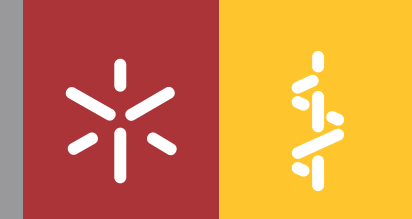


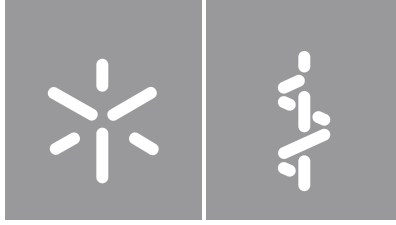


Tiago Miguel Castro Costa

Antimicrobial flexible coating material for the car sharing segment of the automotive industry

Universidade do Minho
Escola de Medicina





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Tiago Miguel Castro Costa

**Antimicrobial flexible coating
material for the car sharing segment
of the automotive industry**

Tese de Doutoramento

Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da

Doutora Alexandra Gabriel Fraga

do

Professor Doutor Nuno João Meleiro Alves Neves

e da

Doutora Helena Pereira da Costa Aguilar Ribeiro

Direitos de Autor e Condições de Utilização do Trabalho por Terceiros

Despacho RT - 31 /2019 - Anexo 3

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Statement of Integrity

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I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Revestimentos flexíveis antimicrobianos para o segmento de partilha de carros da indústria automóvel

A partilha de carros é um segmento da indústria automóvel em rápida expansão, com milhões de utilizadores em todo o mundo. Até 2027, estima-se que só na Europa existirão cerca de 14,7 milhões de utilizadores, traduzindo-se num valor de mercado de 4,56 mil milhões de dólares. Contudo, o aumento deste fenómeno leva a preocupações acrescidas ao nível da higiene e segurança, levando a indústria automóvel a procurar soluções para fazer face a esta situação. A literatura sugere que espaços confinados e multi-partilhados, incluindo superfícies de alto toque no interior do carro, representam áreas importantes para a colonização de microrganismos. Assim, o principal objetivo deste trabalho foi o desenvolvimento de materiais de revestimento flexíveis antimicrobianos para a redução da exposição humana a agentes potencialmente patogénicos.

Este trabalho teve como foco o desenvolvimento de materiais de revestimento flexíveis com atividade antimicrobiana através da modificação das propriedades físicas (topografia da superfície) ou químicas (incorporação de agentes antimicrobianos). Os filmes foram produzidos a partir de formulações de poliuretano com diferentes agentes antimicrobianos (compostos de base fenólica, iônica ou metálica) ou estabilizadores de luz ultravioleta - Aminas Estericamente Impedidas Estabilizadoras à Luz (HALS) vs não-HALS. Os filmes produzidos foram caracterizados ótica, química e fisicamente, enquanto as propriedades antimicrobianas foram testadas contra bactérias, fungos e vírus. As modificações na topografia da superfície, independentemente do tamanho do padrão, não influenciaram na capacidade antimicrobiana do revestimento. No entanto, a incorporação de agentes antimicrobianos teve impacto no crescimento microbiano. Os aditivos com maior potencial antimicrobiano foram os compostos à base de cobre, os compostos iónicos, assim como o HALS Tinuvin 770 DF, que reduziram a carga microbiana em 99%. Em relação ao mecanismo de ação do HALS, este pode ser atribuído à liberação de nitritos por auto-oxidação do Tinivum 770 DF. Importante referir que, a incorporação desses compostos antimicrobianos não alterou significativamente as propriedades originais dos filmes e não foi observada citotoxicidade decorrente da utilização destes compostos.

Esses resultados destacam o potencial uso de materiais antimicrobianos na indústria automóvel para controlar a carga microbiana e reduzir a transmissão de agentes patogénicos entre os utilizadores.

Palavras-chave: estabilizadores de luz ultravioleta; filmes antimicrobianos, partilha de carros, poliuretano e Tinuvin 770 DF.

Abstract

Antimicrobial flexible coating material for the car sharing segment of the automotive industry

Car sharing is a rapidly expanding segment of the automotive industry, with millions of users worldwide. By 2027 it is estimated that Europe alone will have around 14.7 million users in Europe, translating into a market value of \$4.56 billion. However, the increase of car sharing has also brought about concerns in hygiene and safety, prompting the automotive industry to search for solutions to address this emerging trend. Research suggests that confined and multi-shared spaces, including high-touch surfaces in car interiors, represent important areas for the colonization of microorganisms. Considering this, the main aim of this work was the development of antimicrobial flexible coating materials for automotive industry thereby reducing human exposure to potential harmful pathogens.

This work focused on developing flexible coating materials with antimicrobial capacity through the modification of physical (surface topography) or chemical properties (incorporation of antimicrobial agents). Films were produced from polyurethane formulations incorporating different antimicrobial agents (phenolic-, ionic- or metal-based compounds) or UV light stabilizers - pure Hindered Amine Light Stabilizers (HALS) vs non-HALS. The produced films underwent optical, chemical and physical characterization, while their antimicrobial properties were tested against bacteria, fungi and virus. Surface topography modifications, regardless the pattern size, did not influence the antimicrobial capacity of the coating. However, the incorporation of antimicrobial agents did impact microbial growth. The additives with the highest antimicrobial potential were copper- and ionic-based compounds, along with HALS Tinuvin 770 DF, which reduced microbial burden by 99%. Regarding the mechanism of action of HALS, this could be attributed to the release of nitrites through autoxidation of Tinuvin 770 DF. Notably, the incorporation of these antimicrobial compounds did not significantly alter the film's original properties and were not cytotoxic.

These results highlight the potential use of antimicrobial materials in the automotive industry to control microbial burden and avoid the transmission of pathogens among car users.

Keywords: antimicrobial lacquer-films, car sharing segment, polyurethane, Tinuvin 770 DF, and UV-light stabilizers.

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List of Abbreviations/Nomenclature/Acronyms

μg	Microgram or micrograms
μL	Microlitre or microlitres
μM	Micromolar
μm	Micrometre or micrometres
AA	Acrylic acid
ABS	Acrylonitrile butadiene styrene
Abs	Absorbance
ACEA	European Automobile Manufacturers' Association
Ag	Silver
AMP	Antimicrobial peptides
Ar	Argon
ATP	Adenosine triphosphate
ATT	Accelerated aging tests
B2B	Business-to-Business
B2C	Business-to-Consumer
BAC	Benzalkonium Chloride
BMW	Bayerische Motoren Werke AG (Bavarian Motor Works)
BPMPC	2-methacryloyloxyethyl phosphorylcholine-co-butyl methacrylate co-benzophenone
C	Carbon
CA	Contact angle
Ca	Calcium
CFU	Colony-forming units
CHX	Chlorhexidine
CI-BTMP	Bis(N-chloro-2,2,6,6-tetramethyl-4-piperidiny) sebacate
Cm	Centimetre or centimetres
cm^{-1}	Wavenumber
cm^2	Square centimetre
Co	Cobalt
CO_2	Carbon dioxide
Cq	Threshold /quantification cycle
Cu	Copper
CuSP	Copper (II) sulphate pentahydrate
DAF-FM	4-amino-5-methylamino-2',7'-difluorofluorescein diacetate
DDS	Drug delivery systems
DMIC	1,3-didecyl-2-methylimidazolium chloride
DNA	Deoxyribonucleic acid
D-Tyr	D-Tyrosine
EPS	Extra-cellular polymeric substances

<i>et al.</i>	“and others”
FA	Ferulic acid
FTIR	Fourier transform infrared spectroscopy
GADSL	Global Automotive Declarable Substance List
GMI	Global Market Insights
GPS	Global Positioning System
h	Hour or hours
H ₂ O ₂	Hydrogen peroxide
HALS	Hindered amine light stabilizers
HMIMI	1-hexyl-3-methylimidazolium iodide
IC ₅₀	Half-maximal inhibitory concentration
ICVS	Life and Health Sciences Research Institute
ILs	Ionic liquids
ISO	International Organization for Standardization
LDPE	Low-density polyethylene
Log	Common logarithm or logarithm with base 10
MFI	Mean fluorescence intensity
MIC	Minimum inhibitory concentration
min	Minute or minutes
mL	Millilitre or millilitres
mm	Millimetre or milimetres
mN	Millinewton
Mo	Molybdenum
MRSA	Methicillin-resistant <i>S. aureus</i>
MSSA	Methicillin-susceptible <i>S. aureus</i>
N	Nitrogen
nAg	Nano-silver
NCO	Isocyanate
nHAp	Nano-hydroxyapatite
Ni	Nickel
nm	Nanometer
nM	Nanomolar
NO	Nitric Oxide
NOR	Non-radical species/products
O ²⁻	Superoxide
OEM	Original Equipment Manufacturer
OH	Hydroxyl group
OPP	[1,1'-Biphenyl]-2-ol, 2-phenylphenol or o-phenylphenol
p	p-value or probability value
P2P	Peer-to-peer

PA	Polyamide
Pb	Lead
PBAT	Poly(butylene adipate-co-terephthalate)
PBS	Phosphate-buffered saline
PC	Phenolic compounds
PCL	Polycaprolactone
PDA	Polydopamine
PDDA	Poly(Diallyldimethylammonium chloride)
PDMS	Poly(dimethyl siloxane)
PE	2-phenoxyethanol
PEG	Poly(ethylene glycol)
PET	Polyethylene terephthalate
PLA	Poly(lactic acid)
PMMA	Poly (methyl methacrylate)
PMOXA	Poly(2-methyl-2-oxazoline)
POM	Polyoxometalate
PP	Polypropylene
PS	Polystyrene
PUR	Polyurethane
PVC	Polyvinyl chloride
QAC	Quaternary ammonium compounds
R	Antibacterial and antifungal activity
R ₂ NH	Secondary amine
R ₃ N	Tertiary amine
R ₄ N ⁺	Quaternary amines
R _a	Arithmetic average of height of peaks
RNA	Ribonucleic acid
RNH ₂	Primary amine
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
R _q	Standard deviation of height of peaks
RSNOs	S-nitrosothiols
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
R _z	Average of the five highest peaks and the five lowest valleys across a surface profile
Sn	Tin
SNAP	S-nitroso-N-acetylpenicillamine
SSAmC16	Cetyltrimethylammonium 4-styrenesulfonate
TA	Tannic acid
TCH	Tetracycline
Ti	Titanium

TMG Automotive	Têxtil Manuel Gonçalves - Tecidos Plastificados e Outros Revestimentos Para a Indústria Automóvel, S.A.
UV	Ultraviolet
WW	World War
X	Anionic group
Zn	Zinc
Zr	Zirconium

List of Symbols/Special Characters

%	Percentage
<	“Less than”
>	“Bigger than”
±	Plus-minus
≤	“Less than or equal to”
≥	“Bigger than or equal to”
°	Degree

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General Outline and Thesis Organization

The work entitled “Antimicrobial flexible coating material for the car sharing segment of the automotive industry” was developed in the scope of the PhD program in Health Sciences and is a result of a collaboration between the University of Minho (Life and Health Sciences Research Institute (ICVS), School of Medicine and 3B’s Research Group on Biomaterials, Biodegradables and Biomimetics) and Têxtil Manuel Gonçalves - Tecidos Plastificados e Outros Revestimentos Para a Indústria Automóvel, S.A. (TMG Automotive).

This work is organized in 3 chapters. **Chapter 1** is divided in four main subchapters. The first part begins with a brief historical introduction to the automobile industry, highlighting the most important developments in this sector, followed by statistics about automobile use in Portugal and Europe. After that, some health risks for occupants regarding the use of automobiles are addressed and the main strategies used to overcome these issues are presented and discussed. In the second part, it is highlighted the role of inanimate surfaces in the colonization and dissemination of potentially harmful pathogens among humans. This chapter also discusses the strategies used for the development of antimicrobial surfaces and the main antimicrobial agents immobilized on polymeric surfaces, including practical examples reported in the literature. In the third part some commercial antimicrobial solutions for automotive industry are presented. The chapter ends (fourth part) with a description of the aims of this work and of the main projects conducted herein.

In **Chapter 2**, it is described the experimental work develop in the context of this thesis. The first experimental work is entitled “Influence of surface properties on the antimicrobial effect of polymer coated surfaces”. In this work, commercial coated surfaces were embossed with different patterns (imparting smooth and rough textures) to induce different surface properties. The aim of the study was to analyse the effect imparted by surface topography on bacterial proliferation and subsequently assess a possible antibacterial effect. The second experimental work consists in a manuscript entitled “Development of polyurethane antimicrobial coatings by composition with phenolic-, ionic- and copper-based agents”. In this chapter, it is reported the modification of antimicrobial lacquer-films with different antimicrobial agents (phenolic-, ionic- and copper-based compounds) as well as the main results obtained. These results focus (i) on the evaluation of physical/chemical properties and the impact of the incorporation of antimicrobial compounds in lacquer-films and (ii) the biological activity of the produced lacquer-films (antibacterial, antifungal, and antiviral activity as well as its cytocompatibility). The third work is entitled “Antimicrobial properties of hindered amine light stabilizers in polymer coating materials and their mechanism of

action". In this chapter, it is reported the development of antimicrobial lacquer-films incorporated with two commercial UV-stabilizers and the study of the molecular mechanisms underlying the antimicrobial properties of these additives. The biological properties of films were further characterized regarding their antibacterial and antifungal activity and a possible antimicrobial mechanism of action of a specific HALS (Tinuvin 770 DF) was proposed.

Finally, in **Chapter 3**, it will be provided a summary of the main results obtained in this thesis, including in this analysis the main limitations of the results obtained and the future perspectives of development of the lacquers used in automotive surfaces that may be relevant for car sharing.

Chapter 1

General Introduction and Definition of the Problem-Situation

1.1 Car sharing segment in the automotive industry: contextualization and definition of problem-situation

Motorized vehicles, and in particular automobiles, are a crucial element in modern society responsible for the immense flux of goods/products and people across the globe. Automobiles were initially idealized in the XVIII century, and since then they have been continuously improved with new functionalities to provide not only a better user experience, but also improved safety. Nowadays, automobiles are accessible to almost all people, with more than 286 million motor vehicles in circulation in the European Union alone. Considering the increase in mobility, the technological advances, and the environmental concerns, new trends have been emerging in the automobile industry. Car sharing is one of these new trends and can be defined as a service that facilitates car rental for short periods of time. Given the facilities of use, this service is attracting new users every day. The market projections indicate a continued growth worldwide, representing an important marketing opportunity. However, (i) the high contact-ratio among people in confined spaces, aligned with an (ii) increased awareness of hygienic and public-health standards in modern societies and (iii) the identification of automobiles as a potential source for microbial colonization, led the automobile industry to search for viable solutions to mitigate this problem.

In this chapter, a brief description of the automobile history will be made including some statistical data regarding automobile users and vehicles in circulation in Portugal and in Europe. After that, the car sharing market will be contextualized, indicating the main vehicle models in use as well as a forecast for market segment evolution. Finally, emphasis will be given on the automobile as a potential fomite and consequently, a source of health issues for automobile users. The aim of this chapter is to give context to the subject that will be researched in this thesis and to define the aims of this work.

1.1.1 A brief description of automobile history

One of the first prototypes of a motorized vehicle dates from 1769 in France, by Nicolas Cugnot. Cugnot developed a tractor operated by a steam engine. The system was fed by a boiler with hot water providing steam to induce the movement of pistons operating the wheels. Despite this landmark being considered a success, the loss of financial support and an automobile accident in 1771, led to the premature end of this project [1]. Following this first design, many inventors were fully invested in developing and improving steam vehicles, particularly automobiles and

carriages for trains. It was only in 1860 that Etienne Lenoir developed an internal combustion engine with gas for fuel and an electric spark for ignition. This invention was considered one of the first commercial gas engines [1]. In the following years, in Germany, Otto and Langen improved this gas engine by developing the four-stroke cycle, which is still used today. During this same period, Daimler used this four-stroke cycle in a gas vapor engine, which he manufactured in large quantities [1]. In 1885, Karl Benz built the Benz Patent Motorwagen, using a system based on gas-powered internal combustion. It featured wire wheels (unlike the wooden ones on carriages) and a four-stroke engine of Benz's own design [2]. In 1893, in the United States, the Duryea brothers produced a gasoline driven "horseless-carriage" system. This system worked with one-cylinder, a gasoline engine and a three-speed transmission, reaching a maximum speed of 8 miles per hour [1].

In the beginning of twentieth century, the automobile was considered a useful device, although uncommon and accessible only to rich people [1,2]. To change this paradigm, Henry Ford, established the Ford Motor Company in the United States in 1903. Ford established the use of assembly lines to manufacture automobiles in a large number, at an affordable cost. This strategy allowed the automobile system to develop a basic, dependable, and reasonably priced car, with an innovative design. As a result, in October of 1908, Ford launched the Model T [2].

The use of motorized vehicles was potentiated during the World War (WW) I, where vehicles were used for transporting and supplying armies. In this period, a huge number of small manufacturers appeared in an effort to supply the troops. In WW II, the advancement of technology and resources, allowed for the sharp increase of automobile use for military effort. In particular, the British government created automotive industries for the sole purpose of military manufacturing, especially aircraft [1]. Around the same period, automotive designers closed the body of the car to create aerodynamic outline, changed the car interior, and improved the glass system to accentuate the privacy for the consumer [2]. At the safety level, the illumination system and fenders reinforcing the safety and strength of the frame were introduced. The tires were wider and thicker adding to the solidity of the vehicles [2]. Some landmarks in automobile industry are shown in Table 1.

In the 1970s, new concepts were introduced, such as brake light signals, facilitating the communication of information between drivers, as well as different headlight positions to increase night visibility [3]. In the next two decades, the advent of the computer and the beginning of the digital era allowed new developments in the automobile market. For example, in 1981, Honda

developed the first vehicle navigation system called Gyrolocator and Toyota released the NAVIVOM, a navigation system that indicated the destination with an arrow. The navigation systems rapidly spread after the global positioning system (GPS) became available in 1990. The first navigation system with GPS using a colour display was launched in Europe in 1994 by Bayerische Motoren Werke AG (BMW). Later versions of the navigation system included: audio and communication functions and touch screen devices [3].

Table 1 – Some important inventions in the automobile development. Based on Akamatsu, M. *et al.* 2013 [3].

Year	Invention	Observations
1894	Foot pedal and a steering wheel	
1900	Water-pressure gauges	
1900	Speedometer	Introduced to highlight the high-speed capability of the vehicle. In 1901, the state of Connecticut imposed a speed limit of 8 and 12 mph* within and outside the city, respectively.
1910s	Thermometer	Allowing the driver to read the temperature of the radiator in his seat.
1910	Tachometers and clocks	
1920s	Horn buttons	
Final of 1920s	Seat-adjusting mechanism	Allowed the adjustment of the body position to pedals and seat.
1930	Car blinkers	First in a mechanical way, and in 1950 the electric light was introduced.
1949	Seatbelt	Introduced by Nash Motors. Before that, in 1800s, the seatbelt was used in steam-powered horseless carriages, not to protect the passengers in the event of a collision but rather to keep passengers in their seat.

*mph: mile per hour

After 2000s, and in particular after 2015, environmental issues and sustainability politics gained more mediatic attention, leading the automotive industry to focus on the development of more ecologic solutions. Data from 2018 showed that the transport sector depended almost exclusively (>95%) on fossil fuels, being responsible for 24% of global energy-related CO₂ emissions. For these reasons, many efforts were made to develop and accelerate the decarbonization process, using biofuels, electricity, and/or hydrogen, as an alternative to fossil fuels [4]. This awareness of society promoted the development of more clean transportation options to mitigate climate change. Electric vehicles, for instance, provide zero or low emissions, no reliance on petroleum, improved

fuel economy, lower maintenance, and also provide a more comfortable driving experience (acceleration systems, noise reduction, and home and recharging logistics) [5].

All these landmarks show that the automobile industry is in constant growth and in search for new market opportunities to promote a better driver experience with more comfortable spaces and technological resources, to improve the safety for passengers, aligned with international legal standards and customer requirements.

1.1.2 Automobile numbers in Portugal and Europe

Motor vehicles and automobiles are used not only for transporting products but for the mobility of people (daily necessities and leisure), thus contributing to increased vehicle circulation. In fact, in Portugal, motor vehicles in circulation have been increasing since 2015, after the decrease in 2012/2013 related to the economic contraction and the intervention of external financial agencies in Portugal. Statistics indicate that 7.09 million motor vehicles were in circulation in Portugal in 2021, 5.63 million of them were light passenger vehicles, representing more than 79% of all motorized vehicles in circulation. Detailed data about motor vehicles in Portugal between 2010-2021 are available in Figure 1A and 1B. This increase of motor vehicles in Portugal is aligned with data available from the European Union (Figure 1C and 1D). Indeed, in 2021, there were more than 286 million of motor vehicles in circulation in the European Union, of which almost 250 million were light passenger vehicles (Figure 1C and 1D). The last report of the ACEA - European Automobile Manufacturers' Association, launched in January of 2023, but reporting data from 2021, reported an increase of 2.1 and 1.2% of car passengers in Portugal and Europe from 2020 to 2021, respectively.

Interesting data from the ACEA show that light passenger vehicles in the European Union have a prolonged life span, with an average of 12 years of utilization. In Portugal, the average life span of light passenger vehicles is slightly higher with an average of 13.5 years. With the lowest light passenger vehicle life span we find Luxembourg (with an average of 7.6 years of utilization) followed by Denmark and Austria (with 8.5 and 8.7 years, respectively). In the opposite spectrum is Greece (17 years), followed by Estonia and Czech Republic (16.8 and 15.6 years, respectively). These numbers suggest a high dependency on motor vehicles, as well as their great use in the daily routine of European citizens. These data further support the great demand for new developments in the automobile industry related to new lifestyles and requirements of users.

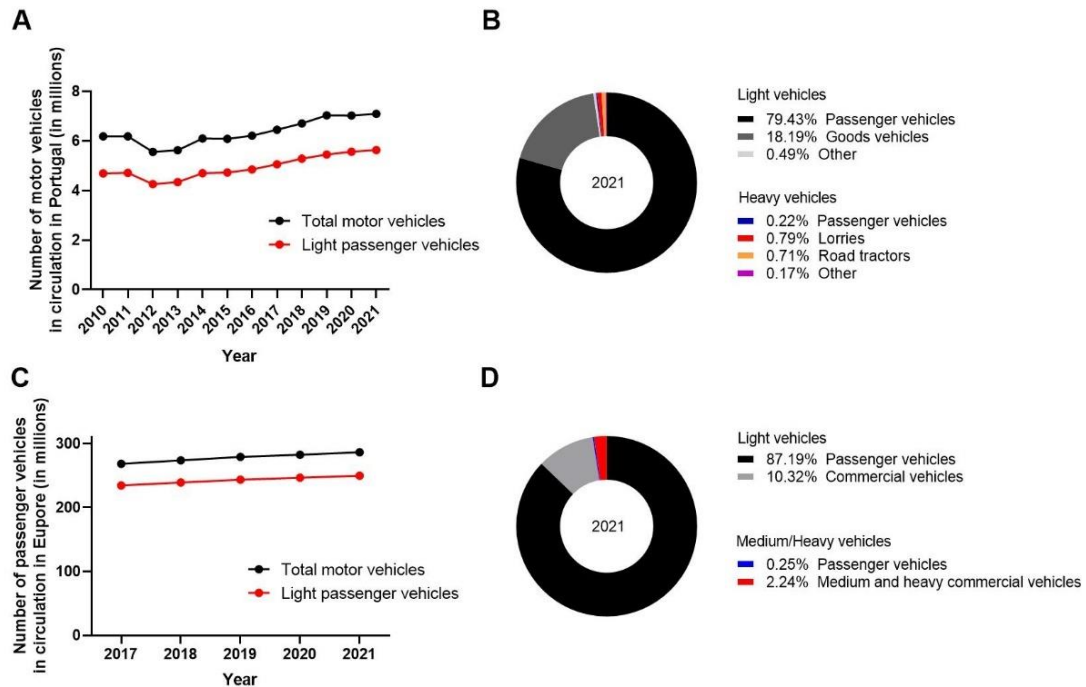


Figure 1 – Motor vehicles in circulation in Portugal and in European Union: (A) Evolution of light passenger motor vehicles in circulation in Portugal between 2010-2021; (B) Distribution of motor vehicles in circulation in Portugal in 2021 by typology; (C) Evolution of light passenger motor vehicles in circulation in the European Union between 2017-2021. (D) Distribution of motor vehicles in circulation in the European Union in 2021 by typology. Graphs (A) and (B) were made with data obtained in <https://www.pordata.pt> access in 16-01-2023, from the National Statistics Institute (INE - Instituto Nacional de Estatística: Stock Road Vehicles Statistic). Graphs (C) and (D) were made with data obtained by the European Automobile Manufacturers' Association (ACEA). *Data for Bulgaria and Malta are not available. **Light commercial vehicles include vehicles up to 3.5 tons and medium and heavy commercial vehicles include vehicles over 3.5 tons.

1.1.3 The future in automotive industry: trends of car sharing segment

As described above, the automobile industry has experienced many changes over time, from the steam-power vehicles to the current electric vehicles with constant innovations. According to Philips, A. *et al.*, 2013 [cited in Szmelter A, (2017)], mobility in the future will be characterized by the following words: zero emission, low carbon, electric, shared, autonomous, green and sustainability [6]. According to Szmelter A, (2017) there are two main changes in the future related to automobiles: (i) the process of using a car will change, with an increase in car sharing and car renting. These changes are influenced by high costs associated with driving and parking, aligned with high flexibility and the great flux in circulation of people within the cities; and (ii) the car will need to be more environment friendly (zero-emission vehicles using renewable fuels like electric, solar or hydrogen cells) [6].

Although car sharing is described as one of the trends in the future in the automobile industry, this segment is already implemented with a large expansion worldwide, attracting new

users and companies every day. Despite this being a recent trend of the automobile industry, the first concept of car sharing dates from 1948 in Zurich. This first initiative was a result of a cooperative formed by a group of private individuals that rented cars. After the period of utilization, the client would return the car to the place of origin. In the following decades, many countries adopted this system, however, many failed because of technological issues and the absence of appropriate control procedures [7]. With the advancement of digital technologies, the automobile industry has been able to disrupt traditional business models, allowing the expansion of new car sharing segments [8]. In fact, in 2008, the first free-floating car sharing scheme, called Car2go, was formed by Daimler [7].

In the car sharing segment three models can be identified, which can vary according to the access service models: business-to-consumer (B2C), business-to-business (B2B) and peer-to-peer (P2P) models. Basically, in a B2C model, a company, with a fleet of cars, rents the car to a final user for short periods of time. In this model, the service provider company assumes responsibility for car maintenance and marketing. The client pays for access to cars at a given daily rate based on the duration of the service or in the distance travelled. Examples of B2C car sharing companies include Zipcar (founded in 2000) and ShareNow, a company that combines the German manufacturers BMW and Daimler. In the B2B model, the service provider company does not own a car fleet. The company provides access to a service for car owners and establishes the connection between them and the final user. Typically, a company signs a contract with the service provider that specifies fees and terms of payment. For the final user, the benefits of this model include a lower cost of travel. In the last model, P2P model, is based on a car ownership group within a defined local group such as friends, neighbours, or a non-profit organization. The use of this model is based on communal interest in sharing cars, instead of profit. This model is generally limited since it is based on local groups, small in size, with limited choices of cars and well-defined routes [7]. Regardless of the model used, car sharing provides clear advantages: (i) a contractual agreement is not necessary, since typical car sharing only requires a membership and subscription; and (ii) does not involve the transfer of ownership of the automobile as well as the responsibility of maintenance and repair [7].

The number of car sharing users has been increasing worldwide in the last two decades (Figure 2). In October of 2018, car sharing was operating in 47 countries in all continents, representing more than 32 million users. Europe is the world's second largest car sharing market, representing 21.2% of worldwide users and 30.6% of vehicle fleets. In Europe, car sharing is

operating in 27 countries, including Portugal, with more than 6.7 million users, representing a growth of 54% when compared to 2016 [9]. According to Global Market Insights (GMI), in 2020, the car sharing market represented a market value of more than 2 billion dollars worldwide (with European market value of 700 million dollars) [10].

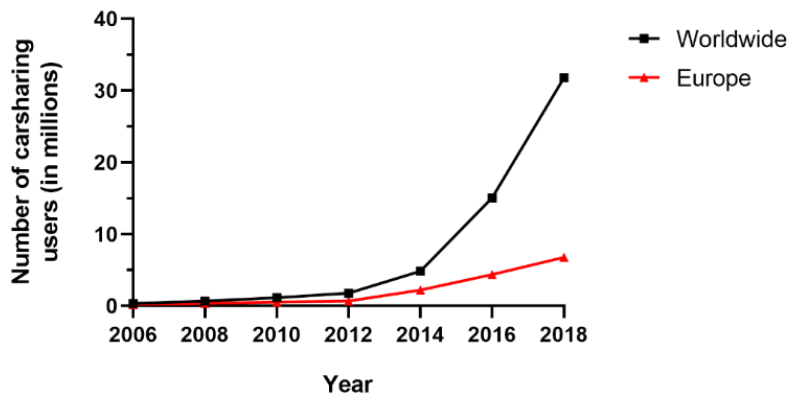


Figure 2 – Car sharing market trends: Evolution of car sharing users in Europe and worldwide between 2006 and 2018. European data were collected from 27 countries (including Portugal), while worldwide data were collected from 47 countries. Graphs were made by data reported by Shaheen, S. and Cohen, A. (2020) [9].

For the future, the forecast indicates a continuous increase in the car sharing market worldwide, driven mainly by: (i) environmental factors - the stringent regulations regarding gas emissions, mainly in Europe and North America; (ii) the technological advances in these platforms; (iii) government incentives and investments by the Original Equipment Manufacturer (OEM) (mainly in United States of America and Germany); (iv) the limited public transportation system and the increase of traffic congestion and (v) the reduction of travel cost [10]. Although car sharing market projections differ among international agencies, all of them indicate a continuous increase in users and commercial value of the segment. For example, GMI estimates a global market of car sharing of about 6.5 billion dollars in 2027 [10] while Statista, an online platform specialized in market and consumer data, shared a more optimistic estimation, with a revenue projected to reach 12.88 billion dollars in 2023 and 16.04 billion dollars in 2027 [11]. In Europe, the estimate indicates a projected value of 3.37 and 4.07 billion dollars, in 2023 and 2027, respectively, with a projected number of 14.7 million users in 2027 [12]. Data from Portugal indicated an expected value of 65.53 million dollars in 2023 and 78.16 million dollars in 2027 [13]. These data are summarized in Figure 3.

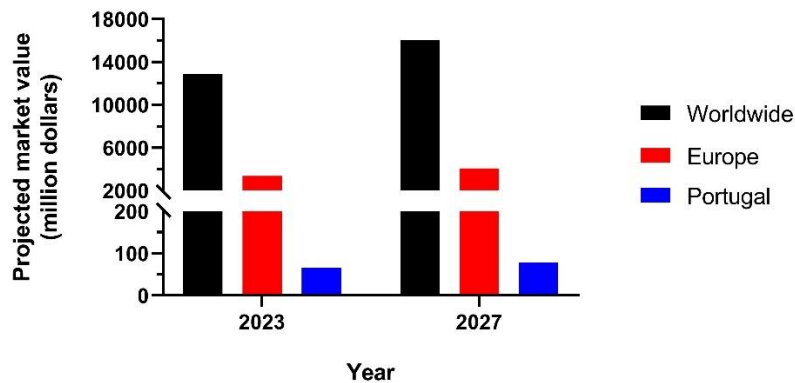


Figure 3 – Projected value of car sharing market: Prediction of car sharing value market in Portugal, Europe and Worldwide in 2023 and 2027. The values are expressed in dollars. Graphs were made by data reported by Statista Market Forecat [11-13].

1.1.4 Automobile as a confined space and a potential risk for health

The human body is an important reservoir of microorganisms, being therefore an important source for microbial colonization of indoor environments [14]. These indoor environments are characterized as multi-shared and confined spaces, in which humans spend 70-90% of their lives [14-16]. Microbial transmission can take place directly by contact between different individuals or indirectly by the exposure of the human body to contaminated areas [14]. Aligned with this, the increased capacity of several pathogens to persist on inanimate surfaces for long periods of time, makes these indoor environments an important source for microbial colonization and a potential route for the spreading of pathogens among humans [17,18]. The most common species found in indoor environments include bacteria: *Staphylococcus*, *Corynebacterium*, *Lactococcus*, *Firmicutes*, and *Actinobacteria* and fungi: *Cladosporium*, *Penicillium*, and *Aspergillus* [14].

Automobiles are one of these indoor environments in which humans spend an important part of their lives, offering important opportunities for transmission of pathogens among users [18]. In fact, population studies indicate that, in developed countries, each individual spends an average of 4.3 years inside a car, considering an average life-expectancy of 8 decades [19]. Despite the efforts to reduce the transmission of microorganisms among vehicle occupants, in particular during the COVID-19 pandemic, mainly through physical separation by using barrier shields and face masks, the risk remains [18].

The main surfaces of fomites are the areas frequently touched by vehicle occupants, including the steering wheel, the centre console, the door latch, the door and window controls, the door handle and the cruise control button. These areas are frequently colonized by bacteria, being the most dominant genera *Staphylococcus* and *Pripionibacterium* species [20]. Within the

Staphylococcus genus the most frequent species identified are *S. epidermidis*, *S. aureus*, and *S. warneii* [20]. Another study analysed the car interior of health care workers and showed that 38% of dashboards and 25% of steering wheels were contaminated, in a total of 50 vehicles analysed. The most common organism isolated were *Bacillus* species followed by *Diphtheroids*, *Micrococci*, *Escherichia coli*, *Klebsiella* species and *Staphylococcus* spp. [21].

All these data suggest that automobile interiors can be an important source of fomites and a potential risk for users. This situation becomes of even more importance given the increased estimations of the carsharing market and the flux of users in the next years. Moreover, the advent of the COVID-19 pandemic has changed car users' perceptions of comfort and safety within automotive vehicles. Whereas in the past automotive interiors needed to be comfortable, attractive, and durable; now there is a new dimension. Aligned with the concept of the car being an extension of a living room, there is also a desire for overall cleanliness and safety against "invisible threats" inside the car. No doubt, the pandemic will have a lasting effect on existing and future mobility concepts, as well as on the materials and technologies used inside vehicles.

One of the strategies to promote this safety against microbial infections is the coating of surfaces with antimicrobial materials. These coatings should be capable of inhibiting the survival and proliferation of pathogens. In that sense, the use of antimicrobial coated surfaces for automotive interiors could be an important strategy to reduce microbiota in cars.

1.1.5 Automotive coatings and its applications

Automobile coatings are materials used to coat automobile surfaces to achieve or promote a property of interest. These coatings should be durable, fulfil the technical requirements imposed by the automotive industry and abided by environmental regulations [22]. The first coatings were applied manually over car paintings, such as varnish to produce shiny surfaces [22]. The first techniques consisted in dipping the material into the solution. After the 1920s, more technical methods were implemented, such as dipping spray equipment. These technologies allowed to decrease the application and drying times and increase the production rates [22]. In the 1970s, new developments were introduced to improve coatings' appearance and durability, such as the application of two coatings with different properties and thickness. These strategies allowed to improve the durability of coatings in all climates (enhancing resistance to temperature and solar exposure). In the 1980s, in Germany, water-based coatings were applied improving the corrosion protection, as well as the effectivity and durability of the colour and gloss of the coating [22].

Nowadays, automobile coatings are applied at large scale, in different components in automobile interior, and different additives are used to diversify the functionalities of the coating, which include mechanical and physical protection (anti-corrosion and anti-abrasion properties, ultraviolet (UV) resistance, flame retardancy); aesthetic effects (colour, anti-reflective properties), self-cleaning properties, water-penetration resistance, thermal isolation [23]; and biocide properties [20-24].

An example of a cross section of a flexible coating material is represented in Figure 4A. The polymeric material is applied over the surface of the substrate, typically a paper roll or a textile layer. The coating materials are obtained by adding successive polymeric layers based with different raw-materials and additives to confer different properties to the material. Finally, a finish lacquer is applied on the surface of the flexible coating with the intent of providing protection against degradation processes, improving the fill and smoothing imperfections, providing a visual and decorative effect (matte or gloss) and conferring resistance to cleaning products or environmental factors [25]. A typical construction of a finish lacquer layer is represented in Figure 4B. The basecoat is in contact with the compact layer and therefore should provide a good adhesion, while simultaneously working as a barrier to prevent the migration of substances from the substrate. The intermediate layer can be composed by one or more layers and is responsible of conferring chemical and physical resistance. Finally, the topcoat is the most superficial layer being responsible for final resistance and aesthetic properties of lacquer layer.

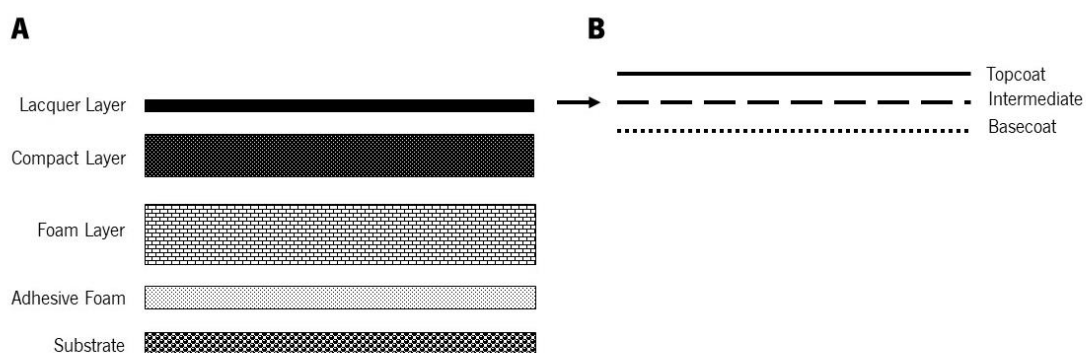


Figure 4 – Cross section of typical flexible coating materials to mimic artificial leather: (A) cross section of typical flexible coating material with different polymeric layer applied over a substrate (fabric or paper) and (B) cross section of typical lacquer layer.

1.2 Antimicrobial surfaces based on polymeric materials: state-of-the-art of production and development of antimicrobial materials and their mechanism of action

Inanimate surfaces play an important role in microbial colonization and contribute to the spread of potential harmful pathogens among humans. Despite the efforts to develop new antimicrobial materials for both medical and non-medical applications, these remain insufficient to avoid the contamination of surfaces and the spread of microbial infections, particularly in hospital settings and on high-touch surfaces. For this reason, there is a great demand for the development of novel antimicrobial materials and strategies to reduce microbial adhesion/growth.

The main objective of this section is to provide a clear and complete overview of the most common strategies used during the development of antimicrobial surfaces, as well as highlight the main antimicrobial agents immobilized on polymeric surfaces. Firstly, a classification of antimicrobial polymers will be described based on their mechanism of action (passive or active action) and the type of polymeric system, distinguishing between biocidal polymers, polymeric biocides, and biocide-releasing polymers. Following this initial classification, the main strategies to promote the antimicrobial property on polymer surfaces will be highlighted, specifically the influence of surface properties on microbial adhesion/growth will be addressed, such as the surface topography, wettability, and surface energy, and finally the incorporation of antimicrobial agents on solid surface. In this last, a great focus will be given to the main antimicrobial agents incorporated in polymeric surfaces, describing their application and mechanism of action.

1.2.1 Microbial Infections as a problem for Healthcare System

Microbial infections are one of the most serious problems in healthcare systems and one of the main primary causes of death worldwide [26,27]. In 2015, the European Center for Disease Control reported that almost 8.3% of hospitalized patients, after two days in intensive care units, acquire at least one healthcare associated infection [28]. The most common bacterial infection in hospitalized patients include *Pseudomonas aeruginosa*, *Staphylococcus spp* and *E. coli*, respectively identified in pneumonia episodes, in bloodstream infections, and in urinary catheter infections [28]. The World Health Organization (WHO) reported an increase in antimicrobial resistance among clinical isolates and this phenomenon is considered one of the most critical threats for the human health, in particular in vulnerable/immunocompromised patients [28].

Despite the cross-transmission of pathogens among humans being mostly associated to medical devices and common high-contact surfaces in healthcare facilities [29], the interaction of

humans with potential harmful pathogens can also occur in other inanimate surfaces outside the medical environment. The cross-contamination rate depends on the type of microorganism, humidity level, size of the inoculum and the initial and final destination surface [29]. The most common inanimate surfaces colonized by microorganisms and, therefore a possible source for transmission, include water purification systems, sanitation facilities, textiles or others touch surfaces [27]. To further complicate this issue, it has been reported that microorganisms are able to survive, depending on the environmental conditions, for several months on inanimate surfaces [29]. Although antimicrobial compounds are commonly used in clinical practice, many infectious diseases remain difficult to control. For this reason, both academic and industrial research have been focused on preventing microbial transmission through the development of materials with antimicrobial properties, for medical, food, and textile industries, to avoid the contamination and colonization of microorganisms on high-touch surfaces [26]. Currently, many touch surfaces are made of polymer material or at least coated with polymer materials. In particular, polymers have been shown to be an attractive material for biomedical applications due their physical and chemical properties, including malleability, resistance, simplicity of production and low cost of raw materials [30]. The most commonly employed polymers include polyvinyl chloride (PVC), polyolefins (polyethylene, polypropylene (PP), polystyrene (PS)), polyurethane (PUR) and silicon [30].

The following sections will be covering the principal aspects related to the development and production of antimicrobial surfaces, focusing on the different antimicrobial agents used in the industry and in the medical field. Additionally, a historical overview of the development of different antimicrobial agents will be presented, along with a description of their main mode of action.

1.2.2 Classification of antimicrobial polymers regarding to mechanism of action and the type of polymeric system

Antimicrobial polymeric materials can be categorized based on their mode of action, as being passive or active systems (Figure 5A). In a passive system, the polymer does not interact directly with the bacteria or cause its death, but rather reduces or prevents bacterial attachment by decreasing the surface affinity of the polymer surface [26,31]. Given the intrinsic hydrophobicity and negative charge of microbial membranes, passive polymers should be hydrophilic, negatively charged, and present low surface energy [26,31]. Examples of polymers with passive action include poly(dimethyl siloxane) (PDMS), poly(ethylene glycol) (PEG), poly(2-methyl-2-oxazoline) (PMOXA), poly(dimethyl acrylamide) and polyampholytes and zwitterionic polymers [2]. PDMS is chemically

inert and despite presenting hydrophobic properties, its passive action is mainly provided by its low surface energy, which hinders the deposition of other materials/functional groups on PDMS surfaces, such as electrolytes for electric applications [31]. Nevertheless, some strategies have been used to overcome the PDMS hydrophobic properties. Specifically, the use of plasma, composed by ionized oxygen at high energy, promotes the oxidation of PDMS by inducing the formation of silanol groups in the final portion of methyl groups ($-\text{CH}_3$), which ultimately exposes hydrophilic regions [32]. PDMS is one of the most used silicon-based organic polymers and has been extensively applied in material science, specifically in microelectronics systems and elastomers, as well as in the medical, cosmetics and food fields [32,33]. Regarding PEG, its passive nature derives from its intrinsic properties, such as high chain mobility, large exclusion volume, and steric hindrance effect of highly hydrated layer [26]. The antimicrobial properties of PEG can also be influenced by their molecular weight and/or its physical state. Indeed, it has been reported PEG's antimicrobial properties against *Streptococcus* sp., *Enterococcus faecalis* and *E. coli* [34].

In active systems, the antimicrobial polymers kill the adherent bacteria (Figure 5A) [26]. Examples of polymer active action include polyethylenimine, polyguanidine, and N-halamine. Specifically, polyethylenimine acts by disrupting the Ca^{2+} channels [26]. The antimicrobial action of polyguanidine, and some derivatives such as polybiguanides, are attributed to electrostatic interaction between the positive charges of the polymer and the electronegative nature of the cytoplasmic membrane, ultimately causing the destabilization and disruption of the cytoplasmic membrane for both gram positive and negative bacteria [35].

Despite the distinction between passive or active system, some polymers can be functionalized with other bioactive compounds changing the initial classification. In these cases, the mode of action also depends on the bioactive agents. For example, PEG can be used as a passive system but also can be combined with other antimicrobial agents such as tobramycin [36], nisin [37] or silver (Ag) nanoparticles [38], acting as an active system.

Regarding the classification based on the polymer system considered, the antimicrobial materials can also be classified as either biocidal polymers, polymeric biocides or biocide-releasing polymers [27] (Figure 5B).

Biocidal polymers are polymers with intrinsic antimicrobial activity [27]. The design of antimicrobial biocidal polymers has the main advantage of avoiding the release of potential harmful biocides to the environment given that the biocide portion is intrinsic to the polymer backbone [27]. These materials are usually based on functional groups derived from the polymer structure [39].

Biocidal polymers generally assume dual structures with amphiphilic properties. The hydrophilic properties are typically derived from the cation groups that can anchor to anionic portions of membranes, while the hydrophobic properties are provided by alkyl chains, which are capable of interacting with negatively charged phospholipid bilayers of the cell membranes [39]. Examples of biocidal polymers include ϵ -poly-lysine [40], polyethylenimine [41,42] and natural polymers such as chitosan [43]. Another important group of biocidal polymers include polycationic polymers, which possess high bioactivity by promoting electrostatic interactions between their cationic structure and the bacterial cell membrane resulting in its disruption [44]. The most cationic groups include quaternary ammonium compounds (QAC), quaternary phosphonium, guanidinium, tertiary sulfonium, pyridinium, and biguanide [39]. Indeed, Sanchez, L. *et al.* (2015) studied the antimicrobial action of the cationic polymer, poly (diallyldimethylammonium) chloride (PDDA), immobilized on poly (methyl methacrylate) (PMMA) [44]. The authors show different antimicrobial efficacy of 8, 7 and 2 colony-forming units (CFU) Log reduction against *E. coli*, *S. aureus* and *Candida albicans*, respectively, which was attributed to the differences in cell wall among these organisms. Regarding these differences between bacteria, the authors suggest that in gram-positive bacteria, after the contact of cationic polymers with the bacterial surface two phenomena occur: D-alanylation of teichoic acids components and a lysylation of phosphatidylglycerol. These are responsible for decreasing the negative charge of the membranes of gram-positive cells, impacting the adsorption of cationic antimicrobials. Regarding the decreased antifungal activity, this was attributed to the dense and complex architecture of the fungal cell wall, composed by an inner skeletal layer with polysaccharides β -1,3-glucan and chitin, which hindered the penetration of PDDA/PMMA [44].

Polymeric biocides are based on polymer backbones with biocide molecules in their architecture [39]. These polymers can be prepared by the attachment of known biocide molecules as functional groups to a polymer structure [27,39]. Usually, these type of materials exhibit lower efficacy than the free biocidal molecules, due to the steric hindrance created by the polymer backbone [39]. Peng, K, *et al.* (2017) describes the production of polymeric biocides based on the synthesis of PUR with tetramethylgermanium chains by the copper-catalyzed azide-alkyne cycloaddition (CuAAC) process [45]. These materials, with 2.1% of tetramethylgermanium functional groups, show high antimicrobial properties against gram-positive and gram-negative strains (99.9% of antibacterial efficacy) [45].

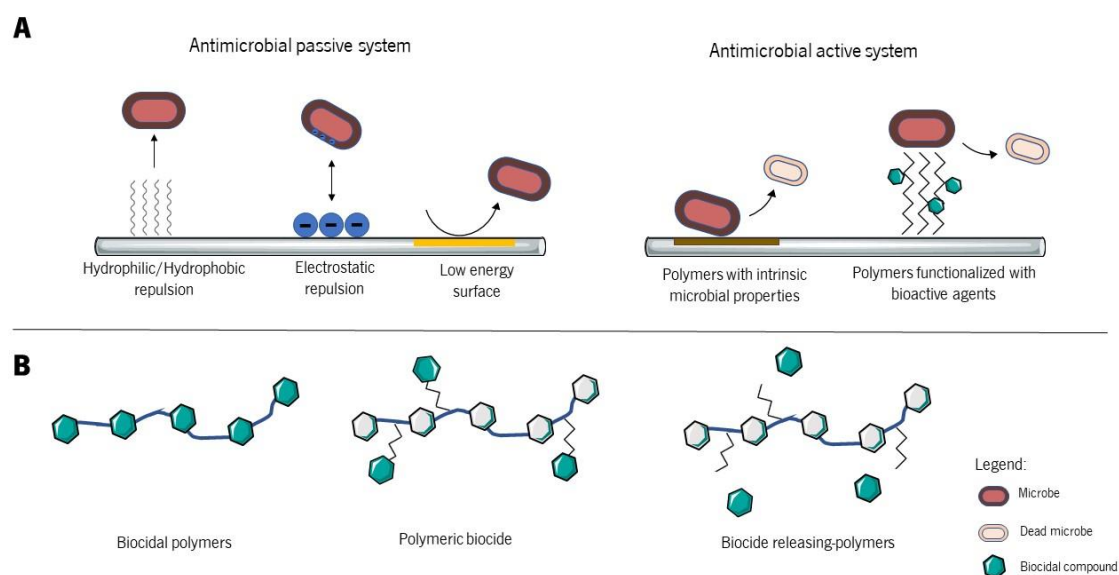


Figure 5 – Different antimicrobial polymer classifications: (A) based on mode of action and (B) based on polymer system. Adapted from Siedenbiedel, F. and Tiller, J. (2012) [53].

Finally, biocide-releasing polymers consist of materials loaded with biocide molecules or ions loaded in the polymer either through physical interactions or chemical bonding [39]. The biocide molecules can be entrapped using different methods (ex: cold spraying or deposition from the active gas phase) or establishing chemical bonds with the reactive groups present on the surface of the polymer [39]. The chemical bonds applied for the attachment of biocidal agents include metal-ion coordination bonds; nitrogen-halogen bonds (N-halamine); hydrolysable functional groups, such as esters and amides; or other specific chemical interactions [39]. This strategy is mainly used to develop drug delivery systems. The biocide-releasing polymers can include antibiotics, such as ampicillin [46], ciprofloxacin [47] or rifampicin [48], and other molecules like carbon monoxide (CO) [49] or metal-based materials (Ag [50], zinc (Zn) or copper (Cu) [51]). An example is reported by Rezić, I. *et al.* (2022) that describes the application of antimicrobial Ag microcapsules, with a core composed of Ag ions and a shell based on sodium alginate and Zn-sulphate 7-hydrate, on the polymer surface by the dip-coating method. The antimicrobial action was obtained through the release of Ag⁺ cations from the microcapsule by diffusion [52]. The biocide-releasing polymers promotes an excellent antimicrobial activity due to their efficacy in releasing high concentrations of biocides near the intended target [27]. On the other hand, the toxicity associated with the biocide-releasing polymers is a great disadvantage, and their antimicrobial efficiency reduces significantly over time [27].

Regardless of the polymeric system adopted, the ideal antimicrobial polymer should be (i) easy and inexpensive to synthesize; (ii) non-toxic or non-irritating when in contact with the skin; (iii) stable during storage and for long-term applications; (iv) resistant to decomposition and degradation, with no release of toxic byproducts to the environment; (v) and have the broadest antimicrobial spectrum possible according to the application of interest [39,43].

1.2.3 Strategies to promote the biocide effect in polymer materials

To obtain the biocide effect in polymeric coatings many strategies can be used including: (i) modification of the polymer surface (surface free energy, polarity, topography); (ii) deposition of the biocidal agent on the polymer surface and (iii) incorporation of biocidal agent in the polymer matrix [54]. These strategies are summarized in Figure 6. The main polymers and biocidal agents used to produce antimicrobial coating materials will be discussed in the next section of this thesis.

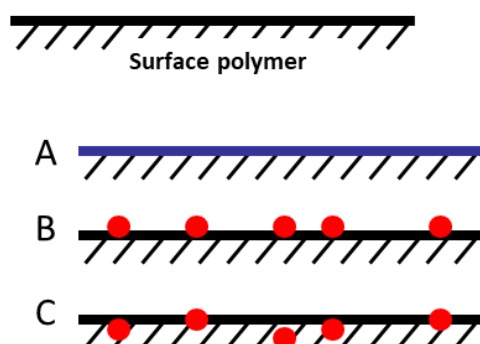


Figure 6 – Strategies to promote the biocide effect in polymer materials: (A) modification of polymer surface properties; (B) deposition of an antimicrobial compound directly on the polymer surface or after a chemical surface modification and, (C) incorporation of the antimicrobial compound in the polymer matrix. Adapted from Sedlarik, V. (2013) [54].

Antimicrobial coatings are used in many applications and are described as being an effective strategy to reduce bacterial adhesion to surface materials and/or kill the microorganisms on inanimate surfaces, reducing the spread of potentially harmful pathogens. This is highlighted by the number of publications and patents related to “antimicrobial coatings” which have been increasing over the past years [55]. In 2016, almost 2500 international patents were granted on antimicrobial coating, with 65% of them registered in United States patent office and 18% in European patent office. Despite these efforts, there is still no ideal solution, and there is a great demand on both academic and industrial partners to continue to develop new antimicrobial coatings for indoor and outdoor applications in order to fulfil health and safety issues [55].

1.2.3.1 Modification of polymer surface properties

The first stage of microbial colonization of a surface is bacterial adhesion, followed by growth, and eventually the spreading and colonization of other surfaces (Figure 7). These events are, in an initial stage, mediated by electrostatic and hydrophobic interactions in association with environmental conditions, such as temperature, oxygen availability, humidity, among others. After this, bacteria can produce specific adhesins and/or receptors that bind to the surface forming an adhesive layer. This layer, also called extra-cellular polymeric substances (EPS), promote bacterial attachment and biofilm growth [56]. Biofilm can be simply defined as a colony of microorganisms embedded in its extra-cellular matrix attached on the substrate growing over EPS substrate. The prevention of biofilm formation is an important issue since it has been described that bacteria when in biofilm are more resistant to antimicrobial compounds [56,57]. As described above, the surface properties of the substrate can affect bacterial adhesion and can inhibit biofilm formation. Understanding the mechanism of interaction between bacteria-substrate can avoid potential bacterial attachment and consequent contamination of surface. In the next sections the main surface properties involved in microbial adhesion to surfaces will be highlighted, such as surface topography, wettability, and surface energy.

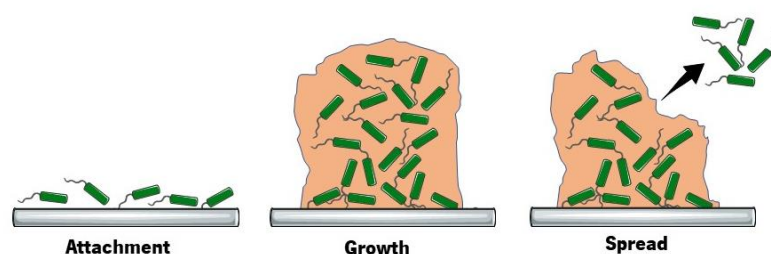


Figure 7 – Schematic representation of various stages of bacterial colonization of inanimate surfaces.

Adapted from J. Hasan and K. Chatterjee (2015) [56].

a) Surface topography

Roughness can be defined as «irregularities in the surface texture which are inherent to the production process» [58]. Roughness can occur in two planes, perpendicular to the surface, described as height deviation, and parallel to the surface, described as texture. This configuration allows to distinguish two typical structures, known as peaks and valleys which can vary in height and spacing (see Figure 8). According to the physical parameters of these structures, some criteria can be recognized to characterize the surface roughness: arithmetic average height (R_a), standard

deviation of the z height values (R_q) and the difference in height between the average of the five highest peaks and the five lowest valleys along the assessment length of the profile (R_z) [58]. Roughness can be achieved by using diverse physical and chemical approaches to imprint surfaces with nanograin, pore, tube, particle, ridge or valley structures. The most important techniques include chemical or physical etching, anodization, self-assembling monolayers, sputter coating, ion beam deposition, and lithographic techniques [59]. Physical etching, also known as reactive ion etching, is a physical process in which partially ionized plasma interacts with the material surface promoting wear of the surface of the material. The plasma ions are accelerated in a defined orientation allowing a directional/anisotropic etching [57]. In contrast, a chemical etching is an isotropic etching in which radicals react with the material surface resulting in volatile products [57,60]. A dual approach combining physical and chemical etching is also possible (Figure 9).

Several studies have evaluated the influence of surface pattern on bacterial adhesion/proliferation. Mann, E. *et al.* (2014) tested two different surfaces (PDMS elastomer or acrylic film) with an embossed Sharklet™ micropattern or a smooth surface as a control. The micropattern surface showed a 99 and 98% reduction in microbial attachment of a methicillin-sensitive and methicillin-resistant *S. aureus* strains, respectively [61]. The authors reported that the embossed surface was more effective in reducing persistence of *S. aureus*, when in comparison with Cu-coated surfaces. In fact, for methicillin-sensitive *S. aureus* the Cu surfaces had no effect in controlling the bacterial burden, while the Sharklet™ pattern exhibited good efficacy, reducing the CFU density in 87 and 97% after 0 and 90 min of contact [61]. The antimicrobial activity induced by rough surfaces can be attributed to the mechanical and structural stress induced by this type of surface. This stress on the peptidoglycan wall and membrane of bacterial cells, promotes its deformation and ultimately bacterial death [62]. In addition to the disruption of mechanical properties of the cell, this architecture system can also affect the internal osmotic pressure of the microorganisms by modifying the surface wettability (see next section) [62,63]. Similarly, Jijie, R. *et al.* (2018) synthesized polymerized styrene films, combined argon gas and styrene vapor. Topographical pattern was obtained by immersing the film in aqueous media for 72 hours to promote the solubilization of the low cross-linked oligomers and their subsequent extraction. The authors confirmed that this type of surface can significantly reduce the number of adherent bacteria; however, there is no effect on the activity of planktonic bacteria [64].

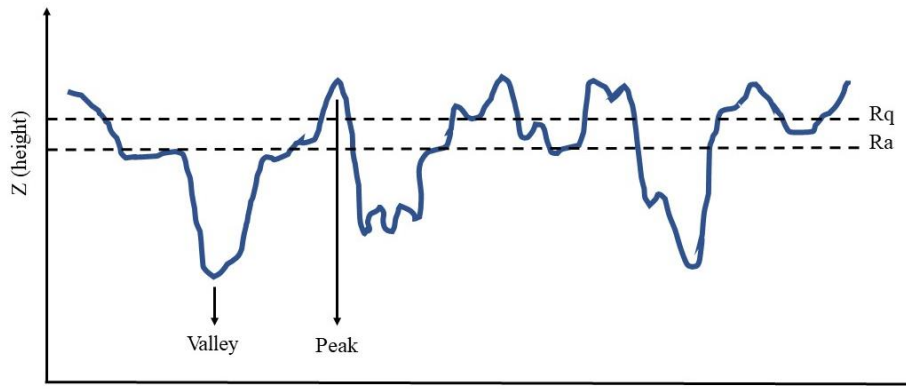


Figure 8 – Schematic representation of a typical cross-section of a roughness surface: Ra and Rq are physical parameters that characterize the arithmetic average and the standard deviation of height of peaks, respectively. Adapted from Whitehead, K. and Verran, J. (2006) [58].

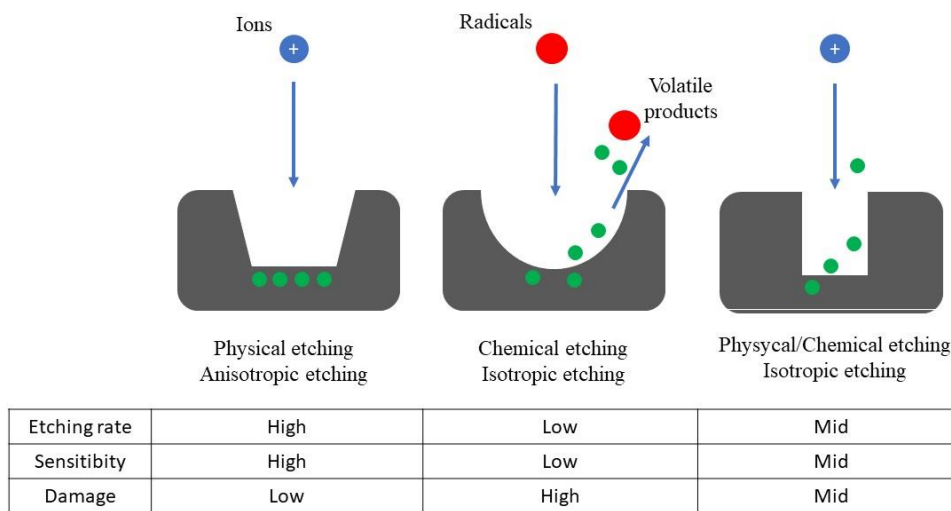


Figure 9 – Schematic representation of plasma dry etching reaction and their mains properties. Adapted from Kamimura, R. and Furuta, K. (2017) [65].

b) Surface wettability

Wettability is the tendency of a liquid droplet to spread or wet a solid surface. This is a property of solid materials mediated by intermolecular interactions that can be easily measured by determining the contact angle (CA) between the material surface and the droplet. Wettability has been shown to influence bacterial adhesion [66]. Some reports show that CA values between 40-130° can potentiate adsorption of bacterial peptidoglycan, promoting cell adhesion and bacterial growth [67]. Although there is no exact angle for the transition between hydrophilic, hydrophobic and superhydrophobic materials, it has been established that surfaces with CA values below 90° are hydrophilic; above 90° hydrophobic and above 150° superhydrophobic (Figure 10).

Superhydrophobic surfaces have excellent water repellence and were shown to be able to avoid bacterial adhesion [67,68]. These superhydrophobic properties are more attractive due to their potential not only for antimicrobial coatings but also in microfluidics, textile, oil–water separation, anti-corrosion coatings and self-cleaning surfaces [68].

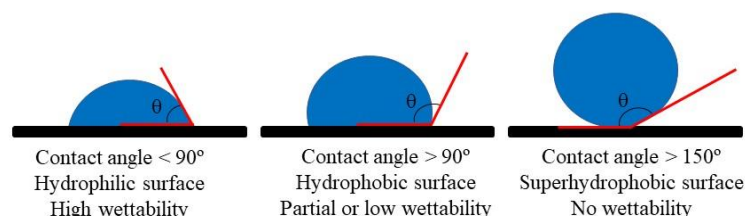


Figure 10 – Relation between contact angle (CA), wettability and hydrophobic or hydrophilic surface:

A CA below 90° exhibit a good wettability; a CA higher than 90° exhibit a partial or low wettability depending on the CA value and CA higher than 150° the is a non-wettable surface. Adapted from Song, J. *et al.* (2021) [69].

The design of synthetic superhydrophobic surfaces includes some techniques, mainly photolithography techniques, surface etching or modifications via nanoparticles. These surface modifications generally result in nanoscale surface roughness and reduced surface energy [70].

Freschauf, L. *et al.* (2012) reported that structurally modified superhydrophobic surfaces of PDMS, PS, polycarbonate and polyethylene, with CA above 140° , are effective in reducing bacterial burden against *E. coli*. Non-modified structures retain up to 10% of the initial inoculum, while for superhydrophobic modified structures this percentage was below 0.1% [71]. This effect on bacterial reduction is also described for non-polymeric materials. Indeed, nanostructured systems based on fluorinated silica with superhydrophobic properties (151 – 167°) reduce adhesion of *S. aureus* and *P. aeruginosa* in 99.0 and 98.2%, respectively [70]. In line with these results, Pham P. *et al.* (2021) [60] developed a series of polymers with different ratios of cationic hydrophilic and hydrophobic groups and tested their antibacterial and haemolytic activity. In general, increasing the ratio of hydrophobic groups was associated with a decrease in the minimum inhibitory concentration (MIC) values. As an example, modification of the PEG-methyl ether acrylate group (hydrophilic monomer) with N-isopentylacrylamide (hydrophobic monomer), doubled the hydrophobic content from 20 to 40% and decreased the MIC value from >256 to 8 – 16 $\mu\text{g/mL}$ against *E. coli* and *P. aeruginosa*. A decrease in MIC value was also shown for the same hydrophilic monomer, but with a different hydrophobic monomer (N-benzylacrylamide). Interestingly, this increase in hydrophobic content did not have an impact on the MIC value against gram-positive strains. The authors attributed this situation to differences in cell wall structure of gram-positive

and gram-negative bacteria [60]. Although both bacteria cell walls are composed by peptidoglycan layers containing amino acid and sugar groups, the structure and thickness are different. While gram-positive bacteria have multiple layers of peptidoglycan in a rigid crosslinked architecture, hindering the penetration of small molecules or monomers, gram-negative bacteria have a thin peptidoglycan layer and an outer membrane composed by lipopolysaccharide molecules, which represents important anchoring sites for polymeric monomers [72] (Figure 11). Similar results were obtained by Tan, J. *et al.* (2022), suggesting that increasing hydrophobicity of the polymer increases the interaction and destabilization of biological membranes, which can potentiate the antimicrobial activity [73].

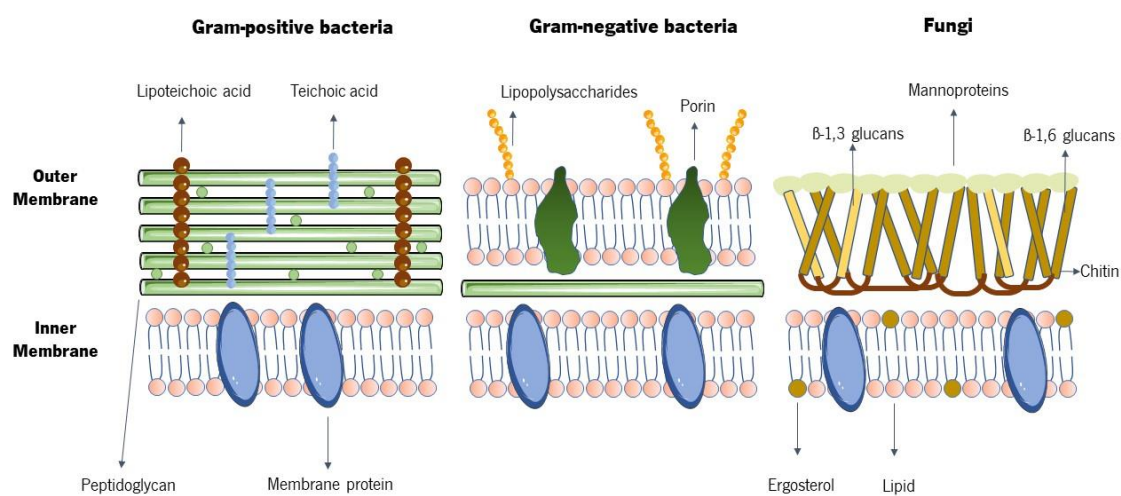


Figure 11 – Differences in biological membranes of gram-positive and gram-negative bacterial and fungi. Adapted from Huang, Y. *et al.* (2020) [74].

Although superhydrophobic surfaces are recognized as an important strategy to reduce protein and bacteria adsorption, surfaces with extreme wettability can also act as anti-adhesive surfaces. In case of highly hydrophilic polymers, the high affinity to water and the capacity to spread on the surface, can promote the formation of an ultra-hydrate layer. This layer can act as a physical barrier and can decrease bacterial adhesion. Some examples of antifouling polymers with high affinity for water, includes PEG and PMOXA [57]. In an interesting work, nisin-functionalized films were developed from a low-density polyethylene (LDPE). LDPE is a hydrophobic polymer, however authors submitted LDPE to a plasma-treatment with Ar/O₂ and a functionalization with acrylic acid (AA) monomers to produce hydrophilic surfaces. These surface treatments show a CA reduction from 101.8° to 44.2°. The antibacterial activity, based on agar diffusion assay, for nisin-LDPE films showed an irregular and undefined border, while nisin-LDPE treated films exhibited a higher

and uniform inhibition zone for all tested bacteria. Despite nisin being considered an amphiphilic molecule, it is composed predominantly by hydrophobic regions. For this reason, the authors suggested that in the presence of a hydrophobic surface, nisin is subjected to a large distortion in their molecular conformation in comparison to when exposed to hydrophilic surfaces. This alteration in their conformation may contribute to nisins's lower activity when functionalized on hydrophobic surfaces [75].

c) Surface energy

Surface energy can be defined as an excess of energy resulting from the interaction of surface-active groups with other chemical groups or molecules, which are in contact or near the surface [76,77]. It is difficult to experimentally quantify surface energy without correlating it with wettability, since these two properties are coupled. In fact, higher surface energy, in general, correlates with a hydrophilic behavior. In contrast, increased hydrophobicity correlates with a decrease of surface energy [78]. As expected, materials with lower surface energy (20-30 mN) were shown to be associated with lower bacterial adhesion compared to materials with higher surface energy [67]. Similarly, Chen, J. *et al.* (2021) reports that urushiol-based benzoxazine Cu polymer exhibits a low surface energy and hydrophobicity and these surface properties provide antibacterial activity against *S. aureus* and *E. coli* as well as antifouling property with low algal cell adhesion (*N. closterium*) for marine applications [79]. In follow-up work, the authors show that poly(1H,1H,2H,2H-perfluorododecylacrylate) and poly(methylpropenoxyfuoroalkylsiloxane) surfaces with a low energy surface exhibit a reduction of bacterial attachment after 2 weeks [80].

1.2.3.2 Deposition/Immobilization of antimicrobial agents on solid surfaces

An alternative approach to the modification of the physical properties of the polymer surface described above is the deposition or immobilization of active agents on polymer surfaces in order to provide the intended antimicrobial properties. In fact, the literature describes that there has been a recent ongoing interest in developing new hygienic/sanitary standard polymer-based coatings with improved antimicrobial and functional performance for a wide range of commercial products to avoid the spreading of microorganisms [81]. This demand has led to the development polymer surfaces immobilized with known antimicrobial compounds, including antibiotics, quaternary ammonium salts, metal-based compounds, or antimicrobial peptides. In the next sections a brief state-of-the-art is highlighted, focusing on the principal antimicrobial agents used on polymeric surfaces.

a) Quaternary ammonium compounds

Amines are one of the most important reactive groups in the chemical field. The amine class can be distinguished as primary (RNH_2), secondary (R_2NH), tertiary (R_3N), and quaternary amines (R_4N^+), according to number of radicals linked to central atom [82]. This last class is generally called cationic quaternary ammonium-based compounds, or simply quaternary ammonium compounds (QAC). Structurally, QAC are ammonium compounds in which four alkyl organic groups are linked to a nitrogen (N) atom positively charged (cation) (Figure 12). R^1 , R^2 , R^3 , and R^4 is the alkyl chain (saturated, unsaturated, branched or straight, cyclic or acyclic) having different number of carbon atoms and an anion group (X) [82,83], which is usually chloride or bromide [83]. The radical or alkyl chain group can be further functionalized with other compounds to improve some properties of QAC.

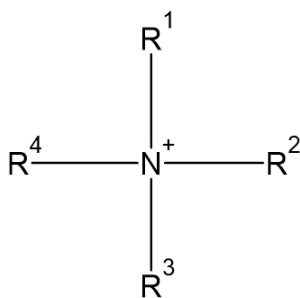


Figure 12 – Structure of quaternary ammonium compounds (QAC). Drawn in ACD/ChemSketch (Freeware) 2022.2.

QAC represent one of the most commercially class of biocides used worldwide. These biocides are used in many in clinical, industrial, agricultural, and environmental fields to control the spread of environmentally transmitted pathogens, but also in personal care products for everyday use such as a disinfectants, deodorants, hair and cleaning products, and antistatic agents [80,84,85]. QAC have an efficient antimicrobial activity and a large spectrum of antimicrobial action against bacteria, fungi, and virus. One of the first publications on these agents dates back to 1915-1916, in which the bactericidal properties of some derivatives of hexamethylene tetramine was studied [82]. In 1935, Domagk developed one of the first QAC-derived of biocides, benzalkonium chloride (BAC) [86]. BAC is composed of a mixture of QAC with benzyl, methyl, and alkyl chains with a variable number of carbons from C8 to C18 [86]. The discovery of BAC is still currently important given that nowadays, it is estimated that 11% of the biocide market in the US is BAC-based, of which 80% was in biocides, sanitizers and disinfectants (data of 2019) [84].

QAC of short and medium chain length show high solubility in polar and protic solvents, such as water and alcohols. For long chains, with C14 or more carbon atoms, water solubility generally decreases or becomes practically insoluble, while the solubility in nonpolar solvents is improved. The thermal properties of QAC can be regulated by the structure of the R-residues and length of alkyl chain, but, at room temperature, QAC are generally solid. Due their ionic properties, QAC exhibit high conductivity and QAC-based solutions are very good electrolytes. Furthermore, the dual structure (polar and nonpolar residues, positively and negatively charged, respectively), make QAC an important group of surfactants, acting as a repellent or surface-active agent against microorganisms [82,84].

As described above, QAC can be used as a biocidal agent in functionalized coating polymer surfaces. Their functional groups allow QAC to be cross-linked into non-leaching biocidal coatings improving their biological properties [81]. QAC directly target the cytoplasmatic lipid bilayer and the outer membrane of microorganisms, by promoting a strong electrostatic interaction between the positively charged headgroup of lipidic layer and the negatively charged bacterial membrane [84,87]. After interaction, the hydrophobic alkyl chain diffuses through the intramembrane region and disrupts the cytoplasmic membrane by changing the permeability of the barrier, causing cell death [81]. Due to their proven biocide and surfactant commercial application QAC-based materials have been attracting more attention for the development of antimicrobial materials. The synthesis of polymers with QAC are generally taken by post-polymerization approaches, where quaternarization of polymers occurs after the polymer reaction, with variable degrees of cationization as a result of the unpredictable impact of steric hindrance from neighbouring groups, or copolymerization approaches, where QAC monomers are grafted into the polymer network [88]. When incorporated in the polymer matrix, the biocidal activity of QAC can be optimized by taking into consideration the functional residues in their structure and the length of the alkyl chain.

Some reports show that QAC with alkyl chain lengths longer than C8 [81] or from C12 to C16 [84] exhibit better antimicrobial activity. In fact, Wynne J. *et al.* (2011) reported the development of an antimicrobial PUR coating system by synthesizing several quaternary ammonium diols with different alkyl length (C4, C6, C8, C10 and C18) which were linked by hydroxyl (OH) functionality groups of the QAC-diol to the polyisocyanate reactive groups present in the PUR material. Accordingly, independent antimicrobial assays corroborated that only a C8, C10 and C18 QAC-diol coating provided a significant antibacterial reduction [81]. Indeed, the C8 QAC-diol coating was reported as having a better antimicrobial activity, with a 3 and 5 Log-scale

reduction against both *S. aureus* and *E. coli* species, respectively. Interestingly, the antimicrobial activity against gram-negative bacteria decreased with the increase of the alkyl length (from 8 to 18 carbons atoms). For C18 QAC-diol coating, the longer alkyl chain length promoted a shielding of the electrostatic forces and was associated with a less intense interaction between bacteria and quaternary ammonium coating [81]. Conversely, other authors show strong antimicrobial activity of quaternized-polyethyleneimine derivatives against gram-positive bacteria with different alkyl chain lengths, but a more limited activity against gram negative bacteria [87]. Nuzhdina, A. *et al.* (2017) reported that, for *S. aureus*, the increase in the length of the alkyl chain promoted hydrophobicity and consequently the capacity of quaternized polyethyleneimine derivatives to penetrate the hydrophobic bacterial membrane. However, for *P. aeruginosa*, the structure of its bacterial cell membrane contributed for increased resistance since the absence of efficient porin channels and the low permeability of the outer membrane influenced the penetration of both hydrophilic and lipophilic antimicrobial agents [87]. Similar results on the influence of alkyl chain length in antimicrobial activity were obtained by Bao, Y. *et al.* (2017), not only for bacteria but also for different fungal strains. Indeed, for *Aspergillus flavus*, a C14 chain length showed the strongest antifungal activity in a range from C10 to C16. The authors suggest that this type of cationic surfactants interact with biological membranes by electrostatic and hydrophobic interaction, and too long or too short chains can promote a decline of hydrophobicity, absorbability, and electron density [89].

To demonstrate the versatile use of QAC, Druvari, D. *et al.* (2016) developed a polymeric-quaternary ammonium coating with dual contact and release antimicrobial action. For this, different series of copolymers were synthesized by free radical copolymerization and incorporated with biocide agents. Two series of copolymers: poly(4-vinylbenzyl chloride-co-acrylic acid) (PVBC-co-AAx) and poly(sodium 4-styrenesulfonate-co-glycidyl methacrylate) (PSSNa-co-GMAx) were prepared. The reactive groups, AA and glycidyl methacrylate (GMA) were covalently (by 4-vinylbenzyl dimethylhexadecylammonium chloride, VBCHAM) or electrostatically attached (by cetyltrimethylammonium 4-styrenesulfonate, SSAmC16) on a polymer backbone to produce P(VBCHAM-co-AAx) and P(SSAmC16-co-GMAx) antimicrobial polymers. Their results show that, varying the composition of binding agents, the antimicrobial activity of the polymers was affected. A SSAmC16 content of 20 and 40% showed a good antimicrobial property with 5.4 and 5.3 Log reduction against *P. aeruginosa* and *S. aureus*, respectively. Moreover, release studies suggested a faster and easier release of biocidal content when in saline solution than in pure water. These

results suggest that coating materials can have a dual antimicrobial action, specifically in antifouling applications [90].

In addition to the antibacterial activity, QAC have also been reported as having antifungal and antiviral activity. Peng, Y. *et al.* (2022) described the antifungal activity of tetrahydrogeranyl quaternary ammonium, composed by different active groups: *N,N*-diethyl, *N,N*-dimethyl, and *N*-morpholine [91]. In the same way, Oblak, E. *et al.* (2013) reported the antifungal properties of QAC composed by chloride and bromide anions with different alkyl chains against [92]. Recently, the United States Environmental Protection Agency (US EPA) recommended the use of QAC to inactivate the SARS-CoV-2 (COVID-19) virus due to the susceptibility of the viral lipid layer to QAC's hydrophobic properties [84].

Other QAC immobilized on polymer substrates and their functions are summarized in Table 2, including their antibacterial, antifungal, and/or antiviral activity.

Table 2 – State-of-the-art of quaternary ammonium compounds (QAC) as an antimicrobial agent immobilized on polymeric substrates.

Compound and polymer substrate	Aim/Application	Microorganism	Reference
QAC-polyurea and PEI coating	Production of a shape-adaptive and contact-killing coating	<i>S. epidermidis</i>	[85]
PEI derivatives synthesized with octyl, dodecyl, or hexadecyl bromides	Development of an easy and simple low-cost non-covalent antimicrobial coating	<i>S. aureus</i> and <i>P. aeruginosa</i>	[87]
PVDF membrane with QAC and an interlayer composed by PDA/PEI and silica	Production of an antifouling membrane	<i>S. aureus</i> and <i>E. coli</i>	[93]
QA chloride and bromide salts combined with a polyethylene surface	Study the physicochemical and antibacterial activity of extruded polyethylene samples with QAC	<i>S. aureus</i> , <i>E. coli</i> , and <i>P. aeruginosa</i>	[94]
Residues of QA bromide immobilized on PUR surfaces	Production of a PUR surface modified with QAC for biomedical applications	<i>S. aureus</i> , <i>P. aeruginosa</i> , and <i>S. epidermidis</i>	[95]
QA salts onto a PUR surface and a PEG/lysine antifouling sub-layer	Development of contact-active antibacterial surfaces for artificial surfaces	<i>S. aureus</i> and <i>E. coli</i>	[96]
PUR coating with QA groups	Production of a polymer coating for HEPA filters	<i>E. coli</i> , <i>S. aureus</i> and <i>P. aeruginosa</i> and resistant strains to vancomycin (<i>S. aureus</i> and <i>E. Faecalis</i>) and methicillin (<i>S. aureus</i>). A clinical isolate of <i>Serratia marcescens</i>	[97]
Nanopatterned PNIPAAm brushes with QA salts	Production of a nanopatterned thermos-responsive material with dual function: biocide activity and release of dead cells	<i>E. coli</i>	[98]

QAC based on benzophenone long chain groups attached to different polymer substrates (PP, PE, PS, PVC, and polyether ether ketone)	Production of antimicrobial coatings on plastic biomedical devices to prevent biofilm formation and preventing the spread of HAI	<i>Arthrobacter sp.</i> , <i>Listeria monocytogenes</i> and <i>P. aeruginosa</i>	[99]
Quaternary dimethylammonium compounds bearing n-alkyl and oxyethylene groups onto PUR resin	Development of a self-decontaminating surface	<i>S. aureus</i> and <i>E. coli</i>	[100]
Tertiary amino groups immobilized on PDMAEMA/PTMSPMA segments	Production of a non-leaching antibacterial glass Surfaces	<i>E. coli</i>	[101]
Poly-quaternary amines ATRP	Development of active anti-fungal surfaces	<i>A. niger</i>	[102]
Bis-quaternary ammonium or quaternary salts synthesized on water-soluble polymer based on poly(aspartate-co-succinimide)	Development of antimicrobial surfaces	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>Salmonella typhi</i> , <i>C. albicans</i> , <i>A. niger</i> , <i>Cryptococcus neoformans</i> and <i>A. flavus</i>	[103]
DMMEP/DCAMA polymers with QA groups	Production of antimicrobial and cytocompatibility copolymers for biomedical applications	<i>S. aureus</i>	[104]

Legend: DCAMA – dipicolyl aminoethyl methacrylate; DMMEP – dimethyl (methacryloxyethyl) phosphonate; HAI – Healthcare associated infection; HEPA – High-efficiency particulate air; PDA – polydopamine; PDMAEMA – poly(2-(dimethylamino)ethyl methacrylate); PE – Polyethylene; PEG – poly(ethylene glycol); PEI – polyethyleneimine; PNIPAAm – poly(*N*-isopropylacrylamide); PP – polypropylene; PS – Polystyrene; PTMSPMA – poly(3-(trimethoxysilyl)propyl methacrylate); PUR – polyurethane; PVC – polyvinylchloride; PVDF – polyvinylidene fluoride; QA – Quaternary ammonium and QAC – Quaternary ammonium compounds.

Despite the successful development of bactericidal products, some bactericidal agents can exhibit cytotoxicity. To overcome this issue, different strategies have been developed. Specifically, Yan, S. *et al.* (2016) developed a hierarchical dual function system for biomedical applications composed by an antifouling bottom layer of PEG and a bactericidal top layer with QAC. This hierarchical system exhibits excellent antibacterial activity at long term applications (7 days), high biocompatibility (inhibiting the platelet adhesion and activation) and low toxicity against mammalian cells. In this system, the high surface density PEG acted as a hydrated barrier to prevent attachment of microorganisms, while the QAC layer exhibited a direct bactericidal or bacteriostatic effect. The use of high-density PEG allowed the reduction of the amount of QAC required to induce antibacterial properties, improving the biocompatibility of this hierarchical system to mammalian cells [105].

b) Metal-based compounds

Proof of antimicrobial action of metal-based compounds dates to ancient times, when Phoenicians, Romans, and Greeks used these agents to store and prevent the contamination of water. Additionally, in the pre-antibiotic era, some Cu-based compounds were used to treat tuberculosis and certain skin problems [106]. Metal-based compounds are essential elements for biomedical applications, in particular in medical devices, such as orthopedic implants, bone fixators and artificial joints. Metals can be used for the development of stents in cardiovascular problems and in dentistry for restorations, orthodontic wires, and dental implants [107]. In addition, due to their physical properties (high strength, elasticity, fracture toughness and high electrical conductivity) [107], metal compounds are an important raw material used in daily equipment and technologies, as well as in the electronic industry.

Metal-based compounds are one of the most used raw-materials in biomedical applications, especially when considering their antimicrobial activity and low toxicity. The basis of the antimicrobial action of metal compounds includes: i) inhibition of metabolic pathways by disruption of proteins and enzymes; ii) Reactive oxygen species (ROS) production by the Fenton-like reaction, in which the ions (for example from Cu) react with bioproducts of aerobic microorganisms (hydrogen peroxide H_2O_2 and superoxide O_2^-) producing hydroxyl radicals that can ultimately lead to peroxidation of the cellular machinery causing cell death [108]; iii) direct damage on cell membranes and iv) prevention of the uptake of important elements for microbial survival [109].

The differential efficacy of nine pure metals was investigated by Yasuyuki, M. *et al.* (2010), including titanium (Ti), cobalt (Co), nickel (Ni), zirconium (Zr), molybdenum (Mo), tin (Sn), lead (Pb), and Zn, by using a film contact-method by placing the inoculum over the test metal surface. The authors conclude that Co, Ni, Cu, Zn, Zr, Mo, and Pb showed increased efficacy in controlling bacterial burden while, Ti and Sn show no significant reduction in bacterial burden [110]. Considering the great potential of metals as antimicrobial agents, next sections will review the the most promising applications of antimicrobial metal-compounds, Cu, Ag, and Zn.

i) Copper-based compounds

As mentioned above, Cu is one of the oldest reported metals to be used as antiseptic agents to sterilize water and treating wounds. The word Cu derives from the Latin word *cuprus*, that is related to Cyprus, where Cu was found in large quantities [111]. The first historical evidence of Cu as a bactericidal agent remotes to 3200 BC, in Smith's Papyrus, and suggested the application of Cu to treat burns, skin diseases and warts [112]. Hippocrates (Greek physician) recommended the application of Cu-containing wound dressings or spray dry Cu powder to treat leg ulcers, and infections [112]. More recently, Cu was used in Europe to control the cholera epidemics in the 19th century. Despite the demonstrated benefits of Cu, the limited understanding of the mechanisms of antimicrobial action, and the advent of antibiotics in the medical field at the middle of the 20th century, led to decrease its application as bactericidal agent [112]. Cu is involved in many biological functions of living organisms [113]. Indeed, over 30 proteins have been shown to contain some elements of Cu. Examples include lysyl oxidase, tyrosinase, dopamine β -hydroxylase, which are involved in catecholamine pathway; and cytochrome-c oxidase which is involved in the respiratory chain acting as a terminal electron acceptor [114]. If Cu is an essential element for cell survival, at high concentration of this element can be toxic and lead to bacterial death. Given its high biocompatibility and antimicrobial properties, Cu has been used as an antimicrobial agent on coated surfaces in hygiene sensitive fields, such as in hospitals to reduce microbial contamination [113].

The generation of ROS released by Cu ions is the main antimicrobial mechanism attributed to Cu [106]. This mechanism causes oxidative stress that not only promotes the loss of structural integrity of bacterial membranes, but also increases its permeability, inducing lipid and protein peroxidation. ROS can also cause the conformational disruption of nucleic acids through the generation of hydroxyl radicals, due to the direct interaction of Cu ions with cellular constituents,

such as enzymes, proteins and ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) [106,115]. In fact, it was suggested that the degradation of DNA, is a secondary effect of Cu ions release from Cu containing surfaces. Wie, X. *et al.* (2014), showed that Cu salt-surfaces effectively kill bacteria more rapidly than Cu-surfaces, probably due to the easier release of Cu ions in the salt form. Indeed, the authors showed that Cu-polymer coatings need 8 h to kill all bacteria, while polymer-CuCl₂ only required 20 min to reach a similar effect. Under electron microscopy, *E. coli* exposure to the polymer surface exhibited a smooth surface without loss of integrity. In contrast, cells presented a shrinkage in their original size with deformation and abnormalities on their surface after contact with Cu-polymer and Cu salt-polymer surfaces. Moreover, bacteria exposed to Cu salt-polymer surfaces showed holes in their membranes promoting the leakage of cell content. The bacterial damage is dependent on the release of the Cu ions. Beyond the damages promoted by Cu ions on membranes, Cu can also bind to DNA and establish cross-linking within the DNA double helix chain causing its fragmentation. At least two binding sites for Cu ions with the DNA chain have been identified [116]. A similar mechanism based on the degradation of viral genome by ROS production from Cu materials was proposed against *Herpes Simplex Virus Type 1* [117]. On the other hand, Martinez, A. *et al.* (2022) suggested antifungal properties for Cu-materials through the action of Cu²⁺ ions on the fungal membrane causing alterations in the morphology and density of the cell wall. Indeed, upon exposure of *C. albicans* to Cu elements, the authors identified a separation of the cytoplasmatic membrane from the cell wall and membrane fragmentation as well as organellar swelling similar to what is observed in a necrotic scenario [118].

Ozkan, E. *et al.* (2016) reported the production of a film based on Cu elements, that combined a dual function: anti-adhesive properties promoted by super hydrophobicity of the surface and the antimicrobial activity of Cu. For this, Cu-nanoparticles were incorporated in a PDMS matrix by an aerosol assisted chemical vapor deposition technique. The coated PDMS surface showed a low-surface energy with a very rough surface. The anti-adhesion assays showed a reduction in the number of adherent gram-positive bacteria of 30% and 80-87% on PDMS and Cu/PDMS surfaces, respectively, relative to a control glass surface. Regarding the antibacterial activity, the authors report a 2.3 Log reduction in *E. coli* bacterial burden after 10 min of exposure to Cu-PDMS surfaces and a 4 Log reduction after 15 min of exposure. For *S. aureus* the bacterial Log reduction was 0.69 after 15 min and 1.4 Log reduction after 45 min and >4 Log reduction after 1 h [119]. Another example of antimicrobial action of Cu was reported by Kredl, J. *et al.* (2016). In this work, the authors developed an inorganic antimicrobial surface based on the

deposition of thin Cu films on acrylonitrile butadiene styrene substrate by atmospheric pressure jet plasmas devices. The materials produced (7% of Cu) showed a reduction of bacterial attachment of 90% [115].

A summary of the of the large spectrum of Cu bioactivity is presented in Table 3.

Table 3 – Examples of antimicrobial applications of copper in polymeric substrates.

Application	Microorganism	Reference
Cu nanoparticle in polymer thin films	<i>E. coli</i>	[120]
Cu (II) ions were immobilized on P4VP	<i>E. coli</i>	[121]
PCL/Cu fibers	<i>C. albicans</i> , <i>C. glabrata</i> , and <i>C. tropicalis</i>	[122]
Cu particles were combined with methylacrylate and ethylmethacrylate or silicon polymer	<i>E. coli</i> , <i>S. parvulus</i> and <i>B. subtilis</i>	[123]
Cu acetate combined with chitosan	<i>F. graminearum</i>	[124]
CuNPs in a PVA colloidal solutions	<i>Corticium Salmonicolor</i>	[125]
Cu oxide into PP layers	<i>Human influenza A virus</i> and <i>avian influenza virus</i>	[126]
Cu and CuO nanoparticles PMMA	<i>HCoV-OC43</i> <i>human coronavirus</i>	[127]

Legend: Cu – copper; CuNPs – copper nanoparticles; CuO – cupric oxide; P4VP – poly(4-vinylpyridine); PCL – polycaprolactone; PMMA – poly (methyl methacrylate); PP – polypropylene and PVA – poly (vinyl alcohol).

ii) Silver-based compounds

For a long time, Ag and Ag-based compounds were used as antimicrobial agents. In the 18th and 19th century, Ag compounds were used in medicine to treat infectious diseases, wound antiseptis applications and treatment of burn wounds [128]. Despite the reduction in the use of Ag compounds in medicine, mainly related to the advent of antibiotics, these compounds have been gaining new interest as an antimicrobial agent, due their attractive physical and functional properties, such as a high thermal and electrical conductivity. Approved in 1968 by the Food and Drug Administration (FDA), Silvadene® is one of the first medical drugs based on Ag extracts indicated to treat burn wounds. This drug is based on a complex with Ag sulfadiazine which acts as a reservoir of ions of Ag which are slowly released to the target [128].

Ag is described as an inert element but becomes easily ionized in the presence of oxygen, moisture, and biological fluids. This ionized form of Ag (Ag^+) is described as having an important role since it can react with thiols, phosphates, and other anionic groups of proteins and cell components [129]. As a result of this interaction, Ag^+ can easily penetrate the cell wall and lead to the denaturation of proteins, which in turn results in the inactivation of metabolic pathways, disruption of the microbial cell membrane, leakage of cellular contents and, ultimately, cell death

[28,129]. Furthermore, Ag ions can act by producing ROS, which can target lipids, proteins, DNA and RNA [128]. The activity of Ag also depends on the release rate of Ag ions, water solubility and lipophilic and redox properties [128]. To prevent this rapid clearance of Ag⁺ ions and have a sustained antimicrobial action, these ions can be immobilized on a polymeric surface. This strategy is particularly useful in long-term applications. Riau, A. *et al.* (2019) described the immobilization of nano-silver (nAg) and nano-hydroxyapatite (nHAp) on PMMA substrate for corneal prosthesis application [129]. In this work, the authors found that a composition with 95:5 nHAp:nAg content prevented biofilm formation of both gram-negative and gram-positive pathogens, and not exhibiting toxic activity to the human corneal stromal fibroblasts [129]. For long-term use (incubation for 28 days), the selected composition was capable of killing >99% of *P. aeruginosa* [129]. Other reports using Ag as a nanomaterial on a polymer matrix on a 70/30 poly (butylene-co-adipate terephthalate)/Poly(lactic acid) (PBAT/PLA) reports a bacteriostatic effect of Ag agents against *Listeria monocytogenes* and *Salmonella enteritidis* [130]. Scuri S. *et al.* (2019) reports the production of two additives, based on silver (I) acyl pyrazolonate, immobilized on polyethylene substrates [28]. The results showed that both materials have a large spectrum of activity, targeting both gram-positive (*S.aureus*) and gram-negative (*E. coli*) bacteria as well as fungus (*C. albicans*), with a minimum Log reduction, of 5, 1.5 and 0.5, respectively [28]. Using the same polymer matrix, Harrasser, N. *et al.* (2016) determined the antimicrobial effect of polyethylene polymeric surfaces treated with Ag. Despite some differences in antimicrobial activity between the techniques used to immobilize Ag ions on the polymer surface, both Ag-coated materials were associated with a reduction of bacterial adhesion of *S. epidermidis* (between 1.2-2.2 Log reduction) [131]. Additional evidence of Ag's antimicrobial action was reported by Khalilpour, P. *et al.* (2019), in which Ag particles were immobilized on SiO_xC_y plasma polymer coating. The Ag/SiO_xC_y was produced by physical vapour deposition technique using hexamethyldisiloxane as a precursor. The antimicrobial assays were performed against *S. aureus*, *S. epidermidis*, and methicillin-resistant *S. aureus* (MRSA) using three different protocols: ASTM E-2810, JIS Z 2801, and proliferation assay. All tests showed a good antimicrobial activity (99.99%) in comparison with non-coated samples. The biocompatibility with L-929 mouse fibroblast cells was tested after direct and indirect contact according to ISO 10993-5. In both cases, Ag/SiO_xC_y showed high biocompatibility [3].

Other polymers used to immobilize Ag-based compounds include poly (vinyl alcohol) [133], poly (N-isopropylacrylamide) (PNIPAAm) [134], polyethylene terephthalate (PET) [135], and PS [136]. Despite this, some non-polymer matrices, such as nanosilica, have also been reported to

immobilize Ag-compounds [137]. At a nanoscale range it is possible to control and improve new functionalities without an influence on the bulk properties [137].

The use of Ag as an antimicrobial agent is clearly evident, as reflected in the increasing number of registered patents over the years. For instance, in just a period of 10 years, the number of Ag-related patents increased over 6-fold, with 200 patents in 2007 to approximately 1300 in 2017, spanning various fields such as medicine, personal care products, household items, industrial applications, and agriculture [138]. However, the accelerated and widespread use of Ag-based products, concerns on their accumulation in ecosystems have been emerging. Chronic exposure to Ag, coupled with elevated environmental concentrations, can have detrimental effects on several environmental communities, including bacteria, fungi and other decomposers, significantly impacting vital biogeochemical cycles involved in the maintenance of a balanced ecosystem [138]. By understanding the mechanisms of resistance and comprehending the true impact of Ag on ecosystems, new solutions or approaches can be developed to minimize or mitigate the excessive use of Ag.

iii) Zinc-based compounds

Zn is one of the most abundant metalloproteins found in the human body. The majority of Zn is localized in myocytes and corresponds to 85% of Zn content in the human body [109]. Zn is also present in the dental plaque and in the saliva [140]. Zn plays an important catalytic (as a coenzyme) and structural role together with a large number of proteins, thus allowing the interaction of the protein subdomains with other molecules [109]. It was also suggested that Zn has an important catalytic role for coenzyme activity and Zn ions can promote proton permeability of the cell membrane, while also having an inhibitory effect on several metabolic pathways, such as glycolysis, glucosyltransferase production and polysaccharide synthesis [140]. Specifically, it has been described that Zn ions can act by reducing the adenosine triphosphate (ATP) synthesis and F-type adenosine triphosphate synthases (F-ATPase) activity due to its ability to inhibit the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenases [140].

The described mechanisms of Zn include: the generation of ROS, disruption of cell membranes, selectively binding to proteins causing the downregulation of gene expression [109]. Accordingly, Tiwari, V. *et al.* (2018) studied the mechanism of zinc-oxide (Zn-oxide) in a multi-drug resistant opportunistic pathogen, *A. baumannii* and confirmed a 4-fold increase in ROS production and a 2-fold increase in bacterial lipid peroxidation after treatment with Zn-oxide. Moreover, Zn-

oxide treatment led to a 1.5-fold reduction in sugars, due to membrane leakage as well as an increased of protein and DNA leakage through cell membrane of 1.4 and 1.3 times higher, respectively when compared to control groups. These results suggest that Zn can act at the cellular membrane level, causing its disruption [141]. The antimicrobial property of Zn is also reported by Vázquez, K. *et al.* (2021). In this work, the authors produce functional fibers based with ZnO nanoparticles into recycled PET (r-PET), with the functionalization resulting in a great antibacterial activity against *B. subtilis* and *E. coli*, and a potent antifungal activity, against *Fusarium graminearum* and *Penicillium* species [142]. Another publication reports the development of an antimicrobial polymer system based on Zn-oxide structures on nanotubes or nanoparticles on acrylic/PE substrate. For the same concentration of Zn (1%) both materials show a potent antimicrobial against *E. coli* and *S. aureus* exhibiting more than 3.18 and 1.27 Log reduction [143]. Despite acting as a substrate for immobilization of antimicrobial materials, the polymers can actually be used to increase the antimicrobial performance of metal compounds. Confirming this observation, Ammar, H. *et al.* (2021) reported an increase in the antifungal activity of Zn nanoparticles against six common fungal species found in food spoilage, when associated in a PEGylated system. This could be due to the high surface charge combined with electrostatic and steric stabilization provided by PEG prevented the aggregation of Zn nanoparticles and improved nanoscale properties over time [144].

iv) Other metal elements

In addition to the metals previously described, other elements are known to have antimicrobial action, including the transition metals of the d-block group, and metalloids from groups 13–16, such as aluminium (Al), gallium (Ga), germanium (Ge), arsenic (As), selenium (Se), antimony (Sb), tellurium (Te), and bismuth (Bi), and Pb [145]. However, some elements may have legal restrictions due to potential health effects. Examples of these elements and their antimicrobial action are summarized in Table 4.

Table 4 – State-of-the-art of metal-based compounds as an antimicrobial.

Metal based compounds	Substrate/Polymer System	Microorganism	Application	Reference
Metal Nanoparticles (Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge and Se)	MNPs immobilized into sol–gels coating	<i>E. coli</i>	Prevention of biofouling of microorganisms, plants and animals on artificial surfaces immersed in a common matrix	[146]
Mg	Mg immobilized on P(ISA-co-DMA)/TA surfaces	<i>S. aureus</i> and <i>E. coli</i>	Production of a biomedical material with dual function: antimicrobial properties and corrosion resistance	[147][4]
Ti and Ti/Au	TiO ₂ and Au-TiO ₂ nanoparticles were encapsulated in polysiloxane by a swell dip-coating technique	<i>S. aureus</i> and <i>E. coli</i>	Production of a functional surface to potentially reduce the HAI	[148]
Au	Produce an organic/inorganic coating based on functionalized PUR with gold nanorods and PEG	<i>P. aeruginosa</i> , and, <i>S. aureus</i>	Production of a hybrid coating with antifouling and bactericidal properties for medical devices to treat infected hernia	[149]
Mn(II), Co(II), Ni(II), Cu(II) and Zn(II)	Base Schiff polymers with formaldehyde and piperazine	<i>E. coli</i> , <i>S. aureus</i> , <i>B. subtilis</i> , <i>C. albicans</i> , <i>M. canis</i> , and <i>C. neoformans</i>	Production of thermally resistant antimicrobial and antifouling coatings for packaging materials	[150]
Mn(II), Co(II), Ni(II) and Cu(II)	Polymeric metal-organic frameworks with AFP	<i>E. coli</i> , <i>P. fluorescence</i> , <i>B. subtilis</i> , <i>S. aureus</i> and <i>S. typhi</i>	Production of metal-organic coordination polymers (p.e. electronic devices)	[151]
Co	Synthesis and stabilization of Co nanoparticles using cellulose, sodium borohydride and hydrogen	<i>S. aureus</i> , <i>E. coli</i> , <i>A. baumannii</i> and <i>P. aeruginosa</i>	Development of materials for treatment of wastewater or medical applications	[152]

Ni	Polyrhodanine/nickel ferrite nanocomposite were synthesized via a co-precipitation approach	<i>S. aureus</i> and <i>E. coli</i>	Design of a new antimicrobial agent	[153]
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Legend: AFP – 5,5'-(piperazine-1,4-diylbis(methylene))bis(2-aminobenzoic acid); Au – gold; Co – cobalt; Cr – chromium; Cu – copper; Fe – iron; Ga – gallium; Ge – germanium; HAI - healthcare-associated infections; Mg – magnesium; Mn – manganese; MNPs – metal nanoparticles; Ni – nickel; P(ISA-co-DMA)/TA – poly(isobornyl acrylate-co-dimethylaminoethyl methacrylate)/tannic acid; PEG – poly(ethylene glycol); PUR – polyurethane; Sc – scandium; Se – selenium; TA – tannic acid; Ti – titanium; V – vanadium; and Zn – zinc.

c) Antimicrobial peptides

The first antimicrobial peptides (AMP) were discovered in 1939, by René Dubos, from a *Bacillus* strain isolated from the soil. Dubos identified the antimicrobial properties of gramicidin when tested in a pneumococcal mouse infection. After this landmark, several AMP were reported, such as tyrocidine (produced by bacteria *Bacillus brevis*), purothionin (identified in the plant *Triticum aestivum*), defensin (isolated from rabbit leukocytes) and lactoferrin (identified in cow milk). Actually, more than 3000 AMP have already been identified and characterized [154]. AMP are bioactive proteins, naturally produced by living organisms and represent an important mechanism of defence in animals and plants. In animals, AMP have already been isolated from fish, amphibians, insects, and mammals, and they mainly act in protection against microbial infections. In plants, AMP are also involved in microbial defence but are also involved in cellular signalling [154]. Considering AMP's antimicrobial properties, broad spectrum of action, high biocompatibility, and reduced capacity to generate resistance, these proteins have been considered as good alternative to antibiotics [155].

A typical structure of AMP comprises 10-50 amino acid residues with a positive charge [156], although some authors consider AMP as peptides of less than 100 amino acid residues [157]. Despite this, AMP have been characterized with two portions: a cationic portion with a positively charged amino acids, such as lysine and arginine (charge between +2 and +9), and a sub-unit composed by hydrophobic residues [157]. AMP can be found in a variety of structures and can be classified as α -helical; β -sheet; with one or more disulphide bridges or even with different chain lengths [156,157]. These diverse structural forms of AMP and different properties (size, geometry, charge, hydrophobicity, and peptide self-association to the biological membranes) enhance their broad spectrum of action [157].

The mechanism of action of AMP is primary attributed to electrostatic interaction of the peptides with lipids present on biological membranes, which are negatively charged. The positively charged residues of AMP can interact with lipopolysaccharides and teichoic acids, in gram-negative and gram-positive bacteria respectively, increasing their permeability and promoting the release of cellular content, ultimately resulting in bacterial lysis [155,156]. The cationic groups can include ammonium, sulfonium and phosphonium ions [158]. On the other hand, the AMP hydrophobic groups can be comprised of linear or cyclic alkyl groups with different chain lengths. It has been suggested that increasing the chain length decreases the antimicrobial activity [158]. Zhang, Qi-Yu *et al.* (2021) describes two modes of action for AMP interaction: non-membrane action and direct

membrane action that can be further subdivided into 4 distinct models of membrane-cavity formation, including the barrel-stave, toroidal-pore, carpet and aggregate models. These models are present in Figure 13.

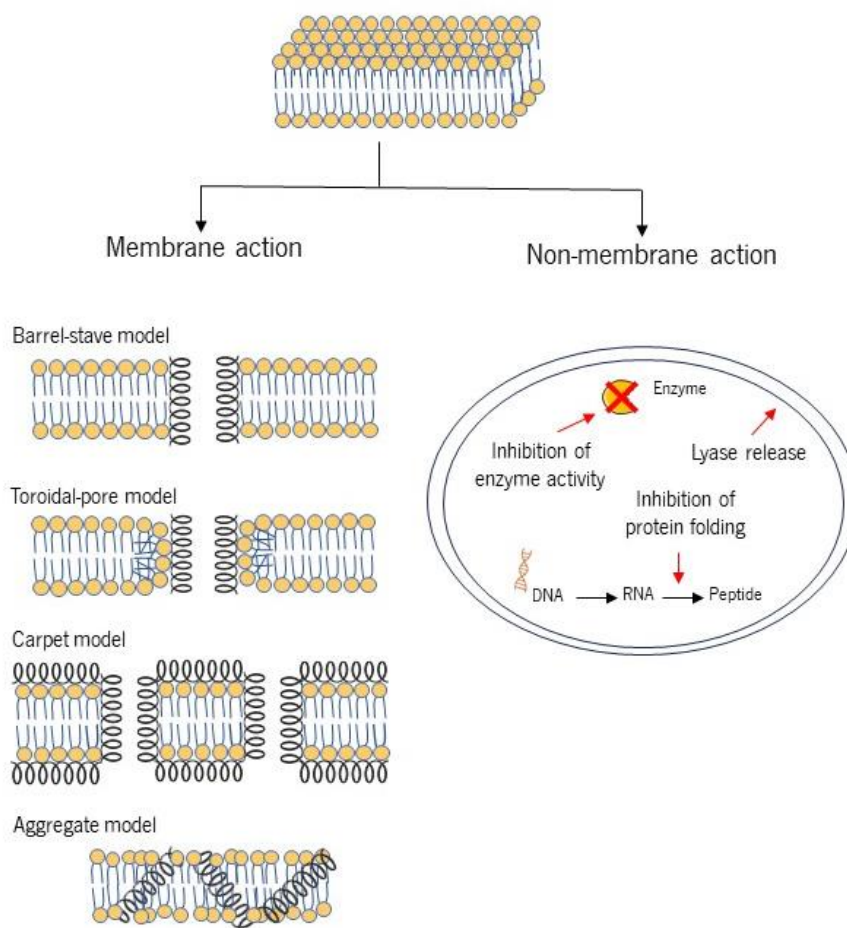


Figure 13 – Proposed models for antibacterial mechanisms of AMP: the direct membrane action promotes the permeability and conformational changes in the cellular membrane and the non-membrane action results in the penetration of AMP into cytoplasm inhibiting the synthesis of DNA, RNA or the enzymatic activity. Adapted from Zhang, Qi-Yu *et al.* (2021) [159].

In a barrel-stave model, AMP bind to the membrane, promote conformational changes, leading to the shift of phospholipid head groups and membrane thinning. The hydrophobic regions of the peptides will be localized close to the hydrophobic regions of the membrane phospholipid, while the hydrophilic regions of the peptide will be positioned in parallel to form the central lumen. The toroidal-pore model is similar to the first model, but the peptides are inserted into a membrane and bind with lipids to form toroidal pore complexes. At high concentrations, AMP induce the bending of the lipids, thus enabling the intersection of peptides and the lipid head groups inside of the lipid hydrophobic zone. The carpet model suggests that in high concentrations of AMP, the

electrostatic effects of peptides act on the anionic membrane causing its rupture in a surfactant-like manner, forming micelle-like structures and ultimately promotes membrane disruption. Finally, in the aggregate model, AMP bind to the membrane and form a channel that allows the intracellular content to leak out, causing cell death. This last mechanism allows the interaction of AMP with intracellular components. In fact, in the same report, Zhang, Qi-Yu *et al.* (2021), suggest that AMP can act within the cytoplasm and interact with intracellular substances, inhibiting the synthesis of DNA, RNA and proteins synthesis [159].

In Table 5 it is provided a list of examples of immobilization of antimicrobial peptides on polymer substrates.

Table 5 – Examples of immobilization of antimicrobial peptides on polymer substrates.

AMP and Substrate	Application	Microorganism	Reference
Hydrophobic peptide with terminal cysteine reactive groups coupled to PEG	Prevention and inhibition of biofilm formation	<i>P. aeruginosa</i> and <i>E. coli</i>	[160]
AMP (labeled with cysteine) immobilized on PUR substrate	Prevention of biofilm formation associated to CAUTIs	<i>P. aeruginosa</i> , <i>S. aureus</i> and <i>S. saprophyticus</i>	[161]
Two arginine/lysine/tryptophan-rich AMP on AGE and PDMS surface	Development of an AMP-based coating substrate to prevent CAUTIs	Bacteria strains: <i>E. coli</i> , <i>S. aureus</i> and Fungi <i>C. albicans</i>	[162]
Antimicrobial peptide KR12 were immobilized on a starch-based macroporous sponges	Production of a homeostatic and antimicrobial material for intraoperative wound	<i>S. aureus</i> , <i>S. epidermidis</i> , <i>E. coli</i> , and MRSA	[163]
Synthetic β -peptide polymer modified with PA beads	Production of a material with dual function: bacterial killing and endotoxin adsorption for production/manufacture of food, water, drinks, and injections	<i>S. aureus</i> and <i>E. coli</i>	[164]
AMP derived from GH12 were conjugated with methacrylate monomers	Development of an oral adhesive for treatment of tooth decay and improve the durability of dental composite restorations	<i>S. mutans</i>	[165]
Cecropin B were covalently immobilized on a pH-responsive PMMA	Development of a bioactive responsive surface for biomedical applications	<i>S. aureus</i>	[166]

Legend: AGE – allyl glycidyl ether polymer; AMP – antimicrobial peptides; CAUTIs – catheter-associated urinary tract infections; MRSA – Methicillin-resistant *S. aureus*; PA – polyacrylate; PDMS – poly(dimethyl siloxane); PEG – Poly(ethylene glycol) and PMMA – poly (methyl methacrylate).

d) Antibiotic-compounds

Despite the use of some natural compounds to treat open wounds in ancient societies, it was only in 1910 that the first antibiotic (commercial name Salvarsan¹) was clinically used by Paul Ehrlich to treat *Treponema pallidum*, the etiologic agent of syphilis. Almost two decades after, in 1928, Alexander Fleming discovered penicillin, an antibiotic that nowadays is still used in the clinic [167]. This landmark opened a new field in microbial natural products and the discovery of new antibiotics increased a lot in mid 1950s in an era called Golden Age [167]. Despite the increase in associated antibiotic resistance, antibiotics remain part of clinical routine and are the first line of treatment in many infectious diseases. There has been a decline in the discovery of new antibiotics and most related clinical trials involve new classes of synthetic, already known, antibiotics. In parallel, many strategies are being tested to deliver antibiotics to the site of action. Drug delivery systems (DDS) are basically an interface or physical substrate that facilitate and control the delivery of the drug to the target site by regulating its interaction with the micro-environment [168]. These strategies aim to limit the systemic circulation to non-target sites, and therefore decreasing secondary effects, while increasing the concentration at the site of interest. In Figure 14, different DDS are presented.

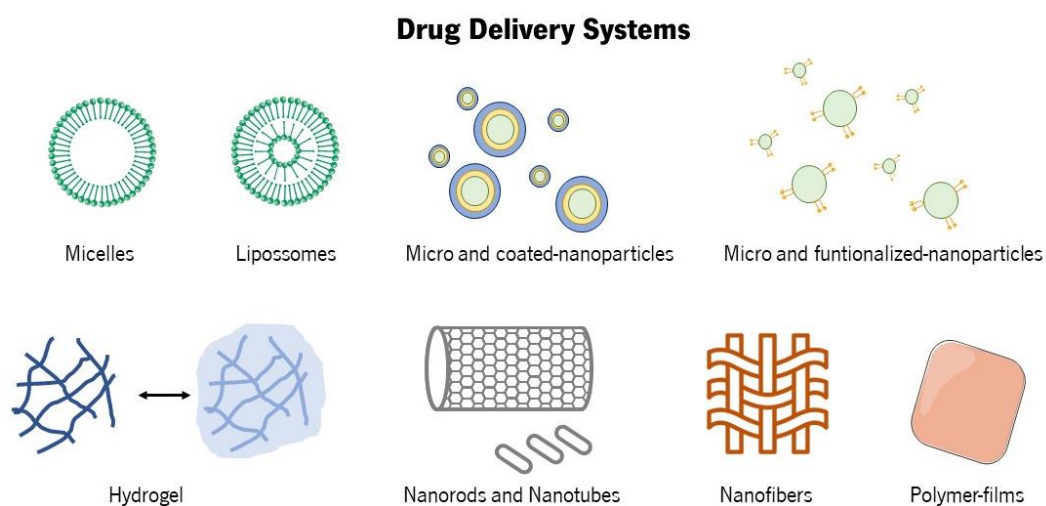


Figure 14 – Schematic representation of different drug delivery systems described in the literature.
Adapted from Vargason, A. *et al.* (2021) [168].

¹ Salvarsan is no longer used in clinical applications.

There are several publications regarding the use of polymer films as a substrate for the immobilization or encapsulation of antibiotics. Bottino, M. *et al.* (2016) reports the use of tetracycline (TCH) incorporated in polymer fibres for dental implants. TCH is an antibiotic that acts by inhibiting protein synthesis which prevents the attachment of aminoacyl-tRNA to the ribosomal acceptor site [169]. In this work, different concentrations of TCH were added into a polymer solution composed by PLA, polycaprolactone (PCL), and type-B gelatin with different ratios. The fibres were produced by electrospinning, followed by modification with Ti disks. The fibre mats showed a sustained drug release of at least 40% after 1 h, associated with significant antimicrobial activity against *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and *Fusobacterium nucleatum* and a moderate activity against *Prevotella intermedia* [170]. Another TCH-based compound (TCH-hydrochloride) was reported by Tamjid, E. *et al.* (2020) incorporated in PCL with antimicrobial activity against both gram-negative and gram-positive bacterial strains [171].

Many antibiotics have also been incorporated in polymer substrates, to produce biocide-releasing polymers. In this context, Monteiro, N. *et al.* (2015) developed functionalized electrospun nanofibers of chitosan with gentamicin-releasing liposomes. The liposomes were distributed at the surface of the nanofiber mesh with a sustained release of antibiotic during 16 h, while exhibiting antibacterial properties against *S. aureus*, *E. coli* and *P. aeruginosa* [172]. In another work Hegde, V. *et al.* (2018) reported a novel implant coating with biocide-release of vancomycin or tigecycline on a poly(ethylene glycol)-poly(propylene sulfide) substrate to treat infection after spinal cord implants. In the presence of bacteria, the ROS production by the host promotes a cascade of events, that allows the release of antibiotics from the polymer [173]. Both systems were able to control de bacterial infection 3 days after infection for vancomycin system while tigecycline system only significantly decreased the bacterial burden 5-10 post-infection [173].

Fosfomycin ($C_3H_7O_4P$) is another important antibiotic used in the clinical practice since 1970, mainly to treat urinary tract infections. This antibiotic acts at the bacterial cell membrane by disturbing the peptidoglycan pathway. Fosfomycin inhibits the enzyme (UDP-N-acetylglucosamine enolpyruvyl transferase) responsible for catalysing the formation of N-acetylmuramic acid, which is a precursor of peptidoglycan. Due to its mechanism of action, fosfomycin has a large spectrum of action and is even used for the treatment of infections with multidrug-resistant pathogens. Furthermore, considering that both gram-positive and gram-negative bacteria require the formation of N-acetylmuramic acid for the synthesis of peptidoglycan, the possibility of resistance for this

antibiotic is low and therefore increases its importance in clinical practice. Considering this, Kudzin, M. *et al.* (2020) produced an antimicrobial coating material with fosfomycin on nanofibers or textile of PLA by electrospinning or melt-blown techniques. The results show, for 0.005% of antibiotic in coating formulation, a 3-4 mm of inhibition growth for *E. coli* and 5 mm against for *S. aureus* [174].

Other antibiotics incorporated on polymers and their mechanism of action are summarized in Table 6.

Table 6 – State-of-the-art of antibiotics immobilized on polymer substrate and their mechanism of action.

Application	Mechanism of action	Polymer substrate	Microorganism	Ref.
Gentamicin, an aminoglycoside antibiotic family	Inhibition of protein synthesis by binding to the 30S ribosomal subunit	PMMA	<i>S. epidermidis</i>	[175]
Imipenem (β -lactams antibiotic) and tobramycin	Imipenem-mediated inhibition of bacterial cell wall synthesis by binding and inactivating transpeptidases (penicillin binding proteins), and the D-alanine carboxypeptidase activity [176]. Tobramycin-mediated inhibition of protein synthesis by binding to the A-site on the 16S ribosomal RNA of the 30S ribosome [177]	Alginate gel layers	<i>P. aeruginosa</i>	[178]
Rifampicin	Blocking of the elongation of RNA chain by binding to the β -subunit of the RNAP [179]	PUR	No applicable	[180]
		Amine-modified polystyrene surface	<i>S. epidermidis</i>	[181]
Rifampicin and minocycline	Minocycline-mediated inhibition of protein synthesis by acting on bacterial 30S ribosomal subunit [182]	Cyclodextrin polymer system	<i>S. aureus</i>	[183]
Ciprofloxacin	Inhibition of DNA replication by inhibition DNA gyrase [184]	Chitosan	<i>S. aureus</i> and <i>P. aeruginosa</i>	[185]
Metronidazole	Inhibition of proton activity and reduction of ATP production, by acting as an electron acceptor [185]	Chitosan-based mucoadhesive vesicles	<i>C. albicans</i>	[187]

Vancomycin, rifampicin, linezolid and daptomycin	Vancomycin-mediated inhibition of peptidoglycan layer maturation by binding to the alanine residues of peptides [188]; Linezolid-mediated inhibition of bacterial protein synthesis [189], and daptomycin-mediated membrane depolarization by insertion into the cellular membrane, with subsequent loss of intracellular components (K ⁺ , Mg ²⁺ and ATP) [190]	PLGA and PCL nanofibers	MRSA	[191]
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Legend: ATP – adenosine triphosphate; DNA – deoxyribonucleic acid; K – potassium; Mg – magnesium; MRSA – Methicillin-resistant *S. aureus*; PMMA – Poly(methacrylic acid); PUR – polyurethane; PLGA – poly(lactic-co-glycolic acid); PCL – Polycaprolactone and RNA – ribonucleic acid.

e) Phenolic-base compounds

Phenolic compounds (PC) are a large family of compounds widely distributed in nature, being one of the most abundant secondary metabolites of plants [192,193]. Structurally, PC can be described as one or more aromatic ring(s) bearing one or more hydroxyl group(s), including functional esters, methyl esters and glycosides [192,194]. PC can be classified in different groups according to their structure, specifically their functional groups and phenolic rings [194]. In the last years, phenolic based compounds have attracted much attention due to their antioxidant and antimicrobial properties [193]. In particular, their aromatic structure, along with hydroxyl substituents, confer PC the protective effect from damages caused by oxygen- and free-radicals, reducing the risk of cancer, cardiovascular and Alzheimer's disease, as well as diabetes [192]. Some examples of phenolic-based compounds and their classification were summarized in Figure 15.

Regarding PC's antimicrobial mechanism of action, it has been suggested that they mainly act on biological membranes, changing their permeability. PC can establish hydrogen bonds with enzymes or induce alterations in the cell wall rigidity, disrupting membrane integrity, causing the leakage of essential intracellular constituents [195]. It has been described that PC can also attach to microbial enzymes, reacting with their sulfhydryl groups or inhibiting protein activity [196]. It is also suggested that an increase of OH groups on PC can increase their reactivity and toxicity for microorganisms. Specifically, caffeic acid can act by donating an electron pair to functional groups on cellular membrane proteins or lipids, leading to alterations in membrane function [197]; carvacrol can insert into the cytoplasmic membrane, act as a protonophore, increase entropy and

stability of the membrane, eventually increasing the proton passive flux across the membrane [198]; while flavonoids can act by inhibiting nucleic acid synthesis, through the insertion of the aromatic ring or establishment of hydrogen bonding to nucleic acids, in order to inhibit DNA and RNA synthesis or by destabilizing the cellular membrane inducing alterations in the membrane fluidity [198]. Despite this general mechanism of action, the antimicrobial activity of PC may vary according to the octanol-water partition coefficient (Log P) related to the hydrophobic and amphiphilic property of the molecule; the number and position of OH groups; and presence of other chemical groups, such as acetate or aldehydes groups [195,196].

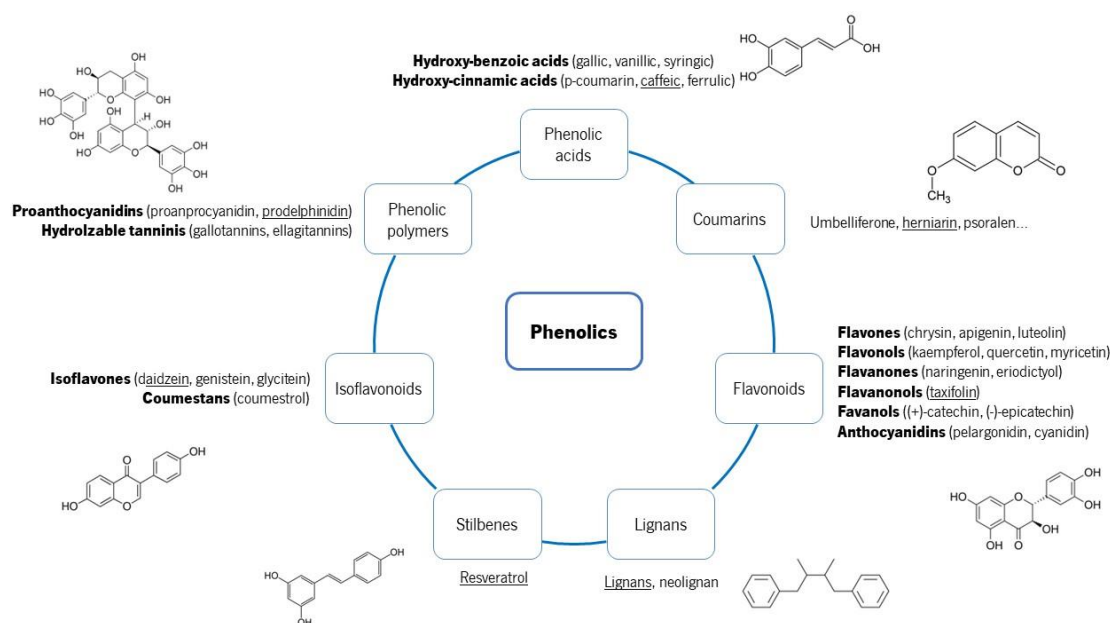


Figure 15 – Classification of phenolic compounds according to their structure: chemical structures of underline phenolic compounds are show. Adapted from Barba, F. *et al.* (2014) [194].

Some examples of PC and their antibacterial properties are shown Table 7. In addition to antibacterial properties, antifungal and antiviral activity of PC were also described. In addition to antibacterial properties, resveratrol has bioactivity against fungi: *Trichophyton spp.* *E. floccosum*, *M. gypseum* (25-50 µg/mL) and *C. albicans* (20 µg/mL), as well antiparasitic activity against *Leishmania amazonensis* [200]. Regarding the antiviral activity it has been suggested that caffeic acid can inhibit the multiplication of HSV-1 by acting on DNA replication [201] and 2-phenylphenol is described to act on viral membranes and denaturing viral protein [202].

Table 7 – Minimum inhibitory concentration of phenolic compound against different bacterial and fungal strains.

Phenolic Compound	Chemical Formula	Microorganism Strains	MIC (µg/mL)	Ref.
Phlorizin	$C_{21}H_{24}O_{10}$	<i>S. pyogenes</i> ATTC 49399	100	[203]
		<i>L. monocytogenes</i> CMCC54004	200	[204]
Naringenin	$C_{15}H_{12}O_5$	<i>S. pyogenes</i> ATTC 19615	50	[203]
Flavone	$C_{15}H_{10}O_2$	<i>S. pyogenes</i> ATTC 19615	50	[203]
Myricetin	$C_{15}H_{10}O_8$	<i>S. pyogenes</i> ATTC 19615	50	[203]
Thymol	$C_{10}H_{14}O$	<i>S. aureus</i> ATCC 29737	200	[205]
		<i>E. coli</i> ATCC 11345	400	[205]
		<i>P. aeruginosa</i> ATCC 12432	400	[205]
		<i>B. cereus</i> ATCC 16754	100	[205]
1,2-Naphthoquinone	$C_{10}H_6O_2$	<i>S. pyogenes</i> ATTC 19615	3.125	[203]
Resveratrol	$C_{14}H_{12}O$	<i>M. tuberculosis</i> H37Rv	100	[200]
		<i>H. pylori</i> ATCC 43504	25	[200]
		<i>N. gonorrhoeae</i> (clinical isolate)	75	[200]
		<i>A. cryaerophilus</i> LGM 10829	50	[200]
		<i>C. albicans</i> TIMM 1768	20	[206]
		<i>S. cerevisiae</i> KCTC 7296	10-20	[206]
Dequalinium chloride	$C_{30}H_{40}Cl_2N_4$	<i>S. pyogenes</i> ATTC 19615	0.39	[203]
Benzoic acid	$C_7H_6O_2$	<i>S. epidermidis</i> NCIM 2493	256	[207]
		<i>P. aeruginosa</i> ATCC 27853	512	[207]
Gallic acid	$C_7H_6O_5$	<i>P. multocida</i> ATCC 29702	250	[208]
		<i>M. haemolytica</i> ATCC 43137	500	[208]
		<i>S. aureus</i> CECT 976	1750	[209]
		<i>C. albicans</i> (not specified)	12.5	[210]
		<i>T. rubrum</i> (not specified)	43.75	[210]
Chlorogenic acid	$C_9H_{10}O_5$	<i>S. epidermidis</i> NCIM 2493	128	[207]
Syringic acid	$C_9H_{10}O_5$	<i>P. aeruginosa</i> ATCC 27853	128	[207]
Caffeic acid	$C_9H_8O_4$	<i>S. epidermidis</i> NCIM 2493	256	[207]
		<i>P. aeruginosa</i> ATCC 27853	512	[207]
		<i>B. cereus</i> EMCC 1080	1833	[211]
		<i>S. aureus</i> ATCC 13565	1666	[211]
		<i>S. typhi</i> ATCC 25566	583	[211]
		<i>F. verticillioides</i> (not specified)	50	[212]
p-Coumaric acid	$C_9H_8O_3$	<i>S. epidermidis</i> NCIM 2493	512	[207]
		<i>P. aeruginosa</i> ATCC 27853	256	[207]
Ferulic Acid		<i>S. epidermidis</i> NCIM 2493	512	[207]

	$C_{10}H_{10}O_4$	<i>E. coli</i> CECT 434	100	[209]
		<i>P. aeruginosa</i> ATCC 10145	100	[209]
		<i>S. aureus</i> CECT 976	1100	[209]
		<i>L. monocytogenes</i> ATCC 15313	1250	[209]
Tannic acid	$C_{76}H_{52}O_{46}$	<i>S. epidermidis</i> NCIM 2493	32	[207]
		<i>P. aeruginosa</i> ATCC 27853	32	[207]
Kaempferol	$C_{15}H_{10}O_6$	<i>S. epidermidis</i> NCIM 2493	128	[207]
		<i>P. aeruginosa</i> ATCC 27853	64	[207]
		<i>E. coli</i> (not specified)	25	[213]
Eugenol	$C_{10}H_{12}O_2$	<i>S. epidermidis</i> NCIM 2493	64	[207]
		<i>P. aeruginosa</i> ATCC 27853	32	[207]
Carvacrol	$C_{10}H_{14}O$	<i>S. Typhimurium</i> ATCC 14028	312	[214]
Chlorophorin	$C_{24}H_{28}O_4$	<i>F. verticillioides</i> (not specified)	6.25	[206]

To evaluate the antibacterial properties of PC, He, W. *et al.* (2021) developed a cellulose membrane incorporating tannic acid (TA) or TA with increasing amounts of magnesium chloride. The anti-biofilm activity was assessed by using an *in vitro* multispecies biofilm model based on a single culture of *S. aureus* and *P. aeruginosa* or a co-culture system with both strains. Using a CFU-method, the authors shown that the cellulose membranes had an inhibitory effect on biofilm formation, with the membrane without Mg exhibiting the strongest antibacterial performance (99.62 and 99.41% reduction against single cultures of *S. aureus* and *P. aeruginosa* and >80% for co-culture systems). The authors suggest that the introduction of Mg decreased the antibacterial properties after 24 h of culturing due the more controlled sustained TA release [215]. Polymers can also be used as a drug release system of bioactive compounds. An example with PC is reported by Gámez, E. *et al.* (2020) in which it is reported the incorporation of thymol on electrospun PCL nanofibers for wound dressing applications [216]. Another example is TA and cationic polylysine (PLYS) grafted on pH-sensitive poly(2-diisopropylaminoethylmethacrylate)-b-poly(2-methacryloyloxyethyl phosphorylcholine) (PDPA-b-PMPC) to produce antimicrobial and antifouling polymer brushes. The produced materials exhibit good antimicrobial activity against *S. epidermidis* and *E. coli*, as well as resistance to microalgal *A. coffeaeformis* attachment [217]. Gou, J. *et al.* (2018) also reported a strong bacterial reduction (close to 100%) when TA incorporated in gelatin hydrogels were used, but also a potent antifungal activity against *C. albicans* with a survival rate near 0% and a halo of inhibition >36 mm [218]. In other reports, Sharma, S. *et al.* (2021) produced active films with ferulic acid (FA) incorporated on PLA and PBAT matrix for food

packaging. 10% of FA-incorporated films showed a CFU reduction of 3.5 Log against *L. monocytogenes* with a CFU reduction of 2 Log for *E. coli* [219]. Another study reported the application of FA on polymeric nanoparticles of PLA and poly(lactic-co-glycolic acid) (PLGA) for ocular applications [220] and chitosan matrices for both food and agriculture applications [221]. In this last work the authors report the antifungal property of FA. The FA-chitosan matrices showed a decrease of radial germination of *A. parasiticus* in 28% (at 168 h) and an inhibition of spore germination of (55-57%) at 30 h [221].

f) Ionic-liquid base compounds

The first reports of ionic liquids (ILs) dates from 1914, when Walden synthesized ethyl ammonium nitrate by reacting concentrated nitric acid and ethylamine. But it was only between 1970-1980, that ILs gained more relevance when a new class of ILs based on dialkylimidazolium and alkylpyridinium as cations and metal halides as anions, were developed. These ILs were called as “first-generation ionic-liquids”. Despite the recognized electrochemical features, these compounds were not stable. The “second generation ionic liquids” gained track in 1992, when water-stable imidazole-based ILs were developed and applied at large scale as lubricants and reaction solvents in the industrial and energetic field [222,223]. The “third generation ionic liquids” comprises biodegradable and natural ions, such as amino acids and choline. This class of ILs are highly accepted for biomedical and pharmaceutical applications due to their biological properties. The last generation, “fourth-generation ionic liquids” were introduced in 2018, and biocompatibility and rearrangement of ILs with other molecules to improve its properties have been reported [223]. Structurally, ILs are composed by an inorganic/organic anion linked to an organic cation (Figure 16). Some organic cations include imidazolium, pyrrolidinium or pyridinium, while anions are halides, hexafluorophosphate, tetrafluoroborate or octyl and methyl sulphate.

ILs are characterized for presenting a high thermal and chemical stability, ionic and electrical conductivity, and a low vapor pressure and melting point [224]. These liquids are molten salts and are liquid at temperatures below 100°C [223]. Despite these general features, some properties can be modulated by the size and the anion/cation portion of ILs. The associated antimicrobial mechanism of ILs remain unclear but some authors suggest that the cationic head groups promote an electrostatic/lipophilic interaction with structural proteins present in the microbial outer layer, disrupting the integrity of biological membranes [224]. The cation can then be readily absorbed into the cell layers, penetrating into the cytoplasm, resulting in the inhibition

of several metabolic pathways by inactivating key enzymes, such as acetyl cholinesterase, AMP deaminase, acylase I, cytochrome c oxidase, glutathione reductase or carboxylesterases [225].

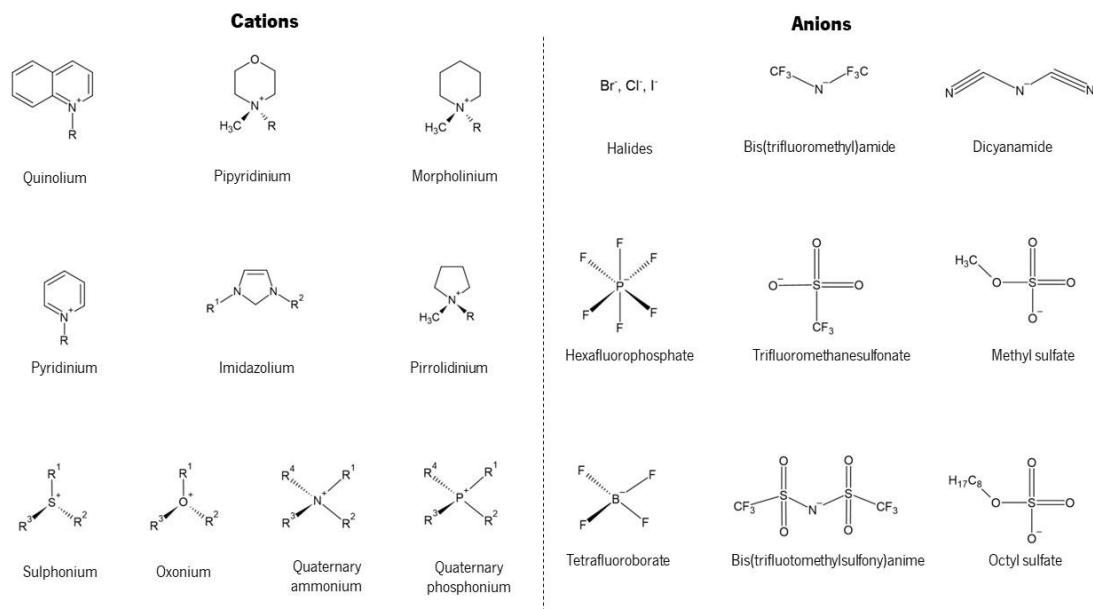


Figure 16 – Typical structures of most used cations and anions used in ionic liquids. Adapted from Nikfarjam, N. *et al.* (2021) [223]. Drawn in ACD/ChemSketch (Freeware) 2022.2.

The antimicrobial properties of ILs depend not only on the anion or cation, but also on the length of the alkyl chain. It is described that antibacterial properties of ILs generally increase by increasing the alkyl chain length. Longer chain lengths are able to better destabilize the cell membranes and penetrate the phospholipid bilayer [223]. This effect is shown by Zheng, L. *et al.* (2020) in which ILs with C12 cause more damage in biological membrane and are able to penetrate into the cytoplasm, when compared to C10 ILs [226]. Garcia, M. *et al.* (2014) reported MIC values of amide-functionalized ILs with C6-C14 alkyl chains. The authors showed that shorter chains (C6) are not effective against gram-positive and gram-negative bacteria and even fungal strains. However, increasing the number of carbons from C8 to C12 the MIC value significantly decreased. In particular, the methylimidazolium bromides-based compound with C8, C10 and C12 showed a MIC value of 777, 178 and 41 μM against *S. epidermidis*, respectively [227]. However, increasing even more the alkyl chain can have the opposite effect. Abbaszadegan, A. *et al.* (2017) reported a lower MIC value for imidazolium and pyridinium based compounds with C12 when compared with the same compounds with C18 [228].

Regarding the incorporation of ILs on polymer substrates, Sasi. R. *et al.* (2021) reported the production of antimicrobial coatings based on 1-hexyl-3-methylimidazolium iodide (HMIMI) on a PP substrate. In this work, the authors showed that HMIMI-PP matrices were capable of preventing the adhesion of *E. coli* with 394.32 mM of ionic-agent [229]. In a related report, Enderle, A. *et al.* (2022) synthesized a new ILs-based on polyoxometalate (POM) incorporated into a PMMA substrate. POM-ILs exhibited antibacterial and antifungal activity against *E. coli*, *B. subtilis*, *L. monocytogenes* and *A. niger*, (p.e. MIC of 125, 1.95, 31.25 and 250, respectively). For 2 $\mu\text{g}/\text{cm}^2$ of POM-ILs incorporated in a coating material, the authors reported 100% of microbial reduction against *E. coli*, while for 8 $\mu\text{g}/\text{cm}^2$ a 100% of microbial reduction against and against *B. subtilis* and *L. monocytogenes* was observed [230]. Other polymer matrices for which incorporation of ILs has been attempted include poly(ethylene oxide) (PEO) and poly(vinylidene fluoride-hexafluoro) blends for energy storage applications, making use of the high electronic conductivity properties of ILs [231]; midblock-sulfonated multiblock polymers for environmental applications - mainly CO₂ capture [232]; and PLA, polybutylene succinate, and PCL for enhancing properties of biodegradable polymers [233].

g) Other antimicrobial agents

Other important classes of compounds incorporated in polymer substrates are disinfectants and antiseptic agents, such as chlorhexidine (CHX), NO-releasing polymers and amino acids. First synthesized in 1950, by the Imperial Chemical Industries in England, CHX is an important disinfectant used for the daily disinfection of medical instruments and surfaces, as well as a preservative for some pharmaceutical and medical products [234,235], due to its large spectrum of action and relatively low toxicity to mammalian cells [234]. Structurally, CHX is composed of two guanidine groups and two 4-chlorophenol rings placed symmetrically linked by a hexamethylene chain [235]. The proposed antimicrobial mechanism of CHX is based on the interaction of cationic portions of CHX with anionic phosphate residues of negatively charged lipids. In this interaction, adsorption of CHX to phosphate groups comprising the bacterial membrane takes place. It has been suggested that CHX perfuses to the cytoplasm by passive diffusion, disrupting the integrity of membrane and causing the release of low molecular weight cytoplasmic components through the cell membrane. Additionally, it has also been reported to inhibit the enzymatic activity of the cell. Finally, CHX can cause the formation of cytoplasmic precipitates with phosphate moieties, such as ATP and nucleic acids [234]. Despite this general mechanism,

Cheung, H. *et al.* (2012) showed that the interaction of CHX with biological membranes can differ according to the bacterial strain. For gram-positive bacteria, damage of bacterial membrane after CHX exposure is preferentially localized at the tip or cap region of bacteria, while for gram-negative bacteria the membrane damage is mainly localized in the trunk. In fact, the interaction of CHX with gram-positive bacteria seems to be more efficient due to their higher negative charge, when compared to gram-negative. It has been reported a concentration of inhibition of 10 $\mu\text{g/mL}$ for gram-positive bacteria and around 50 $\mu\text{g/mL}$ for gram-negative bacteria [235].

Considering this, CHX have been extensively studied as an important agent to control or prevent infection in biocide-releasing polymers. Sjollem, J. *et al.* (2016) reported the production of CHX releasing poly(ethylene glycol) diacrylamide coatings in urinary catheters [236]. Mai, H. *et al.* (2019) described the synthesis of mesoporous silica nanoparticles encapsulated with CHX in a PDMS and PMMA matrix to produce realising coatings for dental restorations [237]. Moreover, CHX and some derivatives were loaded onto biodegradable polymers (polyglycolide-b-poly(glycolide-co-trimethylene carbonate-co- ϵ -caprolactone)-b-polyglycolide) and polyester matrices for surgical sutures and vascular prostheses [238,239].

Another important class of antimicrobial polymers is nitric-oxide (NO) releasing polymers. NO molecules are involved in many biological processes and have been shown to have antimicrobial properties. NO antimicrobial activity is mostly associated to oxidation of lipids, nitration of tyrosine present on the cell wall, nitrosation of amines and thiols altering the extracellular matrix and ultimately damages in DNA [240]. The production of NO releasing polymers is achieved by incorporating NO donors into the polymer matrix, such as diazeniumdiolates, S-nitrosothiols and S-nitroso-N acetylpenicillamine. In this sense, Darder, M. *et al.* (2019) developed a polymer functional coating with Cu-cystine structures for a biomedical application. The release of NO is provided by decomposition of low molecular weight S-nitrosothiols (RSNOs). RSNOs decomposition is promoted by Cu in the crystalline structure of the polymer coating, which is released in the presence of redox species, like ascorbic acid, typically found in the blood. In the presence of physiological concentrations of RSNOs, the amount of generated NO can be around 360 nM. Despite this, the authors showed that the amount of NO release is not enough to promote antibacterial effect [239]. In another work, Li, Q. *et al.* (2017) developed an antimicrobial and antifouling polymer based on 2-methacryloyloxyethyl phosphorylcholine-co-butyl methacrylate co-benzophenone (BPMPC) grafted with NO donor (S-nitroso-N-acetylpenicillamine (SNAP)) by UV-cross-linking. The results showed an increase of NO content release 2-fold higher from the coated

surfaces with SNAP and BPMPC, when compared to the control surfaces at day 0 and almost 3-fold higher at day 14 at 37°C in PBS). The surfaces showed a 99.9% of reduction of viable bacteria on coated surfaces [242]. In other work, Sadrearhami, Z. *et al.* develop NO releasing coatings by grafting NO precursors on polydopamine (PDA) and PEG substrate. The authors observe a cumulative release of NO from PDA-coated substrates until 2 days (in PBS, pH 7.4, 37°C) and an inhibition of biofilm formation of 96% at 24 h and 70% at 36 h against both gram positive and negative strains [243]. Another example is reported by Pant, J. *et al.* which fabricate NO releasing polymers with SNAP, as NO donor in commercial polymers (CarboSil® polymer) [244].

It has also been suggested that some D-amino acids, in particular D-Tyrosine (D-Tyr), can be involved in inhibition of biofilm formation acting as a repellent. To assess this, Milenkovic, J. *et al.* (2014) prepared PVC composites incorporated with natural zeolite silver and D-Tyr. Although the original PVC coated with Ag zeolite already showed a 4.1-4.4 CFU Log reduction of immobilised *A. baumannii*, the addition of D-Tyr increased this value to 6.9 CFU Log reduction, corresponding to a 100% reduction. Although the D-Tyr *per se* did not show significant antibacterial activity, the authors suggested a synergistic effect of D-Tyr and PVC, acting as a repellent and reducing bacterial attachment to the polymer surface [245].

1.3 Antimicrobial solutions for automotive industry

Polymer materials are widely used in a variety of applications, including clothing, paints and glues, daily products (household and personal products), the automobile industry, construction, the medical field, and others. Given that polymer surfaces can act as an important substrate for microorganism colonization and their ability to persist in these surfaces over time, particularly in closed and multi-shared spaces, the development of new antimicrobial materials capable of inhibiting microbial attach and/or killing microorganisms is an important strategy for reducing the risk of infections among humans.

The ideal antimicrobial material should have a broad spectrum of action against pathogens of interest; be produced on a large scale at the lowest possible cost; have a wide range of applications; be low in toxicity to humans and have a low environmental impact. Analyzing the state-of-the-art in antimicrobial polymeric materials, this section intends to highlight a few of the main antimicrobial agents already used in the market or are currently under development.

Nowadays, there has been a great commercial interest in antimicrobial products and additives for many applications. One example is Stayzsafe™, a range of disinfectant products based on BAC or ethanol providing protection against bacteria, viruses, fungus and parasites [246], commercialized by Ultimotive Ltd, that can be applied by the automobile owner and provide elimination of microorganisms and help eliminate odours. Despite this promising strategy this type of products requires a constant and frequent application by the car user, which is not compatible with the lifetime of the car. Additionally, depending on the chemical composition of the disinfectant, these products can induce chemical and physical alterations of the surface (ex: colour changes, stains, wear of the surface) since they may not be specific for the polymer system.

Another example is given by BioCote®, a company that dedicates to the commercialization of antimicrobial additives for plastics, polymers, paints, coatings and textiles. In specific for the automotive industry, their materials were capable of decreasing the microbial burden inside a car, avoiding unpleasant odours and reducing material degradation [247]. The additives are mostly based on metal additives, such as Ag ions and Zn-zeolites, QAC, and iodopropynyl butylcarbamate (IPBC) for antifungal applications. Importantly, these additives are compatible with a variety of polymers, including PC/acrylonitrile butadiene styrene (ABS) blends; polyamide (Nylon PA 6, PA 66); polybutylene terephthalate (PBT); polycarbonate; PMMA; polyoxymethylene; thermoplastic polyurethane (TPU); ABS; polyethylene (PE/HDPE/LDPE); PP and PVC [248].

In addition to the commercial applications described above, there are patents regarding antimicrobial films that can be used in automotive industry. One of these patents entitled “Transparent antibacterial film and preparation method thereof” comprises a transparent antibacterial film prepared from polyester with at least one antibacterial agent (such as Ag, Zn, Cu and Ti) [249]. The patent claims antibacterial activity against *E. coli*, *S. aureus* (including MRSA) and *P. aeruginosa* and antifungal activity against *Aspergillus niger*, *Penicillium pinophilum*, *Chaetomium globosum*, *Gliocladium virens* and *Aureobasidium pullulans*. Importantly, the concentration of antibacterial agents can be adjusted (between 0.1-2% of total weight) according to needs, providing the maintenance of original optical properties (transmittance and haze) of films without addition of antibacterial compound [249]. Another patent entitled: “Water-based paint and antimicrobial leather prepared thereby” describes the preparation of water-based paint comprising an organic polymer and an antimicrobial agent, with the final goal of ensuring the abrasion resistance and noise resistance. The antimicrobial agents include a metal- or metal-oxide compound of Ag, Cu, Zn, Ni, Co, or Pb. The percentage of each compound is variable and can range from 0.3 to 10% in case of zinc-based compounds or 0.2-3% in case of Ag-based compounds [250].

In the examples described above, there are evident concerns that should be taken into consideration during the process of antimicrobial coating development (i) the compatibility of the antimicrobial agent with polymer formulations: the antimicrobial agent should not affect the main properties of the polymer such as colour and viscosity; (ii) the thermal and physical stability of the antimicrobial agents: the antimicrobial agent is processed together with the polymer and therefore it is necessary to guarantee the preservation of the antimicrobial properties even after exposure to extreme conditions (ex: temperature; pressure,...); (iii) the spectrum of action: the antimicrobial agents should provide antibacterial, antifungal, and antiviral properties; (iv) the legal and safety issues; the antimicrobial agents should be included in the Global Automotive Declarable Substance List (GADSL) and fulfil all the requirements related to safety for human health and/or to the environment and; (v) the cost/time of production to the addition of antimicrobial agents: the addition of antimicrobial agents into polymeric solution could represent an increase in the time of production and impact the final cost for clients.

Collectively, these criteria should be taken into consideration during the development of these antimicrobial products. Indeed, the great challenge of this PhD work is exactly this: develop an antimicrobial coating while maintaining the lacquer properties unchanged and cytocompatibility.

1.4 General description of this work

The automotive interior is a potential fomite for microorganism colonization and the development of an antimicrobial coating material for automotive applications is a possible solution to reduce the risk of microbial colonization of high-touch surfaces in automobile interiors and reduce the risk of spreading of infectious diseases among users. Hence, the aim of this PhD thesis was to develop a flexible coating material for automotive interiors with antimicrobial activity, focusing on the superficial layers since these are mainly exposed to human contact and consequently more susceptible to be colonized.

The general aims of this project entail:

1. The study of the antimicrobial effect conferred by material surface properties - topography.
2. The development of flexible coating materials through incorporation of different biocide agents in commercial lacquer formulation used by TMG Automotive, and further validate the physical, chemical, and biological properties of the produced materials.
3. The analysis of the biological properties of UV-light stabilizers as antimicrobial agents in flexible coating materials and identify the mechanism of action of a specific UV-stabilizer (Tinuvin 770 DF).

1.5 References

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Chapter 2

Experimental Work

2.1 Influence of surface properties on the antimicrobial effect of flexible coating materials

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Abstract

Human environments provide excellent ecosystems for the proliferation of microorganisms, making them potential sources of exposure to pathogenic agents. Strategies aimed at minimizing microbial colonization include inhibiting adhesion, inhibiting bacterial growth, or even killing the microorganisms, thus preventing surface contamination. Surface topography can play a key role in influencing bacterial attachment and consequently biofilm formation. By manipulating the surface topography of materials, surface-microorganism interactions can be affected, resulting in an alteration in bacterial adhesion/viability.

In this study, flexible coating materials based on polymer substrates were produced and embossed using a combination of laboratorial and industrial process implemented at TMG Automotive, resulting in different topographic surface properties. These materials were microscopically characterized, and their antibacterial activity was evaluated against two representative bacterial strains.

An analysis of the surface topography of the rough materials revealed a groove width distribution of 0.16 ± 0.04 , 0.09 ± 0.03 and 0.08 ± 0.02 mm for materials with large, medium and fine grain, respectively. Interestingly, the antibacterial assays do not show any significant differences in bacterial proliferation between smooth and rough materials, regardless of the specific patterns, grain widths, and the bacterial strains used. These results indicate that this particular type of pattern, does not exhibit antibacterial effect, probably due to the large groove width compared to the dimensions of bacteria which are typically below that $\leq 2 \mu\text{m}$.

Keywords: antimicrobial coatings; surface properties; topography pattern.

2.1.1 Introduction

Bacteria have demonstrated remarkable adaptability to successfully thrive in diverse environments during millions of years. While many bacteria coexist harmlessly with humans, others are responsible for a wide range of infectious diseases, with significant health consequences for the patient and financial burden on healthcare systems [1,2]. In this sense, surfaces play an important role for exposure of humans to microorganisms, as they can persist for extended periods of time on a wide range of materials, including, paper, cloth, glass, wood and plastics [3]. To mitigate the risk of human exposure, one of the strategies available is to develop antimicrobial surfaces to prevent the microbial attachment and growth. While traditional approaches have relied on the use of chemical agents, recent attention has been focused on the role of surfaces properties

as an alternative means to achieve antimicrobial properties. Microscale topographies have emerged as a promising alternative, as they have been described to induce cellular level responses and affect the cell–substrate interface [4,5]. Although this association between topographic features and bacterial attachment remains unclear, it is known that microorganisms are capable of sensing mechanical signals from the surface. At the microscale level, these topographic features can promote alterations of material properties, i.e. hydrodynamic parameters and physicochemical forces, which play a crucial role in bacterial attachment, specifically by affecting bacterial adhesins or by promoting deformation of the cell membrane [6].

In this study, we aimed to evaluate the antibacterial effect promoted by surface topography of a commercial flexible coating material. For this, we developed a flexible coating material composed of a polyvinyl chloride (PVC) substrate, produced through a knife coating process, and a surface lacquer-film layer of polyurethane (PUR) obtained by a lacquering process. To induce surface alterations and create different textures, the materials were submitted to an embossing patterning process. The assessment of antimicrobial activity of the developed materials was performed using a modified version of an international protocol using two representative gram-positive and gram-negative bacterial strains, *Staphylococcus aureus*, and *Escherichia coli*, respectively. A graphical abstract of this work is present in Figure 1.

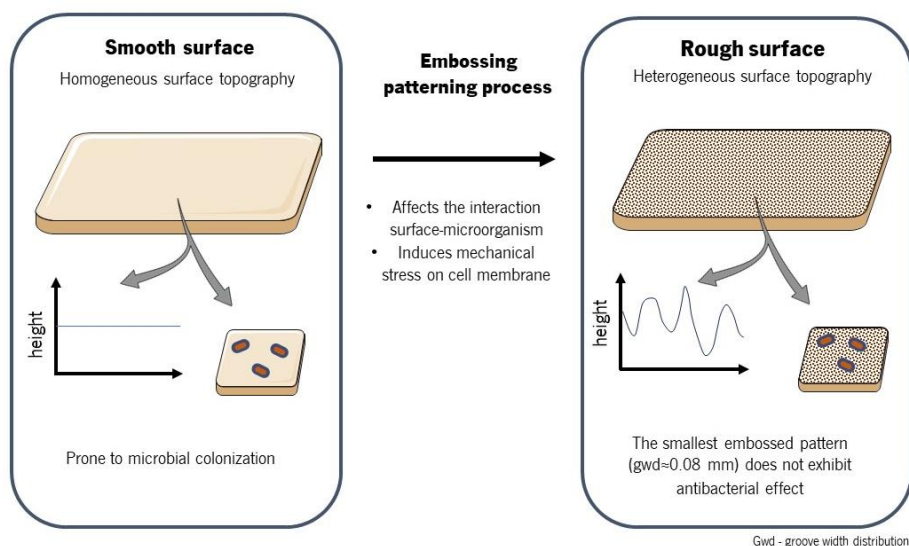


Figure 1 – Graphical abstract of work: “Influence of surface properties on the antimicrobial effect of flexible coating materials”.

2.1.2. Materials and methods

2.1.2.1 Production of flexible coating materials: the flexible coating materials were collected after production following the protocols in use at TMG Automotive. Briefly, the production process begins with the production of PVC pastes (called plastisol). Plastisol is formed from the mixture of polymer and other chemical agents, such as plasticizer, stabilizers, pigments, blowing agents, thickeners and flame retardants [7]. Then the plastisol is processed by a coating technology in which different polymeric layers are applied over a substrate to form a continuous structure in a process called knife coating (Figure 2). In this process, the plastisol is in a liquid state, and is transported by a rotation cylinder at different rates and speeds between the reservoir to the substrate. The substrate moves lengthwise, and it is scraped by the knife, cutting the excess of plastisol. The polymer compound is then spread upon the substrate (paper or a textile layer) to create a uniform surface. The amount of polymeric material applied over the substrate is dependent on the mesh of the cylinder, the thickness provided by the knife, the content of solids in the plastisol and other parameters like viscosity and wettability of the PVC paste [7]. Later, the plastisol is subjected to a cycle of heating and cooling. The increase of temperature promotes the gelation and fusion of the PVC plastisol. At the gelation stage, the resin particles swell upon contact with the plasticizer, followed by the fusion of all plastisol components to promote a homogeneous phase. In addition, the increase of temperature allows the activation of the blowing agent and the expansion of the foam layer. In the end, the substrate is collected by a rotary cylinder.

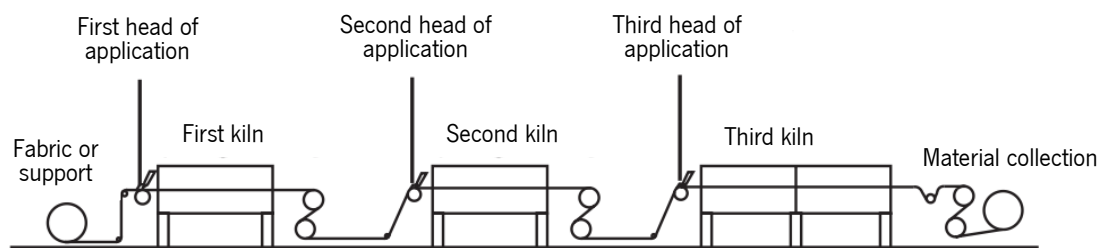


Figure 2 – Schematic representation of the knife coating process of PVC based materials similar to the process present in TMG Automotive: the three head applications allow for the creation of a final material with different layers. Extracted from Rodolfo, A. *et al.* (2006) [8].

After the PVC substrate production, the material was submitted to a lacquering process, through the application of a thin layer of lacquer-film of PUR upon the PVC polymeric layer. In the lacquering process, the lacquer solution is transported by a rotation cylinder from the reservoir to the substrate (polymeric layer). After that, the material is dried for 1 min at 140°C in a laboratory

hot air oven (Werner Mathis AG, LTF-ST 119988) to evaporate the solvents that compose the lacquer solution. The combination of lacquer additives and the process parameters, such as the cylinder mesh, the temperature and the tension of material in the machine rollers, constituted the parameters of the lacquering process. In specific, in this work, three different thin layers of a PUR aqueous formulation (PUR dispersion and additives, such as flame retardants, UV-stabilizers, crosslinker and others) were applied over the PVC polymer substrate. Each layer provided different properties to the final material. The set of lacquer-film layers provided additional technical properties to the based material, such as UV protection and mechanical resistance, as well as aesthetical appearance (i.e. gloss) and good haptics.

2.1.2.2 Embossing process of flexible coating materials: the flexible coating materials were submitted to an embossing process to induce surface modifications. In this process, the material moves lengthwise between two rotating cylinders at high temperature (190-200°C). The cylinders with different designs/engraved mesh press the materials to impart a topography similar to natural leather in terms of visual and touch effects. Three different patterns were induced on materials surface being classified as P1, P2 and P3, according to the size of the grain (large, medium and fine grain, respectively). The patterns were selected according to the embossing textures available at TMG Automotive being representative of commercial applications of flexible coating materials. A flexible coating material without embossing process (smooth surface) was herein used as a control.

2.1.2.3 Characterization of surface topography of flexible coating materials: the surface characterization was done by analysing the measurement of the pattern. For each rough material (P1, P2 and P3) different images were collected by an electronic magnifier with 1x of magnification. After acquisition, the images were analysed in Digimizer Image Analysis Software and the groove width was measured using the software tools. At least 100 independent measurements were registered for each material. Images of smooth flexible coating materials were also acquired as control samples.

2.1.2.4 Evaluation of antibacterial effect of flexible coating materials: the antibacterial assays were based on the protocol described in the International Organization for Standardization (ISO) 22196(E):2011 - Measurement of antibacterial activity on plastics and other non-porous surfaces [9]. The assays were done using a gram-positive bacterial strain *Staphylococcus aureus* (ATCC 25923) and a gram-negative bacterial strain *Escherichia coli* (ATCC 25922). Briefly, the

flexible coating materials cut in 5x5 cm² squares, were inoculated with 0.4 mL of a bacterial suspension (6×10^5 CFU/mL). A polyethylene terephthalate inert cover-film (4x4 cm²) was placed over the material surface to ensure the contact between inoculum and tested materials. Test samples were incubated for 24 h at 35°C, in an atmosphere with high relative humidity. Both materials, embossed and non-embossed samples, as well as cover-films, were previously sterilized by UV-light for 30 min on each side. To validate the initial inoculum dose, and consequently validate the assay, samples of non-embossed material (control samples) were processed immediately after inoculation. After the 24 h incubation period, samples were washed with a wash solution composed by soybean casein, lecithin and polyoxyethylene sorbitan monooleate. Ten-fold serial dilutions of the bacterial suspensions were done in PBS-saline solution. Each dilution, as well as the initial suspension, was plated on agar plate medium composed by yeast extract, tryptone and glucose. The plates were incubated for 40-48 h at 35°C. Following this incubation period, the number of CFUs were counted. The antimicrobial activity (R) was calculated comparing the average of the common logarithm of the number of viable colonies recovered from untreated materials 24 h after inoculation and the average of the common logarithm of the number of viable colonies recovered from treated materials after 24 h of inoculation. In addition, a microbial ratio for treated materials from untreated materials was also analysed.

2.1.3 Results and discussion

The production of antibacterial flexible coating materials represents a new generation of protective coatings, aligning the antibacterial properties with the primary mechanical properties of coatings [10]. The surface manipulation presents a promising strategy to develop antibacterial coatings avoiding the use of chemical products and antimicrobial agents [11]. In this sense, commercial flexible coating material based on PVC substrate and a PUR top lacquer-layer was submitted to an embossing process to induce different surface topographies. The produced materials were further characterized regarding their surface properties (measuring the grain size to assess the structural features), as well as their antibacterial effect.

2.1.3.1 Topography analysis of flexible coating materials: the flexible coating material (smooth material without an embossing pattern) exhibit a homogeneous surface without any noticeable macroscopic defects or imperfections. However, upon closer evaluation at a microscopic level, multiple visible lines can be observed on the material surface (Figure 3A) that can be attributed to the production process given that they are evenly spaced and follow a defined

orientation. In contrast, the rough flexible coating materials exhibit a structure similar to natural leather with an irregular topography, without any defined orientation. These differences are visible by the naked eye and become more pronounced when observed under the microscope. Figure 3A provides a clear depiction of the contrasting surface topography among the different types of flexible coatings analysed. Notably there is a decrease in the grain size from P1 to P3. P1 materials shows a grain width distribution of 0.16 ± 0.04 mm with large areas between the grainy channels (Figure 3B). On the other hand, P2 and P3 materials exhibit smaller patterns with grain widths of 0.09 ± 0.03 and 0.08 ± 0.02 mm, respectively.

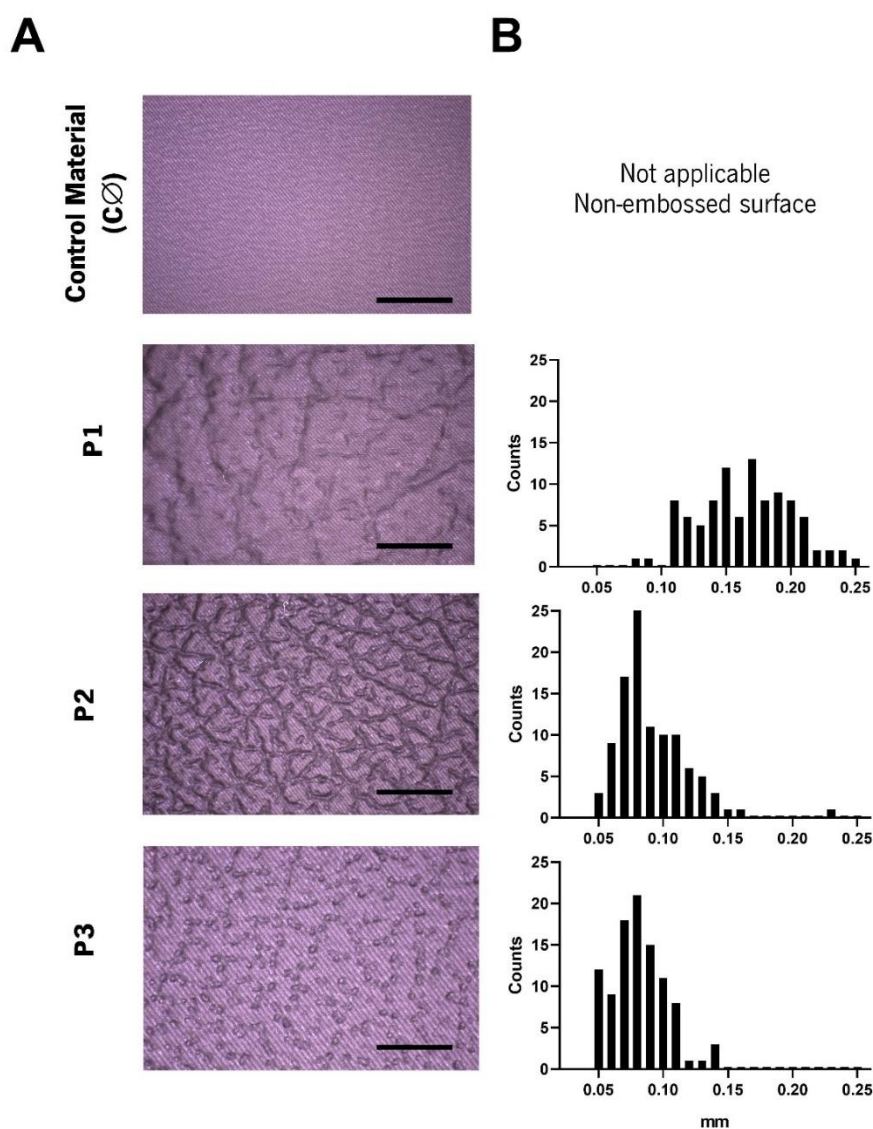


Figure 3 – Topography characterization of flexible coating surface: (A) Microscopic view of different smooth and rough materials and (B) Histogram of groove width distributions ($n > 100$). CØ represents the flexible coating material without embossing (smooth control group) and P1, P2 and P3 represents flexible coating materials after the embossing process (rough test groups) with large, medium and fine grain, respectively. Scale bar = 2 mm.

2.1.3.2 Analysis of antibacterial effect of surface topography of flexible coating

materials: the antibacterial properties of the materials were tested using an international protocol against both gram-positive and gram-negative bacteria. Following an incubation period of 24 h after inoculation, antibacterial analysis revealed no statistically significant differences in the bacterial burden between control and tested materials. These results were consistent across all materials tested (P1, P2 and P3) and for the two bacterial strains tested (Figure 4A and 4B). The R value remained consistently below 0.5 regardless the surface topography, indicating no antimicrobial activity. In fact, in some cases, rough materials exhibited a higher bacterial burden, when compared to smooth materials, particularly for P1 and P3 against *E. coli* (Table 1).

The absence of antibacterial activity associated to the surface topography could be explained by the difference between grain size and the characteristics of the bacteria. *S. aureus*, a gram-positive bacteria and catalase-positive coccus, is an important human pathogen responsible for a variety of infections, including skin infections, endocarditis and pneumonia [12]. Due to their high capacity to acquire resistance to multiple antibiotics, aligned with their capacity to survive on polymeric surfaces for extended periods of time, makes *S. aureus* a microorganism of great interest for biomedical science [12]. Morphologically, *S. aureus* typically appears as spherical cells with a diameter ranging from 0.5 to 1.5 μm , exhibiting well-defined borders [12,13]. On the other hand, *E. coli* is a gram-negative bacterium, commonly found in the human body, characterized by its rapid growth with a generation time of less than 20 min [14]. Structurally, *E. coli* is characterized by a spherocylindrical shape with a length of approximately 2 μm and a width of 1 μm [15]. Considering this information, it becomes evident that the grooves present in our flexible coating materials are nearly 80-fold larger than the size of the two bacteria (comparing the P3 material with a groove size of $\approx 80 \mu\text{m}$ to the average size of bacteria, which is of 1 μm). This size range most likely does not affect on the interaction between bacteria and the material surface, allowing for their attachment and colonization. In fact, the existing literature on the influence of surface topography on the antibacterial activity primarily reports on textured structures in the submicron/nanoscale range as the key contributor to provide antibacterial properties. This represents an important difference to the microscale surface topography obtained for our produced flexible coating materials. A relevant example of this is reported by Xu, L. *et al.* (2022) where the authors successfully produced PUR surfaces with textured patterns at the submicron level using lithography techniques. The surfaces were engineered to have ordered arrays of pillars with 400-700 nm of diameter, 300-700 nm of spacing and 300-600 nm of height. The authors reported an inhibition

of bacterial adhesion ranging from 58.2 to 82.7% for *S. epidermidis* and 74.1 to 84.5% for *S. aureus*, depending on the textured surface [16]. In a similar work, micro- and nano-scale rough surfaces were produced from commercial aluminium alloys, using an etching technique. The authors achieved a surface roughness parameter value for Ra, Rq, and Rz of 154, 189 and 287 nm, respectively, for etching conditions of 1 M of NaOH and 10 min (see section 1.2.3.1). These specific topographic patterns were found to reduce bacterial viability by 97% for *E. coli* and 28% for *S. aureus* [11]. These differences in bacteria viability can be attributed to differences in the bacterial membrane, particularly regarding to the peptidoglycan layer. The peptidoglycan layer present in the bacterial membrane serves as a protective barrier against mechanical and chemical stress [17]. It has been described that gram-negative bacteria have a less rigid cell wall and consequently less resistant to mechanical lysis, compared to gram-positive bacteria [5]. Gram-negative bacteria have a peptidoglycan layer that is 4-5 times thinner, making them more susceptible to irregular surfaces than gram-positive bacteria [17,18].

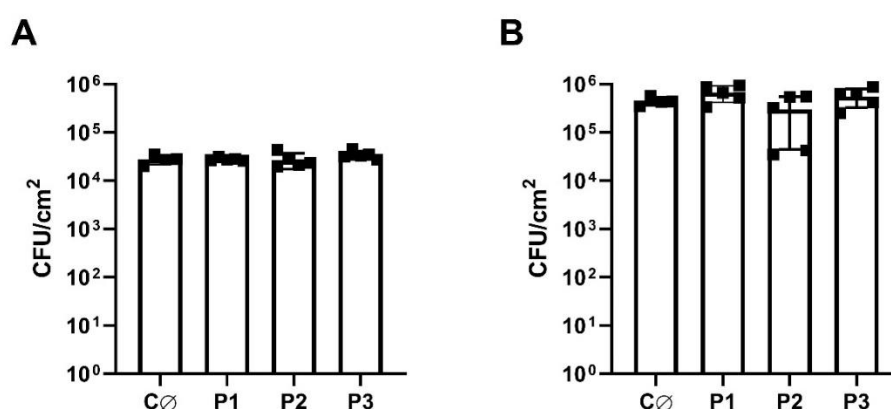


Figure 4 – Antimicrobial analysis of flexible coating materials with different surface topographies: antibacterial analysis of materials against *S. aureus* (A) and *E. coli* (B). CØ represents the smooth flexible coating material (control group without embossing) and P1, P2 and P3, represents the rough surface materials embossed with large, medium and fine grain. Error bars represent the standard deviation (n≥4).

Table 1 – Antimicrobial activity of produced flexible coating materials embossed with different patterns.

Pattern	R value* / Microbial Ratio (%)**	
	<i>S. aureus</i>	<i>E. coli</i>
P1	-0.012 / -1.02	-0.153 / -49.53
P2	0.018 / 1.69	-0.413 / 33.29
P3	-0.097 / -24.29	-0.069 / -25.38

* Antibacterial and antifungal activity (R) is calculated according to ISO 22196:2011, by using the following formula: $R = (Ut - U0) - (At - U0) = Ut - At$ in which *U0*, *Ut* and *At* are the average of the common logarithm of the

number of viable bacteria recovered immediately after inoculation for untreated samples, 24 h of incubation for untreated samples and 24 h of incubation of test samples, respectively. ** Microbial Ratio (MR) is the percentage of viable bacteria recovered from treated samples compared to untreated samples after 24 h of incubation. MR were calculated by the next formula: $MR = 100 - (Ts \times 100)/Cs$, in which Ts and Cs is the average of viable bacteria recovered from treated and untreated samples, respectively. Negative values indicated an increase of bacterial burden comparing to control group and positive values indicates a microbial reduction to control samples.

Collectively these findings demonstrate that a microscale surface pattern created through embossing processes are unable to effectively inhibit bacterial adhesion, due to their size being outside the dimensions that affect bacteria. The patterns used in this study are commonly used in the automotive industry. However, reducing the size of the texture it not possible with the currently available technique, since there is a trade-off between the grain size and the quality of the embossing process. Therefore, it becomes challenging to achieve the desired surface topography on a smaller scale that aligns with the size of bacteria, while maintaining the necessary quality standards for automotive applications.

2.1.4 Conclusion

Surfaces play an important role in microbial colonization acting as reservoirs for microbial growth. One of the available strategies to mitigate these issues and reduce bacterial transmission/infections associated with surfaces, is the development antimicrobial materials. Recently, strategies related involving surface manipulation to obtain antimicrobial effects have gained more attention due to their potential to avoid the use of chemicals and address concerns about resistance. However, based on the results obtained in this study, it is evident that the topography patterns available at TMG Automotive do not possess inherent antibacterial properties. At an industrial level, implementing more refined techniques, such as lithography and etching techniques to induce surface alterations becomes challenging, since these techniques require specialized equipment and are cost-prohibitive, making them incompatible for large-scale production. Nonetheless, the results obtained in this research illustrate the limitations of relying solely on surface topography to achieve antimicrobial effects. The results also emphasize the need for exploring alternative approaches or combining multiple strategies to develop effective antimicrobial materials for industrial applications.

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2.2 Development of polyurethane antimicrobial coatings by composition with phenolic-, ionic- and copper-based agents

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Abstract

Microorganisms can be found in almost all environments with high-touch surfaces being an important fomite for microbial growth. Considering the health issues associated to acquired infection from inanimate surfaces, as well as the raising hygienic concerns, the incorporation of antimicrobial compounds in high-touch surfaces emerges as an effective solution for biomedical and common daily applications.

In this work, we incorporated different antimicrobial agents (phenolic-, ionic- and copper-based compounds) into polyurethane commercial formulations to produce antimicrobial lacquer-films and evaluated not only their physical/chemical properties, but also their antimicrobial activity against bacteria (*Staphylococcus aureus* and *Escherichia coli*), fungi (*Candida albicans*), and virus (SARS-CoV-2).

The incorporation of antimicrobial agents did not affect the performance of lacquer-films and the main properties were maintained, specifically the visual aspect, gloss values, optical properties and chemical stability.

Among the different compounds tested, copper-based lacquer-films exhibited the strongest antibacterial and antifungal activity, with a >4 Log reduction, but not against virus. Importantly, copper-based lacquer-films maintained their cytocompatibility, even at high concentrations. Regarding the ionic lacquer-films, the highest tested concentration also showed a strong antimicrobial action (5 Log reduction) against fungi and gram-positive bacteria, but not against gram-negative bacteria and virus. However, at this concentration, the ionic-containing lacquer-films presented cytotoxic potential. The phenolic-based compounds were not associated with antimicrobial activity, regardless the concentrations tested.

Collectively, these results highlight the potential of incorporating antimicrobial agents in polyurethane surface coatings as a promising strategy to avoid the microbial colonization on inanimate surfaces and, ultimately, prevent the spreading of potentially harmful pathogens among humans.

Keywords: antimicrobial coatings; phenolic-based compounds, ionic-based compounds, and metal-based compounds

2.2.1 Introduction

Inanimate surfaces have an important role in the transmission of pathogens. These surfaces can act as secondary reservoirs for microorganisms and offer important opportunities for

the transmission and dissemination of pathogens among humans [1,2]. This situation is particularly relevant in indoor environments, in which humans spend almost 90% of their lives [3-5]. These indoor environments are characterized as multi-shared and confined spaces in which air (e.g. ventilation systems, air conditioning devices, airborne particles), high-touch surfaces (e.g. door handles, faucets, light switches) and water systems are the most common routes for microbial transmission [6].

Several studies have been published related to the persistence of pathogens on inanimate surfaces, including door handles of public service cars [7], automobile indoor surfaces [8], and frequently touched objects in healthcare facilities [9]. These studies identified Streptococci, Enterococci, *Klebsiella pneumoniae*, *Salmonella ssp.*, *Escherichia coli*, and Staphylococci as the most common contaminating bacteria [10]. Among Staphylococci, methicillin-resistant *S. aureus* (MRSA) have been a rising concern given its generalized dissemination, increased persistence, and resistance to β -lactam antibiotics [11]. Other common group of microorganisms potentially pathogenic for humans are fungi [12,13]. The most common fungi identified on inanimate surfaces include *Aspergillus spp.* [14], *Cryptococcus spp.* [15], *Candida spp.* [1,16], and *Torulopsis glabrata* [1]. Importantly, the concern about viral persistence on inanimate surfaces has been highlighted in the last years, in particular after the COVID-19 pandemic, as an increasing concern for public health [17] and include hepatitis A virus (HAV), rhinoviruses, corona-, noro- and rotaviruses [16].

The persistence of microorganisms on inanimate surface varies according to the type and surface properties, type(s) of pathogen, microbial burden, and environmental conditions [18]. *S. aureus* can persist in low humidity conditions over time. On the contrary, gram-negative bacteria, such as *Pseudomonas spp.*, *Klebsiella spp.* and *E. coli*, improve their survival rates in high relative humidity [1]. Furthermore, bacteria and fungi show different survival rates depending on the type of material surface they encounter, i.e. glass, stainless steel, aluminum, wood and cloth [18-20]. In addition, viruses, such as corona, coxsackie, influenza, or rhino virus, can persist in inanimate surfaces, such as surgical gloves, aluminum, glass, silicon, ceramic, metal or wood [21]. Specifically, *Human Coronavirus 229E* survives on polymer substrate up to 5 days at 21°C [22]. Biofilm formation is also an important factor for survival rate. In a biofilm state, bacteria are at least 500 times more resistant to antimicrobial agents. The biofilm matrix contains water, nutrients and extracellular substances like polysaccharides and proteins, that not only provide a nutritional source but also a certain level of humidity that protects the microorganisms against environmental influences [1].

Considering the ability of microorganisms to persist and survive for long periods of time in inanimate surfaces, the use of antimicrobial compounds emerges as an attractive solution for biomedical and common daily applications. Some examples include anti-fouling materials [23], industrial water systems [24], electronic materials (e.g. keyboards [25]) and medical devices (e.g. catheters, endotracheal tubes [26] and implants [27]).

Polymer materials can be modified to enhance their antimicrobial performance by following different strategies: (i) modification of the polymer surface properties without an antimicrobial agent; (ii) deposition of an antimicrobial compound directly on the polymer surface or after a chemical surface modification; and (iii) incorporation of the antimicrobial compound in the polymer matrix. The first strategy is based on polymer modification of the chemical or physical properties of the surface, including its free energy, polarity, or topography. The second approach involves the immobilization of an antimicrobial agent at the surface material by surface deposition techniques. The last strategy can be carried out by incorporating the antimicrobial agent in the polymer solution or by dispersing the antimicrobial agent in the polymer bulk [28]. Although the development of polymeric coatings with antimicrobial agents is an important strategy to avoid the colonization of microorganisms on inanimate surfaces [29], the selection of antimicrobial compounds is a critical step to produce antimicrobial coatings, since the intrinsic properties of antimicrobial agents can impact the properties of the final product [29] and, ultimately its functionality. Moreover, manufacturing process parameters are a key factor when producing antimicrobial polymeric coatings since materials must withstand extreme pressure and temperature conditions while keeping antimicrobial activity.

The most frequent antimicrobial agents incorporated in polymer materials are quaternary ammonium compounds (QAC) [30], metal based-compounds [31], antimicrobial peptides [32], antibiotics [33], as well as other organic (such as chlorhexidine, povidine-iodine or triclosan) [34,35], and inorganic compounds (graphene based-compounds) [36,37]. Phenolic-based compounds are a large group of molecules based on a hydroxyl group attached to one or more aromatic benzene ring(s) [38]. Due to their antioxidant properties, these compounds represent an important group of metabolites in plants with a wide range of biological properties [39,40]. In materials science, phenolic-based materials have been used as additives in the food packaging industry [41]; as films stabilizers when incorporated in polymeric matrices [42]; in tissue engineering [43]; and as a commercial form as epoxy phenolic resins/compounds [44]. Phenols can be classified in different groups according to the phenol units and the structural elements

linked to aromatic groups. The phenol-based compounds chosen for this study were [1,1'-Biphenyl]-2-ol, mostly known as 2-phenylphenol or o-phenylphenol (OPP), which is a class of hydroxybiphenyl groups in which a biphenyl is substituted by a hydroxyl group at position 2; and 2-phenoxyethanol (PE). PE is not a pure phenol, but rather a derivative from a phenolic compound with a 2-hydroxyethyl group substituting the aromatic ether on oxygen. Both OPP and PE are used as preservatives for surface disinfection, wood treatment, vegetable packaging, textiles, and cosmetics, due to their large spectrum of action against gram-negative and gram-positive bacteria as well as against fungi [45].

In addition to the antibacterial activity associated to phenol-based compounds [46], antiviral (against rabies virus) [47] and antifungal activity (against *Candida albicans* and *Trichophyton rubrum*) [48] has also been described. In particular, it has been shown that OPP and other derivatives of phenolic compounds have potential effect over different types of viruses, such as human immunodeficiency virus (HIV), herpes simplex virus (HSV) or hepatitis B viruses [49].

Ionic-based liquids (IL) are a new class of compounds that have been gaining more attention since the beginning of the century, not only as an alternative green approach to organic solvents in industrial processes [50], but also in detergents and cosmetics [51]. The possibility to physically, chemically, and biologically modify each constituent of ILs independently (cationic head and tail, and anion), makes these compounds interesting molecules for biomedical and industrial applications as antimicrobial agents [52-54]. In fact, it is estimated that there are 10^{18} possible combinations [55]. 1,3-didecyl-2-methylimidazolium chloride (DMIC) is one of those combinations and comprises a heterocyclic group with a chloride anion. Antimicrobial properties of ILs depend on its structure, in particular the cationic organic head group, the tail (usually an alkyl linear chain with a variable length) and an organic/inorganic or biobased anion [55].

Finally, the use of metal based-compound materials has gained great interest not only in the biomedical field but also in physical and materials science due to their intrinsic properties, including good compatibility with biological tissues and bioactivity, high density, high melting point and thermal conductivity [56]. The most common metal compounds used as antimicrobial agents are silver (Ag), titanium (Ti), gold (Au), calcium (Ca), and copper (Cu), magnesium-based compounds [57]. The Environmental Protection Agency (EPA) of the United States has approved Cu's ability to inactivate or kill 99.9% pathogenic bacteria within 2 h [2]. Importantly, Cu's antimicrobial properties are maintained regardless the mode of presentation, whether that be in bulk form [58]; associated to nanoparticles [59-62]; incorporated in coating material alone or

combined with other compounds (e.g. with lactic acid) [63]; or associated to metal-organic frameworks [64].

In this work, antimicrobial polyurethane (PUR) lacquer-films were produced with the incorporation of different antimicrobial agents to avoid the colonization by microorganisms on inanimate surfaces and ultimately prevent the spreading of potentially harmful pathogens among humans. The antimicrobial lacquer-film was obtained by adding phenolic-based compounds such as: 2-phenylphenol (OPP) or 2-phenoxyethanol (PE); an ionic-based compound: 1,3-didecyl-2-methylimidazolium chloride (DMIC); or a metal-based compound: copper (II) sulphate pentahydrate (CuSP) to a PUR-based lacquer formulation. The antimicrobial materials were firstly characterized optically, chemically, and physically. The antimicrobial analysis was performed using the most abundant microorganisms that contaminate indoor surfaces, while the biocompatibility assays of the produced materials were performed with a representative cell line from the skin. A graphical abstract of this work is present in Figure 1.

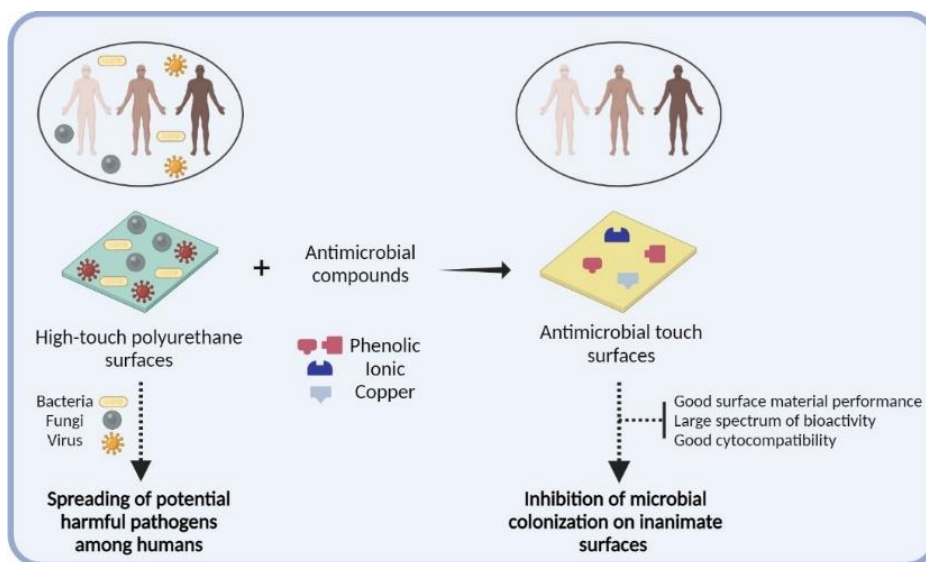


Figure 1 – Graphical abstract of work: “Development of polyurethane antimicrobial coatings by composition with phenolic-, ionic- and copper-based agents”.

2.2.2 Materials and methods

2.2.2.1 Production of PUR-modified antimicrobial films: the base lacquer formulation was obtained by mixing different commercial raw materials, such as waterborne PUR dispersion, rheology modifiers, defoamers, levelling agents, hard modifiers, matting agents (silicone) and a crosslinker agent (polyisocyanate). The isocyanate functional groups (NCO) of the crosslinker react with free amine or hydroxyl groups of urethanes increasing the molecular weight of the pre-polymer. Finally, the antimicrobial stock solutions were added to the base lacquer formulation and mixed to

create a homogeneous solution. The films were obtained by solvent casting. Briefly, the lacquer formulation was spread, with a 30 μm thickness, on polyethylene terephthalate (PET) inert surface and was submitted to two heating cycles: 4 min at 50°C on a digital hotplate (Stuart SD300, R330000718) following 1 min at 140°C in a laboratory hot air oven type (Werner Mathis AG, LTF-ST 119988). The PET surface acts only as a substrate to film deposition. All raw materials were made available by TMG Automotive. Antimicrobial compounds OPP 99% ($\text{C}_{12}\text{H}_{10}\text{O}$, CAS 90-43-7), PE $\geq$ 99% ($\text{C}_6\text{H}_5\text{OCH}_2\text{CH}_2\text{OH}$, CAS 122-99-6), DMIC 96% ($\text{C}_{24}\text{H}_{47}\text{ClN}_2$, CAS 70862-65-6) and CuSP 99% ($\text{CuO}_4\text{S}_5\text{H}_2\text{O}$, CAS 7758-99-8) were purchased from Sigma-Aldrich.

In this work, the highest concentration produced for OPP and PE were 224 $\mu\text{g}/\text{cm}^2$ and 235 $\mu\text{g}/\text{cm}^2$, respectively. These values were obtained after an optimization process to maximize antimicrobial agent incorporation. The phenolic concentrations in lacquer-films were in the upper limit of compatibility between antimicrobial agent and PUR waterborne dispersion. Efforts were made to increase the phenolic concentration in lacquer-films; however, above the mentioned concentrations a rheological modification of lacquer liquid solution was observed (mainly an increase in viscosity), which compromised the production of lacquer-films and their visual appearance.

2.2.2.2 Visual inspection and optical analyses of lacquer-films: a macroscopic inspection of lacquer-films incorporating antimicrobial agents was performed to analyse the surface topography and homogeneity, while gloss analysis was done to evaluate the optical and surface properties using a single angle glossmeter with an incidence angle of 60° (Micro-gloss 60° supplied by BYK Instruments).

2.2.2.3 Transmittance spectroscopy analysis of lacquer-films: transmittance spectra were collected from 360 to 700 nm, with a resolution of 10 nm, from 3 different lacquer-films for each compound in a Datacolor 650™ spectrophotometer. Only the transmittance analysis of lacquer-films produced with highest concentration of antimicrobial agents are presented.

2.2.2.4 Fourier transform infrared spectroscopy (FTIR) analysis of lacquer-films: FTIR analyses were performed on a FT-IR Spectrum Frontier. This technique is based on an interaction between an infrared beam and a diamond or a crystal with a high refractive index. As a result, an evanescent wave is generated, which penetrates a few microns on the surface of the sample and is attenuated by interactions with material, affecting energy absorption. Peak position and intensity are dependent on the various vibrational modes of molecules that comprise the test sample [65].

In this study, FTIR analyses were done on lacquer-films with and without antimicrobial compounds to characterize the chemical influence of these additives on lacquer-films. Spectra were collected at wavenumber range from 400 to 4000 cm^{-1} , with a resolution of 1 cm^{-1} . Only the FTIR analysis of lacquer-films with highest concentration of the antimicrobial compound are presented.

2.2.2.5 Evaluation of antibacterial and antifungal activity of lacquer-films: the antibacterial and antifungal assays were based on the protocol of the International Organization for Standardization (ISO) 22196(E):2011 - Measurement of antibacterial activity on plastics and other non-porous surfaces [66]. Briefly, lacquer-films of 5×5 cm^2 were inoculated with 0.4 mL of a bacterial or antifungal suspension (*S. aureus* ATCC 6538P, *Escherichia coli* ATCC 8739 and *C. albicans* SC5314/ATCC MYA-2876) prepared in a suspension medium (6×10^5 bacteria/mL). An inert cover film (4×4 cm^2) was placed over the material surface to ensure the contact between inoculum and lacquer-film. Test samples were incubated for 24 h at 35°C (or 32°C for antifungal assays) in an atmosphere with high relative humidity. To validate the initial inoculum dose, and consequently validate the assay, samples of untreated material (control lacquer-films) are processed immediately after inoculation. After incubation, the materials were washed with a wash solution composed by soybean casein, lecithin and polyoxyethylene sorbitan monooleate. Ten-fold serial dilutions of washing medium were plated on agar plate medium (based on yeast extract, tryptone and glucose) for bacterial assays and YPD agar medium (composed by peptone, glucose and yeast extract) for antifungal assays. The plates were incubated for 40-48 h at 35°C or 32°C, for bacteria or fungi, respectively. Following this incubation period, the number of colonies forming units (CFU) were counted. The antibacterial activity (R) was calculated comparing the average of common logarithm of number of viable colonies recovered from untreated materials 24 h after inoculation and the average of common logarithm of number of viable colonies recovered from treated materials after 24 h of inoculation. In addition, a microbial ratio (MR) for treated materials from untreated materials was also analysed.

2.2.2.6 Evaluation of antiviral activity of lacquer-films: the antiviral activity was based on the SARS-CoV-2 ribonucleic acid (RNA) degradation assay using a highly sensitive reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method [67]. SARS-CoV-2 samples derived from excess human swabs samples were used. Samples were stored at the University of Minho diagnostic laboratory, approved by the competent Institutional Review Board, Comissão de Ética para a Investigação em Ciências da Vida e da Saúde (CEICVS), with the reference

CEICVS008/2022. Briefly, 0.3 mL of a SARS-CoV-2 sample (with viral loads from 1000 to 3000 copies per mL) was inoculated on lacquer-film surface (3×3 cm²). As control, viral suspensions were also inoculated directly in the PET inert surface (where the lacquer-film is deposited). An inert cover film (2×2 cm²) was placed on the surface of inoculum for all the conditions tested and incubated for 24 h at room temperature in an atmosphere with high relative humidity. After incubation, 150 µL of the viral suspension was collected and inactivated. RNA was further extracted using a semi-automated magnetic-particle processor (KingFisher Flex Purification System; Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer instructions. For RT-qPCR, 10 µL of RNA were mixed with 20 µL of a NZYSupreme One Step RT-qPCR Probe Master Mix, MB414 (©2020 NZYTech, Lda, Lisbon, Portugal). RT-qPCR assays were performed on a QuantStudio™ 6 Pro (Applied Biosystem by Thermo Fisher Scientific) following the protocol previously described [67]. For the antiviral assays, only the highest concentration of OPP, PE, DMIC and CuSP incorporated compounds in the lacquer-films were analysed. The percentage of viral elimination was calculated using Cq values and respective viral quantification load obtained from the control samples (viral sample diluted in water) minus the viral load obtained in the functionalized lacquer-films as previously described [68,69].

2.2.2.7 Cytocompatibility of antimicrobial lacquer-films: indirect cytocompatibility assays were performed using a colorimetric assay based on resazurin reduction and an adaption of protocol of the International Organization for Standardization (ISO) 10993-5:2009 - Biological evaluation of medical devices, Part 5: Tests for *in vitro* cytotoxicity [70]. Resazurin is a non-fluorescent dye used to analyse bacterial or cell viability [71]. This assay is based on the resazurin reduction (blue dye) by reductase or diaphorase-type enzymes from mitochondria of living cells to resorufin (pink colour). The colour change is an indicator of metabolically viable cells [72]. Briefly, lacquer-films (0.31 cm²) were incubated with cell culture media (Dulbecco's Modified Eagle's Medium) supplemented with 10% of fetal bovine serum (FBS) and 1% L-glutamine, at room temperature for 24 h under orbital shaking (100 rpm), to obtain supernatant with lacquer extract. The L-929 fibroblast cell line (1×10⁴ cells/well) was incubated in a 96-well plate for 24 h plate at 37°C in a CO₂ incubator with atmosphere of 5% of CO₂ (Heraeus HERAccl 150 Incubator, Thermo). After incubation period, cell culture media was replaced with the lacquer-extract medium. Plates were incubated for an additional 24 h. A positive control (cells cultured in normal cell culture medium), a negative control (only cell culture medium) and a positive control of cytotoxicity (1% Triton X-100) were also included. After incubation, 0.02% resazurin dye was added. The plates

were further incubated for 3 h to allow for resazurin reduction. The optical density was read spectrophotometrically at 575 and 610 nm. The results were expressed as percentage of cell viability from positive control, by absorbance (Abs) ratio between at 575 and 610 nm for the different conditions, according to the following equation:

$$\% \text{ cell viability} = \frac{\text{Abs sample} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100$$

According to ISO10993-5:2009 protocol, a cell viability below 70% of the positive control is defined as a potential cytotoxic result [70].

2.2.2.8 Statistical analysis: data was reported as mean $\pm$ standard deviation. Statistical analysis was done using GraphPad Prism (version 8.0.2) by testing the normality and the significance values. Bonferroni's multiple comparisons test was used for antibacterial and antifungal lacquer-films analysis and Dunn's multiple comparisons test was used for antiviral lacquer-films analysis. Data was considered statistically significant for $p < 0.05$.

2.2.3 Results and discussion

PUR is one of the most important polymer materials used in coatings, due to its intrinsic properties, high absorptivity, mechanical stability, softness, and flexibility. In addition, the possibility to functionalize PUR-based materials to improve specific properties has made it an attractive material for many biomedical and common daily applications, including hospital environments, packaging, automotive industry, home appliances and infant care [6]. The selection of antimicrobial agents to be incorporated in PUR coating formulations should take into consideration some key criteria: (i) compatibility with processing parameters of the PUR lacquers (thermal stability); (ii) chemical compatibility between antimicrobial compound and the lacquer formulation used, and (iii) spectrum of bioactivity (bacteria, fungi, and virus). With these three criteria in mind, two phenolic-based compounds (OPP and PE); an ionic based-compound (DMIC); and a metal based-compound (CuSP) were selected.

Given that the production process of lacquer-films for surface coatings requires successive cycles of high temperatures to facilitate solvent evaporation, trials were conducted to confirm that the selected compounds maintained their antimicrobial activity, even after being subjected to two consecutive cycles with a minimum temperature of 140°C. OPP, PE, and CuSP are reported to be thermally stable at high temperatures, given their boiling points of 286°C [45], 247°C (according

to supplier), and $>500^{\circ}\text{C}$ [74], respectively. Accordingly, these compounds maintained their half-maximal inhibitory concentration values (IC_{50}), even after being submitted to cycles of high temperatures. In contrast, the IC_{50} of DMIC after two cycles of high temperatures increased; however still presented antimicrobial activity against the strains tested (Supplementary Material). In fact, ILs, such as DMIC, are reported to be stable between $100\text{--}125^{\circ}\text{C}$ [75], but above these temperatures, a partial degradation of the alkyl chain can occur, which consequently impact their properties [75].

2.2.3.1 Evaluation of physical, optical, and chemical properties of lacquer-films incorporated with selected compounds: an important aspect evaluated in this study was the maintenance of the optical, physical, and chemical properties of the lacquer-films after composition with the above-mentioned compounds. The goal of this analysis was to ascertain whether the incorporation of these compounds affected the physical and chemical properties of lacquer-films commonly used as surface coatings. In that sense, antimicrobial compounds were incorporated in a PUR waterborne resin by solvent evaporation to produce antimicrobial lacquer-films. As shown in Figure 2A, macroscopic observation of the lacquer-films with antimicrobial compounds revealed a white hue with a continuous surface. In some conditions it was possible to identify a few white aggregates, most likely due to poor solubility in water or in the PUR resin/formulation. This situation was more visible in lacquer-films with increasing concentrations of OPP (dashed line in Figure 2A). However, some minor clusters could also be identified in other lacquer-films (e.g. lacquer-films of CuSP or DMIC), but in these cases, the unintended visual impact was lower.

Additional optical analysis of the lacquer-films was performed by gloss measurements and transmittance studies. Specular gloss can be defined as a measure between a specular reflectance of a surface relative to the specular intensity reflected at an angle of incidence θ [76]. Gloss is a simple and useful parameter that can be used as an indicator of material durability by monitoring the chemical bonds and functional groups existent in materials [77]; the impact of additives, including pigments [77]; and the surface roughness [78]. Specifically, there is a correlation between surface roughness and gloss, in which the increase of roughness decreases the gloss of the surface for the same angle of incidence [76,79]. In the present study, gloss analysis showed a value of 8.66 ± 0.59 for control lacquer-films (Figure 2B), which was similar to the gloss of lacquer-films with the highest concentrations of PE (8.60 ± 0.45), DMIC (9.16 ± 0.41) and CuSP (9.09 ± 0.41). In contrast, lacquer-films with the highest concentration of OPP presented an increase of 1.5 in gloss values (10.13 ± 0.27), when compared to control lacquer-films, which could be

related to intrinsic fluorescent properties of phenolic-based compounds [80]. The fluorescence can be defined as a radiation emission at lower energy by an object after exposure to a light source. Indeed, in OPP-based materials, this emitted radiation could be detected by the glossmeter [81], explaining the increased values. This increase was not visible in the other tested phenolic-based compound, PE, since its chemical structure is not a pure phenolic compound, as described above.

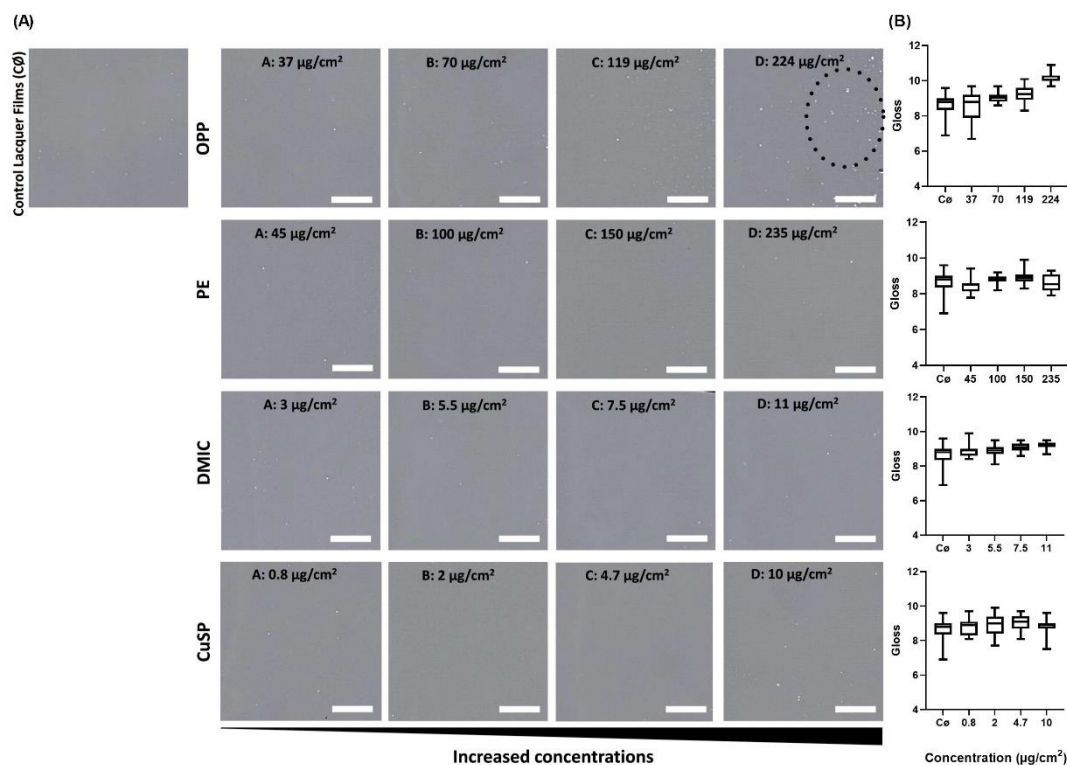


Figure 2 – Analysis of produced lacquer-films produced: (A) macroscopic view and (B) gloss measurements of lacquer-films incorporated with different concentrations of antimicrobial compounds. The gloss values were obtained in three different lacquer-films for each condition, with 12 reads in different locations across each film length. Scale bar = 1 cm.

Transmittance measurements were performed to analyse the optical integrity of the lacquer-films after incorporation of antimicrobial agents. The results showed that the incorporation of antimicrobial agents does not affect the optical properties of lacquer-films produced, since the differences between control and samples are less than 1.5% (Figure 3A). Indeed, the transmittance value at 580 nm for control lacquer-films is 97.5%, while for lacquer-films with OPP, PE, DMIC and CuSP the transmittance values are 98.4, 97.4, 98.1 and 98.0%, respectively.

To assess any possible changes in the chemical bonds of produced lacquer-films induced by the presence of antimicrobial agents, we performed FTIR analysis comparing the normalized FTIR spectrum obtained from the control lacquer-films with lacquer-films with highest concentration of antimicrobial compounds. Firstly, the impact of the incorporation of antimicrobial compounds in

the polymerization process of PUR was investigated. It has been well described in the literature that PUR formation can be monitored by measuring the isocyanate (NCO) content, since it reacts with diols being consumed [82,83]. As shown in Figure 3B, the FTIR spectrum of the lacquer-films, regardless of the addition of antimicrobial compounds, revealed the absence of the characteristic band of NCO (asymmetric isocyanate stretching band at approximately 2250 to 2270 cm^{-1}), confirming a reduction of NCO bands and consequent PUR synthesis.

Further analysis of the FTIR spectrum revealed a spectral overlap among the different samples indicating the absence of chemical alterations in lacquer-films, even after the incorporation of antimicrobial compounds. In Figure 3B, it is possible to confirm some characteristic bands of PUR: (i) band 1 is a large absorption band identified at 3322 cm^{-1} , probably resulting from the overlap of two peaks corresponding to NH stretching (at higher wavenumber) and OH group (at smaller wavenumber) [84-86]; (ii) band 2 is attributed to $-\text{CH}_2$ stretching [84]; (iii) band 3 (1688 cm^{-1}) is assigned by a C=O groups of PUR linkage [85] and (iv) bands in region 4 can be attributed to other modes of vibration of $-\text{CH}_2$ group [84-86]. This lack of specific bands associated to the antimicrobial compounds was expected, given that OPP and PE exhibit the same chemical groups already present in control lacquer formulations and that in the DMIC and CuSP lacquer-films the antimicrobial compounds are present at relatively low concentrations ($\leq 11 \mu\text{g}/\text{cm}^2$ and, $\leq 10 \mu\text{g}/\text{cm}^2$, respectively), which makes it difficult to identify specific bands.

Collectively, these results suggest that the addition of these antimicrobial compounds did not significantly change the chemical nature of the lacquer, with exception of OPP in which high concentrations impacted the gloss values and the surface homogeneity.

2.2.3.2 Antimicrobial performance of lacquer-films modified with the selected compounds: following the validation of the chemical and physical properties of the lacquer-films with the addition of antimicrobial agents, we next evaluated their antimicrobial activity against a representative gram-positive and gram-negative bacterial strains and a fungal strain, following the ISO 22196(E):2011 protocol. According to this protocol, antimicrobial activity is determined by the R value, which is expressed as the difference between the common logarithm of the number of CFU collected in control lacquer-films and the common logarithm of the number of CFU collected in the samples. R values ≤ 0.5 are classified as having no antimicrobial activity; 0.5-1 moderate microbial reduction; 1-2 medium microbial reduction; and ≥ 3 good antimicrobial reduction [87].

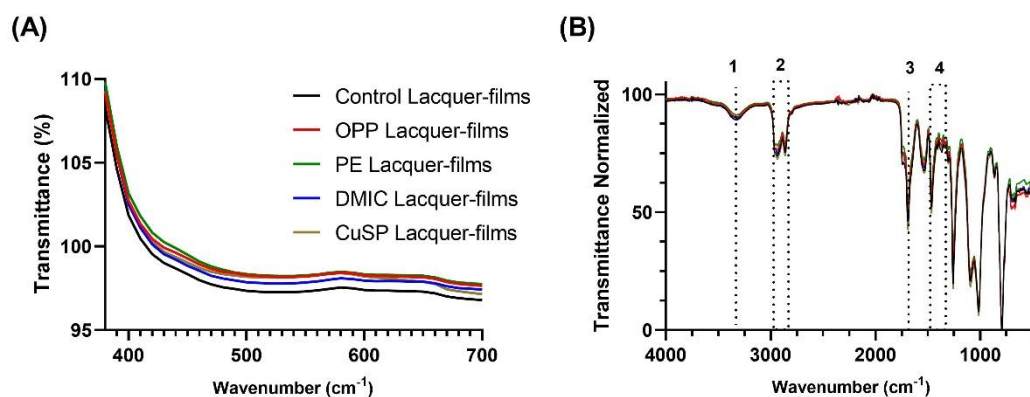


Figure 3 – (A) Transmittance and (B) FTIR analysis of lacquer-films produced with antimicrobial compounds. The lines correspond to the lacquer-films with the highest concentration of antimicrobial compounds produced. Numbers represent specific bands used to analyse the FTIR spectrum.

Regarding the phenolic-based compounds, although OPP and PE lacquer-films were responsible for a significant CFU Fog reduction of *S. aureus* in all concentrations tested (Figure 4), this translated to an R value below 0.5, indicating no antimicrobial activity (Table 1). Additionally, these phenolic compounds showed no antibacterial effect on *E. coli*, as well as no antifungal activity for *C. albicans*, as the microbial load was similar to the control condition. Nevertheless, our data suggests that gram-positive bacteria are more susceptible to phenolic-based compounds incorporated in polymer materials. These results are aligned with Gaikwad, K. *et al.* (2018), which reported an antimicrobial effect of the phenolic compound pyrogallol incorporated in low-density polyethylene (LDPE) against *S. aureus*, but only moderate activity against *E. coli* [88]. A similar result has been reported, showing that films with higher phenolic content (gallic acid equivalent) have an increased bacterial inhibition (>1 Log reduction) against *B. subtilis* and *S. aureus* in comparison with gram-negative strains [89]. In fact, the presence of an outer membrane in the cell wall of gram-negative bacteria plays an important protective role against environmental conditions by stabilizing its inner membrane and protecting its thin peptidoglycan layer [90]. This complex composition of the cell wall in gram-negative strains seems to contribute to their increased resistance to membrane active agents, such as phenolic-based compounds [91]. Despite the high range of applications of phenolic-based compounds for cosmetic [40] and biomedical purposes [38,92,93] their mechanisms of action have not been well described. Their action have been mainly attributed to the inhibition of microbial enzymes, toxins and other virulence factors, by directly altering bacterial metabolism; changing bacterial polarity through alteration of surface electron receptors; and impacting the production of substrates required for growth [94,95]. Most importantly, phenolic-based compounds have been described to act by compromising bacterial cell

wall permeability, suggesting that the presence of the outer membrane of gram-negative bacteria provides protection against phenolic compounds [96]. Indeed, it is suggested that OPP's biocidal effect is associated to the disruption of the diaminopimelate and lysine biosynthesis pathway, leading to the downregulation of amino acid synthesis essential for the peptidoglycan cell wall [97]. PE can also act as a bacteriostatic agent by changing the permeability of the cytoplasmatic membrane through inhibition of DNA and RNA synthesis [98]. In in this work, increased antimicrobial effect of phenolic compounds could not be obtained, since higher concentrations of OPP and PE in lacquer formulations, would significantly change the lacquer properties, specifically viscosity.

Table 1 – Antimicrobial activity of produced waterborne polyurethane-based lacquer-films formulated with different concentrations of antimicrobial agents.

	Concentration ($\mu\text{g}/\text{cm}^2$)	R value* / Microbial Ratio (%)**			
		<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>	<i>SAR-CoV-2</i> ***
OPP	37	0.295 / 48.53	-0.060 / -15.75	0.059 / 11.35	-
	70	0.210 / 38.90	-0.051 / -12.06	0.082 / 17.53	-
	119	0.070 / 13.54	-0.024 / -6.23	0.042 / 7.68	-
	224	0.269 / 46.50	-0.007 / -1.47	-0.027 / -7.51	n.d. / 41.17
PE	45	0.130 / 24.78	-0.075 / -18.98	0.075 / 14.52	-
	100	0.154 / 29.17	-0.009 / -2.19	0.135 / 26.40	-
	150	0.124 / 24.15	-0.035 / -9.49	0.069 / 15.18	-
	235	0.190 / 34.47	0.006 / 1.46	0.022 / 5.28	n.d. / 53.17
DMIC	3	1.506 / 96.83	0.083 / 17.11	0.101 / 16.87	-
	5.5	2.350 / 99.54	0.053 / 11.50	0.104 / 21.14	-
	7.5	5.077 / 100	0.038 / 8.82	0.425 / 59.09	-
	11	5.077 / 100	0.854 / 84.41	5.355 / 100	n.d. / 19.51
CuSP	0.8	0.716 / 76.02	0.038 / 7.67	-0.001 / 0.27	-
	2	2.374 / 99.54	0.579 / 73.50	-0.051 / -11.80	-
	4.7	4.799 / 100	6.573 / 100	0.599 / 74.95	-
	10	4.799 / 100	6.573 / 100	5.135 / 100	n.d. / 13.67

* Antibacterial and antifungal activity (R) is calculated according to ISO 22196:2011, by using the following formula: $R = (U_t - U_0) - (A_t - U_0) = U_t - A_t$ in which U_0 , U_t and A_t are the average of the common logarithm of the number of viable bacteria recovered immediately after inoculation for untreated samples, 24 h of incubation for untreated samples and 24 h of incubation of test samples, respectively. ** Microbial Ratio (MR) is the percentage of viable bacteria recovered from treated samples compared to untreated samples after 24 h of incubation. MR were calculated by the next formula: $MR = 100 - (T_s \times 100)/C_s$, in which T_s and C_s is the average of viable bacteria recovered from treated and untreated samples, respectively. Negative values indicated an increase of bacterial burden comparing to control group and positive values indicates a microbial reduction to control samples. ***Antiviral activity is calculated by percentage of viral reduction according to control group (PET inert surface).

On the other hand, DMIC lacquer-films exhibited an intermediate antibacterial activity against *S. aureus* for the lowest concentration tested ($3 \mu\text{g}/\text{cm}^2$) with a 1.51 CFU Log reduction, and a good antibacterial reduction for the intermediate concentration ($5.5 \mu\text{g}/\text{cm}^2$) with 2.35 CFU Log reduction. These values represent a bacterial reduction of 96.8 and 99.5%, respectively. For the highest concentrations tested (7.5 and $11 \mu\text{g}/\text{cm}^2$) no bacteria were detected, representing a 100% bacterial reduction. Regarding DMIC's antibacterial activity against *E. coli*, only the highest concentration tested ($11 \mu\text{g}/\text{cm}^2$) was able to control bacterial proliferation (0.85 CFU Log reduction and 84.4% of bacterial reduction). These results are in accordance to those reported previously, which describe that gram-positive bacteria seem to be more susceptible to ILs than gram-negative bacteria or fungi [50,99,100], specifically 1-ethylpyridinium docusate and tributyl(2-hydroxyethyl)phosphonium docusate [101]; tetrahexyl-ammonium bromide [102] and methylimidazolium chloride and pyridinium chloride based compounds [103]. Gram-negative bacteria have an additional outer layer which is negatively charged. This layer can act as a barrier for neutral or negatively charged compounds, while cationic compounds can bind to the outer membrane of the cell wall and cause bacterial damage [104]. In that sense, the limited effect of DMIC on gram-negative bacteria could be related to the presence of chloride. Similar results are reported by He. B. *et al.* (2013), in which an ILs with a fumarate anion presents increased antimicrobial properties against *B. subtilis* when compared to *E. coli* [105]. Regarding its antifungal properties, DMIC exhibited a great control on fungal proliferation of *C. albicans*. Specifically, the two highest concentrations of lacquer-films with DMIC had a significant Log reduction. Indeed, $7.5 \mu\text{g}/\text{cm}^2$ and $11 \mu\text{g}/\text{cm}^2$ DMIC lacquer-films showed a 0.43 and 5.36 CFU Log reduction, respectively, representing 59 and 100% fungal reduction (Figure 4 and Table 1). DMIC has also been described to control different fungi strains, such as *A. padwickii*, *A. dauci*, and *A. linicola* [106]. Although the mechanism of antimicrobial control by ILs is not completely understood it has been related to the interaction of cations or anions on the cell surface that promotes structural and dynamic changes causing pathogen disruption [107]. ILs antimicrobial activity is also potentiated

by extending the number of alkyl groups present in chain length rather than the anion/cation combination [50,55].

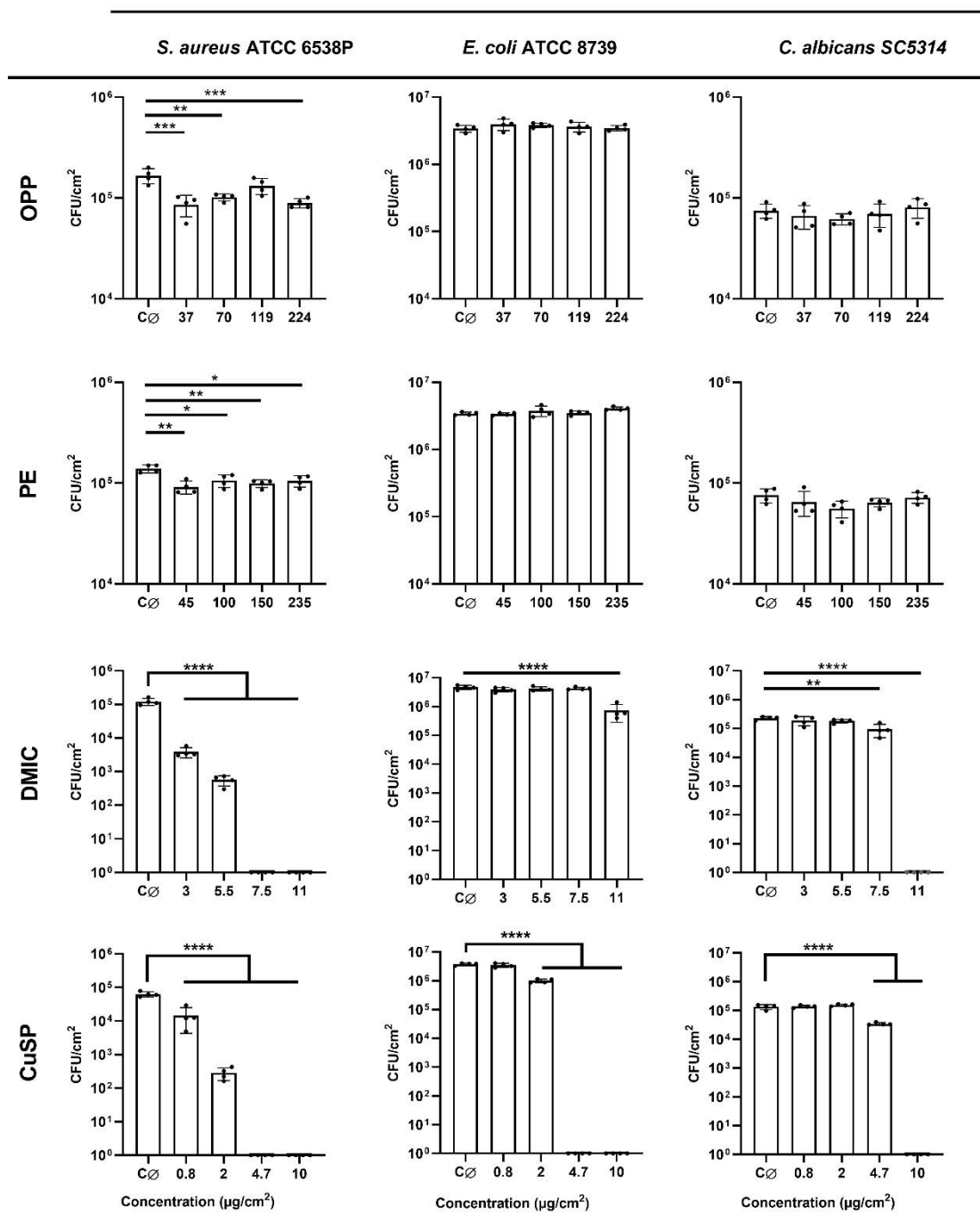


Figure 4 – Antimicrobial activity for OPP, PE, DMIC and CuSP lacquer-films against *S. aureus*, *E. coli* and *C. albicans*. CØ represents the lacquer-films without antimicrobial compound (control lacquer-film). Error bars represent the standard deviation (n=4). Bonferroni's multiple comparisons test: * p<0.05; ** p<0.01; *** p<0.001 and **** p<0.0001. Each condition was tested in quadruplicate in two independent assays.

CuSP lacquer-films exhibited a complete clearance (>4 CFU Log reduction) for the highest concentrations tested (4.7 and 10 $\mu\text{g}/\text{cm}^2$) for both bacterial strains. For the fungal strain, the highest concentration tested (10 $\mu\text{g}/\text{cm}^2$) resulted in >5 CFU Log reduction, while lacquer-films incorporated with 4.7 $\mu\text{g}/\text{cm}^2$ of CuSP showed only a moderate microbial reduction (0.57 Log reduction, representing an almost 75% reduction in the fungal burden). At lower concentrations (0.8 and 2 $\mu\text{g}/\text{cm}^2$), gram-positive bacteria presented a CFU Log reduction of 0.72 and 2.37, respectively (representing a 76.0 and 99.5% bacterial reduction); while for gram-negative bacteria only the 2 $\mu\text{g}/\text{cm}^2$ condition displayed antibacterial activity with a 0.58 CFU Log reduction (73.5% bacterial reduction). Lacquer-films incorporated with 0.8 $\mu\text{g}/\text{cm}^2$ or 2 $\mu\text{g}/\text{cm}^2$ of CuSP showed no antifungal activity. According to this, CuSP lacquer-films showed to be highly effective in limiting both bacterial and fungal growth. These results are aligned with other authors which describe the antimicrobial activity of Cu-based compounds against *S. aureus*, *E. coli* [108] and MRSA [109]. Although the associated antimicrobial mechanisms are not fully elucidated, it is thought that the release of ions from Cu plays an important role, leading to reactive oxygen species (ROS) production [110,111]. Others have suggested that intracellular Cu ions can selectively bind to sulfhydryl groups of respiratory enzymes and disrupt some essential proteins [112]. Cu's proposed mechanism of action justifies its broad spectrum, ranging from several bacteria, such as *Staphylococcus* spp., *E. coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *S. typhimurium* [111], to fungi, such as *C. albicans*, *A. niger*, *A. oryzae*, *Fusarium oxysporum* and *Alternaria alternata* [64,113,114]. Regarding the difference in susceptibility between gram-positive and negative strains, we observed that CuSP based lacquer-films are more effective against *S. aureus* than *E. coli*, which is aligned with what reported by Ude, Z. *et al.* (2019) [115]. In the same work, the authors showed an antifungal activity of Cu-based compounds against filamentous and non-filamentous fungi (such as different *Candida* strains, *T. asahii*, *A. fumigatus*, *A. corymbifera* and *T. mentagrophytes*) [115].

The lacquer-films with and without addition of antimicrobial agents were tested for antiviral activity by evaluating the percentage of SARS-CoV-2 RNA degradation. The exposure of SARS-CoV-2 samples to the lacquer-films *per se* (without addition of antimicrobial compounds), when compared with the control condition (PET inert surface), resulted in the elimination of 75.7% of the virus RNA. However, the lacquer-films incorporated with the different antimicrobial compounds did not enhance viral elimination. In fact, the incorporation of DMIC and CuSP in lacquer-films reduced the initial antiviral activity of the lacquer-film to 19.5 and 13.7%, respectively (Figure 5 and Table

1). This effect could be related to potential interactions with stabilizers of the lacquer formulation. Considering that the waterborne PUR lacquer formulation results from a mixture of different commercial raw materials, it is difficult to pinpoint which compound is responsible for the antiviral activity. The protocol used in this work (RT-qPCR-based detection method) only targets the RNA content by measuring the difference of the copy numbers of nucleic acids in each sample after exposure to viral inoculum. Although the measurement of RNA degradation clearly testifies to virus elimination, the lacquer, as well as the antimicrobial agents tested could target virus viability through the disruption of alternative biological processes. An alternative methodology to test the antiviral activity could be the measurement of number of plaque forming units (PFU).

Collectively, these results highlight the antimicrobial performance of several compounds, with CuSP and DMIC presenting the highest potential and the broadest spectrum.

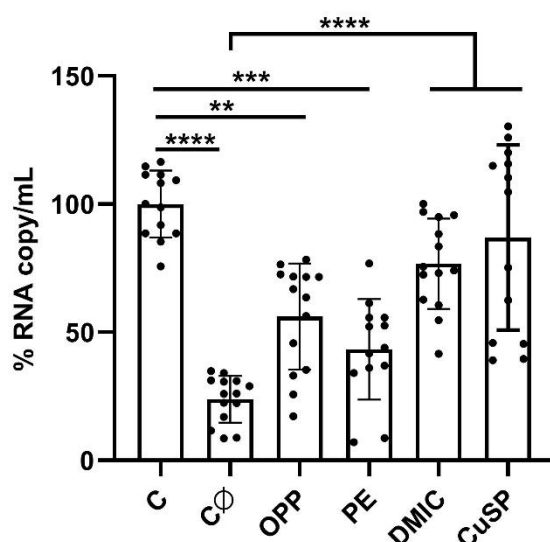


Figure 5 – Antiviral activity of lacquer-films with OPP, PE, DMIC and CuSP, (with 224, 235, 11 and 10 $\mu\text{g}/\text{cm}^2$) against SAR-CoV-2. C represents the control condition (polyethylene terephthalate inert surface where lacquer-films are deposited) and CØ represents the lacquer-films without antimicrobial compounds (control lacquer-film). Error bars represent the standard deviation of four independent essays with replicates. Dunn's multiple comparisons test: ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3.2.3.3 Cytocompatibility performance of lacquer-films: an important parameter in processing materials with antimicrobial activity is the selective action against pathogens of interest, while maintaining cytocompatibility with mammalian cells, in particular for surfaces exposed to human contact. To assess the cytocompatibility of the materials produced, L-929 mouse fibroblasts were incubated for 24 h with medium previously exposed to PUR lacquer-films or PUR lacquer-films incorporated with antimicrobial compounds. Fibroblasts exposed to PUR lacquer alone exhibited $67.2 \pm 13.5\%$ of cell viability, indicating a potential cytotoxic effect against these cells

(Figure 6). Importantly, OPP, PE and CuSP lacquer-films, regardless the concentration tested, exhibited a similar or an increased cytocompatibility, when compared to control lacquer-films. Regarding the phenolic-based compounds, the increased cytocompatibility can be explained by their protective antioxidant activity [116]. Indeed, phenols, specifically their catechol group, can provide protective effect against ROS produced during oxidative stress through its free radical scavenging activity [116,117]. In contrast, lacquer-films with the highest concentrations of DMIC (7.5 and 11 $\mu\text{g}/\text{cm}^2$) significantly decreased cytocompatibility, with only 31.79 ± 21.1 and $25.9\pm 15.6\%$ of cell viability, respectively. The length of alkyl chain and the pair anion/cation seem to play an important role in the cytotoxicity of ILs. ILs with longer alkyl chain length ($n>4$) are more lipophilic and, therefore, tend to be more easily incorporated in the phospholipid bilayers [118]. This incorporation further increases the lipophilic nature of the biological membranes, and results in delocalized charges affecting the physiological function of the cell membrane. Regarding the impact of the anion/cation pair, ILs may contain either bromide $[\text{Br}]^-$, chloride $[\text{Cl}]^-$, tetrafluoroborate $[\text{BF}_4]^-$ or hexafluorophosphate $[\text{PF}_6]^-$, which have also been associated with cytotoxic effect [118]. This information is aligned with our data, since DMIC possesses a longer alkyl chain, as well as the $[\text{Cl}]^-$ anion, and for this reason it may have contributed for its cytotoxicity at higher concentrations. Although the extracts of PUR lacquer were associated with relevant cytotoxicity, cell viability is still within acceptable parameters established in ISO 10993-5. In fact, PUR is described as a polymer with good cytocompatibility in what regards L-929 mouse fibroblasts, MRC-5 human fibroblast [119], and HeLa cell line [120]; and have been used in medical devices [121], chronic wound dressings [122], and water filters [123]. In this work, the lacquer-films with OPP, PE, and CuSP, independently of the concentration tested, did not alter the cytocompatibility of the lacquer-films. In contrast, lacquer-films incorporated with the highest concentrations of DMIC presented a cytotoxic effect [70].

2.2.4 Conclusion

Commercial PUR resins are a versatile material used in many domains of society. In this study, selected antimicrobial agents, OPP, PE, DMIC and CuSP, were incorporated in PUR-based lacquer-films aiming to obtain antimicrobial properties. Transmittance and FTIR analyses showed that the incorporation of the antimicrobial agents did not have an impact on the visual and chemical properties of the lacquer-films. Among the different compounds tested, CuSP lacquer-films exhibited the most promising antimicrobial properties, with strong antibacterial activity against both gram-positive and gram-negative bacteria, and antifungal properties, while maintaining

cytocompatibility. On the other hand, DMIC lacquer-films, although exhibiting good antimicrobial activity against *S. aureus* and *C. albicans*, display reduced cytocompatibility at higher concentrations. Regarding the OPP and PE lacquer-films, they present a null or lower antibacterial and antifungal activity. Furthermore, none of the developed lacquer-films incorporated with antimicrobial agents showed a significant effect on viral load. In future studies, it would be important to evaluate whether the selected compounds maintain their antimicrobial properties when tested against other microorganisms commonly present on inanimate surfaces, such as *P. aureuginosa*, *A. niger* or other representative viruses. In summary, the results reported in this study highlight the potential of incorporating antimicrobial agents in surface coatings as a promising strategy to avoid the attachment and colonization of microorganisms on inanimate surfaces and reduce the exposure of potentially harmful pathogens to humans.

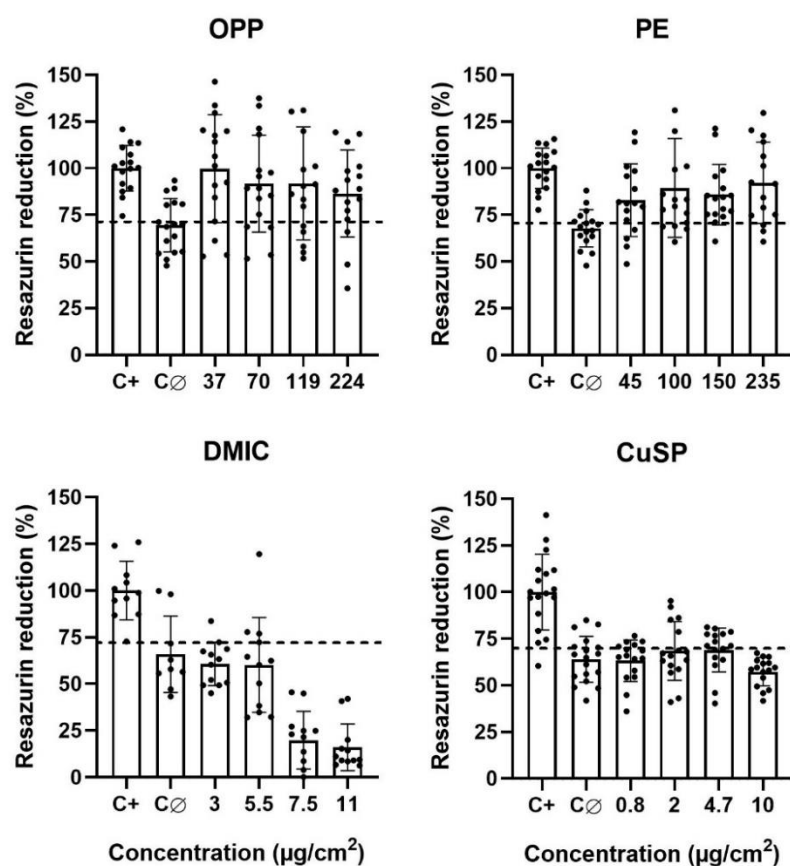


Figure 6 – Viability assay of lacquer-films with antimicrobial compound against L-929 mouse fibroblast cell line using an indirect exposure method. C+ represents the positive control (only cells+medium), CØ represents the control the lacquer-films without antimicrobial compounds (control lacquer-film). Dashed line represents 70% of resazurin reduction and error bars represent the standard deviation. Results are from three independent assays with quadruplicates in each assay.

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2.2.7 Supplementary material: evaluation of thermal stability of antimicrobial agents before and after thermal exposure

To assess the thermal stability and the antimicrobial properties, an half-maximal inhibitory concentration analysis (IC₅₀) of antimicrobial compounds: 2-phenylphenol (OPP), 2-phenoxyethanol (PE), 1,3-didecyl-2-methylimidazolium chloride (DMIC) and copper (II) sulphate pentahydrate (CuSP) was performed before and after exposure at high temperatures.

1. Evaluation of IC₅₀ of antibacterial compounds using a broth microdilution assay:

IC₅₀ determination values for *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 8739 and *Candida albicans* SC5314/ATCC MYA-2876 was performed using a microtiter plate colorimetric assay based on resazurin reduction by living cells to resorufin [1,2]. Antimicrobial compounds were serially diluted in a pre-determined range of concentrations in Muller-Hinton (MH) or yeast extract, peptone and dextrose (YPD) medium in a 96-well plate. 100 µL of microbial suspension prepared in PBS-saline (1x10⁶ CFU/mL) were added in each well. The plates were incubated for 24 h, at 37°C for bacterial strains and at 32°C for *C. albicans*, in a Kelvitron T

Heraeus Incubator. At the end of incubation period, 20 μ L of 0.02% resazurin dye was added to the wells. After 3-4 h of incubation, the optical density was determined spectroscopically at 575 and 610 nm, using the Infinite® M200 NanoQuant Micro Plate Reader, Tecan Systems, Switzerland. IC50 was determined using the GraphPad Prism software (version 8.0.2) adjusting the experimental points at a nonlinear regression (curve fit). A positive control (MH or YPD medium with bacteria or fungi suspension) and a negative control (only medium) were also included.

2. Evaluation of thermal stability of antimicrobial compounds after exposure to high temperatures: to evaluate the thermal stability of antimicrobial compounds, an IC50 analysis of these compounds after thermal exposure was performed and compared with initial values. Briefly, stock solutions of antimicrobial agents were exposed to a thermal program comprised of two cycles of heating at high temperatures ($>140^{\circ}\text{C}$) during 1 min, followed by cooling at room temperature. This thermal program was chosen to correspond to the highest temperature commonly used in the production process of lacquer-films. The vials were sealed, and the weight of solution was monitored in order to guarantee that the solution did not evaporate during the thermal program. After that, IC50 values were determined as described above. Statistical analysis was done using GraphPad Prism by testing the normality and the significance values. Unpaired t test was used for IC50 analysis comparison.

3. Results and discussion:

All antimicrobial compounds used in this work exhibited antimicrobial activity against the selected bacterial and fungal strains, with PE and CuSP presenting the highest IC50 values, while DMIC and OPP the lowest IC50 values, as summarized in Supplementary Table 1. Specifically, DMIC was highly effective against *S. aureus*, with an IC50 value more than 3-fold lower than that against *E. coli* and 8-fold lower than *C. albicans*, for RT analysis. Regarding the phenolic-based compounds, both presented antimicrobial activity, with OPP exhibiting a lower IC50 than PE. On the other hand, CuSP was more effective against *C. albicans* than, *E. coli* or *S. aureus*.

Given that the production of lacquer-films for surface coatings usually involves cycles of high temperatures for solvent evaporation, we sought to determine whether the selected compounds maintained their antimicrobial activity, even after being subjected to two consecutive cycles with a minimum temperature of 140°C . Most of the compounds showed thermal stability, in what regards antibacterial activity, except for DMIC against bacterial strains (Supplementary Table 1). Indeed, IC50 values for OPP, PE, and CuSP were not significantly different from initial

IC50 values, even after thermal exposure, indicating that their antimicrobial performance is kept stable at high temperatures. In contrast, with DMIC, we observed a significant increase in IC50 values, which is more than 3-fold higher after >140°C exposure for both bacterial strains. Despite the increased IC50 value the antimicrobial properties of DMIC were maintained.

Supplementary Table 1 – IC50 analysis of antimicrobial compounds against different bacterial and fungal strains: analysis of IC50 in solution stored at room temperature (RT) and after a thermal program.

		<i>S. aureus</i> (µg/mL)	<i>E. coli</i> (µg/mL)	<i>C. albicans</i> (µg/mL)
OPP	RT	140.4.6±33.1	123.8±31.4	89.5±11.6
	Thermal Program	141.1±12.0	133.6±23.4	84.2±17.1
PE	RT	6654±1104	5009±1140	72865±1592
	Thermal Program	6518±1138	4908±1142	13022±3129
DMIC	RT	0.52±0.08 **	1.50±0.12 *	4.47±1.1
	Thermal Program	1.8±0.45 **	5.68±1.49 *	5.65±2.3
CuSP	RT	589.4±188.3	407.3±182.8	203.8±72.1
	Thermal Program	987±30.1	567.1±39.2	197.8±67.4

Legend: RT – Stored at room temperature without thermal program. The results are expressed as mean ± standard deviation at least 3 independent assays. Unpaired t test: * p<0.05 and ** p<0.01 (RT vs. Thermal Program)

4. References

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2.3 Antimicrobial properties of hindered amine light stabilizers in polymer coating materials and their mechanism of action

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Abstract

The inhibition of polymer degradation is an important parameter to prolong the lifetime of polymeric products, while preserving their properties over time. This objective can be achieved by the use of additives which, either incorporated into the polymer matrix or immobilized at the surface, are capable to confer extended durability and resistance to degradation. UV-stabilizers are known to inhibit photodegradation of materials, keeping their structural integrity and preserving their function and commercial value. In addition, some UV-stabilizers have been attributed antimicrobial activity, which can contribute not only for avoid the dissemination of pathogenic agents among humans but also to inhibit the biodegradation caused by microorganisms.

In this work, two UV-stabilizers (commercialized by BASF), a pure hindered amine light stabilizer (HALS) (Tinuvin 770 DF) or a mixture of HALS/UV-absorber (Tinuvin 5151) were incorporated on polyurethane formulations to develop antimicrobial lacquer-films. The main goals were to evaluate the antimicrobial properties of the produced films, determine the antimicrobial properties of HALS, its underlying mechanism of action, as well as the cytocompatibility of the lacquer-films against a representative skin cell line. For that purpose, a gram-positive bacterial strain *Staphylococcus aureus* (resistant and non-methicillin resistant), a gram-negative strain, *Escherichia coli*, as well as a fungal strain, *Candida albicans* were used.

Lacquer-films incorporated with Tinuvin 770 DF showed having a strong antimicrobial performance with a large spectrum of action against both bacterial and fungi strains, while exhibiting good cytocompatibility. Regarding its mechanism of action, it was shown that there was an association between the amount of reactive nitrogen species (RNS) and Tinuvin 770 DF concentration: with high levels of RNS being associated with high concentrations of Tinuvin 770 DF and consequently bacterial death. These results suggest that RNS produced during autoxidation of Tinuvin 770 DF could be responsible for the antibacterial properties of lacquer-films incorporated with this UV stabilizer. Tinuvin 5151 incorporated in lacquer-films did not exhibit antimicrobial properties and was not associated to the induction of RNS.

Collectively, these results allow for a better understanding of the mechanism underlying the biocidal activity of Tinuvin 770 DF. The results also highlight the commercial potential of the incorporation of a dual function additive in the polymer matrix not only to avoid the degradation of polymers (photo- and biodegradation) but also to inhibit the spreading of potentially harmful pathogens among humans.

Keywords: antimicrobial additives; reactive nitrogen species; Tinuvin 770 DF, and UV-stabilizers.

2.3.1 Introduction

Polymers are composed of very large molecules, called macromolecules, obtained from a large number of covalently linked organic units or monomers [1,2]. The possibility to functionalize polymers with chemical groups [3] aligned with a great diversity of natural-, synthetic-, or semi-synthetic sources [4], has expanded its use worldwide. Applications of these materials are very common in various industries including construction [5], packaging [6] biomedical (tissue engineering and drug delivery) [7], medical devices and implants [8], daily products (household cleaning [9] and cosmetic products [10]) and automotive industry [11].

Commercial polymers are generally composed by one or more additives, like plasticizers, lubricants, UV-stabilizers, flame retardants, tougheners or pigments, to facilitate its processability or to improve specific properties of the final product, concerning durability, lifespan, chemical and physical resistance, stability and aesthetics [1,12-14]. In particular, the exposure to atmospheric gases and UV radiation can induce the photodegradation of polymeric materials, accelerating their degradation process [15], and ultimately diminishing their commercial value and function. In the presence of oxygen, UV radiation can be absorbed by the polymeric material and induce the generation of oxygen-functional groups, such as carbonyl (C=O), carboxyl (COOH) and/or peroxide (O-O) groups [16]. These chemical reactions can result in the loss of integrity of the polymeric chains by scission; loss of chemical groups; or even promote branching, crosslinking and disordered rearrangement of molecular chains [16]. The main manifestations induced by UV-exposure include colour changes, the loss of surface gloss and reduction of mechanical properties. The colour changes mostly result in the yellowing of the material and are a result of the disorganized rearrangement of broken double bonds or by the establishment of new chemical bonds. The loss of surface gloss is derived by surface erosion, due to the removal of fragmented chains at the polymer surface. The most severe damages include the occurrence of cracking on the surface, caused by chain scission. The resulted fragments occupy more volume than the original polymers and induce further stress that leads to the formation of more cracks [16]. To mitigate this problem and avoid/reduce photodegradation of materials, UV-stabilizers are widely used as additives in polymeric formulations [17]. Its function relies on restricting UV penetration into the material to improve durability or delay degradation, significantly extending the polymer lifetime [17].

UV-absorbers and hindered amine light stabilizers (HALS) are the most common UV-stabilizers agents used in polymeric materials. UV-absorbers act by absorbing the incident light and dissipating it into thermal energy by intermolecular transfer of protons [15]. Examples of UV-

absorbers include: benzotriazoles, benzophenones, and hydroxyphenyl triazines [15]. On the other hand, HALS act by inhibiting the autoxidation process and inducing the formation of nitroxide radicals from its parent amines. These nitroxide radicals stop the oxidative degradation of the material by coupling to alkyl radicals [18]. Some HALS are commercialized under the commercial name of Tinuvin, such as Tinuvin 5151, Tinuvin 326, Tinuvin 328, Tinuvin 1130 and Tinuvin 770 DF [17].

In addition to photodegradation, polymeric materials can also undergo thermal, chemical or biological degradation, when exposed to environmental biotic and abiotic factor such as heat, chemical pollutants or even microorganisms. In particular, biodegradation is a complex process mediated by the action of microorganisms in which organic material is disintegrated into organic oligomers [19]. After microbial attachment, the enzymes secreted by the microorganisms can break the polymer chain into even smaller fragments, through hydrolysis and/or oxidation reactions. These resultant polymer fragments can be then used as a substrate for microorganism metabolism and excreted as carbon dioxide (CO₂) and water [20]. In the last years, biodegradation of polymeric materials has been exhaustively studied due to the possibility of decreasing microplastic accumulation in the ecosystems [21]. In fact, due their composition based on carbon and nitrogen, polymers are a potential substrate for heterotrophic microorganisms [14,22,23]. Specifically, it has been described that polyurethane (PUR) based films can be biodegraded by both bacterial and fungal species, including *Staphylococci* and *Pseudomonas* species as well as *Aspergillus spp.* and *Cladosporium spp.* [24]. PUR can be hydrolyzed into polyisocyanate and ethylene glycol fragments. These molecules can be further converted into glycoaldehyde and glycolic acid and act as a substrate for the tricarboxylic acid cycle (TCA-cycle) [25]. Specifically, it has been described that *Comamonas acidovorans* can degraded PUR after 7 days at 30°C and use it as carbon and nitrogen sources [26]. Other microorganisms capable to degrade PUR include bacteria: *Corynebacterium spp.* and *P. aeruginosa* and fungi: *Curvularia senegalensis*, *Fusarium solani*, *Aureobasidium pullulans* and *Cladosporium spp.* [27]. Despite this potential benefit from the point of view of circular economy and carbon neutrality, biological degradation can also contribute to decrease the polymer structural integrity, its function and commercial value [28]. To mitigate this risk, antimicrobial coatings have been developed to avoid the attachment and growth of microorganisms [29] and used in medical devices [30,31], food packing [32], textile industry [33], automobile interiors [34], and marine anti-biofouling applications [35].

UV-stabilizers, such as HALS based compounds, in addition to their primary function, have been described as having both antioxidant and antimicrobial properties [36,37]. Despite this evidence, reports about their specific biological properties are limited. The available literature is mostly based on modified HALS, generally combined with other active molecules [38,39]. Considering the importance of HALS as an additive to protect the polymer material against UV-degradation and their association to antimicrobial activity, we propose to investigate the antimicrobial properties of commercial HALS incorporated in coating materials, particularly lacquer-films, and explore their mechanism of action. In this work, we developed a polymer coating material incorporating different UV-stabilizers: (i) a pure HALS, bis(2,2,6,6-tetramethyl-4-piperidyl)sebacate, $C_{28}H_{52}O_4N_2$, CAS no. 52829-07-9 with the commercial name of Tinuvin 770 DF or (ii) a mixture of HALS and UV-absorber, composed by hydrophilic 2-(2-hydroxyphenyl)-benzotriazole as UV-absorber and a basic HALS, with the commercial name of Tinuvin 5151. The antimicrobial activity of the prepared lacquer-films was tested against representative bacterial and fungal strains and a possible mechanism of action was investigated. A graphical abstract of this work is present in Figure 1.

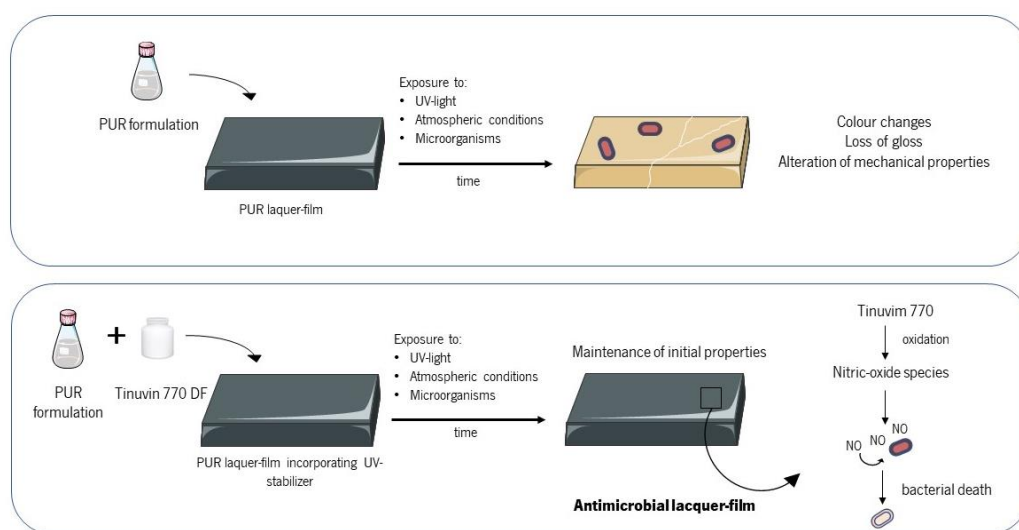


Figure 1 – Graphical abstract of work: “Antimicrobial properties of hindered amine light stabilizers in polymer coating materials and their mechanism of action”.

2.3.2 Materials and methods

2.3.2.1 Production of PUR lacquer-films: two base lacquer formulations were produced according to the UV-stabilizer specifications and solvent system. Solvent-based lacquer formulations were obtained by mixing two PUR commercial resins, a matting agent (silicone) and

Tinuvin 770 DF as a UV-stabilizer, to a final percentage of 96.6, 2.9 and 0.5% (m/m), respectively; while the water-based lacquer formulations were obtained by adding 0.5% (m/m) of Tinuvin 5151 into a commercial waterborne PUR dispersion. Next, both formulations were spread with a 90 µm of thickness onto a polyethylene terephthalate (PET) inert foil, to produce lacquer-films. These films were further submitted to two heating cycles for solvent evaporation: 4 min at 50°C on a digital hotplate (Stuart SD300, R330000718) followed by 1 min at 140°C in a laboratory hot air oven type (Werner Mathis AG, LTF-ST 119988). All raw materials used to produce lacquer-films were purchased from commercial suppliers and are available at TMG Automotive. Before antimicrobial and cytocompatibility assays, the lacquer-films were submitted to a pre-treatment for 336 h (14 days) at 70°C in a drying and heating chamber (Model ED 400, Blinder®) to eliminate volatiles and solvent residues from the polymeric material. Lacquer-films of both solvent- and water-based formulations, without the incorporation of UV-stabilizers were also produced as control lacquer-films.

2.3.2.2 Evaluation of antimicrobial activity of lacquer-films: the antimicrobial assays were based on the protocol described in the International Organization for Standardization (ISO) 22196(E):2011 - Measurement of antibacterial activity on plastics and other non-porous surfaces [40]. The assays were done using two gram-positive bacterial strains, *Staphylococcus aureus* ATCC 6538P and *Staphylococcus aureus* ATCC-BAA-2313 a methicillin-resistant bacteria; a gram-negative bacterial strain *Escherichia coli* ATCC 8739; and a fungal strain *Candida albicans* SC5314/ATCC MYA-2876. Briefly, lacquer-films of 5×5 cm² were inoculated with 0.4 mL of a bacterial or fungal suspensions (6×10⁵ CFU/mL). An inert cover-film (Ref. 132 225 of ©INTERSCIENCE) (4×4 cm²) was placed over the material surface to ensure the contact between inoculum and lacquer-film. Test samples were incubated for 24 h at 32°C for antifungal assays and 35°C for antibacterial assays, in an atmosphere with high relative humidity. Both lacquer- and cover-films were previously sterilized by UV-light for 30 min on each side. To validate the initial inoculum dose, and consequently validate the assay, samples of untreated material (control lacquer-films) were processed immediately after inoculation. After 24 h incubation, lacquer-films were washed with a wash solution composed by soybean casein, lecithin and polyoxyethylene sorbitan monooleate. Ten-fold serial dilutions of the microbial suspensions were done in phosphate-buffered physiological saline solution. Each dilution, as well as the initial suspension, was plated on agar plate medium composed by yeast extract, tryptone and glucose for the antibacterial assays or by a yeast-extract, peptone and dextrose for the antifungal assays. The plates were incubated

for 40-48 h at either 32°C or 35°C, for fungal or bacterial strains. Following this incubation period, the number of CFU were counted. The antimicrobial activity (R) was calculated comparing the average of common logarithm of the number of viable colonies recovered from untreated materials 24 h after inoculation and the average of common logarithm of the number of viable colonies recovered from treated materials after 24 h of inoculation. In addition, the microbial ratio (MR) was also determined. MR is a measure used to quantify the percentage of the microbial burden present in each treated materials relative to the bacterial burden in untreated materials (control samples).

2.3.2.3 Evaluation of half-maximal inhibitory concentration (IC50) by broth microdilution assay: IC50 determination for bacterial strains was performed using a microtiter plate colorimetric assay, based on resazurin reduction. Briefly, 100 µL of bacterial suspension (1×10^6 CFU/mL) were incubated for 24 h in Muller-Hinton (MH) medium with increasing concentrations of Tinuvin solutions, Tinuvin 770 DF with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ at 800 µg/mL or 4-hydroxy-2,2,6,6-tetramethylpiperidine (CAS no. 2403-88-5). After this period, 20 µL of 0.02% resazurin dye was added to the wells. After 3-4 h of incubation, the optical density was determined spectrophotometrically at 575 and 610 nm (Infinite® M200 NanoQuant Micro Plate Reader, Tecan Systems, Switzerland). IC50 was determined using the GraphPad Prism software (version 8.0.2) adjusting the experimental points to a nonlinear regression (curve fit). A positive control (MH medium with bacteria) and a negative control (only MH medium) were also included.

2.3.2.4 Evaluation of antibacterial properties by colony forming units (CFU) counting: bacterial suspensions were incubated for 24 h with increasing concentrations of Tinuvin 770 DF alone or in the presence of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$. At the end of the incubation period, 10-fold serial dilutions were performed in PBS-saline. Each dilution, as well the initial suspension, were plated on nutrient agar medium based on yeast extract, tryptone and glucose. The plates were incubated for 24 h at 37°C, after which CFU were counted. Results are expressed in percentage of bacterial burden from when compared with the positive control (bacteria culture alone).

2.3.2.5 Nitric oxide quantification: as the mechanism of action of Tinuvin 770 DF, as a light stabilizer, involves its autoxidation to a reactive nitrogen species (RNS), we use a specific probe to assess the levels of NO to investigate its possible role in the antimicrobial mode of action. Considering this, after the incubation period of 24 h of bacterial suspensions with increasing concentrations of Tinuvin 770 DF alone or in the presence of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 5×10^{-6} M of 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) was added at each well, and the plates

were incubated for 30 min at 37°C in the dark. The intensity signal of DAF-FM was measured using the FACS LSRII flow cytometer (BD Biosciences®), with excitation laser of 488 nm. The resolution gates were adjusted according to each bacterial strain. At least 3 000 events were recorded for each experimental condition. Flow cytometric analysis was performed using the FlowJo 10.8.1 (Becton Dickinson & Company 2006-2021). The results are expressed as a mean of fluorescence intensity (MFI) for at least 4 independent biological experiments.

2.3.2.6 Cytocompatibility of antibacterial lacquer-films: cytocompatibility assays were performed using the above-described microtiter plate colorimetric assay based on resazurin reduction. An adaption of the International Organization for Standardization (ISO) 10993-5:2009 - Biological evaluation of medical devices, Part 5: Tests for *in vitro* cytotoxicity [41], was used. Briefly, both control and test lacquer-films with different concentrations of Tinuvin 770 DF from 0.5 to 10% (produced according to the methods previously described in section 2.1) were incubated with cell culture media (Dulbecco's Modified Eagle's Medium supplemented with 10% of fetal bovine serum (FBS), and 1% of glutamine) at room temperature for 24 h under orbital shaking (100 rpm), to obtain supernatant with lacquer extractable products. Simultaneously, L-929 fibroblast cell line (1×10^4 cells/well), at maximum passage of 10, was incubated in a 96-well plate for 24 h at 37°C in an incubator with atmosphere of 5% of CO₂ (Heraeus HERAcell 150 Incubator, Thermo). After incubation period, cell culture media was replaced with the lacquer-extract medium. Plates were incubated for 24 h at 37°C. A positive control (cells cultured in normal cell culture medium), a negative control (only cell culture medium) and a control of cytotoxicity (1% Triton X-100) were also included. After incubation, 20 µL of 0.02% resazurin dye was added. The plates were further incubated for 3 h to allow resazurin reduction. The optical density was measured spectrophotometrically at 575 and 610 nm. The results were expressed as percentage of cell viability from control lacquer-film, by the ratio of absorbance (Abs) between at 575 and 610 nm for the different conditions, according to the following equation:

$$\% \text{ cell viability} = \frac{\text{Abs sample} - \text{Abs negative control}}{\text{Abs positive control} - \text{Abs negative control}} \times 100$$

According to the ISO10993-5:2009 protocol, a cell viability below 70% is defined as positive cytotoxic activity [7]. Following the protocol recommendations, a minimum of four test lacquer-films with increasing concentrations of Tinuvin 770 DF were used to assess cytocompatibility.

2.3.2.7 Statistical analysis: data is reported as mean $\pm$ standard deviation. Statistical analysis was done using GraphPad Prism (version 8.0.2) by testing the normality (Shapiro-Wilk test) and the significance values. Unpaired t test was used for antimicrobial lacquer-films analysis. Data was considered statistically significant for $p < 0.05$.

2.3.3 Results

UV-stabilizers have been used for several years with the main purpose of restricting UV penetration into the material and ultimately improve durability and/or delay degradation by photo-oxidation. In addition to these features, some UV-stabilizers, such as HALS, have been associated to microbial growth restriction, although the exact mechanism(s) of action were not fully explored. To further investigate the antimicrobial effect of specific UV-stabilizers, Tinuvin 770 DF and Tinuvin 5151 were incorporated into a PUR commercial resin and formulated to produce lacquer-films. These additives were selected based on the mechanism of action, specifically, Tinuvin 770 DF is a HALS and Tinuvin 5151 is a mixture of HALS and UV-absorber.

2.3.3.1 Antimicrobial performance of produced lacquer-films: our first step was to confirm the antimicrobial activity of the Tinuvin 770 DF and Tinuvin 5151. For that purpose, these UV-stabilizers were incorporated into different commercial PUR resins and formulated to produce solvent and water-based lacquer-films. The lacquer-films were produced using the solvent casting method by spreading the polymer solution onto an inert PET surface. The concentration of both additives follows typical formulations used in TMG Automotive and are aligned with the literature which describes typical concentrations of UV-stabilizers to be around 0.05–2% (m/m) [8]. Before analysis, pre-treatment of produced lacquer-films was carried out by exposing lacquer-films at high temperatures (70°C) for 14 days to eliminate any solvent residues or volatile products from the polymeric material.

The antimicrobial properties of lacquer-films were tested against two gram-positive bacterial strains (*S. aureus* methicillin and non-methicillin resistant strains), a gram-negative bacterial strain (*E. coli*) and a fungal strain (*C. albicans*), using the international protocol, ISO 22196(E):2011. For solvent-based lacquer-films incorporated with Tinuvin 770 DF no viable bacteria were recovered from the test surface, corresponding to an R value >4 for gram-positive strains and an R value >6 for gram-negative (Figure 2A, 2C and 2E). These results show a reduction of over 99.99% for the tested bacterial strains (Table 1). For *C. albicans* the solvent-based lacquer-films showed an R value of 2.19 representing a fungal reduction of 99.31% (Figure 2G and Table

1). Regarding the water-based lacquer formulations incorporated with Tinuvin 5151, the number of viable bacteria and fungi recovered from the control and test lacquer-films were similar, indicating no antimicrobial activity regardless the microorganism (Figure 2B, 2D, 2F, 2H and Table 1).

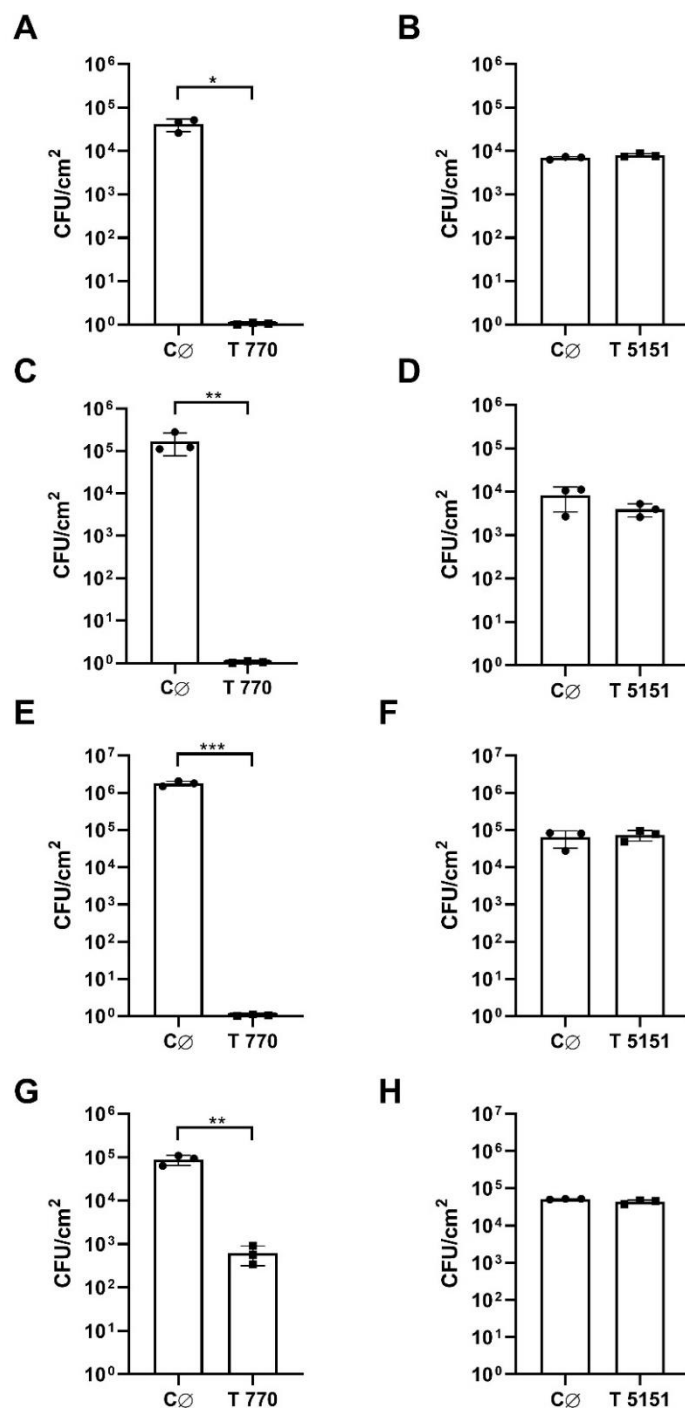


Figure 2 – Antimicrobial activity of lacquer-films: antimicrobial analysis of Tinuvin 770 DF solvent-based lacquer-films against *S. aureus* 6538P (A), *S. aureus* BAA-2313 (C), *E. coli* ATCC 8739 (E) and *C. albicans* ATCC MYA-2876 (G); antimicrobial analysis of Tinuvin 5151 water-based lacquer-films against *S. aureus* 6538P (B), *S. aureus*

BAA-2313 (D), *E. coli* ATCC 8739 (F) and *C. albicans* ATCC MYA-2876 (H). CØ represents the control lacquer-films and T 770 and T 5151 represents the antimicrobial lacquer-films with incorporation of UV-stabilizers. Error bars represent the standard deviation (n=3). Unpaired t test: * p<0.05.

Table 1 – Antimicrobial activity of produced lacquer-films formulated with different UV-stabilizers.

	<i>S. aureus</i> 6538P		<i>S. aureus</i> BAA-2313		<i>E. coli</i> 8739		<i>C. albicans</i> ATCC MYA-2876	
	Rvalue*	MR (%)**	Rvalue*	MR (%)**	Rvalue*	MR (%)**	Rvalue*	MR (%)**
Solvent-based lacquer formulation (Tinuvin 770 DF)	4.60	>99.99	5.20	>99.99	6.25	>99.99	2.19	99.31
Water-based lacquer formulation (Tinuvin 5151)	-0.059	-14.6	0.26	52.1	-0.094	-15.26	0.075	15.38

* Antibacterial and antifungal activity (R) is calculated according to ISO 22196:2011, by using the following formula: $R = (Ut - U0) - (At - U0) = Ut - At$ in which **U0**, **Ut** and **At** are the average of the common logarithm of the number of viable bacteria recovered immediately after inoculation for untreated samples, 24 h of incubation for untreated samples and 24 h of incubation of test samples, respectively. ** Microbial Ratio (MR) is the percentage of viable bacteria recovered from treated samples compared to untreated samples after 24 h of incubation. Microbial Ratio (MR) was calculated as: $MR = 100 - (Ts \times 100)/Cs$, in which Ts and Cs are the average of viable bacteria recovered from treated and untreated samples, respectively. Negative values of MR indicate an increase of bacterial load compared to control group and positive values indicate a microbial reduction to control samples.

2.3.3.2 IC50 analysis of Tinuvin 770 DF: IC50 analysis was done based on resazurin reduction (blue dye) by living cells to resorufin (pink colour) by viable cells. Colour change can be monitored by spectrophotometric techniques (Abs or fluorescence) to measure the metabolic activity of viable cells [42,43]. The reduction rate is an indicator of the metabolic activity of viable cells. Based on findings that solvent-based lacquer-films incorporated with a pure HALS (Tinuvin 770 DF) shows a stronger antimicrobial activity as well as a broader spectrum of action, thus the next step was to determine a possible mechanism of action. In the literature, the impact of HALS on microbial growth inhibition is limited, despite some studies suggesting that the antimicrobial activity could be related to either the action of piperidines by protonation to a quaternary ammonium or the oxidation of nitroxyl free radicals [38]. The piperidine ring is described as an important component of HALS, being essential not only for its function as a UV-stabilizer but also to ensure its compatibility with other additives in the polymeric matrix through the functionalization of the aromatic ring [44]. Another important feature in HALS structure is their secondary amine which can form a nitroxyl radical acting as an intermediate step in the capture of alkyl radicals [45]. Therefore, herein we started by determining the IC50 of the pure HALS Tivuvin 770 DF in

comparison to the non-pure HALS Tinuvin 5151. Furthermore, to further explore the mechanism of action of HALS, we also used 4-hydroxy-2,2,6,6-tetramethylpiperidine to evaluate the specific effect of piperidine ring, which is included in the HALS structure (Figure 3A and 3B). For this analysis, we focused on methicillin-susceptible and methicillin-resistant *S. aureus* strains (MSSA and MRSA), due to their high predominance on inanimate surfaces and their importance in public health as one of the most relevant human pathogenic bacteria [46]. Data demonstrated that Tinuvin 770 DF exhibited an IC₅₀ value of 233.8 and 298.0 µg/mL, against MSSA and MRSA, respectively. Tinuvin 5151 showed an IC₅₀ value 5.5-fold higher (1282 µg/mL) for MSSA, while for MRSA the difference was almost 10-fold (2968 µg/mL) (Figure 4A and 4B).

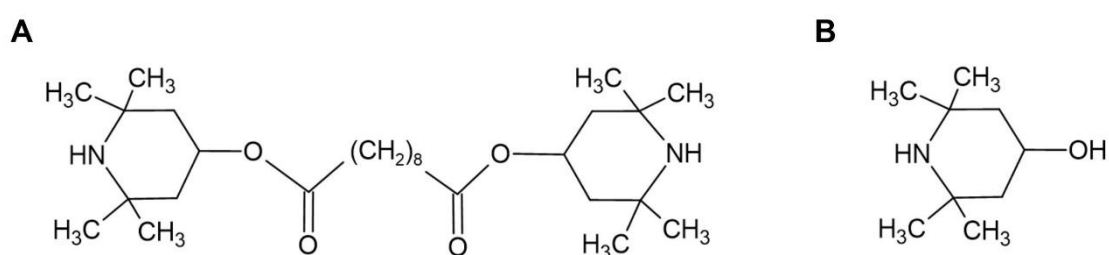


Figure 3 – Chemical structure of Tinuvin 770 DF, bis(2,2,6,6-tetramethyl-4-piperidyl)sebacate (A) and a piperidine ring, 4-hydroxy-2,2,6,6-tetramethylpiperidine (B). Drawn in ACD/ChemSketch (Freeware) 2022.2.

The next step was to evaluate the potential role of piperidines in HALS antibacterial activity. Regarding the IC₅₀ values for the piperidine compound (4-hydroxy-2,2,6,6-tetramethylpiperidine) against MSSA, these were significantly higher (5268 µg/mL) and represent more than 22-fold increase compared to values obtained for Tinuvin 770 DF (Figure 4C). The same pattern was observed for MRSA (Figure 4D), with the piperidine compound's IC₅₀ being more than 26-fold higher (8013 µg/mL) than the IC₅₀ obtained with Tinuvin 770 DF. Overall, these results suggest that the antimicrobial action of Tinuvin 770 DF was not associated to the action of piperidines, at least as isolate compound.

Next, we asked whether the generation of reactive oxygen and nitrogen species (ROS/RNS) could underly Tinuvin 770 DF antimicrobial properties. For that we incubated both MSSA and MRSA strains suspensions with Tinuvin 770 DF in the presence or absence of the ROS/RNS scavenger, CuCl₂·2H₂O [47-49] (Figure 4E and 4F). Importantly, when the ROS/RNS scavenger was added together with Tinuvin 770 DF, there was an approximately 5-fold increase in the IC₅₀ value (1093 µg/mL against MSSA (Figure 4E). Similar results were obtained with MRSA, in which the IC₅₀ of Tinuvin 770 DF alone increased from 289 µg/mL to 584 µg/mL in the presence of the ROS/RNS

scavenger (Figure 4F). It is noteworthy that the concentration of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ used in this study (800 $\mu\text{g/mL}$) did not have a significant effect on bacterial viability (our previous studies show a MIC value of 970 $\mu\text{g/mL}$ for $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ against both bacterial strains). Based on the reduced antimicrobial activity of Tinuvin 770 DF when in the presence of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (Figure 4E and 4F), these results suggest a possible role of RNS in the inhibition of bacterial growth, probably due to copper's (Cu) scavenging action on nitrogen oxide species.

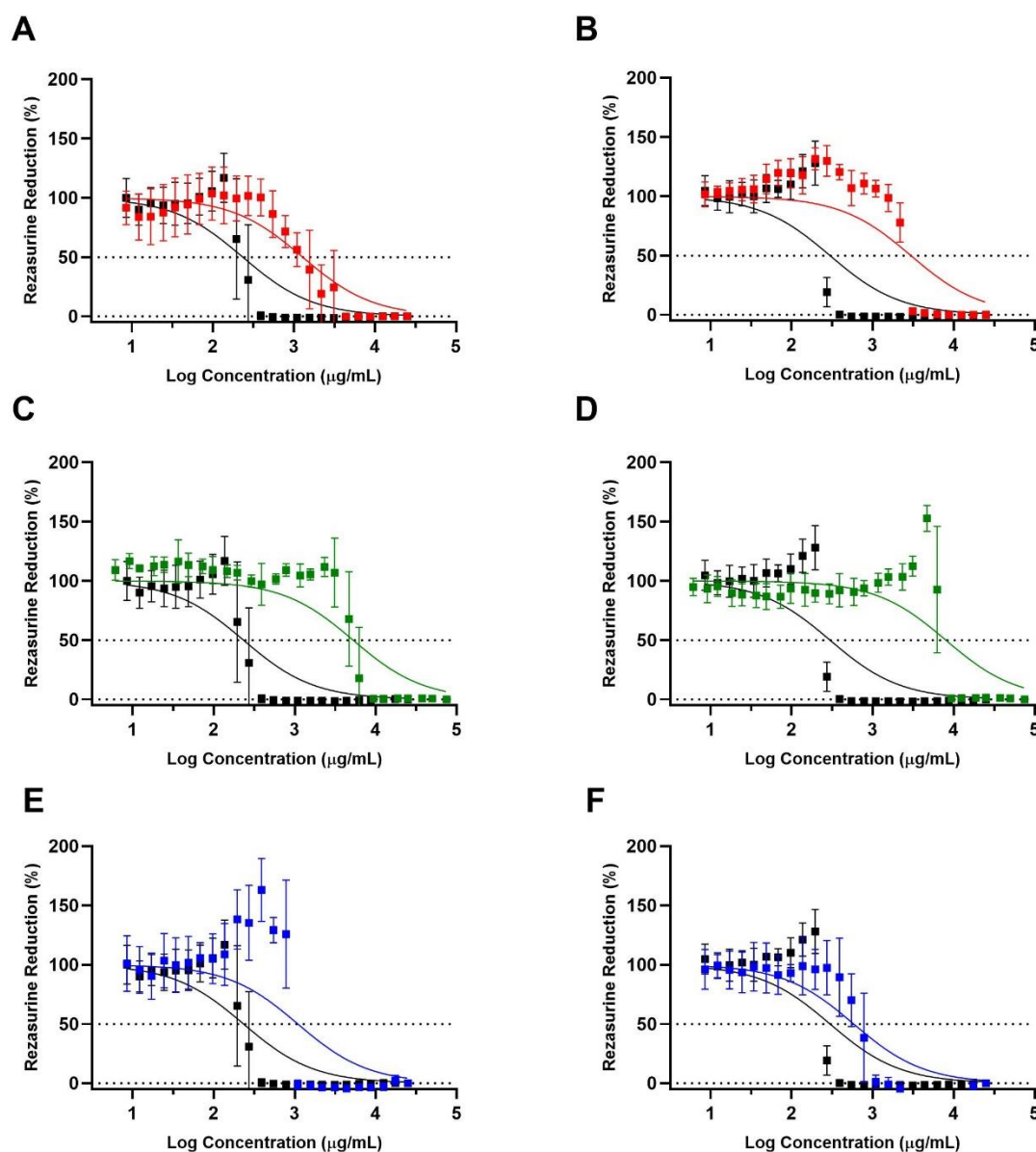


Figure 4 – IC₅₀ analysis of selected compounds: IC₅₀ analysis of Tinuvin 770 DF (dark line) and Tinuvin 5151 (red line) against *S. aureus* 6538P (A) and *S. aureus* BAA-2313 (B); IC₅₀ analysis of Tinuvin 770 DF (dark line) and 4-Hydroxy-2,2,6,6-tetramethylpiperidine (green line) against *S. aureus* 6538P (C) and *S. aureus* BAA-2313 (D), and IC₅₀ analysis of Tinuvin 770 DF (dark line) and Tinuvin 770 DF in the presence of RNS/ROS scavenger (blue line), against *S. aureus* 6538P (E) and *S. aureus* BAA-2313 (F). Horizontal lines represent the resazurin reduction at 50%. Error bars represent the standard deviation for at least 3 independent biological replicates.

2.3.3.3 Impact of antibacterial properties of HALS in the presence of ROS/RNS

scavenger: to further confirm the role of NO in the antibacterial performance of HALS, we evaluated the production of RNS by Tinuvin 770 DF, when incubating MSSA or MRSA strains. In parallel, we determined the bacterial viability. Bacteria suspensions were incubated with increasing concentrations of Tinuvin 770 DF for 24 h, after which bacteria were stained with DAF-FM, a NOS-sensitive fluorescent dye. It to aimed to quantify the relative amount of NO species. Due to NO species high reactivity and short half-lives in biological environments the most effective techniques are based on colorimetric and fluorometric techniques with living tissues or intact cells [50]. In this work, the NO species were evaluated using a flow cytometer by measuring MFI of DAF-FM. Data showed that incubation of MSSA with low concentrations of Tinuvin 770 DF [10-200 µg/mL] resulted in the accumulation of basal levels of NO (MFI~10), while at higher concentrations [400-6250 µg/mL] it was observed a 15-fold increase in DAF-FM MFI (Figure 5A, 5E and 5G). Importantly, this increase accumulation of RNS correlated with a decrease in the number of viable bacteria. Indeed, with lower concentration than 200 µg/mL of Tinuvin 770 DF it was detected viable bacteria, ranging from 4.8 to 83.7% (Figure 5C). However, addition of ROS/RNS scavenger, to the MSSA suspensions treated with Tinuvin 770 DF resulted in clear reduction of the DAF-FM fluorescent intensity, associated with decrease in the accumulation of NO (Figure 5A, 5E and 5G). Furthermore, we only detected the increment in NO accumulation in cell suspensions treated with Tinuvin 770 DF concentrations higher than 800 µg/mL (blue line in Figure 5A). Accordingly, the levels of detected NO inversely correlated with the percentage of viable bacteria (Figure 5A vs 5C). This difference can be also observed by the histogram of MFI of Tinuvin 770 DF and Tinuvin 770 DF with ROS/RNS scavenger ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), when we compare the MFI at concentrations lower than IC50 value (0.2 mg/mL) with MFI at concentrations higher than IC50 value (Figure 5E and 5G, respectively). Below the IC50 value there are low levels of NO species (MFI~12-18), with a high and narrow MFI. Above IC50 value there is a clear shift in the histogram, with an increase of peak width, especially for Tinuvin 770 DF experimental condition. This is an indication of high levels of NO were obtained with Tinuvin 770 DF alone, while combining Tinuvin 770 DF with ROS/RNS scavenger, there are a decrease of levels of NO, confirming the scavenger activity of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$.

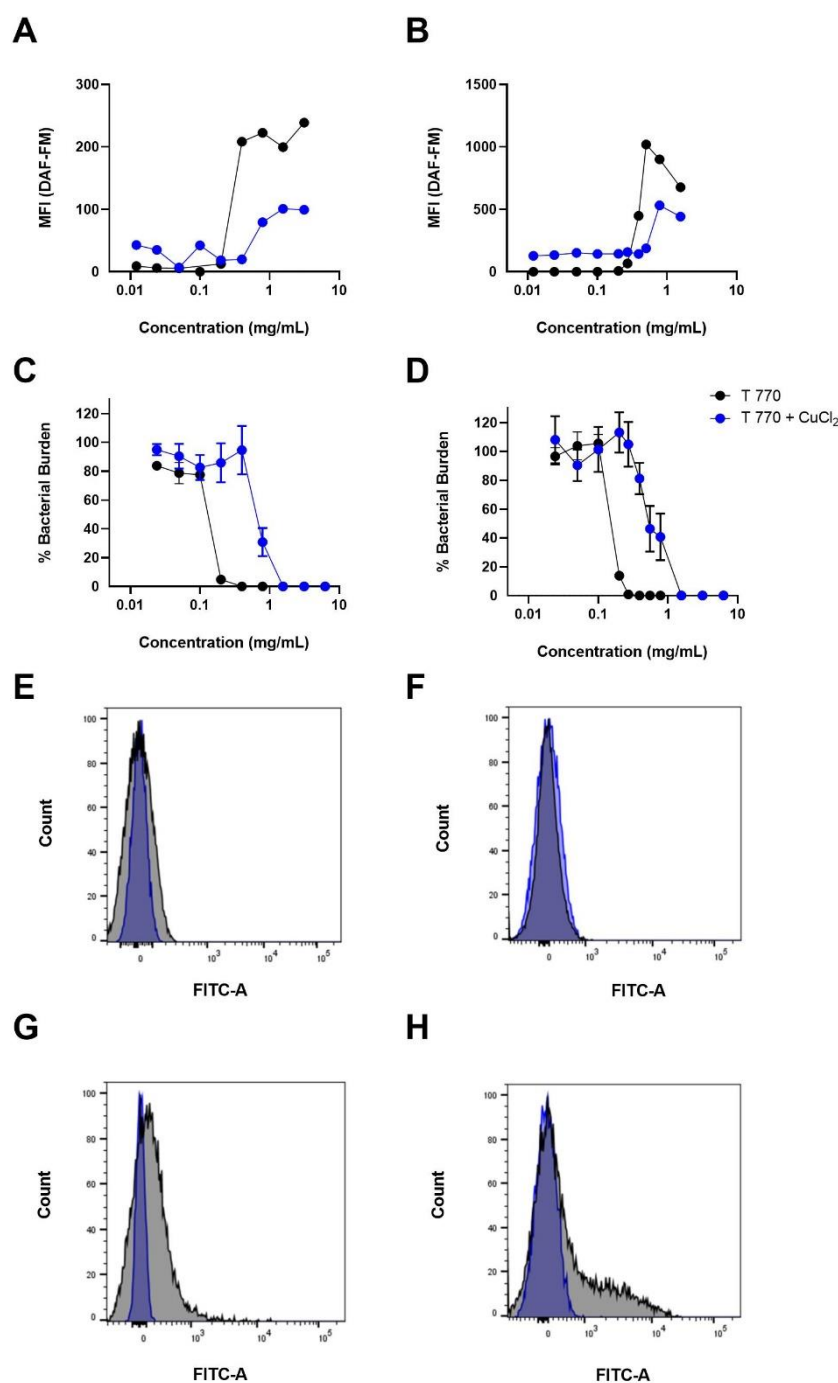


Figure 5 – DAF-FM Nitric oxide quantification and antibacterial performance of Tinuvin 770 DF: MFI of Tinuvin 770 DF (dark line) and Tinuvin 770 DF in the presence of RNS/ROS scavenger (blue line) against *S. aureus* 6538P (A) and *S. aureus* BAA-2313 – MRSA (B); CFU analysis of Tinuvin 770 DF (dark line) and Tinuvin 770 DF in the presence of RONS scavenger (blue line) against *S. aureus* 6538P (C) and *S. aureus* BAA-2313 – MRSA (D); histogram of MFI for Tinuvin 770 DF alone and in the presence of RONS scavenger against *S. aureus* 6538P at 0.2 mg/mL (E) and 1.56 mg/mL (G), and histogram of MFI for Tinuvin 770 DF alone and in the presence of RNS/ROS scavenger against *S. aureus* BAA-2313 – MRSA at 0.27 mg/mL (F) and 0.78 mg/mL (H). Error bars represent standard error of the mean for at least 3 independent biological replicates.

Regarding MRSA, the results were similar to those observed for MSSA. Therefore, at concentrations lower than 200 $\mu\text{g/mL}$, it was observed a residual DAF-FM MFI ($\text{MFI} \sim 10$), while at higher concentrations of Tinuvin 770 DF [270-1560 $\mu\text{g/mL}$] there was a gradual increase in NO accumulation, reaching a maximum MFI value of 1019 (dark line in Figure 5B). In the presence of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, the increase in NO production was only observed for Tinuvin 770 DF concentrations higher than 800 $\mu\text{g/mL}$, with a maximum MFI value of 531 (blue line in Figure 5B). Once again, the levels of NO are inversely correlated with the percentage of bacterial load (Figure 5B vs Figure 5D). The histogram of MRSA followed the same pattern as that described for MSSA. For low concentrations there is an overlap of both histograms indicating a low production of RNS (Figure 5F). In contrast, at concentrations higher than the IC_{50} value it is possible to observe a clear change of the histogram in Tinuvin 770 DF. In this condition there is an increase in the peak width, representing high RNS levels for the Tinuvin 770 DF condition (Figure 5F vs Figure 5H).

Together, the above-described results suggest that there was an increased production of RNS in the experimental conditions where Tinuvin 770 DF is present (originated from its autoxidation process), and this increase of RNS is associated with the antimicrobial activity of this specific HALS. At high levels, RNS can induce oxidative/nitrosative stress ages, affecting the physiological functions and affect cellular structures, including lipids, proteins, carbohydrates, and DNA [51].

2.3.3.4 Cytocompatibility of antimicrobial lacquer-films: until here, the study was focused in the mode of action of Tinuvin 770 DF, however, we also intend to explore its cytocompatibility against a representative skin cell line. For that, cytocompatibility was determined by indirect exposure of L-929 mouse fibroblast to lacquer-extract medium, using an adaptation of ISO 10993-5:2009 protocol [41]. As expected, results demonstrated that control lacquer-films (without Tinuvin 770 DF) had a high cytocompatibility with more than 100% of cell viability (Figure 6). Furthermore, lacquer-films with 0.5, 1.5, and 2.5% (m/m) of incorporated Tinuvin 770 DF also showed cytocompatibility, with percentages of cell viability of 102.7 ± 9.0 , 95.5 ± 6.3 and $92.4 \pm 8.4\%$, respectively. Nevertheless, incorporation of more than 5% (m/m) of Tinuvin 770 DF in the lacquer-films resulted in a significant cytotoxic effect with cells viability near of 0%. Importantly, it should be noted that in the typical commercial formulations Tinuvin 770 DF is used at 0.5% (m/m), for which this UV-stabilizer has an antimicrobial effect, and also high cytocompatibility.

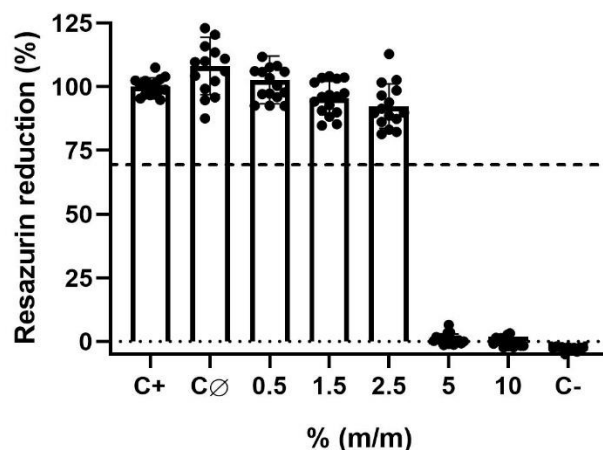


Figure 6 – Cytocompatibility assays of solvent-based lacquer-films with different concentrations of Tinuvin 770 DF against L-929 mouse fibroblast cells: C+ represents the positive control (only cells+medium), CØ represents the control lacquer-films without Tinuvin 770 DF (control lacquer-film) and C- represents the cells exposure to Triton X-100 (negative.control). Dashed line represents 70% of resazurin reduction. Error bars represent the standard deviation at 4 independent biological assays with quadruplicates in each assay.

2.3.4 Discussion

Polymers, due to their versatility, have a wide range of properties and applications. Therefore, polymer-based materials are used in numerous industries, including packaging, automotive, construction, electronics, healthcare, and many others. Some of the polymers' properties are determined by the incorporation of polymer additives during their processing. The main function of these polymer additives is to promote/facilitate polymer processability and/or to prolong polymer function, by maintaining their properties over time [52]. Specifically, additives can be used to (i) control/improve polymer processing, (ii) promote specific properties of interest, (iii) induce an aesthetic effect, and/or (iv) prevent polymer degradation during its processing or during exposure to environmental factors [52]. In fact, the degradation is an importance parameter which can limited the lifetime of polymer and decrease their commercial value. Degradation can be defined as all processes which lead to a decline of polymer properties, and can be caused by heat, light, oxidation, hydrolysis, and exposure to chemicals [52] as well as by the action of microorganisms [53]. Light, and in particular UV-radiation, can promote the loss of structural integrity of polymeric chains and induce discoloration of materials, such as yellowing of polymers, loss of gloss and loss of mechanical properties (e.g. cracking) [54]. To avoid light-associated polymer degradation, UV-stabilizing additives are commonly used. The ideal UV-stabilizer should be (i) soluble or dispersible in the polymer matrix; (ii) non-volatile and non-leaching and (iii) compatible with the polymer and other additives. UV-stabilizers act in various ways: while some

control the amount of radiation that reaches the polymer, others inhibit chemical reactions initiated by the absorption of the radiation. Specifically, the primary methods of UV-stabilization involve: (i) absorbing or blocking UV radiation before it can reach the chromophore(s) in the polymer matrix; (ii) deactivating or quenching the excited species generated by the UV radiation and converting them into stable, non-reactive forms; or (iii) scavenging reactive free radicals generated by UV radiation and converting them into stable, non-reactive forms [16]. Combinations of UV-stabilizers are often used to protect against more than one mechanism or to provide synergistic protection. For example, UV-absorbers do not usually provide adequate UV-protection unless paired with HALS [16]. Regarding the impact of microorganisms on polymer degradation, the main manifestations are the formation of metabolites, such as H_2O , CO_2 , and CH_4 , that impact the structure and function of the original material causing discoloration, mildew, odours, loss of gloss and ultimately structural degradation [13,14,23]. Biodegradation occurs by reduction of polymer chains into low molecular weight constituents, followed by surface and bulk erosion of the polymeric materials [55]. In particular, the biodegradation of PUR occurs by cleavage of urethane bonds and has been reported to occur upon colonization by fungal species, such as *Aureobasidium pullulans* and *Cladosporium* spp. and bacterial strains, such as *P. aeruginosa*, *Bacillus subtilis* and *Acinetobacter calcoaceticus* [56]. Moreover, it has been described that PUR can act as a carbon and urease substrate for fungi and bacteria, increasing its susceptibility to microbial colonization and ultimately biodegradation [57]. Considering this, the development of polymeric materials with additives with dual function (UV-stabilizer and biocide agent) has been gaining interest as an attractive solution to avoid both photo- and biodegradation of polymers.

Interestingly, UV-stabilizers such as HALS, have been reported to have an impact on microbial colonization. Indeed, Abualnaja, K. *et al.* (2016) report the development of UV-stabilizers with dual function: photo-stability and antimicrobial activity. The authors showed that the produced HALS-based compounds containing chromophores, presented a moderate biological activity with antimicrobial reduction of 9% for *C. albicans* and 17 to 24% against *S. aureus* and *E. coli* [39]. Taking this information, in the present work, two commercial UV-stabilizers based on HALS were incorporated into PUR-formulations to produce lacquer-films: Tinuvin 770 DF, a pure HALS and Tinuvin 5151, a mixture of HALS and UV-absorber. The solvent-based lacquer-films incorporated with Tinuvin 770 DF presented a good reduction of microbial load for gram-positive and gram-negative bacteria, as well as for fungal species, showing a broad spectrum of action. On the other hand, the water-based lacquer-films incorporated with Tinuvin 5151 did not show any antimicrobial

activity. In the literature there are some reports related to the antimicrobial activity of HALS but, in general, these HALS were combined with other chemical groups, mainly chloride reactive groups. Li, R. *et al.* (2014) reported the antibacterial properties of chlorinated-HALS (based on 2,2,6,6-tetramethyl piperidinol) incorporated in cotton fabric. The authors showed a strong antibacterial activity against *S. aureus* (100% of bacterial reduction representing 7.1 Log reduction), but moderate antibacterial activity against *E. coli* (83.25% bacterial reduction representing 0.78 Log reduction), after only 10 min of contact. For the same contact time, the unchlorinated-HALS showed a moderate bacterial reduction of 87.3% for *S. aureus* (0.9 Log reduction) and null bioactivity for *E. coli* (6.2% of bacterial reduction representing 0.03 Log reduction) [58]. Following a similar approach, Paine, M. *et al.* (2016) developed a polyester based-material with 2% (m/m) of Tinuvin 770 or a modified Tinuvin 770 with a post-chlorination process (N-chlorinated HALS) with antibacterial activity against *P. aeruginosa*. The authors reported a decrease in protein quantification, associated to an increase in antibacterial activity, regardless the developed material, although the effect was more pronounced with N-chlorinated HALS. Despite this antibacterial activity, the authors were not able to observe antifungal properties against *Cladosporium spp.* [38]. In another work, Chen, Z. and Sun, Y. (2005) reported the production of a HALS based on a bis(N-chloro-2,2,6,6-tetramethyl-4-piperidiny) sebacate (Cl-BTMP) in a polypropylene (PP) substrate. The authors showed that films with 0.1% (m/m) of Cl-BTMP effectively killed gram-positive and gram-negative bacteria after 15 min of contact. Interestingly, the authors reported that both BTMP (concentration between 0.1-4% (m/m)) and PP substrate by themselves did not have antibacterial effect [59]. Despite this demonstration, the mode of action was not explored and can be related to primary function UV-stabilizers. In fact, both compounds (Tinuvin 770 DF and Tinuvin 5151) act with distinct mechanisms. While Tinuvin 770 DF acts as a UV-stabilizer by capturing free-radicals produced by reactions with UV-light [60], Tinuvin 5151 is a mixture of HALS and UV-absorber, acting by dissipating the absorber energy into thermal energy [61].

Despite the described biological activity of HALS-based compounds, the antimicrobial mechanism of pure HALS is not well elucidated but can be related to their primary function as a UV-stabilizer, avoiding photo-degradation of polymers. Photo-degradation begins with the cleavage of the homolytic polymeric bonds (R-R) [62], a process that can occur either by (i) photochemical cleavage of aldehydes and ketones (known as Norrish type I reaction) [63,64] or through (ii) a hydrogen abstraction by an atmospheric radical, both resulting in the formation of a polymeric radical (R•). In turn, this R• can react with the atmospheric oxygen to form a peroxy radical

(ROO•). Finally, this ROO• can abstract a hydrogen atom from the polymer backbone inducing the hydroperoxide (ROOH) formation and more R•, promoting the degradation of the polymer [62]. As described above, HALS are long term stabilizers that act by trapping the free radicals produced during the photo-oxidation process, protecting the polymer against photo-oxidative damages. This protective effect arises from the scavenger ability of HALS to form nitroxide radicals, in a process known as the Denisov cycle [62,65,66]. The Denisov cycle begins with the oxidation of HALS into nitroxide radicals (RNO•) by peroxides or hydroperoxides. The RNO• can react with polymeric alkyl radicals (R•) forming non-radical products (NOR). After this, new RNO• can be generated from hydroxylamine ethers with alkylperoxy and acylperoxy radicals, continuing the cycle [62]. In other words, the polymer is protected by HALS through the inhibition of free radical activity during thermal and/or oxidative reactions [66]. A simplified HALS based Denisov cycle is presented in Figure 7. Specifically, in the case of Tinuvin 770 DF, the amine 2,2,6,6-tetramethylpiperidine is oxidized into the 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO), forming a nitroxide that reacts with the R• generated during photodegradation. This results in an alkoxylamine that in turn reacts with a peroxy radical to re-establish the nitroxide species, as well as nonradical products [62]. Given the formation of NO-radicals during the Denisov cycle, and the role of these radicals with a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria [67], fungi, and viruses [68], we hypothesized that NO-radicals may be involved in the antimicrobial mechanism of Tinuvin 770 DF. This hypothesis will be discussed herein.

NO is a natural gas and a small molecule with lipophilic and hydrophilic behaviour, being able to cross biological membranes [69,70]. NO is involved in many biological processes and can act as a signalling molecule that promotes cellular growth (proliferation, migration and, differentiation) and metabolic activity; but can also act as an effector molecule that actively kills invading microorganisms and tumour cells during the inflammatory process [71]. Indeed, at high concentrations, NO has been shown to have an antimicrobial effect by oxidation of lipids, proteins and nucleic acids, altering their structural and functional properties and promoting oxidative and nitrosative stress [50,69]. Due to their potential in controlling microbial growth, NO-releasing polymers have been developed for antimicrobial applications [71] and include PET and silicone elastomer (SE) [72]; poly(ether-block-amide) (PEBA) and nylon [73]; PUR-based materials with selenium [74]; and polyvinyl chloride (PVC) substrate [75].

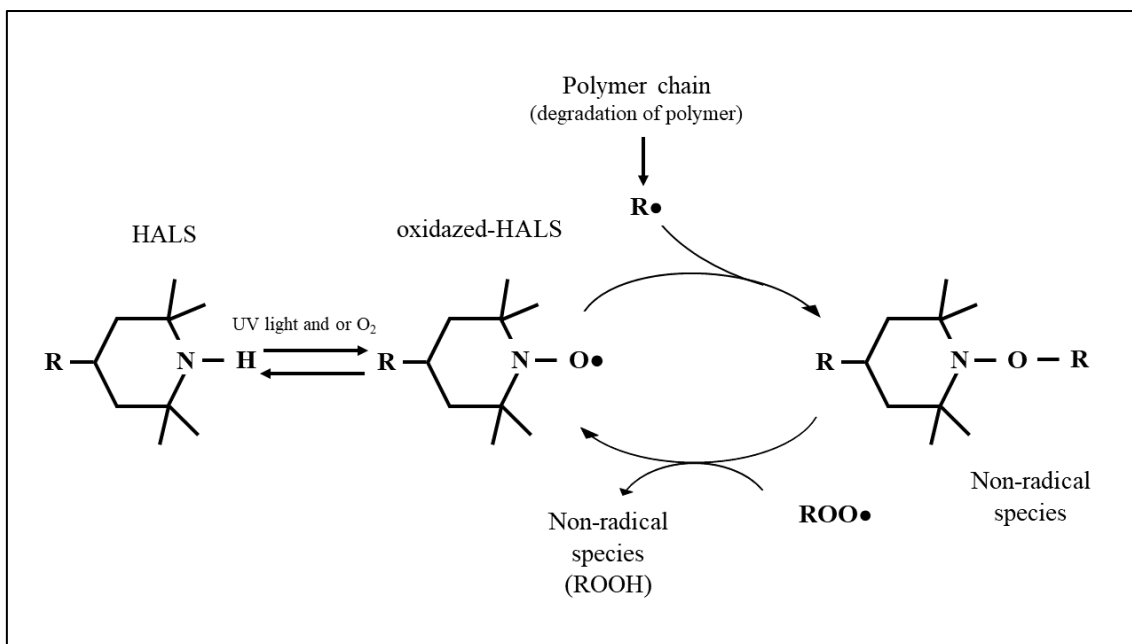


Figure 7 – Mechanism of action of HALS based on Denisov Cycle: HALS acts by eliminating free radicals resulting from polymer degradation. The nitroxide reactive species (formed by oxidation of HALS through light or in the presence of oxygen: RNO • radicals) react with other radical species to form intermediates non-radical species. In turn, these intermediate products are capable to react with other radical species to regenerate the nitroxide. Adapted from Yousif, and Hasan, A. (2015) [54]; Hodgson, J. and Coote, M. (2010) [62], and Babaghayou, M. *et al.* (2021) [66].

In this work, we used a NO-specific probe to analyse the NO-content after exposure of the bacterial suspension (*Staphylococcus* species) to a range of concentrations of Tinuvin 770 DF. During this exposure period, DAF-FM, a non-fluorescence compound, crosses the cellular membranes by passive diffusion and is converted to a fluorescent triazole derivative in the presence of RNS, in particular NO species [50,76]. Aligned with high selectivity and sensibility, DAF-MF is used as a probe to assess of cellular NO production/levels in biological systems [77]. We focus on antimicrobial activity against MSSA and MRSA due their high incidence as a one of the most important pathogens for humans as well as their high persistence on inanimate surfaces for a large period of time [46]. *Staphylococcus* species are responsible for the colonization of a large range of surfaces (including healthcare facilities [78] and also in common daily surfaces: fitness gym facilities [79], automobile interiors [34]) and cloths, plastics, steel, glass or flooring materials [46]. As the results show, there is an association between NO-content and bacterial viability. High values of MFI are associated to higher Tinuvin 770 DF concentrations, where bacteria are not viable. On the other hand, lower Tinuvin 770 DF concentrations (dark line in Figure 5) exhibit a relatively low NO-content (low MFI signal) and high bacterial viability. This suggests that for low Tinuvin 770 DF concentrations, the NO produced does not have toxic effect and could probably be used by bacteria in their metabolism. In fact, NO is naturally present in microorganisms, being produced by nitric

oxide synthases through L-arginine as the substrate [80] as well as involved in many bacterial pathways such as anaerobic respiratory [81]. This “transition zone” between physiological action and antibacterial properties of NO is difficult to establish and depends on the local concentration achieved. It is described that NO levels higher than 1 μM induces a possible cytostatic effect by promoting nitrosative stress [82].

In the presence of a NO-scavenger there is a decrease in the antibacterial activity of Tinuvin 770 DF given that it increases the necessary concentration to obtain the complete elimination of the bacterial load. Specifically, against *S. aureus*, Tinuvin 770 DF shows complete bacterial elimination at a concentration of 0.39 mg/mL, while for this same Tinuvin 770 DF concentration the presence of NO-scavenger increases the bacterial load to 94.7% (Figure 5E). This observation indicates a possible protective effect of NOS-scavenger, by capturing the NO content produced from Tinuvin 770 DF.

A critical that should be taken into consideration during the development of polymeric materials is their cytotoxic effect, specifically applications with the potential of contacting the skin. The cytotoxic activity of Tinuvin 770 DF lacquer-films was tested by exposing mouse fibroblast L-929 cell line to lacquer-extract medium for 24 h at 37°C. The results show that the produced PUR-based lacquer-films exhibited no cytotoxic potential, up until 5% (m/m) of Tinuvin 770 DF. This concentration is 2.5-fold higher than the top concentration limit of HALS generally used in commercial formulation [16]. These results are aligned with literature which describes that both PUR and HALS are biocompatible. Specifically, PUR-based materials are described as biocompatible materials, being used in pharmaceutical and biomedical applications such as drug delivery applications, cardiovascular devices, scaffolds for tissue engineering and bone regeneration, and wound dressing [83-86]. The safety concerns about PUR are generally associated with unreacted (free monomers) or volatile products subsequent to their synthesis [87]. Regarding biocompatibility of Tinuvin 770 DF the literature is limited. However some HALS have been described as being biocompatible, as is the case for Tinuvin 123 (Bis(1-octyloxy-2,2,6,6-tetramethyl-4-piperidyl)sebacate; CAS Nr. 129757-67-1) for S-G epithelial cell line and human gingival fibroblasts [88].

The selection of additives is a crucial step in the polymer industry, since additives can modify specific functional properties, such as tensile strength, transparency, thermal stability, and ultimately impact the final function of the material [89]. For the selected UV-stabilizers, we tested physical and chemical parameters to evaluate the impact of incorporation of Tinuvin 770 DF in

solvent-based PUR lacquer formulations. Based on the results obtained for the timing of runoff (to analyse the impact on viscosity of liquid solution), FTIR analysis (to verify the chemical stability) and gloss analysis, we concluded that, as expected, the incorporation of Tinuvin 770 DF did not impact the properties of the prepared PUR-based lacquer formulations and films (Supplementary Material).

2.3.5 Conclusion

In conclusion, the results reported in this work highlight the potential of incorporating additives with dual function in polymeric materials. In particular, the Tinuvin 770 DF can act as a UV-stabilizer avoiding the photo-degradation of polymer materials, as well as a biocide agent by inhibiting bacteria growth and ultimately reducing polymers surface contamination. Moreover, we highlight a potential antimicrobial mechanism of HALS, based on formation of NO species, during the photo-oxidation of Tinuvin 770 DF. Our results contribute to better understand the antimicrobial mechanism of HALS and possibly to expand the commercial application of Tinuvin 770 DF as a UV-stabilizer and biocidal agent.

2.3.6 References

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2.3.7 Supplementary material: antimicrobial properties of hindered amine light stabilizers in polymer coating materials and their mechanism of action

Macroscopic, physical and chemical properties of PUR-formulations upon incorporation of Tinuvin 770 DF additive.

1. Rationale: the main function of additives in the polymeric industry is improving the processability of materials or to provide them a specific property of interest. In this work, we incorporated a UV-stabilizer, Tinuvin 770 DF, to produce a lacquer-film with dual functions: stability to UV-light (to avoid the photodegradation of material) and antimicrobial (to avoid the biodegradation of the material and inhibiting the colonization and growth of potential harmful

microorganisms to humans). Specifically, we carried out a comprehensive evaluation to assess both the physical and chemical impact of the incorporation of Tinuvin 770 DF in PUR-based formulations (PUR-formulations), as it is essential to ensure the processability of the polymeric formulation, while achieving the desired properties in the final product. Since Tinuvin 770 DF is a powder additive, this evaluation allowed not only the assessment of its dispersion/solubilization in the solvent system being used, but also the analysis of its impact on lacquer viscosity, gloss, and the stability of additive in the presence of the other lacquer constituents.

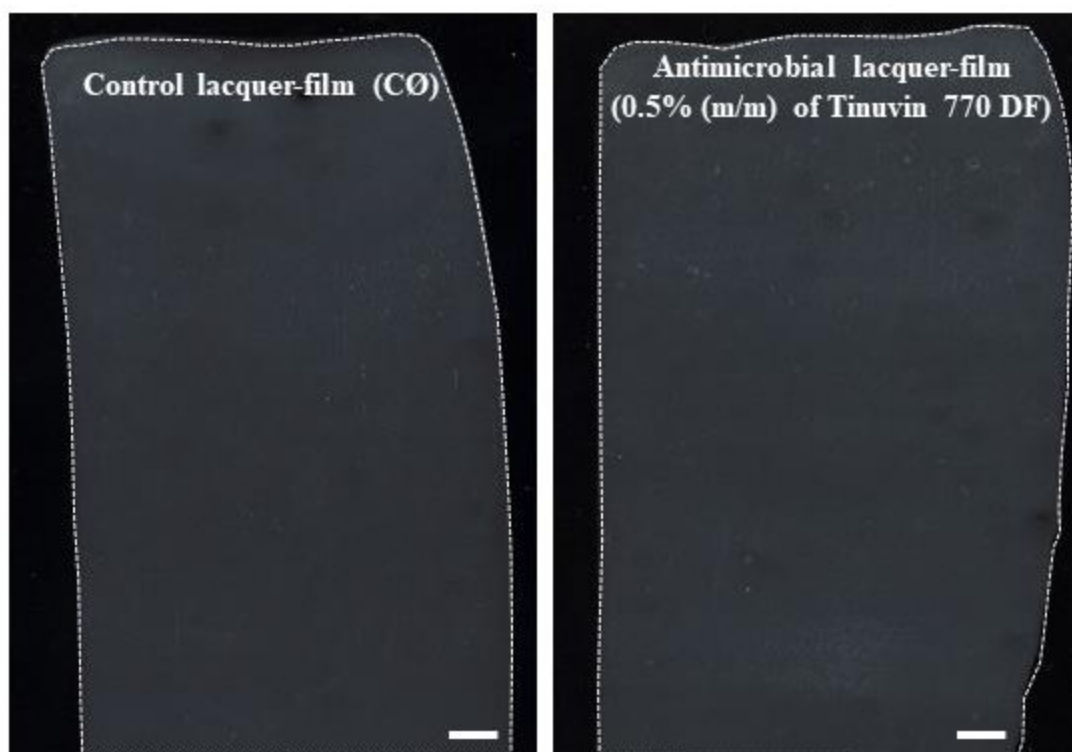
2. Methodology: the liquid lacquer-formulations were produced and deposited on a PET inert surface to obtain lacquer-films, according to the protocol described in the Material and Methods section. For liquid lacquer-formulations, the runoff time and FTIR analysis was evaluated. For lacquer-films, macroscopic alterations, FTIR analysis and the gloss value was evaluated. Control lacquer-formulations (without Tinuvin 770 DF) were prepared, characterized, and used as a reference for comparison.

2.1 Runoff time of lacquer formulations: runoff time or flow time can be described as the time it takes for a liquid to start to flow through the orifice of the cup ford until the time the flow breaks [1]. The runoff time allows to study the resistance and velocity of a fluids (in this study, PUR-based lacquer-formulations) and is an indirect parameter to monitor the viscosity of a fluid. In this work, equal volumes of lacquer-formulations (control and incorporated with Tinuvin 770 DF) were placed on a ford flow cup and the flow time (in seconds) was measured for each experimental condition and over time. During the experiment, the room temperature was monitored.

2.2 Fourier transform infrared spectroscopy (FTIR) analysis of lacquer-formulations and lacquer-films: in this study, FTIR analyses were done on lacquer-formulations and lacquer-films to assess the impact of incorporating Tinuvin 770 DF on chemical properties of the prepared formulations/films. FTIR-spectra were collected at wavenumber range from 400 to 4000 cm^{-1} , with a resolution of 1 cm^{-1} , over time, in a FT-IR Spectrum Frontier. In addition, FTIR-spectra of Tinuvin 770 DF (powder) were also collected.

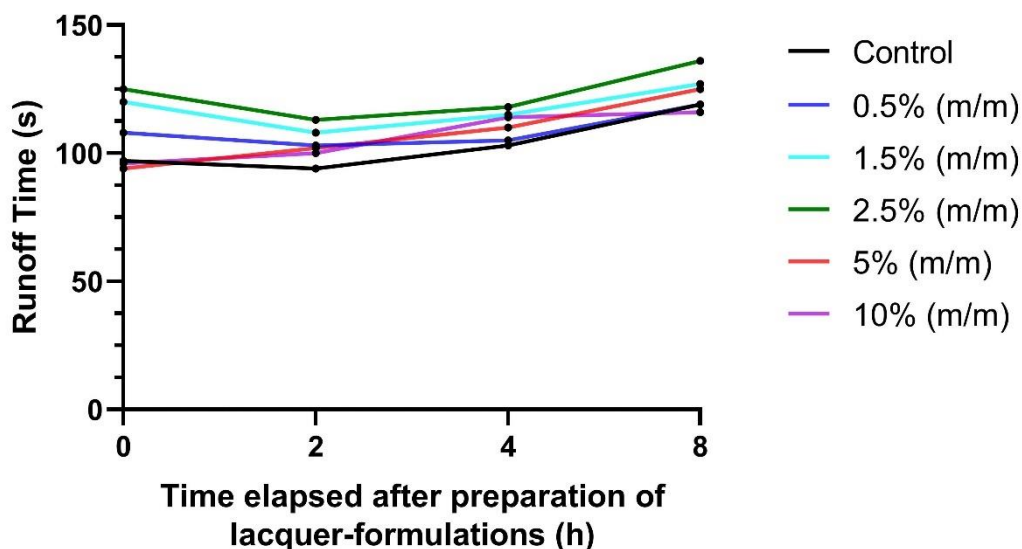
2.3 Gloss analyses of lacquer-films: to evaluate the optical and surface properties of the prepared lacquer-films, gloss was measured, using a single angle glossmeter with an incidence angle of 60° (Micro-gloss 60° supplied by BYK Instruments). For each experimental condition at least 8 independent reads were recorded in different locations across the surface of the lacquer-film.

3. Results and discussion: as represented in Supplementary Figure 1, both control and antimicrobial lacquer-films showed a regular white cast surface, with well-defined borders. As expected, there were no significant differences between these films, indicating that the incorporation of Tinuvin 770 DF did not impact the visual appearance of control lacquer-films.



Supplementary Figure 1 – Macroscopic view of produced lacquer-films: control lacquer-films (CØ) and antimicrobial lacquer-film (incorporating 0.5% (m/m) of Tinuvin 770 DF). Dashed lines represent the borders of applied lacquer-films on PET substrate. Scale bar = 1 cm.

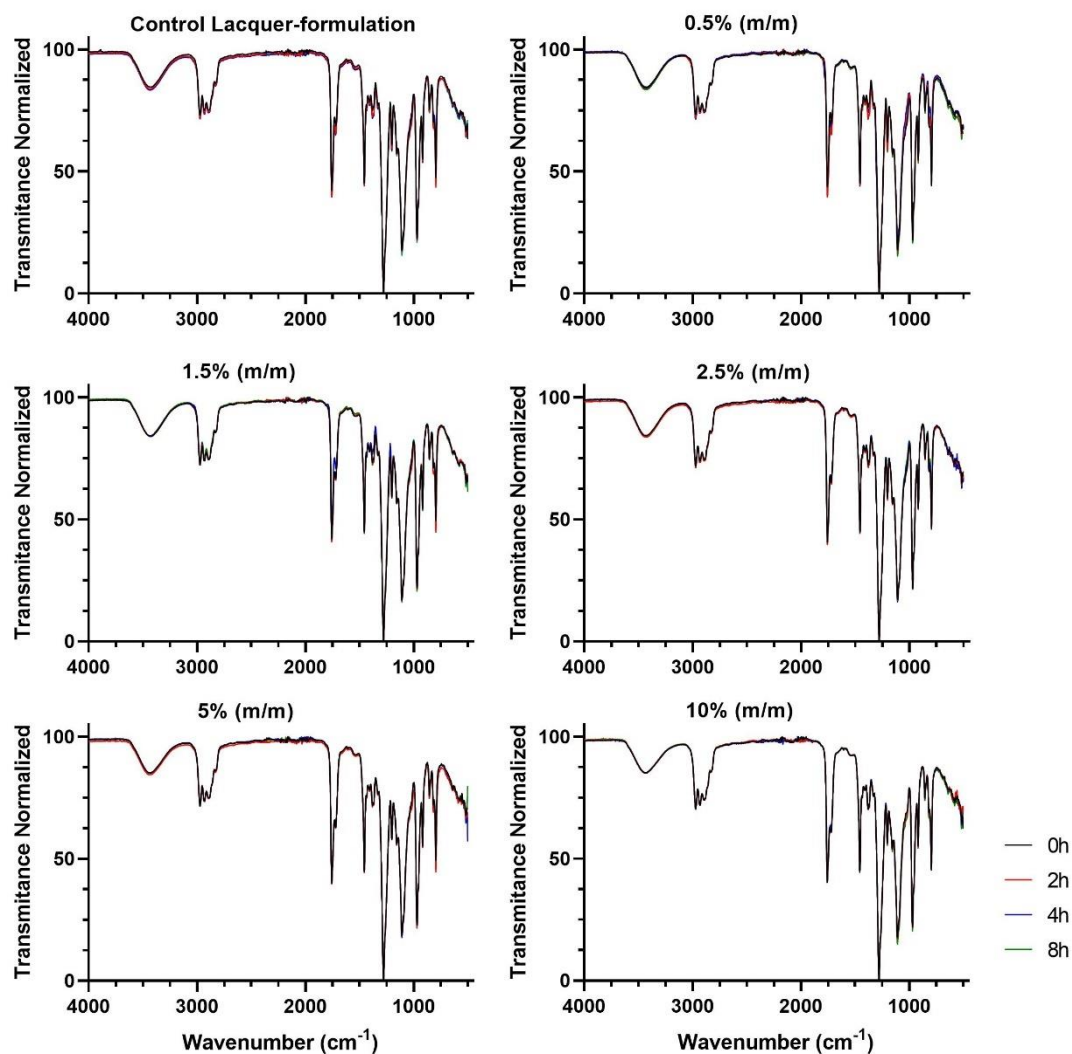
All lacquer formulations showed an increase in runoff time, over time, regardless the concentration of Tinuvin 770 DF, in particular after 2 h. Between 0 and 2 h, the behaviour of the lacquer formulations varied according to the mass concentration of the additives, as depicted in Supplementary Figure 2. The control lacquer formulation without Tinuvin 770 DF, showed an increase of 22 s in runoff time from 0 to 8 h, while the lacquer-formulation with 0.5% (m/m) of Tinuvin 770 DF, which represents the original formulation of the antimicrobial lacquer-films used in this work, only revealed an 11 s increase in runoff time. These results are important as they show that the addition of Tinuvin 770 DF does not significantly impact on lacquer viscosity, at least up to 8 h after production.



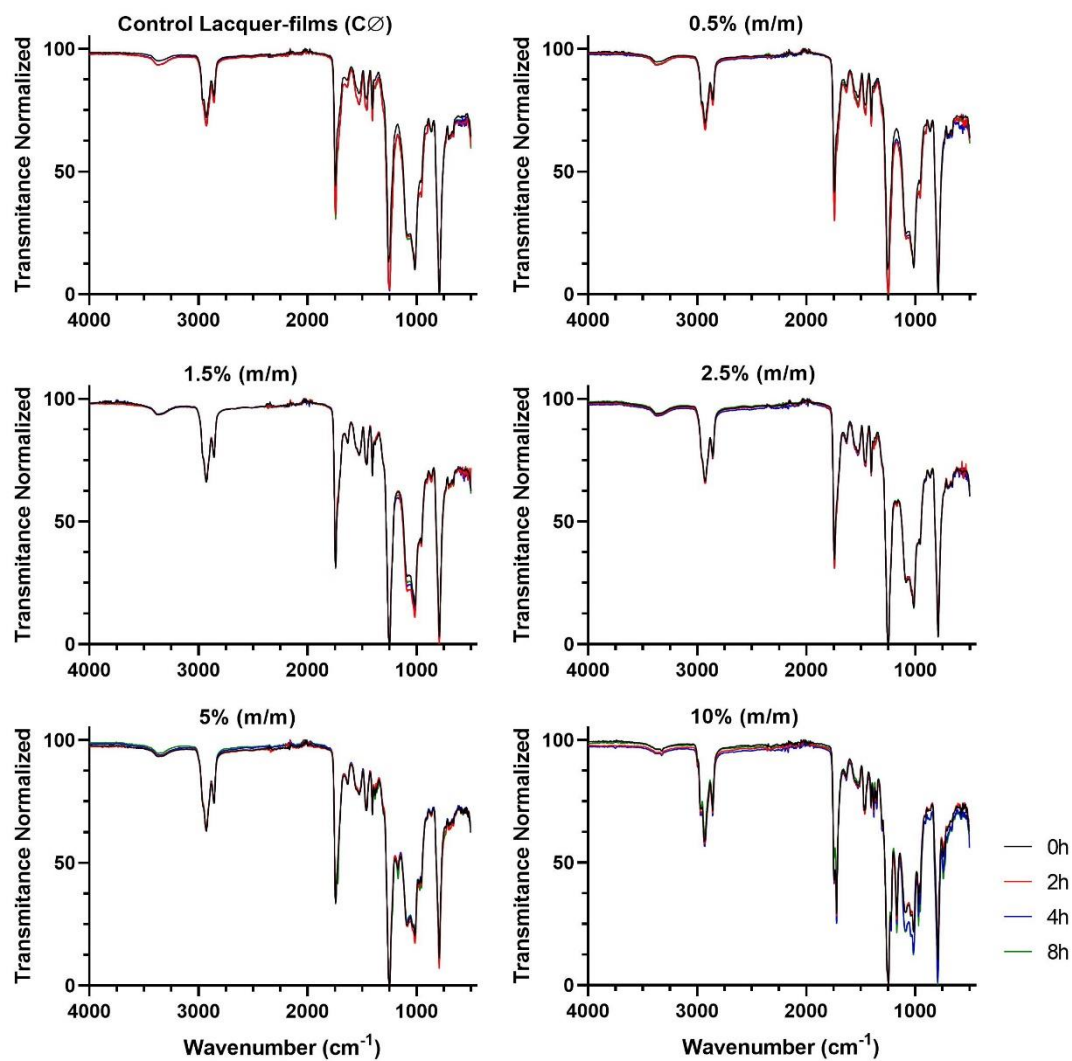
Supplementary Figure 2 – Runoff time of PUR-based lacquer-formulations: Control represents the PUR lacquer formulation without incorporation of Tinuvin 770 DF, and 0.5, 1.5, 2.5, 5 and 10% (m/m) represents the lacquer-formulations with increasing concentrations of Tinuvin 770 DF.

Regarding FTIR analysis no significant differences in the spectrum were observed between control and lacquer-formulations incorporated with Tinuvin 770 DF, regardless the concentration tested over time, at least up to 8 h after production of the lacquers (Supplementary Figure 3 and 4). However, for the highest concentrations of Tinuvin 770 DF (5 and 10% (m/m)) there are differences in the FTIR spectrum compared to lower concentrations and control lacquer-films, where additional specific bands between 1100 and 1400 cm^{-1} are detected. This zone is called the “fingerprint region” and it is frequently used to distinguish specific molecular vibrations, being important to characterize chemical structures [2]. In this fingerprint region it is possible to identify specific bands, which were attributed to Tinuvin 770 DF (Supplementary Figure 5). These bands are not identified in control lacquer-films as well as in the lacquer-films with lower concentration of Tinuvin 770 DF (<2.55% (m/m)) provably due the low concentration. Specifically, the following absorption bands were identified: (i) at 1165 cm^{-1} , attributed to -C-O and/or -CH₂ stretching [3]; at 1302 cm^{-1} attributed to -CN stretching [4] and at 1350 cm^{-1} attributed to C-N ring vibration [5], which are identified by number 1, 4 and 5 in Supplementary Figure 5. These bands are observed in spectra of Tinuvin 770 DF and in lacquer-films with highest concentrations of this additive, confirming the incorporation of Tinuvin 770 DF in the lacquer-films. As expected, the intensity of the bands increased with Tinuvin 770 DF content. This occurrence is more evident in band identity at 1350 cm^{-1} .

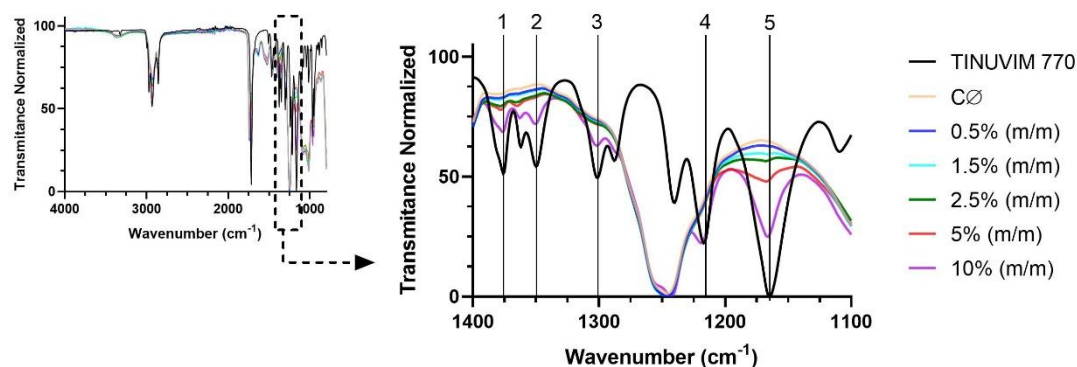
According to these results is possible to assure that incorporation of Tinuvin 770 DF at lower concentrations (until 2.5% (m/m)) does not impact the chemical stability of both liquid and solid lacquer formulations.



Supplementary Figure 3 – Evolution of FTIR spectra of lacquer-formulations: control and lacquer-formulation incorporated with increasing concentrations of Tinuvin 770 DF (0.5, 1.5, 2.5, 5 and 10% (m/m)), 0, 2, 4 and 8 hours after production.



Supplementary Figure 4 – Evolution of FTIR spectra of produced lacquer-films: control and lacquer-films formulated with increasing concentrations of Tinuvin 770 DF (0.5, 1.5, 2.5, 5 and 10% (m/m)), 0, 2, 4 and 8 hours after production.



Supplementary Figure 5 – FTIR analysis of lacquer-films produced and Tinuvin 770 DF in pure state (powder): comparison between FTIR spectra of lacquer-films with different concentrations of Tinuvin 770 DF 4 h after production and Tinuvin 770 DF (powder). CØ represents the control lacquer-films (without Tinuvin 770 DF) vs the lacquer-films with increasing concentrations of Tinuvin 770 DF (0.5, 1.5, 2.5, 5 and 10% (m/m)). Numbers represent specific bands used to analyse the FTIR spectrum.

The gloss analyses showed that the incorporation of Tinuvin 770 DF can influence the gloss value of the lacquer-films, as presented in Supplementary Table 1. Specifically, the control lacquer-films showed a gloss value of 6.63 while lacquer-films with 10% (m/m) of Tinuvin 770 DF shows a reduction of gloss value of 7.8%. The general trend of lower gloss values observed for increasing % (m/m) of additive may be attributed to a matte effect given by the solid particles (white to cream crystalline granules) of Tinuvin 770 DF. Regarding the concentration of Tinuvin 770 DF used in this study and in a commercial context (0.5% (m/m)), this did not significantly impact gloss values, once its below to 5%.

Supplementary Table 1 – Gloss measurements of lacquer-films incorporated with different concentrations of Tinuvin 770 DF.

	Tinuvin 770 DF concentration (% (m/m))*					
	CØ	0.5	1.5	2.5	5	10
Gloss value	6.63±0.11	6.32±0.23	6.41±0.15	6.81±0.24	6.31±0.21	6.11±0.06

* The values are expressed as the mean±standard deviation, with at least 8 reads in different locations across each film length. CØ represents the control lacquer-films (without Tinuvin 770 DF).

4. Conclusion: the preservation of macroscopic, physical and chemical properties of polymers after incorporation of additives is an important factor in material science and development, given that these additives can modify the functional and structural properties of the final material and ultimately impact their function. In this work, specific macroscopic, physical and chemical

properties were tested after the incorporation of Tinuvin 770 DF in a PUR-based formulations. For lacquer-formulations there is no significant changes either in runoff time or in the FTIR spectra. For lacquer-films only the highest concentrations (5 and 10% (m/m)) impacted the physical and chemical properties. However, these concentrations of Tinuvin 770 DF are not usually used in commercial applications. Typically, UV-stabilizers are used in the range of concentration between 0.05 and 2% (m/m) and the concentration used in the lacquer-films tested in this work contains 0.5% (m/m) of Tinuvin 770 DF.

Collectively, we conclude that the incorporation of 0.5% (m/m) of Tinuvin 770 DF into PUR-based formulations does not impact the macroscopic, physical and chemical properties of the base formulation tested in the framework of this study.

5. References

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Chapter 3

General Discussion and Concluding Remarks

3.1 TMG Automotive presentation and its strategic vision

The origin of TMG dates from 1937 through the foundation of *Fábrica de Fiação e Tecidos do Vale*, in Vila Nova de Famalicão (Portugal) by Manuel Gonçalves. In 1965, this family owned company was transformed into an anonymous society, adopting the current name, Têxtil Manuel Gonçalves, S.A.. Aligned with the strategic vision of the founder: “*Tecnologia e Qualidade de mãos dadas*”, the company from early stages bet on diversifying their business areas such as TMG Textiles, TMG Automotive, TMG Energy, TMG Retail (by Lightning Bolt), and wine production and distribution (by Casa Agrícola de Compostela). Furthermore, the TMG group has important participations in several other economic activities related to transports/electromechanics (by Efacec), and in the financial area, as co-founder of Portugal Millennium BCP. Focused on its Automotive business, it is registered under the globally recognized quality management standard for the automotive industry IATF 16949.

TMG Automotive had its origins in 1952, upon the creation of an industrial line division dedicated to the production of plasticized fabrics from the original company, and in 1971 as the Artificial Leather Division of TMG, begins the supply of materials for the coating of automotive interiors fostered by SAAB and the Swedish market. TMG Automotive was the first company of the group to obtain the certification of the Research, Development, and Innovation Management System (SGIDI) by the NP4457:2007 standard. Currently, the company specializes in the manufacturing of flexible coating materials, specifically designed for car interiors, including seats, head and arm rests, door panels, gaiters and instrument panels. Some Original Equipment Manufacturer (OEM) supplied with these automotive components are well-known companies of the automotive industry, such as Volvo, BMW, Ford, Porsche, Mercedes, Renault, Stellantis and Jaguar Land Rover.

As part of the TMG group's commitment to “Deliver solutions to empower our clients' success”, TMG Automotive has been dedicated to developing innovative market solutions in response to current trends, prioritizing constant product innovation to meet the demanding requirements of the clients, while upholding the high-quality standards, complying with national and international legislation and fulfilling the commitment to sustainability.

Driven by the market demand for antimicrobial solutions in car interiors, TMG Automotive and the University of Minho, in particular the ICVS and 3B's, have collaborated to develop an innovative antimicrobial flexible coating material tailored for the car sharing segment of the automotive industry.

3.2 Antimicrobial solutions for car interiors

Considering the final application of flexible coating materials produced by TMG Automotive, it is essential to acknowledge that these materials are constantly exposed to human contact. The significance of this situation becomes particularly evident in car sharing segments, where numerous users share the same space and come in contact with these surfaces. To mitigate these concerns and contribute to decreased microbial contamination of automobile high-touch-surfaces, different approaches were carried out in the context of this PhD, with a common goal of developing antimicrobial lacquer-films based on an established commercial formulation of PUR commonly used in the automotive industry. The first approach involved studying the influence of surface topography properties on bacterial growth (subchapter 2.1). In the second approach, different antimicrobial additives were incorporated into polymeric formulations (subchapter 2.2 and 2.3).

In the work presented in subchapter 2.1, flexible coating materials were submitted to an embossing process with different patterns (large, medium and fine grain) in order to induce a possible antibacterial effect of different surface topographies. The surface topography pattern has been recognized as an important factor in bacterial attachment. It has the potential to promote deformation in cell membranes or even influence hydrodynamic parameters and physicochemical forces that are responsible for bacterial adhesion [1]. Numerous studies have investigated the impact of surface roughness on bacterial adhesion and biofilm growth across several materials, including ceramics, polymers and metal surfaces using a wide range of microorganisms such as *Lactobacillus spp.*, *Staphylococcus spp.* and *P. aeruginosa* [1]. However, our findings revealed that the embossed patterns used in our study had no significant impact on bacterial growth against *S. aureus* and *E. coli*. This lack of effect is likely due to the substantial size difference between the groove width of the embossed patterns and dimensions of the bacteria, rendering the topography less influential in inhibiting their growth than what has been previously described. In fact, the smaller pattern reported in our study (0.08 ± 0.02 mm) falls far from the submillimetric pattern (below $0.2 \mu\text{m}$) which has been described to have the capability of effectively inhibiting bacterial adhesion [2].

In subchapter 2.2 and 2.3, our efforts focused on incorporating different chemical compounds to the coating material and test their antimicrobial capacity. The first task was the selection of antimicrobial agents. This selection was based mainly on its inclusion in Global Automotive Declarable Substance List (GADSL). GADSL is a comprehensive inventory that includes all chemical substances and materials used in the manufacturing and assembly of automotive

components. This document was developed to facilitate the communication among automakers and suppliers and to ensure compliance with international regulations governing the use of potential hazardous substances in the automotive production. For this reason, it was essential that all the antimicrobial agents used in this work were included in this list to guarantee compliance to relevant legislation. In light of these requirements, a panel of different antimicrobial agents (phenolic-, ionic-, and metal-based compounds) were selected based on their classification according GADSL: all biocide agents have been approved or authorized to be used as a biocide for product-type 9, which include fibers, leather, rubber, and polymerized materials preservatives [3]. Additional criteria were taken into consideration upon the selection of the antimicrobial agents: (i) thermal properties: antimicrobial agents should exhibit stability at high temperatures to ensure compatibility with manufacturing process at TMG Automotive; and (ii) spectrum of microbial action: antimicrobial agents should have a broad range of effectiveness against the target bacteria and fungi. In addition to the use of individually selected antimicrobial agents based on GADSL, commercial UV-light stabilizers were also included in this study based on their possible dual mechanism of action, such as HALS.

These compounds were incorporated in aqueous or solvent-based PUR systems, according to their properties, to produce lacquer-films and found that the inclusion of these specific agents generally did not affect the primary physical and chemical properties of the films, except for high concentrations of the phenolic-based compound OPP. In terms of antimicrobial properties, Cu-based lacquer-films exhibited the most promising biological performance, displaying antibacterial and antifungal activity, while maintaining cytocompatibility comparable to control lacquer-films. Ionic-based lacquer-films showed good antimicrobial performance against gram-positive bacteria and fungi, but only moderate activity against gram-negative strains. However, it should be noted that, despite this promising antimicrobial activity, high concentrations were associated with decreased cytocompatibility against the L-929 cell line, thus limiting its applicability for commercial use in high touch-surfaces. On the other hand, both tested phenolic agents were not associated with a significant microbial reduction. None of the lacquer-films exhibited notable antiviral activity against SARS-CoV-2, regardless of the concentration tested.

We also discovered that certain UV-stabilizers, specifically the pure HALS Tinuvin 770 DF (in commercial concentration), exhibited excellent antimicrobial activity against both gram-positive and gram-negative bacteria as well as fungi. Additionally, our findings indicate that antibacterial activity of Tinuvin 770 DF is associated with NO production.

The above-mentioned data are significant as they provide evidence supporting the use of antimicrobial agents in the production of biocidal coatings in the automotive industry. Both biocide agents are allowed or at least are possible to be used in the manufacturing of components for the automotive interiors.

3.3 Limitations and future perspectives

The results obtained in this work suggest that it is possible to develop antimicrobial lacquer-films through the incorporation of known compounds and, highlights the antimicrobial potential of already available commercial lacquer-film. Despite these positive results, some aspects should be taken into consideration:

(i) the antimicrobial action of the different lacquer-films was assessed against a limited number of microorganisms and always in a microbiological monoculture. However, as described in chapter 1, the literature reports a diverse microbial community capable of colonizing touch surfaces, beyond those that were used in this work. For example, Al, H. *et al.* (2017), identified a microbial community of six different bacterial species present in high-touch surfaces in confined and shared spaces, including *E. faecalis* (26%), *S. aureus* (14%), *E. coli* (10%), *S. pyogenes* and *P. alcaligenes* (4%), *S. agalactiae*, *K. oxytoca*, *K. pneumoniae*, and *P. aeruginosa* (2%) [4]. It would be important to further expand our current panel of microorganisms and include additional potentially pathogenic bacteria, as those described above, as well as the most commonly found fungi and virus. It could be also interesting to assess the antimicrobial properties of lacquer-films against polycultures of microorganisms, involving the simultaneous presence of multiple microbial species. In fact, it is described that the MIC value can be increased in the presence of polycultures, as described for *P. aeruginosa* and *C. albicans*, since the presence of polymicrobial communities can increase the prevalence of genetic mutations and resistance mechanisms [5]. This approach would provide a more comprehensive understanding of the lacquer-films' efficacy in real-world scenarios, where diverse microorganisms may coexist. Another crucial aspect to consider is the formation of biofilms, and the possibility of bacteria developing resistance to antimicrobial compounds in biofilm phase [6,7]. While the general aim of this work is preventing microbial attachment on lacquer-films and consequently inhibit biofilm formation, it could also be important to assess the antimicrobial properties of selected agents against microorganisms in a biofilm phase, as this could provide valuable insights into the lacquer-films' effectiveness in combating biofilms and preventing the development of antimicrobial resistance.

(ii) the antimicrobial assays conducted in this study focused on non-aged material, rather than on material at different aging-states. It is worth noting that in this PhD work, the antimicrobial properties of lacquer-films were studied within a maximum timeframe of 2 months after lacquer production. However, given that the average life expectancy of passenger vehicles in the European Union is 12 years [8], it is crucial for the automotive industry to ensure that automobile surfaces maintain their initial properties over-time.

A strategy employed to assess the effect of time on the properties of the material is the use of accelerated aging tests (AAT) to simulate the effects that occur during natural ageing [9]. AAT are carried out in specific laboratorial equipment with pre-determined conditions (e.g. time or number of cycles) and allows to simulate, in a shorter amount of time, one or more effects that characterize ageing due to real exposure.

The typical conditions used in AAT are those found during real exposure, such as humidity, high-temperature or temperature variations, UV-radiations, exposure to atmospheric agents (acid, alkaline, saline) and/or mechanical stress [9]. Examples of AAT described in the literature for polymer materials include relative humidity (r.h.) test by sample exposure to 85°C and 85% r.h. for 1000 h in specific climatic cabinets [10]; thermal aging involves polymer material exposure to 120-150°C for 35 days [11]; and UV-aging test by material exposure to UV wavelength irradiation at 340 nm and 125 W for 144 h [12]. Aligned with this, it would be important to assess the lacquer-films after exposure to different AAT to evaluate if their long-term antimicrobial performance remains effective over time and ultimately ensure their reliability in real-world automotive applications.

(iii) although the impact of the incorporation of antimicrobial compounds in a commercial formulation was assessed by physical, chemical, and optical techniques, additional laboratorial assays are required for validation according to specific automobile regulations. Indeed, flexible coating materials produced at TMG Automotive are submitted to a set of tests to analyse the mechanical properties of the materials and to evaluate if they fulfil the technical requirements imposed by the automotive industry. These tests include mechanical strength, flexibility, thermal and UV resistance, cleaning resistance, burning tests, emissions, odour tests and colour tests. For future work it will be important to further assess if the incorporation of the tested antimicrobial compounds (specifically CuSP and DMIC), affects any of the parameters mentioned above. As Tinuvin 770 DF has been used as a UV-stabilizer in serial production materials at TMG Automotive,

this characterization is performed as part of standardized procedures, creating a database for future studies.

(iv) considering that the incorporation of antimicrobial compounds was only carried out in PUR topcoat formulations, two future pathways can be pursued. Firstly, it would be valuable to test the efficacy of the antimicrobial compounds using other PUR formulations. Although the PUR formulations used in this work represents the most commonly used formulations at TMG Automotive, testing the antimicrobial action of the selected antimicrobial compounds in other lacquer formulations, namely focused in more sustainable and environmentally friendly chemistries would be important. This is particularly relevant for phenolic compounds that presented limited antimicrobial action with the formulation used and also changed the properties of lacquer-films. Secondly, it would be worthwhile to explore the incorporation of antimicrobial compounds into other layers that comprise the flexible coating materials. Although the topcoat layer has a more direct contact with humans, it is also important to guarantee that the deeper underlying layers also have antimicrobial action, especially when considering the wear that the topcoat can suffer over time. Incorporating antimicrobial compounds in the intermediate layer or in the basecoat could allow a prolonged antimicrobial performance of the materials even after physical, chemical, or mechanical degradation of the topcoat. To ensure the long-lasting antimicrobial properties of the materials, the antimicrobial agents could be incorporated in time-controlled drug delivery systems (DDS) or immobilized within the polymer backbone.

(v) the antimicrobial lacquer-films have the capability to avoid/inhibit the microbial growth, reducing the human exposure to potential microorganisms. While it is expected that a car interior treated with antimicrobial lacquer-films will create a “cleaner” environment, issues related to potential development of microbial resistance should be considered. To mitigate this, it is important to assess not only the microbial properties of lacquer-films, but also the concentration of antimicrobial agents incorporated. Regarding the first question, the material should be effective in eliminating all target microorganisms, resulting in a total reduction of microbial burden. This approach minimizes the likelihood of any surviving microorganisms acquiring resistance. Secondly, it is important to optimize the antimicrobial concentration, aiming for lowest possible effective concentration to achieve the desired antimicrobial effect. This allows to avoid unnecessary exposures to antimicrobial agents. In all cases, an exact mechanism of action of these compounds should be investigated, allowing for more informed decision-making regarding their use. Additionally, it is important to exercise careful vigilance after introducing these products in the

market. Monitoring their efficacy, potential adverse effects, and any signs of emerging resistance will help ensure their continued effectiveness and safety.

In addition to the above information, it is important to acknowledge that the lacquer-films were produced at a laboratory scale, although mimicking the industrial processes. To facilitate a possible commercial application, it is necessary to transition from laboratory-scale production to a large-scale production using industrial processes. However, this transition can present challenges as certain parameters of production can affect the properties of the lacquer-films (specifically for CuSP and DMIC). Aside from technical issues, other factors such as time and costs of production, environmental impact, and legal requirements must all be carefully considered before producing a flexible coating material at an industrial scale and introducing these products to the market. Ultimately, it will be essential to ensure that the industrial production process maintains the desired antimicrobial properties, while meeting quality standards, efficiency, and sustainability requirements.

3.4 Conclusion

The forecasts indicate an increase of users of the carsharing segment and consequently an increased market worldwide. For this reason, as a strategic market sector, R&D companies of automobile industries have made an effort to develop antimicrobial solutions for car interior. The use of antimicrobial coatings in automobile touch surfaces can reduce the risk associated to transmission of potential microorganism among users keeping the hygienic standards. The results obtained in this work highlight the great potential of developing antimicrobial lacquer-films from PUR-formulations designed to meet the most demanding automotive specifications, and ultimately add value and functionality to the coating materials for automotive interiors. Our results clearly highlight the successful development of lacquer-films capable of inhibiting microbial colonization, effectively reducing the potential exposure to potential harmful pathogens, while maintaining cytocompatibility. Importantly, we have shown that antimicrobial activity, with a broad spectrum of action, can be attained through the incorporation of known antimicrobial agents into commercially available PUR-formulations, but can also be achieved through the incorporation of already available commercial formulations, further enhancing the practicality and feasibility of implementing these solutions. In this case, a deeper insight into the antimicrobial mode of action of HALS was a major step forward for the comprehension of synergistic effects among different components of coating formulations. This advancement contributes to creating a safer and more hygienic environment for

carsharing users, aligning with the industry's commitment to ensure customer satisfaction and well-being.

3.5 References

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