared [144]. All these features make PEDOT:PSS and other PEDOT-based polymers a common option in biological and medical applications [149, 150]. Further details about the properties of the available conducting polymers as well as examples of applications can be found in the literature [133, 138, 144].

1.6.2 History of conducting polymers

The history of conducting polymers can be traced back to the isolation of aniline by F. F. Runge in 1834 and C. J. Fritzsche in 1840, with reports of the appearance of a blue color upon oxidation [144, 151]. Remarkable was also the work of H. Letheby, who electropolymerized aniline into polyaniline over a platinum electrode, although without uncovering its electrically conducting properties [144, 151].

The possibility of an electrically conducting polymer was put in the spotlight in 1962 with the theoretical study by J. A. Pople and S. H. Walmsley, who discussed the presence of solitons in polyacetylene and how this could originate an electrical conductivity [152]. Finally, in 1963, the possibility of an electrically conducting polymer was proven real by the work of D.E. Weiss and collaborators on polypyrrole [144, 152]. Some years later, in 1967, a lecture at the 18th Meeting of CITCE (Comité International de Thermodynamique et Cinétique Electrochimiques) (later called ISE, International Society of Electrochemistry) by R. Buvet, published a year later, reported the electrically conductive character of polyaniline [151].

An important event that encouraged investigation in conducting polymers was the discovery in 1973 of the conducting inorganic polymer poly(sulfur nitride) [152]. Another milestone was the report of the electrical conductivity of polyacetylene, further increased upon doping with several halogens, by H. Shirakawa and collaborators in 1977 [151]. For this and other contributions, Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa received the Nobel Prize in chemistry in the year 2000 [144, 151].

Research in conducting polymers continued, resulting in new materials and fabrication techniques. One important example is the work of Diaz and collaborators at IBM on the electropolymerization of polypyrrole in 1979 and polyaniline in 1980 [153, 154]. Another is the synthesis of polythiophene, pioneered by researchers like Yamamoto, Lin and Koßmehl [144, 155–160].

Once conducting polymers became a mature technology, a race began for a fully processable, commercially viable material. Success was achieved by Jonas and collaborators in Bayer AG as a result of the synthesis of PEDOT [144]. A first patent was filled on 22 April 1988 describing the chemical synthesis of PEDOT, followed by another one on novel

applications and by a third one on its electrochemical polymerization [144]. Further research was then performed under a collaboration between Bayer and Agfa-Gevaert on anti-static coatings for photographic purposes, which led to the invention of the highly processable PEDOT:PSS by Jonas and Krafft. The patent on PEDOT:PSS was filed in 1990 [144]. Subsequently, upon public dissemination of the discoveries on PEDOT and PEDOT:PSS, a frantic investigation on these materials began [161]. Further details can be found in recent publications [144, 158–162].

1.6.3 Structure of conducting polymers

The electrical properties of conducting polymers arise from their chemical structure, which is characterized by alternating single and double bonds along the polymer backbone, forming what is termed a conjugated system. This conformation causes the orbitals in the carbon atoms of the backbone to undergo sp^2 hybridization, with the three sp^2 orbitals undergoing σ bonds with adjacent atoms (for example, one hydrogen atom and two adjacent carbon atoms) and the remaining p orbital (p_z orbital) available to form a π bond. In a conjugated system, the available p_z orbitals overlap between neighbouring atoms, creating a system of connected p orbitals along the polymer chain. This results in a region where electrons are not anymore associated to any particular atom, therefore becoming “delocalized”, and can move with a certain degree of freedom along the polymer backbone. A representation of the conjugated system formed in trans-polyacetylene is shown in figure 5.

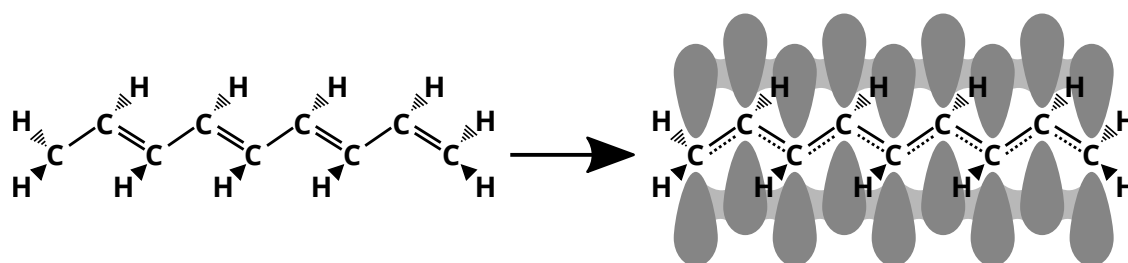


Figure 5. Representation of the conjugated system formed in trans-polyacetylene.

Despite the existence of delocalized electrons, the conductivity of an undoped conducting polymer is still rather low. For example, $10^{-10} \text{ S cm}^{-1}$, in the range of insulating materials like glass, is obtained for undoped polyaniline while $10^{-5} \text{ S cm}^{-1}$, in the range of semiconducting materials like undoped silicon, is obtained for undoped trans-polyacetylene [136]. Upon doping, however, conducting polymers acquire conductivities several orders of magnitude higher, reaching values similar to metals. For example, conductivities in the range of 10^3 S cm^{-1} are obtained for doped polyaniline, while values close to 10^5 S cm^{-1} are obtained for doped trans-polyacetylene. It should be noted, however, that while only a few parts per million are needed to increase the conductivity of inorganic semiconductors like silicon, dopant percentages in the order of 10 % to 50 % are typically used to achieve similar increases in conductivity in conducting polymers [163–166].

Although termed “doping” in analogy to inorganic semiconductors, doping in conducting polymers vastly differs from that of inorganic semiconductors. In conducting polymers, doping is better understood as a redox process. To achieve “p-doping” electrons are removed from the conducting polymer, which becomes oxidized and positively charged. To compensate this charge imbalance, a counter ion, in this case a negatively charged species, forms an ionic complex with the polymer, rendering the construct electrically neutral. Conversely, to achieve “n-doping” electrons are added to the conducting polymer, which renders it reduced and negatively charged. In this case a positively charged counter ion will form an ionic complex with the polymer so the whole construct is electrically neutral. Commonly, the doping agent, responsible for the oxidation (in p-doping) or the reduction (in n-doping) of the polymer, becomes the counter ion once it has been, in turn, reduced (in p-doping) or oxidized (in n-doping), although that does not need to be necessarily the case. It should also be noted that p-doping is by far the most common type of doping process in conducting polymers. Although n-doping can be achieved by, for example, employing alkali metals, the rapid re-oxidation of n-doped conducting polymers upon exposure to ambient oxygen has so far severely limited their applicability [167]. Further details about polymer doping and the role of counter ions are covered in section 1.6.5.

1.6.4 Charge carriers in conducting polymers

Despite sharing the common denomination of “semiconductors”, inorganic and organic semiconductors present numerous differences in the underlying physical mechanism of electrical conduction. One important difference, already mentioned, is in the relative doping percentage as well as the mechanisms behind it. A second difference, related to the first one, is the way the charge carriers provided by doping behave.

In inorganic semiconductors, the movement of electrons through the semiconductor does not affect the surrounding crystal lattice. However, a local distortion in the lattice, termed “relaxation”, occurs in conducting polymers as electrons move along the conjugated system in the polymer chain, locally changing the polymer conformation [166, 168]. The electron and its accompanied distortion are treated jointly and modeled as a “quasiparticle”, so it can be easily described in the developed mathematical framework of the theory of solids. Therefore, these quasiparticles constitute the charge carriers in conducting polymers as electron and holes constitute the charge carriers in inorganic semiconductors.

Three types of charge carriers exist in conducting polymers: solitons, polarons and bipolarons. Solitons appear in conducting polymers with a degenerate ground state. A degenerate ground state exists when interchanging the double and single bonds of the conjugated polymer results in the same ground state energy. The most prominent example of a conducting polymer with a degenerate ground state is trans-polyacetylene. This is shown in figures 6.a and 6.b. Due

to this degeneracy, the energy of the system does not depend on the position of the soliton, so the soliton (also termed “solitary wave”) is free to move along the polymer chain [168]. The movement of positive, neutral and negative solitons along the trans-polyacetylene chain, with the soliton separating the two possible polymer conformations, is depicted in figures 6.c-e. A soliton can be described in the energy band model as a energy level in the band gap. A soliton can contain zero electrons (positive soliton), one electron (neutral soliton) or two electrons (negative soliton). An energy band model depicting a soliton is illustrated in figures 7.a-c.

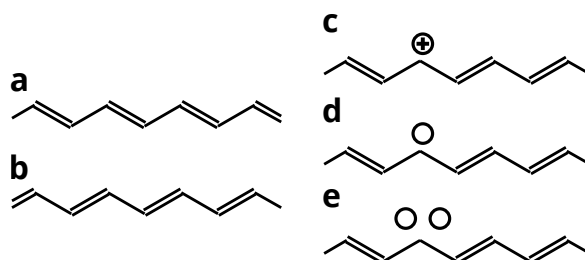


Figure 6. Degenerate ground state of trans-polyacetylene (a and b) and positive (c), neutral (d) and negative (e) solitons moving along the trans-polyacetylene chain.

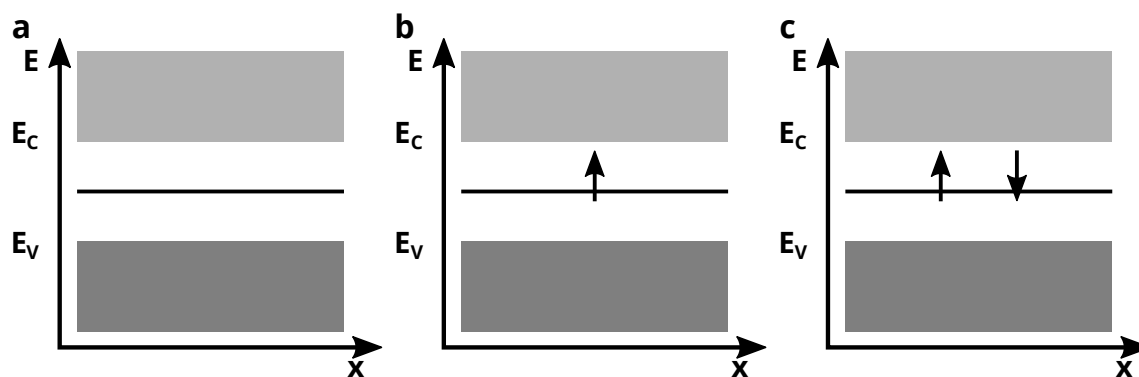


Figure 7. Charge carriers in a conjugated polymer: positive (a), neutral (b) and negative (c) solitons.

Most conducting polymers, like, for example, cis-polyacetylene, polyphenylene, polyaniline, polypyrrole and polythiophene, have a non-degenerate ground state with two variants, the aromatic state, of lower energy, and the quinoid state, with larger energy [168]. Figures 8.a and 8.b illustrate the case of polythiophene. Contrary to the case of trans-polyacetylene, now the soliton separates two regions of different energies (the aromatic and quinoid states) as shown in figure 8.c, which impedes its free movement and prevents its role as charge carrier [168]. To stabilize the structure two solitons can then couple, keeping a minimum number of quinoid-form monomers between them so the energy of the structure is minimized.[168] When a positive and a neutral soliton are coupled, the resulting quasiparticle is termed polaron, while when two positive solitons interact the quasiparticle is termed bipolaron. This is illustrated in figures 8.d and 8.e. In terms of energy states, polarons and bipolarons can be described as two energy levels in the band gap, with each state able to hold up to two electrons. This is illustrated in figures 9.a and 9.b. Interestingly, the energy levels for a bipolaron are located further away from the valence and conduction bands edges compared to the case of a polaron. This is due to the larger lattice relaxation for bipolarons compared to the case of polarons [163, 169].

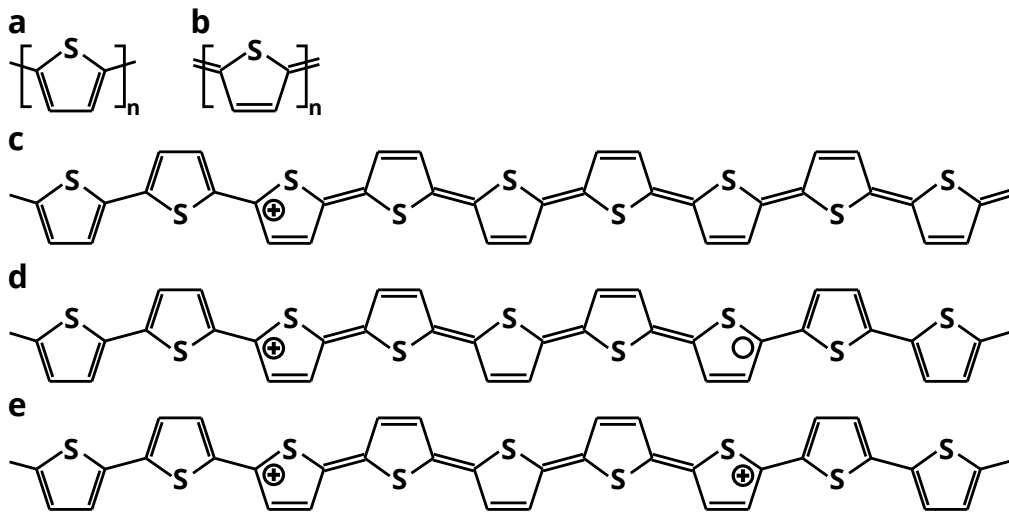


Figure 8. Non-degenerate ground state of polythiophene: aromatic (a) and quinoid (b) forms. A Soliton (c), a polaron (d) and a bipolaron (e) in polythiophene.

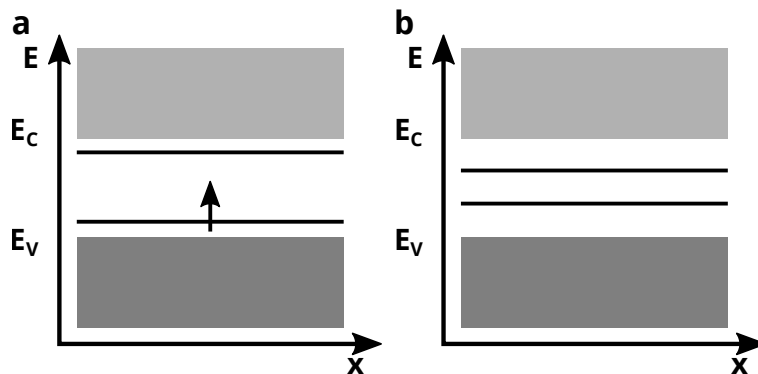


Figure 9. Charge carriers in a conjugated polymer: polaron (a) and bipolaron (b).

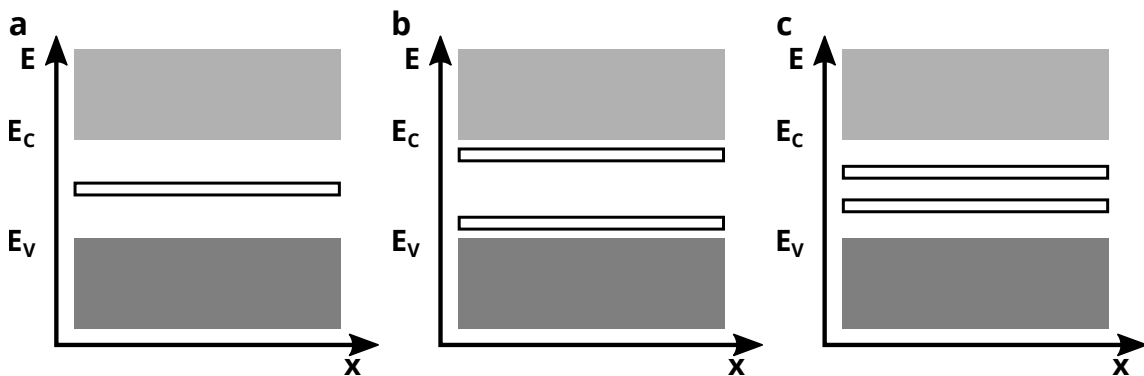


Figure 10. Representation of energy bands corresponding to soliton states (a), polaron states (b) and bipolaron states (c).

Finally, once single energy levels have been created by the presence of solitons (for conjugated polymers with a degenerated ground state) or polarons and bipolarons (for conjugated polymers with a non-degenerated ground state), further doping will cause more energy levels to appear. This eventually leads to the formation of energy bands in the band gap, creating a band structure similar to that of doped inorganic semiconductors.[169] This is shown in figures 10.a-c, where the energy of the highest occupied molecular orbital (HOMO)

corresponds to the upper edge of the valence band and the energy of the lowest unoccupied molecular orbital (LUMO) corresponds to the lower edge of the conduction band.

1.6.5 Synthesis of conducting polymers

1.6.5.1 Chemical polymerization

Chemical polymerization is a versatile procedure that can be adapted to obtain every conducting polymer from its corresponding monomer [138]. Besides, the procedure can be easily scaled up to achieve large production quantities [132]. The resultant polymer is normally in powder form and can be diluted or dispersed in an appropriate liquid. This can then be used as a “conductive ink” to coat the desired substrate with techniques such as spin-coating, bar coating, dip coating, screen printing or inkjet printing [138]. Two main types of routes exist for the chemical synthesis of polythiophenes: transition metal-catalyzed polymerization and oxidative coupling polymerization [170].

In transition metal-catalyzed polymerization, halogens such as bromine are placed at positions 2 and 5 of the thiophene monomer, therefore performing the role of a Grignard reagent. Upon addition of catalysts such as Ni(II) or Pd(II), a metal-catalyzed cross-coupling reaction such as Kumada coupling is produced [159, 170]. This was employed by Yamamoto and by Lin and Dudek in 1980 for the first chemical synthesis of unsubstituted polythiophene [159]. The synthesis of unsubstituted polythiophenes was later refined by Wudl employing iodine at the 2 and 5 positions, which permitted to obtain a more purified polymer. However, unsubstituted polythiophenes revealed insoluble in any solvent with the exception of a mixture of arsenic trifluoride/pentafluoride, which limited their processability and therefore their applicability [159, 171]. In the search of a processable polythiophene, alkylthiophenes (with the alkyl chain in the 3 position) were investigated. The first chemical synthesis of an environmentally stable and processable poly(alkylthiophene) was performed by Elsenbaumer and collaborators in 1985, discovering that an alkyl chain longer than a propyl group rendered the polymer soluble in many common solvents.[134] A transition metal-catalyzed polymerization, similar to that used for unsubstituted thiophenes, was employed [159]. The lack of symmetry of the 3-alkylthiophene monomer prompted the study of the orientation of the monomers in the polymer chain, a property termed regioregularity. This was firstly studied by Elsenbaumer and collaborators, who found that high regioregularity produced more conductive polymers [159, 172]. Synthesis routes to produce regioregular “head-to-tail” polymers were later developed by McCullough and by Rieke [159]. It is also worth noting that the transition metal-catalyzed polymerization is able to produce neutral (undoped) polymers [144]. The transition metal-catalyzed polymerization has been used for the polymerization of many thiophene derivatives beyond unsubstituted thiophene and poly(alkylthiophene), with PEDOT being a notable example [144].

The oxidative coupling polymerization method was first described by Sugimoto and collaborators in 1986 [159]. This method only involves the mixture of the thiophene monomer and an oxidant (typically Fe(III) from iron(III) chloride salts) in an appropriate solvent. Although simpler than transition metal-catalyzed polymerization, this method does not produce regioregular polymers. However, this is not a problem for symmetric monomers like 3,4-ethylenedioxythiophene (EDOT). The polymerization mechanism also differs from the previous method. While the transition metal-catalyzed polymerization was based in the direct coupling of carbon atoms, the oxidative coupling polymerization method involves the formation of radicals by the oxidant followed by radical-to-radical coupling [173]. Although undoped polymer has been obtained at low yields, with this method doped polythiophene is typically produced [144]. The oxidant (typically Fe(III)) has the role of doping agent, responsible for removing electrons from the polymer chain, while ions present in the reaction mixture counter the charge of the doped (oxidized) polythiophene [144]. Examples include FeCl_4^- counter ions from the oxidation with iron(III) chloride salts and tosylate counter ions from the oxidation with Fe(III)-tosylate, a Fe(III)-sulfonate salt [144]. A very well known example is PEDOT:PSS, where the EDOT monomers are oxidized with sodium peroxodisulfate (iron(III) sulfate is also normally included as catalyzer to control the reaction rate) and poly(styrenesulfonic acid) is used as counter ion [144].

Further information about the chemical synthesis of thiophenes can be found in several specialized sources [136–138, 144].

1.6.5.2 Electrochemical polymerization

Electrochemical polymerization, also termed electropolymerization, of conjugated polymers was pioneered by Diaz and collaborators in their work on polypyrrole in 1979 [153]. The electrochemical synthesis of polythiophene was achieved shortly after its chemical synthesis [174, 175]. An historical analysis of the electropolymerization technique has been recently published [162].

Polythiophenes can be prepared by either cathodic or anodic electropolymerization, although the anodic method is by far the most commonly used [143]. Anodic electropolymerization induces the formation of the conjugated polymer by oxidation of the monomer with an electrical current, with negative ions present in the polymerization mixture forming a complex with the polymer as counter ions. Although simple in practice, the mechanism leading to polymer formation is considerably complex [138, 176–178]. Due to the applied electrical current, the monomers are oxidized, leading to radical cation formation. Subsequently, monomers are joined together through radical-radical coupling followed by deprotonation [138, 178]. Radical formation, radical-radical coupling and deprotonation processes repeat, leading to chain propagation and polymer formation. After polymer formation, the applied

voltage triggers its electrochemical oxidation, prompting the incorporation of negative ions from the polymerization mixture to generate an electrically neutral complex [177]. Additional processes, however, seem to affect the procedure, like parallel chemical polymerization, polymer branching and crosslinking [177]. Another interesting effect is the potential polymer degradation by overoxidation from the applied voltage used during polymerization [177]. This is particularly important for polythiophenes, since the voltage values required for monomer oxidation directly cause polymer overoxidation, a phenomenon that has been termed the “polythiophene paradox” [138, 178]. This problem has, however, been greatly mitigated in some substituted polythiophenes such as PEDOT, where the needed voltage for monomer oxidation has been diminished [138, 178].

To perform an electrochemical polymerization, a polymerization solution is prepared containing the monomer to polymerize, a supporting electrolyte to confer an appropriate electrical conductivity to the solution, and an appropriate solvent. Important parameters to consider include the potential window of solvent and electrolyte, so they do not undergo redox reactions for the potentials needed for monomer oxidation, as well as the solubility of the electrolyte in the selected solvent. Details about the effects of the solvent and the supporting electrolyte as well as of factors like temperature and monomer concentration can be found elsewhere [138, 143, 178]. A potentiostat in a standard 3-electrode configuration is generally employed [138], with the synthesized polymer depositing over the working electrode as it becomes insoluble upon chain elongation [138, 141, 143]. The difficulties of polymer deposition on conducting oxides prompt the use of working electrodes that do not oxidize along with the monomer, such as those made of noble materials like platinum or gold [138, 143]. Similarly, the auxiliary electrode also needs to be inert and withstand the cathodic reactions originated by polymer formation at the working electrode [138]. Typically, platinum is used for the auxiliary electrode [138, 179]. Common Ag/AgCl electrodes or saturated calomel electrodes are used as reference [179]. Three electrical schemes are commonly used for electropolymerization: potentiostatic, galvanostatic and potentiodynamic [138, 141, 178]. During potentiostatic electropolymerization the voltage is specified, which allows to maintain the integrity of the synthesized polymer by using voltage values away from the overoxidation potentials. During galvanostatic electropolymerization the electrical current is specified, which allows a precise control of the rate of polymer deposition. During potentiodynamic electropolymerization the applied voltage constantly cycles between a low and a high potential limits. This can produce undoped polymers and can also generate morphologies different than the other two methods [138, 141, 178].

1.6.5.3 Other polymerization methods

Although not as frequently used as the chemical and electrochemical methods, there are alternative synthesis strategies worth mentioning. A relatively popular method is vapor

phase polymerization of monomers like pyrrole and EDOT, where the evaporated monomer is passed through a solid substrate impregnated in an oxidant. Although initial studies were performed with FeCl_3 , typically iron(III)-sulfonates, like iron(III)-tosylate, are generally used [138, 180, 181]. A related, more recent technique is oxidative chemical vapor deposition (oCVD). First, the EDOT monomer is evaporated and deposited onto the substrate. Then, the oxidant FeCl_3 , chosen for its relatively high vapor pressure, reacts with the previously deposited EDOT monomer, generating a PEDOT film [182]. Other polymerization techniques include photochemical polymerization, where the polymerization is initiated employing visible light, and microwave and radiofrequency plasma polymerization, using ionized gasses to achieve polymerization [138, 183, 184].

1.6.6 Functionalized conducting polymers

Novel functionalities could be achieved by functionalizing a conducting polymer with biologically active molecules. One typical option is based on the use of 3-substituted alkylthiophenes employing a carboxyl moiety, like 3-thiophenecarboxylic acid or 3-thiopheneacetic acid, as well as other strategies [143, 185, 186]. However, 3-substituted alkylthiophenes often have poor solubility and are difficult to electropolymerize due to a high oxidation potential, which complicate their practical use [143, 144]. Conversely, PEDOT is highly processable, has a low oxidation potential and also a good conductivity, but lacks available groups to allow its functionalization [144, 187]. However, several alternatives exist.

One typical method is the use of electrochemical polymerization, where biologically active negatively charged molecules can be included in the polymerization mixture as part of the supporting electrolyte and incorporated as counter ions, forming an ionic complex with PEDOT [144]. However, molecules incorporated as counter ions during electropolymerization are linked only electrostatically, which makes them prone to be released unintentionally, specially upon the dedoping process triggered by chemical or electrochemical polymer reduction [144, 178].

Another option for PEDOT functionalization is based on the physical entrapment of bioactive molecules. Several reports describe the physical entrapment of bioactive molecules into PEDOT using vapor phase polymerization [188–190]. Besides, two different approaches for the entrapment-based functionalization of PEDOT:PSS dispersions have also been described [191, 192]. One approach is based on the mixture of PEDOT:PSS and poly(vinyl alcohol) (PVA). This provides available hydroxyl groups, so the silane coupling agent 3-glycidyloxypropyltrimethoxysilane (GOPS) can be used to bind proteins and polypeptides to the conducting polymer [191]. The other approach is based on the mixture of PEDOT:PSS with carboxymethylated dextran (CMD), providing active groups for functionalization via amide bond formation. A conductivity enhancer (glycerol) and the crosslinking agent GOPS

are also added to the mixture to provide good electrical conductivity and high stability in aqueous environments [192]. None of these three entrapment-based strategies, however, employ a defined complete chain of covalent bonds between the polymer and the bioactive molecule, relying instead, to a greater or lesser degree, on the physical confinement into the polymer matrix. As a result, unintended release of the molecules from the polymer, upon factors like polymer swelling due to water absorption, can be of importance. While this problem can clearly affect composites functionalized with the vapor phase polymerization, surfaces using the two PEDOT:PSS dispersion functionalization approaches described are also not free of risk. In the PVA methodology no covalent bond exists between PEDOT:PSS and PVA, which compromises the stability of the whole composite. This is mitigated in the CMD approach through the use of GOPS as a crosslinker that holds the composite together, although leakages can still appear due to the lack of a defined bond between the polymer and every bioactive molecule.

One reported strategy for covalent functionalization is the post-polymerization treatment of PEDOT with fluorinated thiol vapors, producing a nucleophilic reaction that incorporates covalently bonded thiol groups that can be used for polymer functionalization. However, the electrical conductivity of the polymers resulted severely compromised after this process [193, 194].

An alternative to provide covalently functionalized, fully electroactive conducting polymers is the use of chemically functionalized EDOT variants. These monomers present an available reactive group covalently coupled to the polymer backbone, resulting in a chemically stable and fully defined functionalization [187]. These monomers can then be polymerized with standard methods like electrochemical polymerization [195], which permits a fine control of the ratio of functionalized/non functionalized polymers via the proportion of functionalized and standard monomers in the polymerization mixture. Besides, functionalization via the reactive group can be performed either pre- or post-polymerization depending on the particular experimental conditions.

The aim of providing a simple functionalization mechanism has rendered EDOT variants with “click” chemistry-based functionalization a popular option. One typical strategy is the use of the azide-alkyne Huisgen cycloaddition “click” chemistry, which can be performed with an EDOT variant containing either the azide or the alkyl side of the reaction [196–201]. Another reported strategy is the use of the thiol-ene “click” chemistry, where a thiol and an alkene join and form an alkyl sulfide. Monomer variants with either the thiol or the alkene side of the reaction have been prepared [202–204]. Functionalization strategies besides “click” chemistry have also been reported, like halogenated EDOT variants for functionalization via nucleophilic substitution [205] or variants with an available carboxyl group for functionalization through amide bond formation [206, 207].

Another possible alternative is the use of hydroxymethyl 3,4-ethylenedioxythiophene (EDOT-MeOH), which presents available hydroxyl groups [208]. Although this EDOT variant has been traditionally employed solely as starting point for further monomer modifications [187, 201, 205], its available hydroxyl group makes it a candidate for functionalization using silane coupling agents [209]. This includes, for example, the use of aminosilanes to couple metal atoms via co-ordinate bonding as well as functionalization through the formation of amide bonds.

1.6.7 Conducting polymer-based electrochemical devices

The multifaceted nature of conducting polymer, with properties of metals, semiconductors and organic polymers, results in a rich electrochemistry that can be utilized to create a variety of electrochemical devices. Here we will analyze some of these devices.

1.6.7.1 The two-electrode architecture

Among the most elemental architectures is the two-electrode electrochemical cell [210, 211], shown in figure 11. Here, a voltage difference is applied between two separated conducting polymer-based electrodes immersed in an electrolyte. Electrons are drawn from the conducting polymer anode, positively biased, which becomes electrochemically oxidized. Meanwhile, electrons accumulate in the conducting polymer cathode, negatively biased, which becomes electrochemically reduced. To maintain charge neutrality in the polymer electrodes, a flux of ions is established, with cations migrating towards the cathode and anions migrating towards the anode.

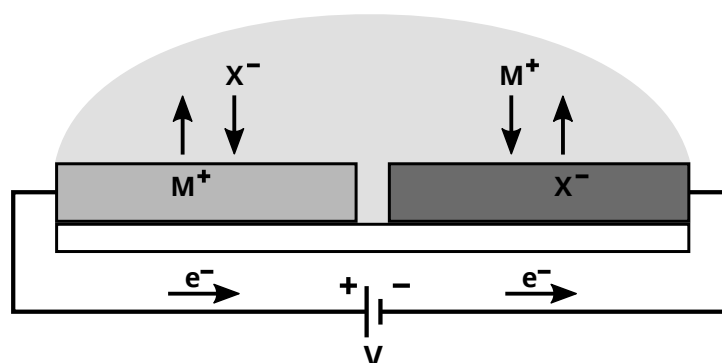


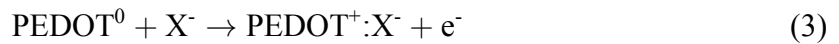
Figure 11. Front view of a two-electrode architecture. Cations are indicated with M^+ and anions with X^- .

Using PEDOT:PSS as example, we can describe the electrochemical reaction occurring in the anode by equation (2):



where M^+ is a positively charged ion that leaves the PEDOT matrix. It should be noted that PSS is a relatively large counter ion that normally remains within the PEDOT matrix. If a

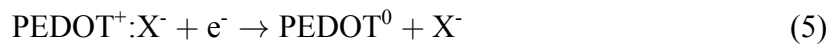
mobile counter ion is employed, the reaction is described by equation (3):



where X^- is a mobile counter ion that is incorporated into the PEDOT matrix. Similarly, the electrochemical reaction in the cathode can be described by equation (4):



where M^+ is a positively charged ion that is incorporated into the PEDOT matrix. Similarly, for mobile counter ions we have the reaction described by equation (5):



where X^- is a mobile counter ion that leaves the PEDOT matrix.

When a constant voltage input is used, the circulating currents are only maintained for a limited time [211]. This is not a surface effect, as conducting polymers do not develop relevant double layers [212], but of bulk origin. The cause is the completion of the redox reactions, which leads to the dedoping of the cathode and the loss of electrical conductivity in the system. This can have important implications for the use of the two-electrode architecture within biological systems.

1.6.7.2 The single electrode architecture

While the two-electrode architecture generates the two extreme electrochemical states for a given input, the single electrode architecture provides a continuous range of electrochemical states. As shown in figure 12, the voltage now drops across two opposite points of the same conducting polymer electrode [210, 213–216]. We can therefore consider each point of this surface as an electrode set at a certain potential, so that a specific equilibrium is reached by the redox reactions of section 1.6.7.1 at that location. The result is a redox level that follows the spatial distribution of the applied voltage difference, leading to a continuous electrochemical gradient on the electrode.

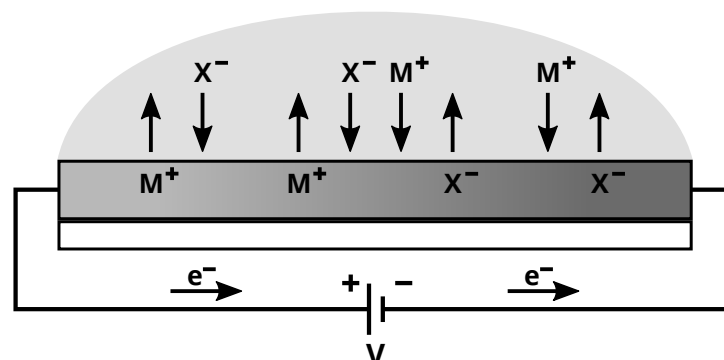


Figure 12. Front view of a single electrode architecture. Cations are indicated with M^+ and anions with X^- .

1.6.7.3 The organic electrochemical transistor

Transistors are electronic devices where the current flowing through two terminals is controlled by an input signal in a third terminal. This allows operations such as amplification and signal modulation, making transistors one of the fundamental building blocks of both analog and digital electronics. By using the two-electrode and single electrode architectures, an organic electrochemical transistor (OECT) can be generated, so the properties of inorganic transistors and conducting polymers are combined [210,217–219]. In the OECT configuration shown in figure 13, the transistor channel, located between the source and the drain, uses a single electrode architecture, while gate and channel form a two-electrode architecture. By altering the electrochemical state of the gate, the redox state of the channel can be modified, leading to subsequent changes in the source-drain current. This has important implications in the development of sensors [220], with redox processes occurring in the gate being detected by changes in the transistor channel current. Moreover, this also provides a versatile method to establish configurable electrochemical gradients in the transistor channel [217], using the source-drain voltage to control the steepness of the gradient and the gate voltage to control the mean redox level of the channel. This makes OECTs an interesting platform to study how biological systems are affected by the redox state of the solid substrate [219]. Conducting polymer-based organic bioelectronics is expanding at a rapid rate, leading to sophisticated devices that interact with biological systems in unprecedented ways [221].

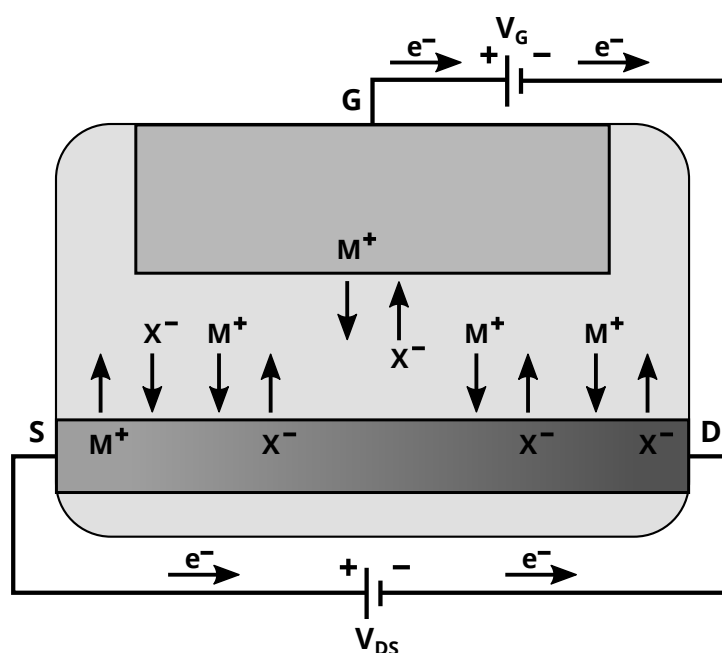


Figure 13. Top view of an organic electrochemical transistor architecture. Cations are indicated with M^+ and anions with X^- . V_G corresponds to the gate-drain voltage difference and V_{DS} corresponds to the source-drain voltage difference.

2 AIMS

Several studies have highlighted the suitability of electrically conducting polymers to interact with eukaryotic cells and tissues. However, little is known about the response of these materials when interfacing a bacterial system. This thesis aims at elucidate how conducting polymer and bacteria can influence each other, and how these interactions can led to clinical and industrial applications. Specifically, the aims of this thesis are:

- To study available conducting polymer fabrication techniques and the properties of the resultant films in the context of bacterial attachment and biofilm formation.
- To evaluate the influence of the doping agent and the electrochemical state of conducting polymers in bacterial attachment and biofilm formation.
- To evaluate the influence of bacterial attachment and biofilm formation in the properties of conducting polymer films, studying whether this can be used to implement conducting polymer-based bacterial sensors.
- To investigate the chemical functionalization of conducting polymers with biocide agents and to evaluate the performance of the resulting materials.
- To evaluate whether an external electrical input can be used to further improve the biocidal character of conducting polymers functionalized with biocidal agents.

3 RESULTS AND DISCUSSION

3.1 PAPER I. ELECTRICALLY CONDUCTING POLYMERS MODULATE BIOFILM FORMATION

Electrically conducting polymers constitute an interesting technology for the development of surfaces to modulate bacterial attachment and biofilm formation. First, their electrical conductivity adds a new dimension respect to non-conducting “passive” materials, allowing on-demand modifications of the material with an electrical input. Second, their potential for chemical tailoring, via selected counter ions or through chemical bonding, allows the incorporation of a wide palette of biologically active compounds to further affect bacteria. Third, their compatibility with mass-production techniques such as dip coating and roll-to-roll printing allows the development of commercially viable medical devices.

PEDOT was employed in the study due to its high electrical conductivity and its chemical stability. Three counter ions were tested: heparin, DBS and the chloride ion. Heparin is a highly negatively charged and hydrophilic molecule typically used to obtain hydrophilic catheter coatings with reduced protein fouling. DBS is an amphiphilic molecule typically used as detergent. The chloride ion was used as control, as its small size and lack of biological activity at the incorporated concentrations are not expected to severely alter the properties of standalone PEDOT. The PEDOT:Hep, PEDOT:DBS and PEDOT:Cl composites were fabricated with the electrochemical polymerization method, which allowed us to finely control the amount of formed polymer and to minimize chemical residues. As working electrode, Orgacon was used.

First, we investigated the influence of the polymerization time and current in the synthesized surfaces. Different values of opacity, electrical conductivity, charge storage capacity and hydrophobicity were observed in each type of composite as the charge employed in the electropolymerization procedure varied. Marked differences across composites were found at high polymerization charges, particularly in surface hydrophobicity. PEDOT:Heparin showed lower hydrophobicity than the PEDOT:Cl controls, indicating the preservation of the original hydrophilicity of the heparin molecules. Conversely, a largely increased hydrophobicity was obtained in PEDOT:DBS, likely due to the long hydrophobic tails of DBS. Taken together, this revealed how the properties of conducting polymer surfaces can be altered using different polymerization parameters and counter ions.

Next, we investigated the use of the PEDOT composites to modulate *S. Typhimurium* biofilm growth. We focused our attention on the effects of the electrochemical state of the composites and on whether these effects would be affected by the employed counter ion. To prevent other surface properties from interfering, we employed fabrication parameters that minimized differences across composites in the studied surface properties. Custom-made biofilm

culturing devices were created. Two surfaces of the same composite were glued to culturing wells, creating two-electrode electrochemical cells. Using an external constant voltage of 0.5 V, oxidized and a reduced surfaces were generated within the same bacterial culture, which minimized phenotypic differences in bacteria colonizing each electrode. Besides, electrical unswitched surfaces, with no applied voltage input, and polyester surfaces were used in additional wells.

A study performed using crystal violet revealed large biofilm growth in the oxidized surfaces, similar to the case of polyester. Conversely, diminished biofilm formation was found in reduced and unswitched surfaces. No significant differences were found among the three composites, indicating the lack of effect of the counter ions for the employed fabrication and experimental conditions. Interestingly, no significant differences between anode, cathode and unswitched surfaces were found in experiments performed on indium tin oxide (ITO), an electrically conductive metal oxide with no major electrochemical activity in the potential window between -0.5 V and 0.5 V. This discarded galvanotaxis towards a particular direction of the generated electrical field [222] as cause of the observed behavior. Electrostatic interactions from accumulated charges due to the external voltage input were also discarded as relevant factors, as charge compensation in electrically biased conducting polymers renders them electrically neutral and with a greatly diminished double layer [212]. Taken together, these results highlight the role of bacterial physiology rather than physicochemical surface properties in the observed modulation of biofilm growth.

We also investigated whether bacteria affected the polymer composites. Experiments with unswitched PEDOT:Cl revealed a dark purple band along the air-liquid interface in surfaces exposed to bacterial cultures, while no changes were observed for surfaces exposed to plain, non-inoculated culture medium. This denoted a bacteria-driven reduction of the conducting polymer at the location of the biofilm, indicating the role of PEDOT as redox mediator in the transport of electrons generated during bacterial colonization.

The obtained results allowed us to propose a model for the interaction between *S. Typhimurium* and the conducting polymer composites. The externally oxidized surface acted as a renewable electron sink, with the received electrons being transferred to the reduced composite by the electromotive force of the external power source. This makes the oxidized surface an optimal electron acceptor, favoring bacterial respiration and metabolism and constituting an advantageous environment for bacterial growth and biofilm formation. Conversely, the externally reduced surface is saturated with electrons, which makes it a poor electron acceptor. This hinders bacterial respiration and metabolism, making the reduced surface a comparatively adverse environment for bacterial growth and biofilm formation. Meanwhile, the unswitched surface is in a semi-oxidized state, permitting the transfer of a certain, but limited, amount of electrons. These available electronic states are,

however, quickly filled in the early phase of bacterial colonization due to the lack of an external electromotive force that removes the transferred electrons. This results in bacteria encountering an electron-saturated surface during most of the colonization process, leading to results similar to the case of an externally reduced surface.

In summary, our data indicate that conducting polymers can modulate biofilm growth via the control of available electronic states, a mechanism not previously described. This can have applications in the development of antibacterial surfaces for medical devices and in the food industry. Besides, the change in the chromic response as a result of bacterial electron transfer can be used as visual indicators of bacterial contamination. Interestingly, our conducting polymer system present similarities with recent *in vivo* studies where *S. Typhimurium* was found to induce gut inflammation for the generation of electron acceptors with which outcompete the local microbiota [223, 224]. This points to the utility of our developed system to generate biomimetic devices for the study of host-pathogen interactions occurring during bacterial infections.

3.2 PAPER II. IMAGE PROCESSING ALGORITHM TO DISCOVER CONSISTENT PHENOTYPIC PATTERNS IN THE BIOFILM ARCHITECTURE

Biofilms are not homogenous bacterial ensembles, but heterogeneous communities with carefully positioned parts and specialized local microniches. This highlights the importance of the biofilm architecture beyond the mere amount of biofilm mass. To investigate whether the different electrochemical states of PEDOT and the employed counter ion affected the biofilm architecture, a fluorescence confocal microscopy study was performed. Custom-made biofilm culturing devices similar to those in in paper I were employed, using PEDOT:Cl, PEDOT:Hep and PEDOT:DBS as conducting polymer composites. After cultivation, *S. Typhimurium* biofilms were stained with the LIVE/DEAD BacLight Bacterial Viability Kit. A series of confocal microscopy stacks were recorded along biofilms at the air-liquid interface for the three types of composites in the oxidized, reduced and unswitched state. The three-dimensional representation of the confocal stacks, obtained with ImageJ [225–228], revealed thick, extensive biofilms for the oxidized states of PEDOT:Cl and PEDOT:Hep, while in the other cases biofilms appeared generally thinner and more fragmented. Interestingly, oxidized PEDOT:DBS presented a very different architecture, with biofilms largely composed of scattered cell clumps possibly trapped in the extracellular matrix.

Although the study of individual confocal microscopy 3D images provided a general idea of the biofilm architecture in the different composites and electrochemical states, the variability encountered on the images prevented us from precisely determining the architectural features for each condition. To obtain an objective characterization of the formed biofilms, a

custom-made image processing tool was developed. In the first part, implemented as a macro for imageJ [225–228], the green channel, corresponding to the live bacterial population, and the red channel, corresponding to the dead bacterial population, were separated. The images were then binarized, followed by the calculation of the amount of foreground pixels for every recorded focal plane in each channel. This information was stored in a data file.

In the second part, implemented as a script for the R language, the stored file was processed. For each biofilm sample, the script generated a two-dimensional representation where the calculated pixels of each channel were plotted against the number of focal plane. This provided the distribution of the live bacterial mass and of the dead bacterial mass respect to the biofilm height. In analogy with the analysis of strata in geological formations, this plot was termed the biofilm stratogram. The use of the stratogram allowed us to define a series of parameters to objectively characterize the biofilm architecture. First, the bacterial mass of each population was defined as the area under the curve. This allowed us to easily calculate the rate of live and dead bacterial mass and the total cell mass. In the stratogram, the value of the abscissa in each vertical coordinate corresponds to the bacterial mass at the corresponding focal plane. We therefore defined the coverage as the sum of the maximum value of the abscissa for each bacterial population, which was used to characterize the horizontal biofilm growth. The biofilm thickness, defined as the number of focal planes, was used to characterize the vertical biofilm growth. Finally, the relationship between the horizontal and the vertical dimensions of the biofilm was characterized with the biofilm density, calculated as the ratio between cell mass and thickness. Moreover, the 2D nature of the stratogram also allowed us to group individual plots and calculate average stratograms, which made our strategy different from other previous solutions [229–231]. This allowed us to investigate consistent patterns in the biofilm architecture from the average shape of the curves rather than relying on individual samples deemed representative.

The average stratograms were then calculated for each composite type and electrochemical state. In addition, the average architectural parameters were calculated from the individual stratograms. The oxidized surface showed the largest cell mass for each composite, while the reduced state presented the minimum value. The unswitched case had an intermediate value for the three composites. This agrees with the model presented in paper I, where the unswitched composite, in a semi-oxidized state and with no external electromotive force to remove the transferred electrons, can accept more electrons than externally reduced polymers but less than the externally oxidized case. Oxidized PEDOT:Cl and oxidized PEDOT:Hep presented large coverage, thickness and density, while oxidized PEDOT:DBS presented an altered morphology characterized by a large thickness but a low coverage and density. This is explained by the long upper tail present in the stratogram, which corresponds to the cell clumps observed in the 3D images. Low coverage, thickness and density were found in the reduced and unswitched cases. Additionally, all the surfaces presented live/dead ratios of

approximately 70%, and no larger concentration of dead bacteria was found in the parts of the biofilm closer to the surface. Similar features were obtained in common polyester surfaces, which indicate a lack of bactericidal effect from the conducting polymer composites or the electrical signal applied.

Taken together, our results provided a deeper understanding of the modulation of biofilm growth by conducting polymer composites, expanding the results of paper I. Furthermore, they showed how the use of biofilm stratograms can benefit the analysis of the biofilm architecture through the discovery and quantification of patterns of structural features.

3.3 PAPER III. BIOFILM GRADIENTS ALONG THE CHANNEL OF AN ORGANIC ELECTROCHEMICAL TRANSISTOR

Here, we investigated whether our studies in paper I and paper II could be expanded with more sophisticated conducting polymer-based architectures in order to achieve a more precise control of biofilm growth. One interesting device is the organic electrochemical transistor (OECT), which, as described previously, can produce externally controllable electrochemical gradients with a relatively simple architecture. PEDOT was selected as conducting polymer, while chlorine was used as counter ion to minimize the influence of factors different from the electrochemical state. First, PEDOT:Cl surfaces were created by electroporation on top of Orgacon working electrodes. To study the modulation of *S. Typhimurium* biofilm growth transistors had to be designed with the channel located along the air-liquid interface. We therefore employed OECTs in a vertical configuration instead of in the more classical horizontal arrangement [219]. Transistors were manually patterned and glued to wells of 12-well plates. The gate contact was accessed through a hole drilled at the bottom of the well, while the source and drain were accessed from the top of the well.

We then characterized how the voltage inputs affected the electrochemical state of the transistor channel by analyzing the electrochromic response. In OECTs addressed with $V_G = 0\text{ V}$, $V_{DS} = 2\text{ V}$, the channel acquired a light brown color around the source area. This response gradually shifted in the central part of the channel and acquired a light blue color in the drain area. This indicated the formation of an electrochemical gradient in the transistor channel, transitioning from an oxidized state in the source area to a semi-oxidized in the drain area. Next, we studied the effect of the gate voltage by employing $V_G = 0.5\text{ V}$, $V_{DS} = 2\text{ V}$. The source area presented a light brown color as in the previous case. Conversely, the drain area acquired a dark purple color, denoting the electrochemical reduction of the polymer to its neutral state. This illustrated how different electrochemical gradients can be obtained in the channel of the OECT with different input signals.

To analyze whether different electrochemical gradients would be translated into different patterns of biofilm formation, OECTs were inoculated with *S. Typhimurium* cultures and

electrically biased. After the bacterial cultivation, the transistor channels were stained with the LIVE/DEAD BacLight Bacterial Viability Kit and analyzed with fluorescence confocal microscopy. To objectively characterize the biofilm architecture, the acquired images were processed with the biofilm stratogram tool developed in paper II. When OECTs were addressed with $V_G = 0\text{ V}$, $V_{DS} = 2\text{ V}$, large biofilms were obtained in the source area. Biofilm growth decreased along the channel, with biofilms on the drain area showing reduced cell mass and thickness. Interestingly, the proportion of dead cell mass increased in the direction of the drain area. Large biofilms were again found in the source area when the input $V_G = 0.5\text{ V}$, $V_{DS} = 2\text{ V}$ was used. However, a much more marked decrease in biofilm growth was found in this case, with large decreases in cell mass, coverage, thickness and density in the direction of the drain. Besides, an increase in the proportion of dead cell mass was again found in the direction of the drain. These results are in agreement with the previous electrochemical characterization, where a larger electrochemical reduction was obtained around the drain area when the gate voltage was increased.

Taken together, the obtained results further confirm the conclusions of paper I and paper II and illustrate how conducting polymer devices can be used to achieve a precise control of biofilm formation. These types of devices can be useful in applications such as the biocatalyzed generation of chemicals, tuning biofilm growth to achieve the desired synthesis rate in each compound. Besides, the versatility of these devices in patterning biofilm growth could be used, as mentioned in paper I, to create biomimetic systems to explore host-pathogen interactions during bacterial infections.

3.4 PAPER IV. ELECTROENHANCED ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES

Here, we propose the use of electrically conducting polymers to produce commercially viable devices where electrical signals and surfaces functionalized with bactericidal compounds can be used to prevent biofilm colonization. To synthesize our functional electrically conductive material, the commercially available monomer EDOT-MeOH was employed. This monomer is typically used in the synthesis of complex functionalizable constructs, as discussed in section 1.6.6. We, however, focused our study in its use as an actual functionalizable material via its hydroxyl moiety. As biocidal agent, AgNPs were selected. This allowed us to build our solution upon a commercially viable technology, so the transition into an actual medical product is facilitated.

First, EDOT-MeOH was electropolymerized on Orgacon working electrodes using PSS as counter ion. To couple the AgNPs, the PEDOT-MeOH:PSS surfaces were first amino-functionalized with the (3-aminopropyl)triethoxysilane (APTES) silane linker. This strategy is typically employed to functionalize glass and metal oxides, where hydroxyl groups

are usually generated via oxygen plasma or chemical pretreatment. No pretreatment was needed, however, for PEDOT-MeOH:PSS due to the intrinsic presence of hydroxyl moieties. Finally, the AgNP functionalization was achieved incubating the amino-functionalized PEDOT-MeOH:PSS surfaces in a citrate dispersion of 50 nm diameter silver nanospheres. This resulted in the formation of coordinate bonds between the AgNPs and the amine groups from APTES, generating the PEDOT-MeOH:PSS-AgNP composite (referred to as “AgNP composite”).

The produced surfaces were then inspected using scanning electron microscopy (SEM), which revealed the presence of AgNPs. This was further confirmed with absorbance measurements, which showed similar surface plasmon resonance (SPR) responses in the AgNP composite and in the original AgNPs suspension. Moreover, spatial absorbance scans revealed the presence of AgNPs across the whole polymer surface, indicating that the employed chemical strategy succeeded in generating a macroscopic AgNPs coverage. The SEM and absorbance characterizations were also performed on surfaces produced similarly to the AgNP composite but lacking the APTES linker. Responses similar to the PEDOT-MeOH:PSS plain conducting polymer were obtained in this case, which allowed us to discard the role of the physical entrapment and physisorption of AgNPs in the AgNP functionalization.

To characterize any effects that electrically addressed AgNP composites might exert on bacteria, we next investigated the electrochemical response of our produced material. A cyclic voltammetry study was performed, revealing the presence of a voltage-triggered release system, with surface-bound AgNPs being converted into released silver ions when a voltage input above a certain threshold was applied.

To test the bactericidal activity of our prepared materials, *S. aureus*, commonly involved in device-associated infections, was selected as bacterial model. Custom-made biofilm culturing devices were prepared by gluing two parallel strips of the material under study to a glass square, forming the bottom of the culturing recipient. Then, a glass ring was glued on top. The strips were enclosed only partially by the ring, so an external electrical addressing could be applied when appropriate. With this design, the surfaces under study were located at the bottom solid-liquid interface, where *S. aureus* biofilms are formed in static conditions. As electrical input, a 5 Hz, 4 V_{pp} (peak-to-peak voltage) square wave voltage between -2 V and 2 V was selected. This provided an alternating polarity that prevented the fully oxidation and reduction of any of the two electrodes, preventing the decay of the electrical current. Besides, due to its low frequency, the input remained similar to a constant signal in terms of maximum ideal energy and propagation dynamics.

We then proceeded with the evaluation of the antibacterial properties of our developed materials, using crystal violet to evaluate biofilm formation. Extensive, thick biofilms were

found on non-addressed PEDOT-MeOH:PSS, indicating that the plain conducting polymer did not possess antibacterial properties. Conversely, thin, damaged biofilms were found for the non-addressed AgNP composite as result of the bactericidal character of the AgNPs. We then assessed whether the bactericidal properties of the AgNPs could be enhanced by applying the selected square-wave voltage input during biofilm cultivation. Large, thick biofilms were found for the addressed plain polymer, indicating that the voltage input did not generate any antimicrobial effect. In contrast, only minimal traces of biofilm growth were found on the addressed AgNP composites. This reduction in biofilm growth, considerably higher than the individual effects of AgNPs and the electrical input, indicated a synergistic effect due to the electroenhancement of the bactericidal effect of AgNPs.

To clarify the mechanism behind the observed synergistic effect, we measured the amount of released silver ions in the addressed AgNP composite devices. We used sodium nitrate as supporting electrolyte, which prevented the formation of water-insoluble silver complexes and allowed us to evaluate the maximum amount of released silver ions. Interestingly, the concentrations measured were considerably lower than the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for the same bacterial strain in similar conditions. This suggests an electrically-triggered increase of bacterial sensitivity towards silver, together with the electrically-triggered release of silver ions, as the origin of the observed bactericidal effect.

We then analyzed how bacteria affected our prepared surfaces. Similarly to paper I, *S. aureus* originated a change in color in the PEDOT-MeOH:PSS plain conducting polymer, which turned dark purple due to its electrochemical reduction. This bacteria-triggered electrochemical reduction also affected the electrical response of the custom devices, as shown in real-time measurements of the circulating electrical current in custom devices addressed with the designed square-wave voltage input. In addressed plain conducting polymer devices, where large biofilm growth occurred, a large decrease in current was observed during the experiment. Conversely, no decrease in current was observed in addressed AgNP composite devices, where bacterial colonization was severely limited. Taken together, this indicates the utility of our system also as real-time bacterial sensor, detecting increases in electrical resistance due to bacteria-driven polymer electrochemical reduction.

By combining the on-demand, electroenhanced bactericidal action and the real-time bacterial detection features of our system, smart, responsive antibacterial coatings could be generated. This would lead to devices that exert their bactericidal action only when needed, contributing to prevent bacterial resistance, as well as to the incorporation of advanced features such as remote patient monitoring.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Bacterial biofilms are ubiquitously present in many aspects of the human life. With a leading role in aspects as diverse as device-associated infections, food contamination, wastewater treatment and the generation of energy, biofilms are both a threat and an opportunity. Recent studies have shown the high complexity inherent to biofilms, where interrelated local bacterial niches lead to carefully crafted electrochemical configurations. This suggests the control of the biofilm electrochemical milieu as an effective method to influence biofilm growth.

With properties of metals and semiconductors and a rich chemistry provided by their organic nature, conducting polymers represent an interesting technology to develop novel electrochemically active devices to interact with bacterial biofilms. This thesis reports the use of several PEDOT-based materials for the control of biofilm growth, analyzing how bacteria and the material influence each other.

Several PEDOT-based materials were used as electron acceptor by *Salmonella* and *S. aureus*. This induced a change of color in the material, which indicates its possible use in colorimetric sensors to monitor bacterial contamination. This can have large implications in areas like food packaging and sterility assurance for medical devices. Another implication is the control of biofilm growth through the number of available electron acceptors in the material, which can be achieved with simply an external voltage input. While this constitutes an interesting, novel strategy for the prevention of biofilm formation, it is also promising for applications benefitting from biofilm formation. As shown by the formation of biofilm gradients along the channel of electrochemical transistors, a sophisticated control of biofilm growth can be achieved with conducting polymer-based electrochemical devices. This represents an interesting opportunity in areas like the biofilm-catalyzed production of chemicals, where electrochemical circuits could be used to modulate biofilm growth depending on certain environmental conditions as well as on external commands.

Interesting similarities were found between *Salmonella* colonization of conducting polymers and *in vivo* studies of gut inflammation during infection. This highlights the potential of conducting polymers to be used in advanced biomimetic organ-on-a-chip devices to study host-pathogen interaction during infection, closely mimicking *in vivo* conditions while maintaining the operational advantages of an *in vitro* system.

For all these applications to be pursued, however, a more detailed study of the physiological changes of bacteria colonizing conducting polymers is needed. Of particular importance is the study of the role of chemotaxis towards particular redox states and the role of aerobic and anaerobic respiration in the use of conducting polymers as electron acceptors by bacteria.

The study of biofilms also requires of software tools to objectively analyze microscopy images

and reveal patterns in the biofilm architecture. One possibility has been presented in this thesis, based on averaged 2D curves that summarized the 3D architecture of biofilms. A large number of options are, however, still unexplored. Major benefits could be achieved by the integration of several existing visualization and data analysis techniques, combined to provide a comprehensive picture of the biofilm architecture.

Finally, we also explored the combination of conducting polymers and AgNPs to develop an efficient electroactive antibacterial coating for clinical devices. We developed a simple, novel functionalization strategy based on the use of PEDOT-MeOH:PSS and a silane linker, generating a PEDOT-MeOH:PSS-AgNP composite with an adequate nanoscale and macroscale AgNPs coverage. An almost complete prevention of biofilm colonization was achieved in AgNP composites electrically addressed with a square-wave input voltage. This result cannot be explained by the simple additive effect of AgNPs and the electrical input, indicating the presence of a synergistic effect that led to the electroenhancement of the biocidal properties of AgNPs. In addition, the bacterial reduction of the conducting polymer allowed our system to function as a real-time electrochemical bacterial sensor. This indicates a possible use as dual sensor-effector system, generating a bactericidal response when bacterial colonization is detected. Although aspects such as host toxicity, bacterial resistance and long-term behavior remain to be analyzed, our system constitute an interesting platform for the development of commercially available active antibacterial coatings for clinical devices.

5 MY SCIENTIFIC CONTRIBUTION

Electrically conducting polymers are enormously promising materials. With properties of metals, semiconductors and organic polymers, they have a big potential for innovative applications. Combining disciplines such as electrical engineering, material science, chemistry and microbiology, the interdisciplinary work presented in this thesis aims at unraveling the interactions between conducting polymers and bacteria, treating aspects from both basic research and technology development with the objective of generating potential clinical applications.

My work has contributed to rethink bacterial attachment on abiotic surfaces, shifting away from a conception where bacteria merely act as passive elements subjected to physico-chemical interactions and highlighting the active role of bacterial physiology. My work also highlights the importance of electrochemical processes in biofilm formation, and shows how the electrochemical state of the substrate can influence the biofilm physiology. This opens a new range of possibilities for applications requiring either prevention or promotion of bacterial colonization, as well as for the construction of biomimetic devices and bacterial sensors. Besides, this investigation also involved the development of several custom-made software tools to comprehensively evaluate biofilm formation. These tools can contribute to elucidate the role of the biofilm architecture in bacterial colonization.

In terms of applications, my work illustrates how electrically conducting polymers can be easily functionalized to effectively prevent bacterial colonization using bactericidal compounds and an external electrical signal. Moreover, this can be combined with the bacterial sensing properties of conducting polymers to generate dual sensor-effector systems. This indicates the suitability of the conducting polymer technology to develop commercially available, smart antibacterial coatings that prevent biofilm contamination of medical devices, therefore contributing to protect the patients' health.

Taken together, my work has opened novel ways of addressing bacterial attachment and biofilm formation from a basic research perspective, while also generating outputs that are of high applied value.

6 POPULAR SCIENCE SUMMARY

Medical devices like catheters and respiratory tubes are important tools to treat medical conditions and improve the patient's health. Their use, however, comes with the risk of bacterial contamination, as the plastic surface of these devices is ideal for bacteria to attach and proliferate. Moreover, bacteria attached to implants often form organized structures called biofilms, which serve as protection against antibiotics and other treatments. As patients needing these devices are normally in a weakened state, the risk bacterial contamination represents an important health problem. To solve this situation, several antibacterial materials have been proposed, but they are either not completely effective or their production at large scale for commercial use is not well established.

An interesting strategy is the use of electrically conducting plastics. These novel materials, with properties of both traditional plastics and electrical conductors, open a new range of possibilities. Successfully used in new revolutionary products like wearable electronics and plastic solar cells, these materials can implement complex electronic functions in flexible devices at low fabrication costs. In this thesis we explore the use of electrically conducting plastics to fabricate materials that prevent bacterial contamination.

We found that these materials can be used to directly influence the bacterial behavior. By applying an external electrical signal, the material changes and affects the bacterial physiology, promoting or preventing attachment and formation of biofilms depending on the input signal used. Furthermore, employing custom developed image processing tools we found that the applied signals also affected the structure of the biofilm. These results have important implications not only to prevent bacterial contamination, but also for situations where biofilms are beneficial, like in microbial fuel cells and bioproduction of chemicals. Interestingly, we also found that bacteria can influence several properties of the material, such as its color and the way electrical signals propagate. This can be used to develop color-based bacterial sensors as well as advanced real-time electronic detectors, with applications spanning from clinical devices to smart food packages.

To test the feasibility of these materials in the prevention of bacterial contamination of clinical devices, we then went one step further and combined the classical bactericidal technology of silver nanoparticles with the conducting polymer technology. Using a specially designed electrical input signal, the bactericidal effect of silver nanoparticles was greatly enhanced, resulting in a higher effectiveness than silver nanoparticles not electrically enhanced. Combined with the sensing properties of our materials, this technology can be used to generate smart medical devices able to detect and prevent bacterial contamination.

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