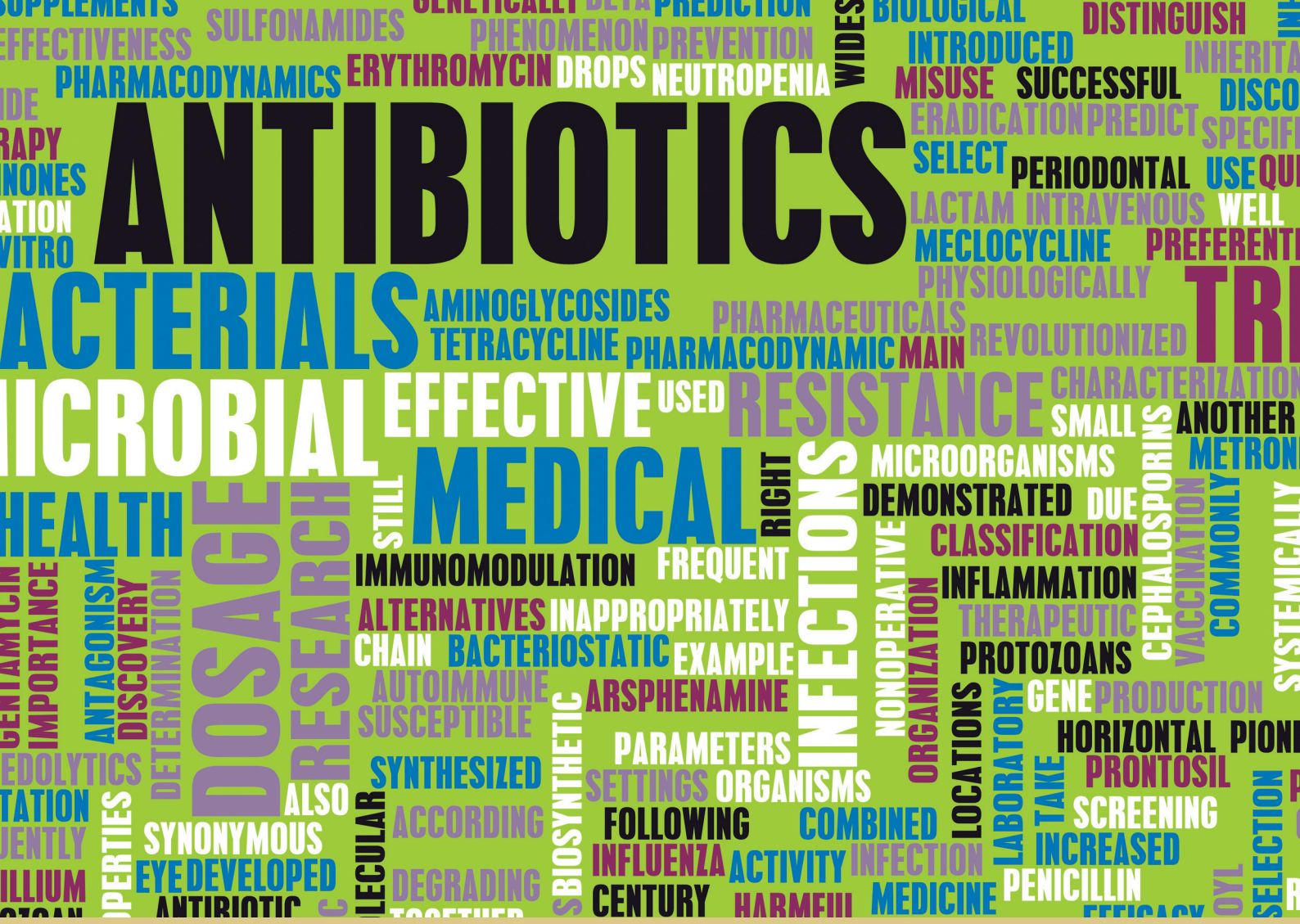

Maria do Céu Esteves Amaral Teixeira

NANOSTRUCTURES AND NANOMATERIALS FOR ANTIMICROBIAL PEPTIDES (AMPs) DELIVERY

Dissertação de Mestrado em Tecnologias do Medicamento orientada pela Professora Doutora Eliana Maria Barbosa Souto
e apresentada à Faculdade de Farmácia da Universidade de Coimbra

Julho de 2016

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RESUMO

A resistência antimicrobiana é, actualmente, uma das principais preocupações económicas e de saúde pública a nível global e, não obstante os esforços na investigação e desenvolvimento de novos fármacos, estes têm-se revelado pouco eficazes na resolução deste problema. Uma abordagem tecnológica baseada na Nanomedicina para o desenvolvimento de novas formulações pode ajudar a superar algumas limitações terapêuticas, tanto de fármacos antigos como daqueles mais inovadores, criando novas adições ao arsenal terapêutico de agentes antimicrobianos.

A descoberta de novas moléculas antimicrobianas, no início do século XX, foi um marco histórico no campo da farmacologia permitindo a redução da taxa de morbidade e mortalidade por doenças infecciosas, que eram, ao mesmo tempo, a principal causa de morte a nível mundial. O uso generalizado e indiscriminado de antibióticos potentes nas últimas décadas conduziu, todavia, a um aumento dramático no nível de resistência microbiana, sendo hoje uma das principais ameaças à saúde pública mundial. Há uma longa lista de bactérias resistentes a medicamentos, que inclui a resistência a e.g. sulfonamidas, penicilinas, macrólidos, meticilina, vancomicina, ou até mesmo aqueles resistentes a múltiplos fármacos. As infecções bacterianas resistentes a fármacos podem, por conseguinte, conduzir ao aumento da dose administrada, com risco aumentado de toxicidade, períodos de hospitalização mais longos, traduzindo-se no aumento da mortalidade. Os antibióticos são geralmente classificados de acordo com seu mecanismo de acção, designadamente, mediante a interferência na síntese da parede celular, no ciclo de reprodução da célula, e/ou na estrutura da membrana bacteriana. Alguns microorganismos podem ser intrinsecamente resistentes a alguns medicamentos antimicrobianos, influenciando o seu espectro de acção, ou adquirir esta resistência em consequência da exposição excessiva a esses tipos de fármacos. Os mecanismos específicos de resistência adquirida antimicrobiana são multifactoriais, e estes incluem a diminuição da absorção e aumentou o efluxo de fármaco a partir da célula microbiana, a expressão de genes de resistência de codificação bombas de efluxo ou modificação do substrato para o agente antimicrobiano, a modificação covalente da molécula do fármaco antimicrobiano provocando inactivação, aumento da produção de um inibidor competitivo de antibiótico, tolerância das células a fármacos que se mantêm metabolicamente inactivas, ou a formação de biofilmes. Devido aos recentes avanços no campo das nanotecnologias, bem como a síntese de novos biomateriais, uma das principais estratégias da resistência antimicrobiana parece ser o

desenvolvimento de novas tecnologias farmacêuticas e sistemas de distribuição de fármacos baseados em nanopartículas – designada por "Nanomedicina". Estes sistemas visam melhorar e/ou modificar as características físico-químicas das moléculas conhecidas com propriedades antimicrobianas, que também podem oferecer uma solução para ultrapassar estes mecanismos de resistência.

O desenvolvimento de novos sistemas de administração e cedência de fármacos permite melhorar e/ou modificar as características físico-químicas de moléculas com propriedades antimicrobianas conhecidas. Com características físico-químicas únicas, os nanomateriais são sensíveis e selectivos para a detecção de sinalização bacteriana podendo, também, exibir propriedades antimicrobianas intrínsecas. Além disso, a utilização de nanopartículas para a administração e cedência de fármacos antimicrobianos, e a incorporação de nanomateriais antimicrobianos em dispositivos médicos e em implantes, pode prevenir a adesão microbiana e, por conseguinte, a infecção. Todos estes factos são importantes no combate à resistência farmacológica, comprometendo os mecanismos de resistência antimicrobiana.

Os péptidos antimicrobianos (AMPs, do inglês "Antimicrobial Peptides") são moléculas pequenas, com ca. 5-100 aminoácidos de comprimento, e com potente e largo espectro de acção antimicrobiana. Eles são parte do sistema imune inato, o que pode contribuir para um risco mínimo de desenvolvimento de resistência. Estas características contribuem para o reconhecimento destas moléculas como sendo novas moléculas, promissoras quanto ao desenvolvimento de novos fármacos antimicrobianos. Devido à sua natureza, estas moléculas são, contudo, dispendiosas, apresentando muitas vezes propriedades antigénicas. Também a sua estabilidade é limitada causando a diminuição da biodisponibilidade. O uso de nanoestruturas e nanomateriais para a cedência de AMPs parece ser uma abordagem promissora, com vista ao aumento da sua biodisponibilidade e a diminuição dos efeitos colaterais e, por conseguinte, risco de citotoxicidade.

O objectivo deste trabalho consiste na revisão do estado da arte sobre as vantagens da concepção de novos sistemas de cedência e distribuição de AMPs, visando a melhoria da biodisponibilidade antimicrobiana, tendo em conta os mais recentes desenvolvimentos em nanotecnologia. Além de uma abordagem conceptual e da exposição dos conceitos teóricos, também é proposta uma avaliação dos avanços mais recentes sobre esta temática.

Palavras-chave: Resistência antimicrobiana, Péptidos antimicrobianos; Biodisponibilidade antimicrobiana, Nanotecnologia, Nanoestruturas, Nanomateriais, Nanomedicina

LIST OF PUBLICATIONS

This work was partially published (Chapters I, III and IV) in:

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ABSTRACT

Antimicrobial resistance is, nowadays, one of the major global economic and healthcare concern and, despite the efforts in research and development of new molecular entities, the pipeline for new drugs tends to grow on empty. A Nanomedicine based technological approach on the development of new formulations may overcome some therapeutic limitations of both old and innovative drugs, creating new additions to the antimicrobial therapeutic arsenal.

The discovery of new antimicrobial molecules, in the early 20ies, was a landmark in the field of pharmacology allowing the reduction of morbidity and mortality from infectious diseases, which were at the same time, the main cause of death worldwide. The widespread and indiscriminate use of powerful antibiotics in recent decades has led, however, to a dramatic increase in microbial resistance, being nowadays a major threat to global public health. There is a long list of drug-resistant bacteria, including the resistance to e.g. sulfonamides, penicillin, macrolides, methicillin, vancomycin, or even those resistant to multiple drugs. The drug-resistant bacterial infections may therefore lead to an increase of the dose with an increased risk of toxicity, longer periods of hospitalization, resulting in increased mortality. Antibiotics are usually classified according to their mechanism of action, in particular, by interfering with cell wall synthesis, the reproduction of the cell cycle, and/or with the bacterial membrane structure. Some microorganisms may be intrinsically resistant to some antimicrobial drugs, influencing their action spectrum of acquired resistance as a result of excessive exposure to these types of drugs. The antimicrobial acquired resistance specific mechanisms are multifactorial, and include decreased absorption and increased the drug efflux from the microbial cell, the expression of coding genes of resistance efflux pumps or modification of the substrate to the antimicrobial agent, covalent modification of the antimicrobial drug molecule causing inactivation, increased production of a competitive inhibitor of antibiotic, cell tolerance to drugs which remain metabolically inactive, or the formation of biofilms. Due to recent advances in nanotechnology, as well as the synthesis of new biomaterials, one of the major strategies of antimicrobial resistance seems to be the development of new pharmaceutical technologies and distribution of nanoparticle-based drug delivery systems – so-called "Nanomedicine". These systems are aimed to improve and/or modify the physicochemical characteristics of the molecules with known antimicrobial properties, which can also offer a solution to overcome these mechanisms of drug resistance.

The development of new drug delivery systems aims to improve and/or modify physicochemical characteristics of known molecules with antimicrobial properties. With unique physicochemical characteristics, nanomaterials are sensitive and selective in the detection of bacterial signaling and may also possess intrinsic antimicrobial properties. In addition, nanocarriers can be used for antimicrobial drug delivery and also for the incorporation of antimicrobial nanomaterials in medical devices and implants can prevent microbial adhesion and infection. All these facts are important against antimicrobial resistance by compromising bacterial mechanisms of resistance.

Antimicrobial peptides (AMPs) are small peptide based molecules, 5 to 100 amino acids length, with potent and broad-spectrum antimicrobial properties. They are part of the innate immune system which can represent minimal risk of resistance development. These characteristics contribute to the description of these molecules as promising new molecules in the development of new antimicrobial drugs. Due to their nature these drugs are, however, expensive and often antigenic. Also their stability is limited causing a decreased bioavailability. The use of nanostructures and nanomaterials for the delivery of AMPs seems to be an excellent approach to increase their bioavailability and decrease side effects and cytotoxicity.

The aim of this work is to revise the state of the art on the approach that combines the advantages of the design of new drug delivery systems for the improvement on antimicrobial bioavailability, taking into account the recent developments in nanotechnology for antimicrobial peptides delivery. In addition to a conceptual definition and clarification, a review of recent advances on this topic is also proposed.

Keywords: Antimicrobial resistance, Antimicrobial peptides, Antimicrobial Bioavailability, Nanotechnology, Nanostructures, Nanomaterials, Nanomedicina

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LIST OF ABBREVIATIONS

A

AAMPs – Anionic antimicrobial peptides

Ag – Silver

AgNPs – Silver nanoparticles

AIDS – Adquired immunodeficiency syndrome

AMPs – Antimicrobial peptides

AP-57-NPs-H – AP-57 nanoparticles hydrogel

Au – Gold

AuNDs – Gold nanodots

AuNPs – Gold nanoparticles

B

BBB – Blood-brain barrier

C

CAMPs – Cationic antimicrobial peptides

CDC – Center of Disease Control

c-di-GMP – Cyclic di-guanosine monophosphate

CF-patients – Cystic fibrosis patients

CFU – Colony forming units

CLSM – Confocal laser scanning microscopy

CNS – Central nervous system

CS – Chitosan

CS-NPs – Chitosan nanoparticles

CS-TPP – Chitosan tripolyphosphate

CW – Continuous-wave

D

DMPG – Dipalmitoyphosphatidylglycerol

DNA – Desoxyribonuceic acid

DPPC – Dipalmitoyphosphatidylcholine

DT – 1-dodecanethiol

E

EPR – Electron paramagnetic resonance

EtBr – Ethidium bromide

F

FDA – Food and Drug Administration

G

GRAS – Generally recognized as safe

H

HPMC – Hydroxypropyl methylcellulose

I

ISMN – Isosorbide mononitrate

J

K

L

LPS – Lipopolysaccharide

LTA – Lipoteichoic acid

LTP-NPs – L-Tyrosine polyphosphate nanoparticles

M

MBC – Minimum bactericidal concentration

MDCAMPs – Multidomain cationic antimicrobial peptides

MDR – Multidrug resistant

MFS – Major facilitator superfamily

MIC – Minimum inhibitory concentration

MLV – Multilamellar vehsicles

MRAB – Multidrug resistant *A. baumannii*

MRSA – Methicillin-resistant *Staphylococcus aureus*

MSN – Mesoporous silica nanoparticles

N

NaTPP – Sodium tripolyphosphate

NDA – New drug approval

Ni – Nickel

NIR – Near-infrared

NLP-NPs – Nisin-loaded pectin nanoparticles

NMR – Nuclear magnetic ressonance

NO – Nitric oxide

NPs – Nanoparticles

NRAMPs – Non-ribosomally synthesized peptides

O

P

P(X)s – Dmyristoylphospholipids

PABA – Para-aminobenzoic acid

PBP – Penicillin binding protein

PCA – Poly (cyanoacrylate)

PCL – Poly (carprolactone)

PEG – Polietileneglycol

PELDOR – Pulsed electron-electron double resonance

PGA – Poly (glycolic acid)

PLA – Poly (lactic acid)

PLA-NPs – Poly (lactic acid) nanoparticles

PLGA – Poly (lactide-co-glycolide)

PLGA-NPs – Poly (lactide-co-glycolide) nanoparticles

PLLA – Poly (L-lactic acid)

PLLA-L35-PLLA – Poly (L-lactic acid)-Pluronic L35-poly (L-lactic acid)

PVP – Polyvinyl pyrrolidone

Q

R

RAMPs –Ribosomally synthesized peptides

RNA – Ribonucleic acid

RND – Resistance nodulation cell division family

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

S

SC – Sodium caseinate

SCC – Silver carbine complexes

SDS – Sodium dodecyl sulphate;

SFT – Surfactin

Si – Silicon; Silica

SiNPs – Silica nanoparticles

SiNS – Silica nanospheres

SLN – Solid lipid nanoparticles

SMR – Small multidrug resistance family

T

TATFAR – Transatlantic Taskforce on Antimicrobial Resistance

TC – Transition temperature

TEM – Transmission electron microscopy

TLN – Triple layerd nanogel

U

V

VRE – Vancomycin-resistant *Enterococcus*

VRSA – Vancomycin-resistant *S. aureus*

W

WHO – World Health Organization

X

Y

Z

ZnONPs – Zinc oxide-nanoparticles

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CHAPTER 1

Introduction

The discovery of antimicrobial drugs or antibiotics, in the early 20th century, was an historical milestone in pharmacology allowing the decrease of morbidity and mortality from infectious disease, which were, at the same time, the main cause of death worldwide [1]. Despite this glorious fact, the widespread and indiscriminate use of potent antibiotics in the past decades, has led to a dramatic increase on the microbial resistance rates, causing one of the major concerns nowadays, and listed by the World Health Organization (WHO) as a top 3 threats to global public health [2]. There is a long list of identified drug-resistant bacteria, including sulfonamide-resistant, penicillin-resistant, methicillin-resistant, macrolide-resistant and vancomycin-resistant, or even multidrug-resistant. As a result, drug-resistant bacterial infections can cause a use of higher drug dosage, higher toxicity treatments, and longer hospitalization periods, ultimately translated in an increased mortality. This impacts negatively both medicine and society in general [3]. The natural step on fighting these issues would be the discovery and development of new antimicrobial molecules to add to the current therapeutic arsenal. Despite the efforts in research, antimicrobial resistance has receiving particular attention, and as a consequence of low return of investment, the pipeline for new drugs tends to grow on empty [1] (Figure 1).

Antibiotics are generally classified according to their mechanism of action on eradicating microbes, for instance, interference on cell wall synthesis, interference on cell

reproduction cycle, and bacterial membrane structure disruption. Some pathogens may be intrinsically resistant to some antimicrobial drugs, influencing their spectrum of action, or acquire this resistance in consequence of overexposure to these types of agents [4]. Specific mechanisms of acquired resistance are multifactorial including: decreased uptake and increased efflux of drug from the microbial cell, expression of resistance genes coding efflux pumps or a modified version of the substrate to the antimicrobial agent, covalent modification of the antimicrobial drug molecule causing inactivation, increased production of a competitive inhibitor of antibiotic, drug tolerance of metabolically inactive persister cells, biofilm formation and swarming [3]. Due to recent advances in technology, as well as in new biopharmaceutical knowledge on old and new materials, one of the main strategies against antimicrobial resistance seems to be the development of new pharmaceutical technologies and drug delivery systems. These systems aim to improve and/or modify physicochemical characteristics of known molecules with antimicrobial properties, which can also offer a solution to overcome and escape these mechanisms of resistance.

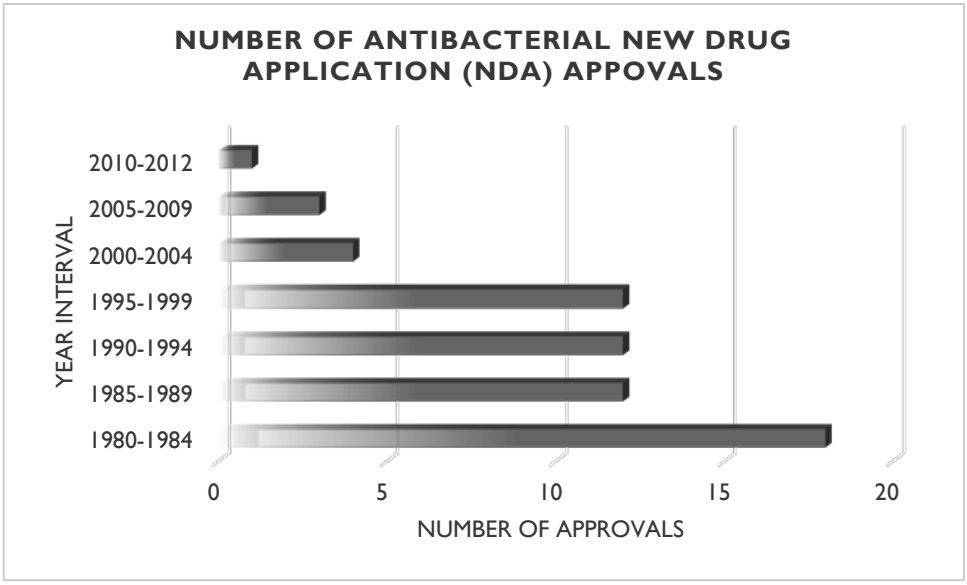

Figure 1 - Number of antibacterial New Drug Application (NDA) in the past 30 years. The number of new antibiotics developed and approved has steadily decreased in the past three decades, leaving fewer options to treat resistant bacteria (adapted from.[5]).

Nanomedicine is currently a well-established approach directly implied with the design and development of nanostructures with unique therapeutic and diagnostic properties [6]. Nanotechnologies have also shown great potential in almost every aspect on the management of microbial infection with more than 10 nanoparticles (NPs) -based products marketed for bacterial diagnosis, antibiotic delivery and medical devices in 2014 (Table 1). With unique physicochemical characteristics, nanomaterials are sensitive and selective in the

detection of bacterial signaling and may also possess intrinsic antimicrobial properties. In addition, nanocarriers can be used for antimicrobial drug delivery and the incorporation of antimicrobial nanomaterials in medical devices and implants can prevent microbial adhesion and infection. All these facts are important in fighting antimicrobial resistance by compromising bacterial mechanisms of resistance [7]. Focusing on nanotechnology-based drug delivery systems, these offer a good strategy to improve the therapeutic index, by the decrease of dosage and frequency of administration. In addition, drug delivery systems, based on nanotechnology, promote intracellular drug delivery, mitigating the development of drug resistant bacteria, and also allowing targeted organ accumulation, by functionalized surface modifications, limiting systemic side effects, as well as immunosuppression [2]. Although this promising outcomes, the main challenge on establishing clinical use is related to the evaluation of interactions of nanoantibiotics with cells, tissues, and organs and their possible toxic effects [1].

Table 1 - Marketed nanotechnology-based products for antimicrobial management (adapted from Zhu *et al.* [7]).

	Name	Company/Sponsor	Composition	Application
Diagnosis	*Verigene*®	Nanosphere	Oligonucleotide-conjugated Au nanoparticle	Bacterial infection and drug resistance diagnosis
Drug Delivery	*Abelcet*®	Enzon Pharmaceutical	Amphotericin B—lipid complex	Fungal infection
	AmBisome®	Gilead Sciences	Liposomal amphotericin B	Fungal infection
	Fungisome®	Lifecare Innovations	Liposomal amphotericin B	Fungal infection
Medical Device	*SilvaSorb*®	AcryMed	Ag nanoparticle-embedded hydrogel	Wound dressing
	Acticoat®	Smith & Nephew	Nanosilver-coated high-density polyethylene mesh	Wound dressing
	ON-Q SilverSoaker®	I-Flow	Ag nanoparticle-coated polyvinylchloride	Catheter for the delivery of local anesthetics
	VentriGuard®	Neuromedex	Ag nanoparticle-embedded nonmetallic porous materials	Ventricular catheter for cerebrospinal fluid drainage
	AGENTO I.C.®	C.R. Bard	Ag nanoparticle-distributed hydrophilic polymer	Endotracheal tube
	LogiCath AgTive®	Smiths	Ag nanoparticle-embedded polyurethane	Central venous catheter
	Silverline®	Spiegelberg	Ag nanoparticle- and insoluble silver salt-incorporated polyurethane or silicone	Central venous catheter

Antimicrobial peptides (AMPs) are small peptide based molecules, 5 to 100 amino acids length, with potent and broad-spectrum antimicrobial properties. They are part of the innate immune system which can represent minimal risk of resistance development. These

characteristics contribute to the description of these molecules as promising new molecules in the development of new antimicrobial drugs. Due to their nature these drugs are, however, expensive and often antigenic. Also their stability is limited causing a decreased bioavailability [2].

Several types of nanostructures and nanomaterials have shown potential on the pharmaceutical field. They have also been studied as potential drug carriers with application in the delivery of AMPs, promising antimicrobial molecules that, due to their nature and physicochemical characteristics, have limited bioavailability. Therefore, in addition to a conceptual understanding and clarification, a review of recent advances and studies in the matter is proposed.

CHAPTER II

Nanotechnology as a tool against antimicrobial resistance

Bacteria show resistance to antibiotics drugs through a variety of mechanisms. Moreover, the development of even new mechanisms of resistance have resulted in the simultaneous development of resistance to several antibiotic classes creating very dangerous multidrug-resistant (MDR) bacterial strains[8, 9]. However, when bacteria are drug resistant it does not mean that they stop responding to antibiotic, but that occurs only at higher concentrations [10, 11]. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent, in particular antibiotics, and proliferate and spread under the selective pressure of use of that drug. One approach to address this challenge is to design analogs of drugs [12, 13] that are already in clinical use and that have activity against resistant organisms. However, bacteria are constantly succeeding to develop resistant mechanism to new antibiotic drugs as well as to their analogs [14, 15]. The prevalent examples of such bacterial pathogens are vancomycin resistance by *Enterococcus* (VRE), MDR *Pseudomonas aeruginosa*, Drug-resistant Non-typhoidal *Salmonella*, drug-resistant *Salmonella Typhi*, Drug-resistant *Shigella*, methicillin-resistant *Staphylococcus aureus* (MRSA), drug-resistant *Streptococcus pneumonia*, drug resistant tuberculosis. These bacterial pathogens cause severe illness. Threats in this category require monitoring and in some cases rapid

incident or outbreak response. Therefore, there is an urgent need in developing new therapeutic approaches[16].

Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds such as antibiotics by manipulating their size to alter their effect. This chapter aims to first establish antimicrobial resistance as a serious global health concern, clarifying microbial drug resistance mechanisms, and second to present evidence in how nanotechnology may be considered a tool against this issue. Thus, here is presented a summary of the evidence and studies collected in the review work of Huh and Kwon [1], Pelgrift and Friedman [3], Brooks and Brooks [2], Diab *et al.* [17] and Shimanovich and Gedanken [16].

2.1. Antimicrobial Resistance

The emergence of MDR pathogens is an increasingly significant global economic and healthcare crisis [5]. Listed by the WHO as one of the top 3 threats to global public health [18, 19], more than 2 million Americans suffer from an antibiotic resistant infection at a direct cost of over $20 billion [20] with over 23,000 dying annually [5]. Analogous worldwide statistics are staggering, prompting intense multidisciplinary efforts by scientific and clinical communities to develop innovative products and tools to address the threat. The USA Center of Disease Control (CDC) has recently classified emergent resistant species as urgent, serious, or concerning [5].

Resistance has developed to virtually every class of antibiotics in current use. Development of bacterial resistance to a given antibiotic is anticipated to evolve within an average of 50 years after initial use. Resistance to certain antibiotics (e.g. tetracyclines, etc.), often develop in at least one bacterial species within a year of drug USA Food and Drug Administration (FDA) approval [21] with clinically significant levels of resistance appearing within months to years [13, 22]. The prevalence of bacterial MDR now vastly outpaces the advent of new antibiotic classes and alternatives [23]. Since the report on antibiotic resistance published by the Infectious Disease Society of America (IDSA), only 2 new antibiotics (telavancin in 2009 and ceftaroline fosamil in 2010) have been introduced to the market. Considering the rising inventory of MDR microbes, antibiotic stewardship, as defined by a number of preventative measures, is not just a formal and practical strategy, but must now be implemented out of necessity [24]. Recently, the Transatlantic Taskforce on Antimicrobial Resistance (TATFAR) outlined the most pressing needs to fight antimicrobial resistance. These include (i) appropriate therapeutic use in human and veterinary medicine; (ii) prevention of

drug-resistant infections; and (iii) strategies for improving the pipeline of new antimicrobial drugs [25]. The IDSA has mirrored these recommendations along with providing additional surveillance measures [23, 26]. While each of these recommendations is commonly accepted as necessary for infection control, several barriers to antibiotic stewardship programs remain, including lack of clinician participation [27], an absence of formal diagnostic standards, and non-uniform reporting guidelines [24]. Nevertheless, appropriate antibiotic use is critical as are prescreening microbiological tests with appropriate antibiotic follow-up [28] and stringent hand-washing guidelines and enforcement. The use of combinations, particularly those with non-antibiotic adjuvants, offers a more effective long-term solution to address multidrug resistant variants via *de novo* drug delivery. Regardless, each strategy requires a major change in antibiotic-prescribing patterns [29]. Ultimately, antibiotic resistance is not just a medical crisis, but must encompass a worldwide societal change at all levels to combat the evolution of antibiotic resistance [2].

2.1.1 Mechanisms of antimicrobial drug resistance

Development of drug resistance occurs in (at least) three steps: (i) acquisition by microbes of resistance genes; (ii) expression of those resistance genes; (iii) selection for microbes expressing those resistance genes. First, bacteria acquire resistance to single and multiple drugs through horizontal gene transfer by transformation, conjugation, and transduction [30]. Bacteria can also acquire resistance genes by spontaneous mutation of existing genes [31]. MDR is acquired when a bacterial cell already containing one type of drug resistance gene acquires another type of drug resistance gene [30, 32]. Second, in response to exposure to antimicrobial drug, microbes express the resistance gene [32]. Third, resistance becomes widespread when there is selection for microbes that express resistance genes against the antimicrobial drug. This selective pressure in favor of resistance occurs whenever microbes are exposed to the drug but not eradicated (either by the microbicidal effects of the drug itself, or by microboistatic effects of the drug followed by killing by the host's immune system) [30]. A schematic representation of some specific mechanisms of antimicrobial drug resistance is showed in Figure 2.

In any setting that creates this selective pressure in favor of drug resistance (such as poor patient compliance, or use of a time-dependent antibiotic with long half-life), the development of that resistance actually is increased by longer duration of use of the drug [32]

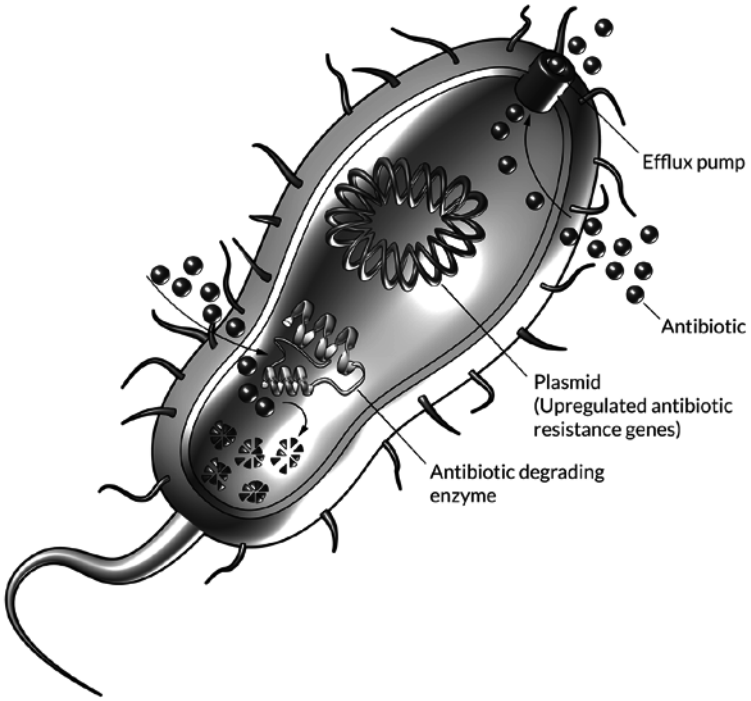

Figure 2 - Three of the main antibiotic resistance strategies used by bacteria (adapted from Brooks and Brooks [2]).

In addition, microbiostatic drugs, which inhibit but do not kill microbes, are more likely than microbicidal drugs to allow some microbial cells to live and therefore develop resistance when exposed to drug [33].When a patient on an antimicrobial drug takes an insufficient number of doses or misses scheduled doses (often due to poor patient compliance), there is increased selective pressure in favor of drug resistance, because the offending microbes are exposed to drug but not completely eradicated [34]. Poor patient compliance is especially a problem for drugs with short elimination half-lives, because these drugs have short dosing intervals, and the number of doses required for microbial eradication is high [3, 34].

2.1.1.1. Decreased uptake and increased efflux of drug from the microbial cell

Two important resistance mechanisms are reduced uptake and increased efflux of drug. Decreased uptake of antimicrobial drugs and/or use of transmembrane efflux pumps prevent the concentration of antimicrobial agent from increasing to toxic levels within the microbial cell [3, 32]. Many bacteria have reduced uptake and/or increased efflux mechanisms that act on multiple drug classes [3, 32]. For example, the low sensitivity of *P. aeruginosa* to antibiotics is often attributed to this mechanism [3, 32]. Gram negative bacteria, like *P. aeruginosa* and *E.*

coli, have also an outer membrane surrounding a periplasmic space (which contains a peptidoglycan cell wall), which surrounds an inner membrane.

The multi drug efflux pump of *P. aeruginosa* consists of an inner membrane H^+/drug antiporter protein bound to a linker protein in the periplasmic space, which itself is bound to an outer membrane channel protein [35]. *P. aeruginosa* becomes multidrug resistant when a mutation occurs in the regulatory protein that normally represses genes coding for efflux proteins, resulting in overexpression of those efflux proteins [35]. Another example of drug efflux is in *E. coli*. *E. coli* expresses at least nine pumps that use the transmembrane proton gradient as an energy source to expel multiple types of antibiotics, thereby conferring MDR to *E. coli*. These proton-dependent efflux pumps are divided into three families: Major facilitator superfamily (MFS), small multidrug resistance family (SMR), and resistance nodulation cell division family (RND). The most well understood pump is an RND pump called AcrAB/TolC. In drug-sensitive bacterial cells, the acrR protein represses expression of proteins comprising the AcrAB/TolC pump. However, when repression by acrR is released (e.g. due to an acrR gene mutation), the pump proteins are expressed, thereby causing antibiotic efflux which makes the bacterial cell drug resistant. As referred, *E. coli* is Gram negative and therefore has an inner membrane and an outer membrane which enclose a periplasmic space. The AcrAB/TolC pump consists of the inner membrane protein AcrB bound to the AcrA protein in the periplasmic space, which is bound to the outer membrane protein TolC. Drug efflux occurs when AcrA changes conformation, thereby bringing AcrB and TolC in close proximity to each other, which creates a passage from the cytoplasm to the extracellular space [3, 32].

Many bacteria express resistance genes that allow for reduced uptake and/or increased efflux of specific types of antibiotic drugs, including tetracyclines, sulfonamides, quinolones, aminoglycosides, chloramphenicol, macrolides, and streptogramins [3, 30, 35].

2.1.1.2. Resistance genes that codify for an altered version of the antimicrobial substrate binding site

Another mechanism of antimicrobial drug resistance is expression of resistance genes that code for an altered version of the substrate to which the antimicrobial agent normally binds. The antimicrobial drug usually has lower binding affinity for this altered version than the wild-type version, resulting in reduced antimicrobial activity [3, 32].

These types of resistance genes confer resistance to antibiotics such as beta-lactams, glycopeptides (including vancomycin), sulfonamides, quinolones, macrolides, aminoglycosides,

tetracyclines, linezolid, and rifampin. For example, the MecA resistance gene confers resistance to β-lactams. The MecA gene codes for PBP2A, which is an altered penicillin binding protein (PBP) that has low affinity for β-lactams and therefore confers resistance to all beta-lactams [3, 30, 35]. MecA is expressed in MRSA [3, 35]. Penicillin-resistant *S. pneumoniae* also expresses PBP with low affinity for β-lactams, but through a different genetic mechanism than MecA expression [36]. Resistance to glycopeptides, including vancomycin, is conferred by the vanA resistance gene. The vanA gene codes for D-alanine–D-lactate ligase, which changes terminal D-ala–D-ala domain of the peptidoglycan precursor (which is both the substrate of the PBP transpeptidase domain and of vancomycin) to D-ala–D-lactate [3, 30, 35]. Vancomycin has 1000 times lower affinity for D-ala–D-lactate than D-ala–D-ala, so the vanA gene confers resistance to vancomycin [3, 30, 35]. Both VRE and vancomycin-resistant *S. aureus* (VRSA) express vanA [3]. Resistance to sulfonamides is conferred by expression of altered bacterial dihydropteroate synthetase (which is the substrate to which sulfonamides bind) [3, 37]. Bacteria using this resistance mechanism include *S. pneumoniae*, S. *pyogenes*, *Neisseria meningitidis*, and *E. coli* [3, 37]. Quinolone resistance can be due to altered topoisomerase IV or DNA gyrase, which both bind quinolones. Topoisomerase IV is the substrate which quinolones bind and inactivate in Gram positive bacteria [38]. Mutations in the parC or parE genes, which code for subunits of topoisomerase IV, result in altered topoisomerase IV for which quinolones have low affinity, thereby conferring quinolone resistance in Gram positive bacteria [3, 30, 35]. DNA gyrase is the substrate which quinolones bind and inactivate in Gram negative bacteria [38]. Mutations in the gyrA or gyrB genes, which code for subunits of DNA gyrase, result in altered DNA gyrase for which quinolones have low affinity, thereby conferring quinolone resistance [3, 30, 35]. In a more recently discovered mechanism of quinolone resistance, the plasmid-encoded proteins QnrA and QnrB bind topoisomerase II and DNA gyrase, thereby blocking binding by quinolones [39]. Resistance against macrolides, aminoglycosides, tetracyclines, linezolid, and rifampin can also be due to resistance genes coding for altered antibiotic binding sites.

2.1.1.3. Covalent modification of antimicrobial drug molecules

Microbes can also express drug resistance genes that code for enzymes that covalently modify the antimicrobial drug, thereby reducing its antimicrobial activity [3, 32]. Covalent modification of drug is used as a resistance mechanism against β-lactams, aminoglycosides, chloramphenicol, tetracyclines, macrolides, quinolones, and streptogramins [3, 30, 35].

For example, β-lactamases hydrolyze the β-ring of β-lactams, thereby inactivating the antibiotic activity of the β-lactam molecule and conferring resistance [3, 30, 35]. Resistance using β-lactamases can occur due to horizontal gene transfer of β-lactamase genes on plasmids or transposons, or due to decreased activity of repressor proteins which normally prevent transcription of beta-lactamase genes on the bacterial chromosome [3, 35]. Hundreds of different β-lactamases have been discovered so far [3, 32].

Two different classification systems are used to categorize the different types of β-lactamases. The molecular classification system, which categorizes β-lactamases based upon amino acid sequence, divides β-lactams into classes A, C, and D which are all serine hydrolases. Class B β-lactamases are metallo-enzymes that use a zinc prosthetic group to catalyze hydrolysis. The functional classification system categorizes β-lactamases by their molecular targets as well as the molecules that inhibit them. These include group 1 which are cephalosporinases; group 2, which includes broad-spectrum β-lactamases, extended spectrum β-lactamases, serine carbapenemases, and β-lactamases that are resistant to β-lactamase inhibitors; and group 3, which includes the metallo-β-lactamases.

Covalent modification of drug also confers resistance against chloramphenicol, tetracyclines, macrolides, quinolones, and streptogramins [3, 30, 35]. Resistance genes coding for acetyltransferases, which acetylate and thereby inactivate chloramphenicol, are the most common acquired mechanism of chloramphenicol resistance [3, 30, 35]. *Streptomyces venezuelae* ISP 5230, which synthesizes chloramphenicol, also expresses resistance enzymes that O-phosphorylate chloramphenicol [3, 32]. Enzymatic modification and inactivation of tetracyclines (using the TetX enzyme) and macrolides are also resistance mechanisms against these drugs [3, 35]. Mutation of an aminoglycoside resistance gene coding foran N-acetyltransferse can generate a new gene that also causes fluoroquinolone resistance. This new gene codes for an N-acetyltransferse that acetylates an NH2 group of the fluoroquinolone molecule, thereby inactivating it [40, 41]. Streptogramins, which bind to the 50S ribosomal subunit and inhibit protein translation, are divided into type A and type B streptogramins [3, 32]. Currently, streptogramins are used in treatment of VRE and VRSA [42]. Streptogramin resistance genes code for enzymes that covalently modify the streptogramin molecule [3, 32].

2.1.1.4. Increased production of competitive inhibitor

Bacteria can also achieve antibiotic resistance by synthesizing a molecule that is a competitive inhibitor of the antibiotic. For example, one mechanism of sulfonamide resistance is increased synthesis by bacteria of para-aminobenzoic acid (PABA), which competes with the sulfonamide

drug for the binding site of bacterial dihydropteroate synthetase [3, 43] This mechanism of sulfonamide resistance is used by *S. aureus* and *N. meningitidis* [3].

2.1.1.5. Drug tolerance of metabolically inactive persisters

The presence of metabolically inactive persisters in an infecting population of bacteria can result in tolerance to antibiotics and recurrence of infection after antibiotic treatment. In a population of bacterial cells, a tiny fraction (~1 in every 106 cells) randomly switches on expression of toxin–antitoxin genes, which cause their metabolic activity to slow or stop [3, 32]. These cells are called persisters, and their slower metabolic activity makes them more tolerant to antibiotics [3, 30, 32]. Therefore, when an infecting population of bacterial cells is exposed to antibiotics, most of the cells are drug-sensitive and are eventually eradicated, whereas the few persisters remain unaffected [3, 32]. This gives the appearance that the infection is cured. However, at some point, the persisters randomly switch back on their metabolic activity and resume growth, causing the infection to recur, despite the previous antibiotic treatment [3, 32].

2.1.1.6. Biofilms

Tolerant bacterial cells have the ability to survive in harsh conditions by several mechanisms, becoming one of the recent studied mechanism of antibacterial drug resistance. One of the most important form and survival strategy of tolerant cells is biofilm formation. Biofilms are immobile bacterial populations attached to surfaces [44]. These microorganisms are usually embedded in polymeric matrix. Bacterial biofilms can develop in medical devices and implants, such as catheters, components of cardiac pacemakers, artificial heart valves and joints [45]. With cells protected by an extracellular matrix, biofilms are highly tolerant to antimicrobials and are a major cause of chronic infections. In addition to the protection by the extracellular matrix biofilm, antibiotic resistance is also attributed to the slow growth of biofilm cells. Even though some antibiotics have been shown to effectively penetrate biofilm matrix they are not effective against these slowly growing cells, especially the dormant subpopulation known as persister cells. Since most AMPs target cell membrane, they may be more effective against these dormant cells compared to antibiotics [44].

Biofilm formation on biomaterial surfaces is a developmental process that includes the following main steps: (i) transport of bacterial cells to the surface and their initial and reversible adhesion; (ii) irreversible attachment; (iii) microcolony formation; (iv) biofilm maturation and differentiation, and (v) cell detachment with propagation of infection (Figure

3). Once implanted, the biomaterial surface is first covered with a layer mostly composed of proteins called a conditioning film. Adhering bacteria can grow and divide, forming microcolonies that are considered the basic organizational units of a biofilm. Entrapment of other planktonic bacteria in the extracellular matrix also occurs, resulting in a multi-layered and mature biofilm. Once established, biofilms are less susceptible to antimicrobial treatment and to the host immune system than their planktonic counterparts [46]

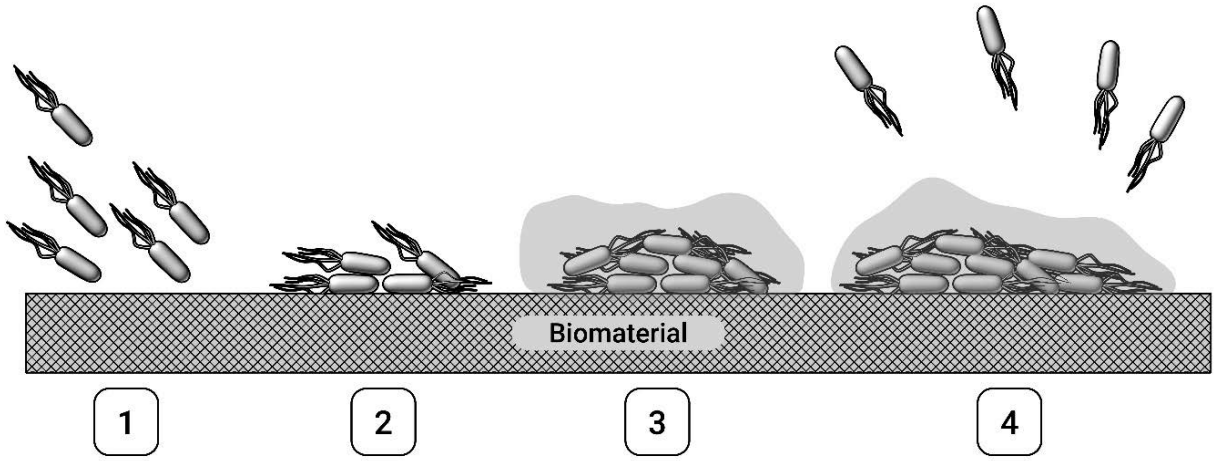

Figure 3 – Schematic representation of the biofilm formation process. (1) Initial adhesion and irreversible attachment, (2) Microcolony and extracellular matrix early formation, (3) Biofilm maturation and differentiation, (4) Single cell migration from the biofilm. (adapted from Alves and Pereira [46]).

A potential antibiofilm drug that can either facilitate the dispersion of preformed biofilms or inhibit the formation of new biofilms *in vivo* is needed. Biofilm formation can result in tolerance of bacteria to very high concentrations of multiple antibiotics, resulting in chronic infections despite antibiotic treatment [1, 30, 45]. Bacteria that form biofilms include *S. aureus* and *P. aeruginosa* [30, 45]. Biofilm formation occurs in the pathogenesis of many infectious diseases, including gingivitis, otitis media, and lung infections, including those in cystic fibrosis (CF) [3, 32].

2.1.1.7. Swarming

Swarming is another mechanism of antibiotic tolerance. Swarming is considered to be a type of multicellularity in bacteria and operates by the following mechanism: Planktonic bacterial cells differentiate into elongated cells with multiple flagella, called swarm cells. These swarm cells stay in close proximity to each other and migrate on surfaces as a single unit, analogous to a raft. These swarm cells are also tolerant to antibiotics. Subculturing swarm cells in liquid medium causes them to dedifferentiate back into planktonic bacteria which no longer have

tolerance to antibiotics [3]. Tolerance to multiple antibiotics has been demonstrated in swarm cells of *Bacillus subtilis*, *Serratia marcescens*, *E. coli*, *S. typhimurium*, *P. aeruginosa*, and *Burkholderia thailandensis* [3].

2.1.1.8. Obligate and facultative intracellular microbes

Intracellular microbes are protected from many antimicrobial drugs due to the limited ability of the drugs to enter the host cell [1, 47]. Obligate intracellular bacteria include *Mycobacterium leprae* [48], *Chlamydia*, and the non-*Bartonella Rickettsiae* (which are *Rickettsia*, *Ehrlichia*, and *Coxiella*) [3]. Facultative intracellular bacteria include other *Mycobacterium species*, *Listeria*, *Neisseria*, *Brucella*, *Francisella*, *Salmonella*, and *Legionella* [3].

2.2. Nanoantibiotics: Nanostructures and Nanomaterials for infection control

Nanomaterials, which either show antimicrobial activity by themselves [49] or elevate the effectiveness and safety of antibiotics administration [50], are called "nanoantibiotics" and their capability of controlling infections *in vitro* and *in vivo* has been explored and demonstrated. Unlike many antimicrobial agents currently being used in the clinic, antimicrobial NPs may not pose direct and acute adverse effects, although potential toxicity upon long-term exposure is questionable. Most importantly, antimicrobial NPs tackle multiple biological pathways found in broad species of microbes and many concurrent mutations would have to occur in order to develop resistance against NPs' antimicrobial activities. Preparation of antimicrobial NPs could be cost-effective, compared with antibiotics synthesis, and they are quite stable enough for long-term storage with a prolonged shelf-life [51]. In addition, some NPs can withstand harsh conditions, such as high temperature sterilization, under which conventional antibiotics are inactivated. Antibiotics delivery using nanomaterials offer multiple advantages: i) controllable and relatively uniform distribution in the target tissue; ii) improved solubility; iii) sustained and controlled release; iv) improved patient-compliance; v) minimized side effects; and vi) enhanced cellular internalization [6, 7].

2.2.1. Antimicrobial Nanostructures and Nanomaterials

Antibacterial NPs consist of metals and metal oxides, naturally occurring antibacterial substances, carbon-based nanomaterials, and surfactant-based nanoemulsions [49]. Antimicrobial mechanisms of nanomaterials include: i) photocatalytic production of reactive oxygen species (ROS) that damage cellular and viral components; ii) compromising the

bacterial cell wall/membrane; iii) interruption of energy transduction; and iv) inhibition of enzyme activity and DNA synthesis[30] (Figure 4).

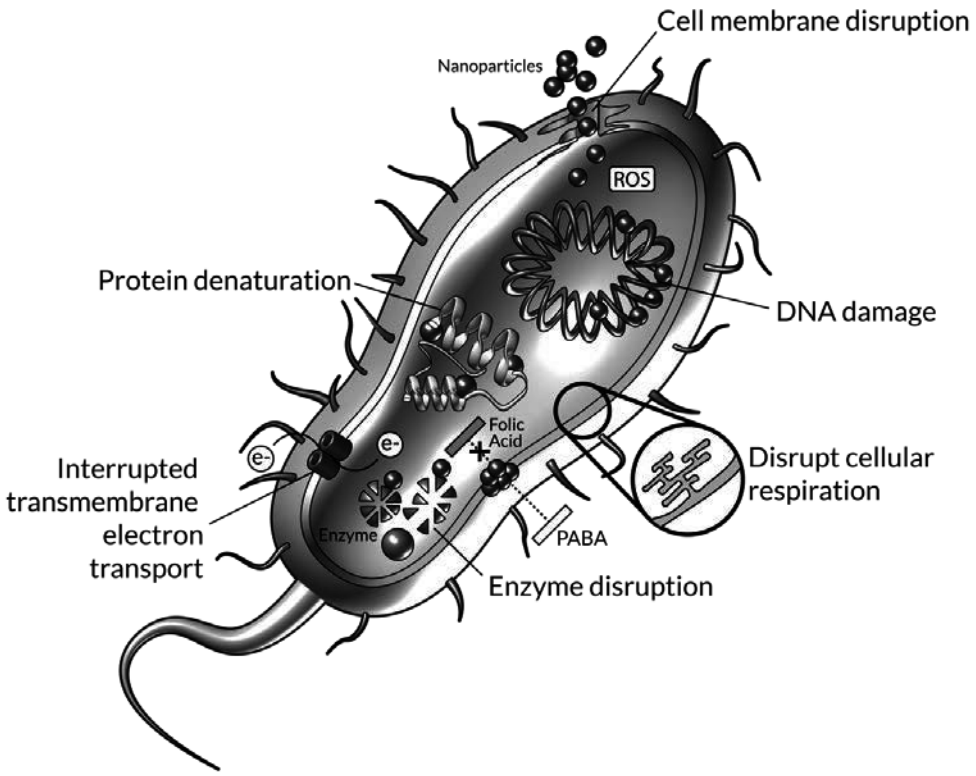

Figure 4 – Mechanisms of action of nanoantibiotics (adapted from Brooks and Brooks [2]).

Table 2 also summarizes nanomaterials with their antimicrobial mechanisms, and potential clinical and industrial uses.

Table 2–Antimicrobial nanostructures and nanomaterials. Antimicrobial mechanisms and potential applications. (adapted from Huh and Kwon[1]).

Nanomaterial	Antimicrobial mechanism	Clinical and industrial applications
Ag NPs	Release of Ag^+ ions; disruption of cell membrane and electron transport; DNA damage	Dressing for surgical wound and diabetic foot; coatings for medical devices; portable water filters; antibacterial agent; antifungal agent
ZnO NPs	Intracellular accumulation of NPs; cell membrane damage; H2O2 production; release of Zn^{2+} ions	Antibacterial creams; lotions and ointment; surface coating of medical device; mouthwash
TiO2 NPs	Production of ROS; cell membrane and wall damage	Antibacterial agent; food sterilizing agent; air purifiers; water treatment systems

Au NPs	Interaction with cell membranes; strong electrostatic attraction	Photothermal therapy with near infrared light; adjuvant treatment after serious infections antibacterial agent; antifungal agent
Chitosan	Increased permeability and rupture of membrane; chelation of trace metals; enzyme inactivation	Drinking water disinfectants; bacteria immobilizer; microbiocide in biomedical products
Fullerenes	Destruction of cell membrane integrity; enhancing activity of infiltrating neutrophil	Potential disinfection applications
CNTs	Cell membrane damage by ROS; oxidation of cell membrane proteins and lipids	Antibacterial agent; biofouling-resistant membranes; water filter; surface-coating
NO-releasing NPs	NO release and production of ROS	Infected wound and diabetic foot treatment
Nanoemulsion	Membrane disruption; disruption of the spore coat	Antimicrobial inhaler; anti-biofilm agent; nasal application; vaccine delivery agents

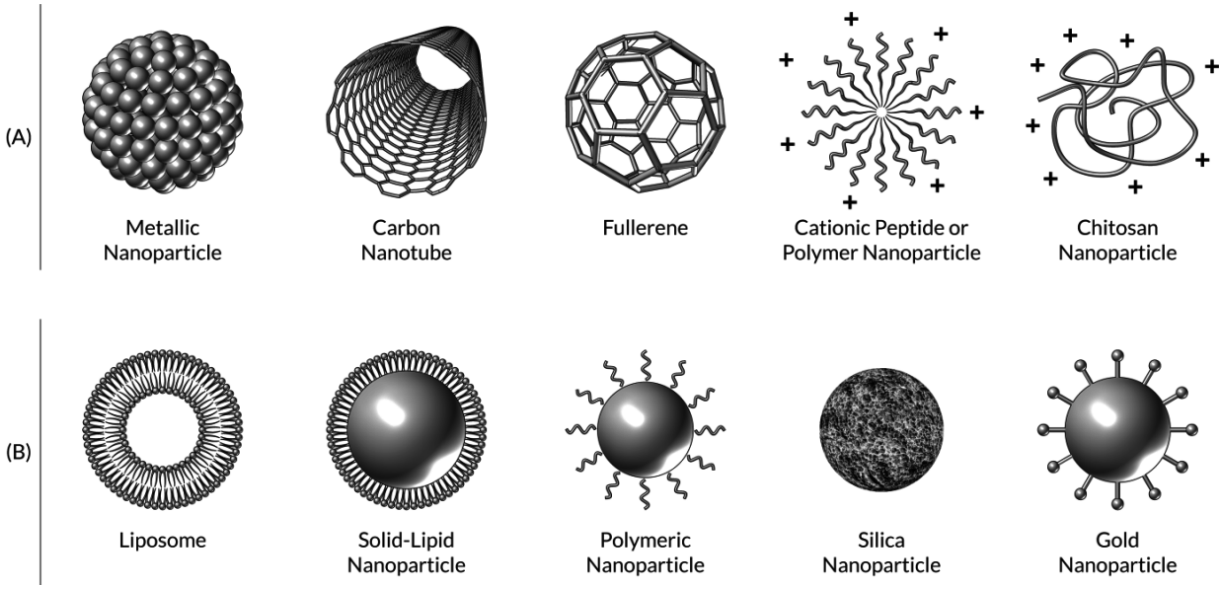

Figure 5 - Schematic representation of (A) nanomaterials with inherent antimicrobial properties, and (B) nanoparticle-based antimicrobial drug (adapted from Zhu *et al.*[7]).

2.3. How can nanoantibiotics help to bypass bacterial drug resistance?

All over the world, researchers developed different nanotechnology-based approaches with the aim to overcome the currently known bacterial resistance mechanisms to antibiotics. In this subsection, evidence of how nanosystems can overcome bacterial mechanismas of resistance is presented.

2.3.1. Alteration of bacteria's efflux pump activity

In this regard, recently-reported advances could be mentioned. Khameneh *et al.* developed piperine-containing nanoliposomes as a vector for gentamicin. The liposomal formulation was specifically developed to fight MRSA, which is widely recognized as a nosocomial pathogen [52]. The encapsulation of gentamicin in classical nanoliposomes or piperine-containing nanoliposomes resulted in a dramatic decrease of minimum inhibitory concentration (MIC) values of 16- and 32- folds, respectively. Similarly, minimum bactericidal concentration (MBC) values were also reduced 4- and 8- folds for encapsulated gentamicin in classical nanoliposomes or piperine-containing nanoliposomes, respectively. These hopeful results were attributed to the piperine inhibiting effect on the bacterial efflux pump. This argument was confirmed using ethidium bromide (EtBr) fluorescence assay. The fluorescence of this compound occurs only when it is bound to nucleic acid. Accordingly, bacterial suspension was incubated with EtBr for 30 min in the presence of: i) bare nanoliposomes (without piperine); ii) piperine-containing nanoliposomes or iii) piperine in its free form. After centrifugation and washing of bacteria, the loss of fluorescence was checked in order to investigate the efflux of EtBr outside bacterial cells. Consistently, a gradual decrease of fluorescence during the assay period was observed in the first case, *i.e.* in the absence of piperine. However, in the presence of piperine the fluorescence was significantly enhanced indicating a significant inhibition of the efflux pump [52]. Therefore, the enhanced antibacterial activity of gentamicin encapsulated in piperine-containing nanoliposomes is likely to be the consequence of an increase in its intracellular concentration. It is of note that piperine in its free form was less effective in inhibiting the efflux pump than the liposomal one, as demonstrated by the EtBr fluorescence assay [17].

2.3.2. Antibiofilm activity

Nitric oxide (NO)-releasing NPs were found to prevent the formation of bacterial biofilms and to eradicate already formed biofilms. Some examples of recent breakthroughs in this domain are presented hereafter. Jardeleza *et al.* encapsulated isosorbide mononitrate (ISMN), as NO donor into different liposomal formulations with the purpose toenhance the antibiofilm

activity against *S. aureus*'s biofilms [53]. NO-releasing multilamellar vesicles (MLV) efficiently eliminated *S. aureus*'s biofilms *in vitro*. A five minutes' exposure to 60 mg/mL ISMN-loaded MLV induced an almost complete eradication of the biofilms. Paradoxically, the authors observed that at low concentrations NO-releasing MLV enhanced the formation of biofilms, which is in accordance with previously obtained results [54]

Duong *et al.* developed nanoparticulate NO-core cross-linked star polymers as new therapeutics able to combating biofilms that are frequently formed during long exposure of the body to medical devices and catheters [55]. These systems were found to release NO in a controlled and slowed-down manner in bacterial cultures and showed great efficacy in preventing both cell attachment and biofilm formation in *P. aeruginosa* over time. This study unveiled, in part, the inherent mechanisms of NO's antibiofilm activity. Accordingly, NO-releasing NPs inhibits the switch of planktonic cells in contact with a surface to the biofilm form by continuously stimulating phosphodiesterase activity. Thus, NO-releasing NP maintained low intracellular concentrations of cyclic di-guanosine monophosphate (c-di-GMP) in the growing bacterial population, thereby confining growth to an unattached free-swimming mode [55].

The dual delivery of two antibiotics *via* their co-encapsulation in nanoliposomes is another proposed strategy to bypass resistance mediated by biofilm formation. For instance, Moghadas-Sharif proposed vancomycin/rifampin-co-loaded nanoliposomes as a new therapeutic against *S. epidermidis* [56]. This strategy was based on two points. First, combination therapy of vancomycin and rifampicin helps avoid the emergence of rifampin-resistant strains. Indeed, numerous studies have already reported the antibiofilm activities of rifampin in combinations with other antibiotics [57, 58]. Second, rifampicin fails alone to eradicate bacterial biofilm [59]. Nevertheless, the developed liposomal combination was ineffective to eradicate *S. epidermidis*' biofilm. The authors attributed this result to the lack of liposomal adsorption or low penetration into the bacterial biofilm [56]. A more adjusted formulation with enhanced penetration behavior into the biofilm may lead to the initially expected effect.

2.3.3. Enhanced penetration through biofilms

Several research papers reported the improved penetration across bacterial biofilms as a plausible reason behind the enhanced antibacterial activity of encapsulated antibiotics against resistant bacteria. For instance, liposomal encapsulation of polymyxin B was first described by Alipour *et al.* as a strategy to enhance its antibacterial activity against *P. aeruginosa* resistant

strains [60]. As they expected, lower MIC values were observed for liposomal formulations with respect to that of the free drug. In an attempt to elucidate the involved mechanisms, the researchers focused on the drug uptake and more precisely on its penetration across the biofilm formed by the polymyxin B-resistant *P. aeruginosa* strain. They used a coupled immunocytochemistry-transmission electron microscopy (TEM) imaging technique. Accordingly, a clinical strain of *P. aeruginosa* resistant to polymyxin B was incubated either with free or liposomal polymyxin B at sub-MIC concentrations (*i.e.* 64 and 16 μg/mL, respectively). Untreated bacteria were used as control. Penetration efficiency into biofilms was checked at predetermined intervals of 0, 4, 8 and 16 h at 37°C. TEM studies showed that the uptake of polymyxin B-loaded liposomes by the resistant strain was higher than that of the free drug [29] [60]. It is important to mention that the treatment with both free drug and empty liposomes did not display a superior effectiveness with regard to the free drug indicating that the enhanced activity can only be attributed to the entrapped form. Furthermore, the superiority of liposomal aminoglycosides was demonstrated on *in vivo* chronic *Pseudomonas* infection model [61]. Consistently, mucoid *P. aeruginosa*-containing agar beads were instilled intratracheally to Sprague-Dawley female rats. After the establishment of infection, animals were treated by inhalation over 14 days. Two treatment regimens were used; tri-weekly dosing schedule with free or liposomal amikacin at 6 mg/kg per dose and compared with the classical aminoglycoside regimen, *i.e.* a twice daily dosing of free tobramycin at the same dose (6 mg/kg/day). Finally, animals were killed and lungs were homogenized. Homogenates were subsequently cultured on agar plates. Then, colony- forming units (CFU) were counted in order to assess the effectiveness of the treatment. The researchers found that "free amikacin was relatively ineffective in the reduction of CFU under these conditions, while bacteria were undetectable in a large proportion of the group treated with liposomal amikacin" [61]. Interestingly, the thrice-weekly treatment with the liposomal amikacin was as effective as the twice-daily treatment with free tobramycin. Although, tobramycin showed a lower MIC value than amikacin against the planktonic form of *P. aeruginosa* [61]. The authors explained the observed enhanced effectiveness of liposomal amikacin by the enhanced penetration through biofilm and by the drug sustained release pattern. The researchers have demonstrated the drug sustained- release profile from liposomes in CF-patients' sputa [61]. They also checked biofilm penetration on *in vitro* 4 days-grown biofilms produced by a mucoid form of PA01, prepared using rat lung models with chronic infections. For this aim, fluorescently labeled liposomal amikacin was used and biofilm penetration was imaged by confocal laser scanning microscopy (CLSM) [61].

2.3.4. Protection against enzymatic degradation and inactivation

Nanoparticulate delivery systems provide a physical barrier shielding the entrapped antibiotic from aggregation and inactivation with polyanionic compounds, such as bacterial endotoxins *e.g.* LPS and LTA. Additionally, encapsulation may protect antibiotics against enzymatic degradation by β-lactamases, macrolide esterases and other bacterial enzymes [62]

Two decades ago, Lagacé *et al.* demonstrated that liposomal encapsulation of ticarcillin or tobramycin reverse the resistance of *P. aeruginosa* strains towards these both antibiotics [63]. Growthinhibition of ticarcillin- and tobramycin- resistant strains was achieved using ticarcillin and tobramycin liposomal formulations at 2 % and 20 % of their respective MIC. Liposomal formulations were as effective against the β-lactamase -producing strains as β- lactamase non-producing ones. Recently, Alipour *et al.* demonstrated the versatility of liposomal encapsulation in protecting tobramycin or polymyxin B from inhibition by LPS, LTA, neutrophil-derived DNA, actin filaments (F-actin) and glycoproteins *e.g.* mucin, common components in the CF-patients' sputa [64]. Being polycationic, tobramycin and polymyxin B can bind to these polyanionic compounds and thereby have their bioactivity reduced. The authors postulated that "liposomes are able to reduce the antibiotic contact with polyanionic factors in the sputum and to enhance bacteria-antibiotic interactions" [64]. *In vitro* stability studies revealed that liposomal formulations were stable after an 18 h-incubation at 37°C with i) a supernatant of biofilm-forming *P. aeruginosa*; ii) a combination of DNA, F-actin, LPS and LTA or iii) an intact or an autoclaved patients' sputum. No significant differences with respect to control (before incubation) were observed. Furthermore, the antibacterial potency of liposomal antibiotics was checked after both short (3 h) and prolonged (18 h) exposure to a combination of DNA/F-actin or LPS/LTA at different concentrations. It was found that for both free and liposomal drugs the antibioactivity was reduced in a concentration- dependent manner. However, much higher concentrations (100 to 1000 mg/L) and (500 to 100 mg/L) of LPS/LTA and DNA/Factin, respectively, were needed to inhibit liposomal forms in comparison to free drugs. The authors explained this finding by the increased viscoelasticity induced by the high concentrations of polyanionic elements that may hinder the interaction of liposomes with bacteria. Indeed, the early leakage of antibiotics from liposomes cannot be used as a plausible cause of the inactivation of liposomal antibiotic because *in vitro* stability studies showed that liposomal vesicles were not disrupted [64]. To further confirm the superiority of liposomal forms, the authors studied the bactericidal activity of liposomal formulations *versus* free forms against *P. aeruginosa* found in CF-patients' sputa. The antibacterial activities of

liposomal formulations were 4- fold higher when compared to the free drugs, despite the presence of different bacterial strains in the patient's sputum. It is of note that liposomal tobramycin reduced growth at a high concentration (128 mg/L), whereas liposomal polymyxin B did it at a markedly lower concentration (8 mg/L). The dissimilar activities of tobramycin and polymyxin B was attributed to their different sites of action.

2.3.5. Intracellular bacterial killing

Obviously, the intracellular location reinforces bacterial resistance as it shields them from both humoral and cellular host defenses and also from the action of therapeutic agents. Indeed, intracellular bacteria, such as *M. tuberculosis* and *L. monocytogenes,* use cells of the innate immune system, not only as reservoirs to launch recurrent infections but even more as vectors enabling them to invade other sites of the body [65]. On the other hand, most of antibiotics, *e.g.* aminoglycosides, β-lactams and glycopeptides, have restricted cellular penetration while others can readily diffuse, *e.g.* fluoroquinolones and macrolides. Unfortunately, these latter suffer from low intracellular retention [66]. Accordingly, a small number of available antibiotics are effective against intracellular infections. To fight intracellular infections, NP are promising vectors allowing antibiotics to target macrophages and to reach bacteria located in intracellular compartments. In this field, a recent review article has already highlighted the role of NP for targeting intracellular infections [67].

2.3.6. Specific targeting and sustained-release

Inherent toxicity of antibiotics is a crucial drawback that led to limit or even to stop the use of some of them, such as aminoglycosides and lipopeptides known for their neuro- and nephrotoxicity [68]. Therefore, specific targeting to bacteria would counteract drug toxicity, since it enables to avoid non-selective and uncontrolled delivery to host cells. To date, few works reported the design of NP with a specific targeting to bacteria for therapeutic purposes. Some examples are presented hereafter. Qi *et al.* elaborated mesoporous silica NPs (MSN) as nanocarriers of vancomycin (Van) in order to specifically target gram positive bacteria over macrophage-like cells [69]. The specific recognition was based on hydrogen bonding interactions of Van with the terminal D-alanyl-D-alanine moieties of gram positive bacteria. Cell viability assay showed a good biocompatibility of Van-MSN with human embryonic kidney and human hepatocytes. Tang *et al.* have recently described the design of a nanoparticulate carrier loaded with a fluorescent dye, and called it "nanoprobe" for diagnostic purposes [70]. The surface of the nanoprobe was grafted with a bacterial ligand, *i.e.* concanavalin A, and

therefore displayed a high affinity to bacteria. The developed nanoprobe was shown to rapidly detect and quantify the extent of bacterial colonization on wounds and catheters in real time.

Prolonged or sustained release of the loaded antibiotic is of great importance for antibiotics with time-dependent action, such as lipoproteins, β-lactams, glycopeptides and some fluoroquinolones. The importance of the sustained-release profile was highlighted by Meers *et al.* [61]. Thanks to the prolonged release of amikacin from liposomes, this latter was as effective, when administered triweekly, as free tobramycin administered twice-daily and despite the fact that MIC of tobramycin is lower than that of amikacin. Additional examples of antibiotic-loaded polymeric NPs were recently reviewed [71].

2.3.7. Downregulation of bacteria oxidative-stress resistance genes

Bacterial adaptation to oxidative and nitrosative stress could be considered as a resistance mechanism to host defenses [72]. Indeed, innate immune cells generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as superoxide and peroxynitrite, respectively, in order to kill phagocyted bacteria [73]. Consistently, pathogenic bacteria resist to host-mediated oxidative stress by up-regulating the expression of their antioxidant enzymes[74]. Importantly, it was claimed that many antibiotics exert their bactericidal effects *via* the production of hydroxyl radicals, regardless of their molecular targets [75]. Recently, it was found that metal NP, namely zinc oxide-NP (ZnONPs), exerts by themselves bactericidal effects on gram positive bacteria and gram negative bacteria [76]. A synergistic killing effect on acid fast bacteria (*i.e. Mycobacterium bovis*-BCG) was also observed for ZnONPs when used in combination with rifampicin [76]. Moreover, ZnONPs effectively killed MRSA clinical strains [76]. Several mechanisms were found to be involved in ZnO-NPs antibacterial activities. Most importantly, ZnO-NPs were found to down-regulate the transcription of oxidative stress resistance genes in *S. aureus*. Strictly speaking, the treatment with 300 µg/mL of ZnONPs decreased the transcription of peroxide stress regulon *kata* and *perR* genes by 10- and 3.1-folds, respectively, when compared to untreated bacteria [76]. These results highlight the importance of ZnO-NP in fighting drug-resistant bacteria. It is of note that ZnO-NP induced oxidative stress response on macrophages, as ROS and NO production was markedly increased, thus reinforcing their bacterial killing capacity [76].

2.4. Nanoantibiotics delivery

2.4.1. Systemic versus local antibiotic delivery

Traditionally, systemic antibiotic administration has been the foundation of clinical therapies to address the ever-present infectious onslaught. Unfortunately, poor penetration to ischemic or post-operative tissue, inappropriate prescribing patterns, systemic toxicity, and poor patient compliance, have predominated the conversation and limited the usefulness of certain antibiotics. Furthermore, systemic administration is often not effective, as it does not provide local tissue concentrations sufficient to kill bacteria prior to incurring serious side effects, such as renal and liver damage. Sub-therapeutic or sub-inhibitory antibiotic concentrations are known to inadvertently exacerbate infectious complication and promote antibiotic resistance [2, 77]. Local delivery of current antibiotics and other antimicrobial biologics (e.g., antimicrobial peptides (AMPs), anti-quorum sensors, bacteriophage, etc.) may preserve and extend their efficacy in the evolutionary race between antimicrobial development and bacterial resistance. In fact, systemic toxicity, and to a lesser extent, antibiotic resistance is rarely seen for local applications of the same drugs, achieving locally higher concentrations and overcoming the reducing effects of lowered bacterial metabolism [78, 79]. Thus, device integrated, local delivery strategies to mitigate the unacceptable consequences of systemic antibiotic delivery (e.g., development of multi-drug resistant bacteria, systemic toxicity, and rising healthcare costs, etc.) are urgently needed to keep pace with the rising demand for medical devices. The concept of locally and sustainably delivering an anti-infective agent is not new. Vancomycin, tobramycin, amoxicillin, gentamicin, cefamandol, caphalothin, and carbenicillin have all been incorporated into commercially available local release systems [2, 80].

2.4.2. Nanoparticles against intracellular bacteria

NPs have also been used to combat intracellular bacteria. NPs, including liposomes, are small enough to be phagocytosed by host phagocytes which contain intracellular microbes. Once inside the host cell, these NPs can release drugs that then combat these intracellular microbes [47, 67]. In addition, NPs can release high concentrations of antimicrobial drugs inside of infected host cells while keeping the total dose of drug administered low [81]. The high local dose at the site of infection kills the intracellular bacteria before they can develop resistance, while the lower total dose decreases the probability that bacteria outside of the site of action of the NPs will develop drug resistance [81].

NPs can combat intracellular microbes in alveolar macrophages. Intracellular microbes that are phagocytosed and proliferate intracellularly in alveolar macrophages include *L. monocytogenes*, *M. tuberculosis*, *L. pneumophila*, and *Chlamydophila pneumoniae*. As discussed above, living inside of host cells protects these microbes from many antibiotics [1, 3]. Attachment of mannose to NPs containing antimicrobial drugs allows them to be targeted to alveolar macrophages, which heavily express mannose surface receptors [1, 3]. Liposomes containing ciprofloxacin and conjugated with mannose were administered by the pulmonary route and shown to have high selectivity for alveolar macrophages [1, 3]. It was also shown *in vivo* that mannose-conjugated liposomes lead to significantly higher concentrations of antimicrobial drugs in alveolar macrophages relative to type II pneumocytes [1, 3].

2.4.3. Nanoparticles that target antimicrobial agents to the site of infection

NPs can target antimicrobial agents to the site of infection, so that higher doses of drug can be given at the infected site, thereby overcoming existing resistance mechanisms with fewer adverse effects upon the patient [82]. As with NPs targeting intracellular bacteria, NPs targeting the site of infection can release high concentrations of antimicrobial drugs at the site of infection, while keeping the total dose of drug administered low. Again, the high local dose at the site of infection also kills the infecting bacteria before they can develop resistance, while the lower total dose decreases the probability that bacteria outside of the site of action of the NPs will develop drug resistance. NPs can be targeted to sites of infection passively or actively. Passively targeted NPs selectively undergo extravasation at sites of infection, where inflammation has led to increased blood vessel permeability. Actively targeted NPs contain ligands (e.g. antibodies) that bind receptors (e.g. antigens) at sites of infection. The antimicrobial action of NPs can also be activated by certain stimuli, such as reactive oxygen species (ROS) or low pH at the site of infection. Drug release can also be regulated by magnetic guidance or radio frequency, in order to target drug release by NPs to the site of infection [47]. NPs can be conjugated with antibodies against a given antigen on the surface of the target microbe. For example, AuNPs conjugated with antibodies against protein A have high selectivity for killing *S. aureus* [47].

Aptamers can also target the site of infection. Aptamers are a type of nanoparticle composed of DNA or RNA oligonucleotides which are folded into a 3-dimensional structure and bind with high affinity to specific antigens, such as peptides and small molecules [1, 3]. Aptamers have been shown *in vitro* to have antibacterial activity against both Gram positive and Gram negative bacteria expressing beta-lactamase [1, 3]. Aptamers also have antiviral

activity, including inhibition of HIV reverse transcriptase and inhibition of replication of Vaccinia virus [1, 3]. Silver carbene complexes (SCCs) inside biodegradable NPs can also target the lungs to treat pulmonary infections. SCCs contained in biodegradable NPs are small enough to accumulate in the lung after administration using a nebulizer. Once inside the lung, these NPs release and maintain therapeutically effective concentrations of SCCs at the site of infection in the lung but not elsewhere, thereby limiting adverse effects [47]. L-Tyrosine polyphosphate NPs (LTP-NPs) containing SCCs are one example of this type of biodegradable nanoparticle [47]. *In vitro*, free SCCs and SCCs incorporated into LTP NPs have antimicrobial activity against MRSA, multidrug resistant *A. baumannii* (MRAB), *P. aeruginosa*, *B. cepacia*, *K. pneumoniae*, and *C. albicans* [47]. In a study by Hindi *et al.,* two inhaled doses given over 72 h of LTP-NPs containing SCCs decreased bacterial burden in the lung, decreased bacteremia, and increased survival by 25% in mouse models with P. aeruginosa pneumonia [83]. The mechanism of action of these NPs was thought to be slow sustained release of intact SCC followed by release from the carbene of free Ag^{+}, which then has antimicrobial effects at the site of infection in the lung [83].

2.4.4. Synergistic effect of multidrug complexes

When chemically or physically combined with certain other chemical agents, some antibiotic drug molecules form complexes that may be slowly soluble in body fluids. This slow dissolution rate provides the extended release of the drug which accompanied with synergistic pharmacological action. This phenomenon is of great interest for super-bugs treatment. Among antibiotic based complexes the highest enhancing effect was observed for chitosan (CS) and metal-based complexes (for metals such as: silver (Ag), zinc (Zn), cupper (Cu), magnesium (Mg) and titanium (Ti)) due to their antibacterial nature. A better nanocarrier platform is actually represented by the antibiotic-contained NPs. Instead of encapsulating the antibiotic into nanocarriers, a core–shell nanoparticle structure made up of (i) antibiotic nanoparticle as the core and (ii) lipid or polymer layer as the shell potentially represents a more clinically effective nanoscale antibiotic delivery system. The antibiotic nanoparticle core ensures high antibiotic loading, whereas the lipid or polymer shell can be functionalized for a wide range of therapeutic functions. This combination was found to be active against several resistant bacteria colonies. Recognizing that a majority of antibiotics are amphiphilic and soluble in aqueous media, whether their acidic or basic solution, the most effective envelope for antibiotic core was found to be an amphiphiles polyelectrolite. In particular polysaccharides with antibacterial activity such as chitosan and dextran are well-established agents for antibiotic

complexation for drug delivery as well as for antibacterial resistance applications. While the polyelectrolyte envelope provides positively charged surface, which enables complex internalization into the bacteria body, the antibiotic core completely released from the complex in the vicinity of the cells' fluids and its antibacterial activity preserved against bacteria's resistant mechanisms [16].

2.4.5. Infection-activated delivery systems

An interesting approach for antibiotic delivery has been the development of systems that differentially deliver therapeutic agents to infection sites. When bacterial infection happened, bacteria will secrete many virulence factors, such as phospholipase, phosphotase, lipase, toxins, protease, acidic pH and so on, to develop unique microenvironments [84]. Strategies utilizing the unique microbial infection environment as a molecular cue to activate drug release or facilitate their binding to the bacteria can achieve such an ambitious goal. These new strategies can improve antibiotic targeting and activity with fewer side effects and can overwhelm drug resistance mechanisms with high, sustained local drug concentrations.

Xiong *et al.* have developed a lipase-sensitive polymeric triple-layered nanogel (TLN) for the differential delivery of antimicrobials to bacterial infection sites [85]. The TLN contains a bacterial lipase-sensitive PCL interlayer between a cross-linked polyphosphoester core and a shell of PEG. The PCL molecular fence protects the drug inside the polyphosphoester core with minimal drug release prior to reaching sites of bacterial infection, thus eliminating potential adverse side effects. However, once the TLN senses lipase-secreting bacteria, the PCL fence of the TLN is degraded by lipase to release the drug. Using *S. aureus* as a model bacterium and vancomycin as a model antimicrobial, it was demonstrated that the TLN released almost all the encapsulated vancomycin within 24 h, but only in the presence of *S. aureus*, significantly inhibiting bacterial growth. The TLN further delivered the drug into bacteria-infected cells and efficiently released the drug to kill intracellular bacteria [71, 85].

2.5. Advantages and disadvantages of nanoantibiotics

The use of NPs as delivery vehicles for antimicrobial agents suggests a new and promising paradigm in the design of effective therapeutics against many pathogenic bacteria [86]. Antimicrobial NPs propose several clinical advantages. First, nanocarriers can be engineered to be activated by stimuli (e.g., chemical, magnetic field, heat, and pH) for targeted delivery as well as biological sensors [87, 88]. For example, amoxicillin was freeze-dried in formulation with CS and polyvinyl pyrrolidone (PVP) for acid-responsive release of antibiotics [89]. This

system could be particularly useful for treating abscess which is frequently acidic and reduces the potency of conventional antimicrobial therapy. Generally, most molecules poorly cross the blood brain barriers (BBB), a tight barrier to protect the brain from the penetration of xenobiotics. However, it was also reported that antimicrobial NPs made of certain materials and at varying particle sizes were capable of efficiently targeting infectious diseases by overcoming anatomic barriers (e.g., BBB) [90]. Second, NPs can be molecularly tailored for versatile physico-chemical properties in order to minimize side effects generated upon systemic administration of traditional antimicrobial agents (e.g., hepatotoxicity of cephalosporins, and ototoxicity and nephrotoxicity of aminoglycosides) [91]. Nanocarriers seem to be able to reduce the side effects by improving the solubility and stability of antimicrobial agents [92]. Third, NP-based antimicrobial drug delivery is promising in overcoming resistance to traditional antibiotics developed by many pathogenic bacteria [86]. Fourth, administration of antimicrobial agents using NPs can improve therapeutic index, prolong drug circulation (i.e., extended half-life), and achieve controlled drug release, enhancing the overall pharmacokinetics [93]. Many studies demonstrated greater efficacy of antimicrobial NPs than their constituent antibiotics alone. For example, vancomycin-capped AuNPs exhibited 64-fold improved efficacy against VRE strains and Gram-negative bacteria such as E. coli over vancomycin alone [94]. In addition, antimicrobial NPs can be prepared and administered in convenient and cost-effective ways via various routes with lowered administration frequency [51]. NP-based antimicrobial drug delivery can achieve improved solubility and suspension of drugs, and concurrent delivery of multiple agents for synergistic antimicrobial therapy [95]. Thus, antimicrobial NPs are of great interest as they provide a number of benefits over free antimicrobial agents (Table 3).

Although nanoantibiotics promises significant benefits and advances in addressing the key hurdles in treating infectious disease, there are foreseeable challenges in translating this exciting technology for clinical use. These include thoroughly evaluating the interactions of nanoantibiotics with cells, tissues, and organs, which consequently recalibrates doses and identifies proper administration routs to obtain desired therapeutic effects [87]. Profound knowledge about the potential toxicity of nanoantibiotics is also required to warrant successful clinical translation [96]. It has been shown that intravenously injected NPs can be accumulated in colon, lung, bone marrow, liver, spleen, and lymphatics [97]. Inhaled NPs also can enter the systemic circulation and reach lung, liver, heart, spleen, and brain [96], which is particularly facilitated for small size NPs because of efficient cellular uptake and transcytosis across epithelial and endothelial cells into blood and lymph circulation [98]. Potential toxicity of

nanoantibiotics to human health is not known much at the moment although it likely shares the nanotoxicity of various non-antibiotic nanomaterials [96, 99]. Many recent studies suggest the possibility of multi-organ nanotoxicity that therapeutically administered antimicrobial NPs may generate. For example, free radical-mediated oxidative stress generated by the interaction of antimicrobial NPs with cells may result in hepatotoxicity and pulmonary toxicity [100].

Table 3 - Advantages and disadvantages of antimicrobial nanodelivery systems over free antimicrobial agents (adaptedfrom Huh and Kwon [1]).

Antimicrobial NPs		**Free antimicrobial agents**	
Advantage	Targeted drug delivery via specific accumulation	***Disadvantage***	No specific accumulation
	Lowered side effects of chemical antimicrobials		High side effects of chemical antimicrobials
	Low antimicrobial resistance		High antimicrobial resistance
	Extended therapeutic lifetime due to slow elimination		Short half-life due to fast elimination
	Controlled drug release		Usual pharmacokinetics of free drugs
	Broad therapeutic index		Narrow therapeutic index
	Improved solubility		Sometimes poor solubility
	Low immunosuppression		Immunosuppression
	Low cost		High cost
Disadvantage	Accumulation of intravenously injected nanomaterials in tissues and organs	***Advantage***	Absence of nanomaterials in the whole body
	High systemic exposure to locally administrated drugs		Low systemic exposure to locally administrated drugs
	Nanotoxicity (lung, kidney, liver, brain, germ cell, metabolic, etc.)		Absence of nanotoxicity
	Lack of characterization techniques that are not affected by NPs' properties		Well-established characterization techniques

Various metabolic changes suggest mitochondrial failure, and enhanced ketogenesis, fatty acid β- oxidation, and glycolysis, contributing to hepatotoxicity and nephrotoxicity [100]. The toxic effects of antimicrobial NPs on central nervous system (CNS) are still unknown, and the interactions of NPs with the cells and tissues in CNS are poorly understood, and very small

steps are being taken towards that understanding [90, 101] Besides, some classes of NPs can affect the circulatory system by altering heart rate [102] as well as reproductive system by increased detachment of seminiferous epithelium [103] and possible spermatotoxicity [96, 104]. Table 5 presents potential multiorgan nanotoxicity that has been implicated to be generated by therapeutically used antimicrobial NPs. NPs exhibit size-specific properties that limit the use of currently available *in vitro* assays in a universal way, and there is no standardized definition for NP dose in mass, number, surface area, and biological specimens (e.g., blood, urine, and inside organs) [97, 105]. This means that there is a high demand to develop new characterization techniques that are not affected by NP properties as well as biological media [1].

Table 4 - Potential toxicity of therapeutically used NPs.(adapted from Huh and Kwon [1])

Toxicity type	Mechanism for toxicity
Pulmonary toxicity	Acute inflammatory change; granuloma formation; oxidative stress
Renal toxicity	Renal glomerulus swelling; proximal tubular necrosis; mitochondrial failure; enhanced ketogenesis, fatty acid beta-oxidation, and glycolysis
Hepatotoxicity	ROS generation; mitochondrial dysfunction; GSH depletion; LDH leakage
Neurotoxicity	Reduced neuro viabilities; exacerbation of cytoskeletal and blood-brain barrier (BBB) disruption; diminished ability to form neuritis in response to NGF; mild cognitive impairment, edema formation
Spermatotoxicity	Sperm fragmentation; partial vacuolation of seminiferous tubules and cellular adhesion of seminiferous epithelium; suppressed proliferation of Leydig cell
NP-protein interactions	Abnormal protein functions generated by structural and conformational changes upon adsorption to NPs; raising protein potential for autoimmune effects
Others	Embryo neurotoxicity; embryo death; metabolic alkalosis

Abbreviations
GSH, glutathione; LDH, lactic dehydrogenase; NGF nerve growth factor.

CHAPTER III

Antimicrobial Peptides (AMPs) – new add on the therapeutic arsenal

Antimicrobial peptides (AMPs) are natural small oligopeptides with a varying number of amino acids, from 5 to 100. AMPs have a broad spectrum of action targeting microorganisms from viruses to parasites. AMPs are also referred in literature as host-defense peptides, because they are synthetized molecules that take action on the defense mechanisms against biological threats of the living organism of origin. The discovery of AMPs dates back to the first half of the 20th century, when in 1939 Dubos extracted an antimicrobial agent from a soil *Bacillus* strain, proven to be effective in mice pneumococci infection. This extract was then fractioned allowing the identification of gramicidin. Despite some systemic toxicity, gramicidin has shown to be effective in the topical treatment of wounds and ulcers. The first animal-originated AMP to be reported is defensin, isolated from rabbit leukocytes in 1956 [44]. Nowadays more than 2000 AMP have been described and current molecular developments can be consulted in a series of databases available in the web, including natural identified molecules, as well as peptidomimetic molecules and analogues, pharmacologically designed, thanks to the use of bioinformatics [106]. Current FDA approved AMPs with well stablished use include bacitracin, colistin and polymyxin B, only for topical administration [2].

Despite the lack of consensus on the influence of peptide sequence in biologic activity of AMPs, some common characteristics seem to be important and fairly related to their antimicrobial property. The main ones are primarily charge, with 90% of AMPs being cationic, and secondly hydrophobicity or amphipathicity, influencing solubility profiles and consequently bioavailability [107]. Associated with structural characteristics, these are also the main physicochemical properties that also should be take into account on the design of new synthetic AMPs [44].

Table 5- Currently available AMPs databases (adapted from da Costa *et al.*[106])

Database	Description
Collection of antimicrobial peptides (CAMP)	Holds experimentally validated and predicted AMP sequences
AMPer	Database and automated discovery tool for gene-coded AMPs
Antimicrobial Peptide Database (APD)	Contains mostly AMPs from natural sources (~98 % of the entries)
Yet Another Database of Antimicrobial Peptides (YADAMP)	Mostly focused on bacterial AMPs
BACTIBASE	Data repository of bacteriocin AMPs
PhytAMP	Database dedicated to antimicrobial plant peptides
RAPD	Database containing recombinantly-produced AMPs
HIPdb	Experimentally validated HIV inhibitory peptides
Bagel2	Bacteriocin mining tool
Peptaibol	Database for peptaibols (unusual peptides)
PenBase	Database devoted to penaeidins
Defensins KnowledgeBase	Information and database dedicated to defensins
CyBase	Database specialized in cyclotides

In addition, a comparison of AMPs pharmacological properties as antimicrobial agents with conventional antibiotics is summarized in Table 2. AMPs demonstrate significant advantages such as potency and board spectrum of activity, as well as an additional activity to modulate the immune system responses, and low resistance rates. They also show some limitations that impair their safe therapeutic use, such as sensitivity to proteolysis, influencing stability, and undefined toxicological data for systemic use [108]. In order to overcome these limitations some strategies have been developed, maximizing the proven therapeutic potential of AMPs. There are numerous methods for obtaining AMP delivery systems. AMPs could be immobilized into a variety of materials or onto a variety of surfaces and still retain their

antibacterial activity. Also, AMPs can be targeted through loading them in nanoparticulate systems with selective delivery capacities, include polymers, liposomes, hydrogels, nanospheres, nanocapsules, and carbon nanotubes [107]. As practical examples on of these type a strategies are the studies of Mishra *et al.* on the development of an AMP-coated surface, specifically immobilizing Lassioglossin-III onto silicone-based catheters and its activity against urinary tract infections [109], and also of Water *et al.* on encapsulating plectasin, a cationic AMP, into polymeric NPs and the evaluation of their efficacy on *S. aureus* strains [110].

Table 6- Properties of AMP's and conventional antibiotics (adapted from Mok and Li [108]).

Properties	Conventional antibiotics	Antimicrobial Peptides AMP's
Spectrum of activity	Usually selective	Broad-spectrum (many with antibacterial, antifungal, antiviral, and anti-parasitic activities)
Uptake mechanism	Requires specific uptake mechanism	Non-specific, charge-dependent mechanism; self-promoted uptake
Cellular targets	Usually has one or one class of targets	Multiple targets, including the membrane and intracellular targets
Rate of killing	Dependent on growth rate (slow for cells in the lag phase)	Fast
Stability	Dependent on the antibiotic (half-lives can range from hours to days)	Short half-life due to sensitivity to proteolysis
Additional activities	None	Can neutralize endotoxins and modulate immune responses
Resistance rates	Resistance detected after a few passages at sub-MIC	Resistance is not readily induced and is only detected after multiple passages (~30) at sub-MIC; some species may be intrinsically resistant to the peptides, including those with impermeable outer membranes and those that produce proteases
Toxicology	Relatively safe	Safe for topical use; safety for systemic use is undefined
Manufacturing costs	Most are inexpensive (many range from $1-20 per gram)	Expensive ($50-400 per gram)

For better understanding these unique molecules, it is important to revise the main classes in which they are subdivided and also their mechanism of action in microorganisms. Also, potential targets and applications, as well as future perspectives on the implementation and use of AMPs are discussed.

3.1. Classification of AMPs

The establishment of a consensual classification system for AMPs has received some controversial discussion, mainly due to the large number of molecules identified and the lack of data on their full biochemical and structural characterization. Classification may vary by author or simply by complexity degree. A simple way to distinguish the main classes of AMPs is taking into account their biological source. Several species, from prokaryotes to higher eukaryotes, synthesize AMPs. Therefore, AMPs can be classified as either non-ribosomally synthesized peptides (NRAMPs) or ribosomally synthesized peptides (RAMPs). NRAMP synthesis catalyzed by peptide synthetases takes place in the cytosol of bacteria and fungi, mainly possessing antibacterial properties, whereas RAMP synthesis occurs in the ribosomes of the eukaryotic cells, also possessing antiviral, antiparasitic, antineoplastic and immunomodulatory activity. Main examples of NRAMPs are polymyxin B, bacitracin, vancomycin, gramicidin A, and cyclic peptides (daptomycin, dalfopristin, quinupristin and cyclosporin A), whereas nisin is a gene-encoded RAMP [111]. Only some NRAMPs are currently FDA approved and marketed.

Other two class division may be achieved considering molecule charge property at neutral pH. Thus AMPs are subdivided in cationic antimicrobial peptides (CAMPs), and anionic antimicrobial peptides (AAMPs). As referred, the majority of AMPs are of cationic nature, despite the identification of AAMPs in invertebrates, vertebrates, and plants. AAMPs are part of various vital organs of the body, including respiratory tract, the brain, the epidermis, the epididymis, blood, and the gastrointestinal tract. As examples of AAMPs there are bovine chromacins and kappacin, human dermcidin, and the amphibian temporin [107].

Nevertheless, authors have proposed more elaborated classifications of AMPs, based on their biochemical characteristics, conformation and structure, or even taking account biological activity and targets. Some clarification on this matter is discussed downward.

3.1.1. Biochemical classification

This classification is based mainly based on amino acid composition, dividing AMPs into the following classes: (i) linear, (ii) cysteine-rich peptides, and (iii) peptides rich in specific amino acids, like glycine, proline, arginine or histidine. Examples of AMPs are given in Table 3 [107].

Table 7- AMPs examples based on biochemical properties (adapted from Narayana and Chen [107]).

Class	Example

Linear	Cecropins, Clavanin, Piscidins, Styelin, Magainins, Dermaseptins, Buforins-II Pexiganan, LL-37
Cysteine-rich	
Single	Esculentin, Bactenecin-1, Thanatin,
Double	Tachyplesin, Androctonin, Protegrin-1
Three	Defensins: α- (HNP3), β- (TAP), θ- (SapecinA)
Four	Drosomycin, Hepcidin
Specific amino acid-rich	
Proline	Drosocin, Metchnikowins, Pyrrhocoricin, Metalnikowin
Glycine	Diptericins, Attacins
Arginine	Penetratin
Histidine	Histatin
Tyrosine	Indolicidin

3.1.2. Structural classification

Recently, a classification based on structure-function relationships studies with Nuclear Magnetic Resonance (NMR) has been proposed, it classifies AMPs into three structural groups: alpha-helical peptides, beta-sheet peptides and extended or loop peptides [4].

Alpha-helical AMPs are characterized by the propensity towards the formation of alpha-helix structures, and these peptides display highly cationic and amphipathic properties, being mainly active against Gram-positive bacteria and fungi. However, helical content may be also correlated to an increased hemolytic activity and cytotoxicity. Some examples of alpha-helical AMPs are cecropins, magainins, temporins, buforins and clavanins [4, 10].

Beta-sheets AMPs are often stabilized by disulfide bridges between cysteine residues. This group is mainly formed by beta-hairpin peptides and defensin mini-proteins. Examples include protegrin-1, thanatin and lactoferricin B [4] .

Extended and loop AMPs are not folded into regular alpha-helix or beta-sheet structures. The extended activity or cyclic character is due to the presence of some specific amino acid residues, for instance, His, Pro, Cys, Arg and Trp. This type of AMPs examples are histatins, proline rich isolates from insects, hepcidin, and indolicin [4].

3.1.3. Biological activity classification

Bahar *et al.*, in their 2013 review have categorized AMPs based on their biological target and mode of action. Hence the classes proposed are antibacterial peptides, antiviral peptides, antifungal peptides, and antiparasitic peptides. Despite different target, AMPs tend to act in a common manner, disrupting cell wall or viral envelope, causing disintegration and intracellular components leakage. In case of antibacterial and antifungal peptides, there is also evidence of

intracellular targeting, inhibiting some important pathways inside the cell such as DNA replication and protein synthesis [44].

3.2. Mechanisms of action

AMPs are unique molecules that can mimic endogenous host peptides, exerting their action in several fronts and targets. Studies have shown that AMPs demonstrate different antimicrobial action mechanisms. The most recognized and explored one is the disruption of cell integrity. However, recently, intracellular active AMPs have been shown also to interact with targets inside the cells, interfering in DNA synthesis and other critical biochemical pathways. Furthermore, the immunomodulatory functions of some AMPs have significant contribution to their efficacy as antimicrobial agents [4, 44, 108] (Figure 4).

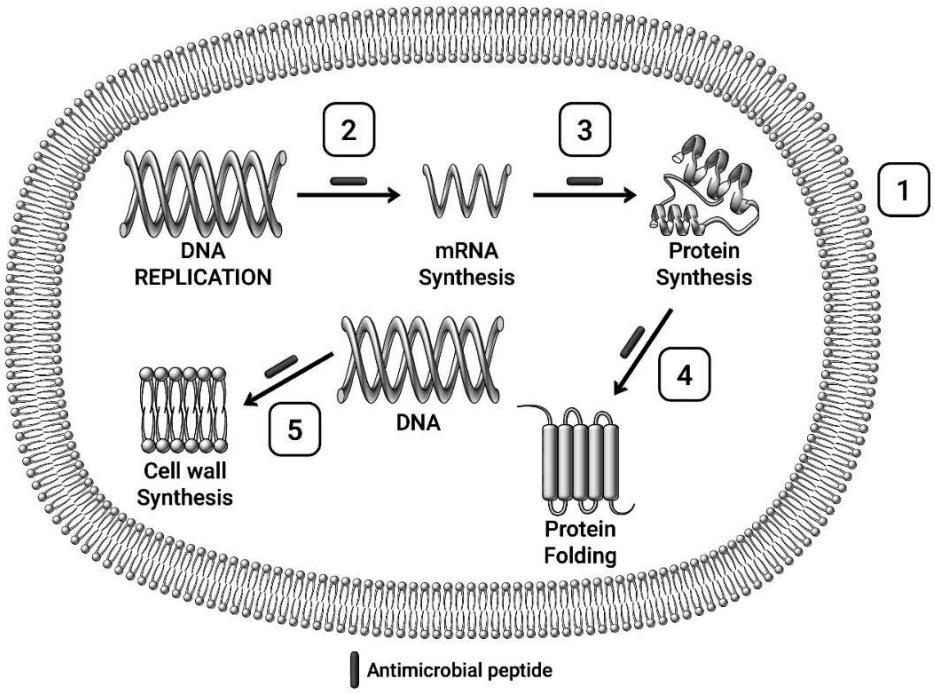

Figure 6– Mechanisms of Action of AMPs. Potential targets (1) Bacterial cell wall (2) DNA Synthesis (3) Protein Synthesis (4) Protein Folding (5) Cell wall Synthesis. (adapted from Cruz *et al.*[4]).

These combination of cellular and biological actions may offer increased security by decreasing dosage, however, action in multiple targets and immunomodulatory response may raise some toxicity or immunogenicity issues.

3.2.1. Membrane disruption and permeation

Membrane disruption and permeation is the first line of action of AMPs in microorganism eradication. This type of mechanism is, primarily, strongly dependent on the interaction of AMPs with the cell wall. Peptides usually bind to the target cell be ion exchange, suggesting a non-specific membrane interaction. Subsequently, mechanisms of disruption and permeation are strongly dependent on peptide physicochemical characteristics. Accordingly, several models have been proposed (Figure 5), that differ mainly in the mode of attachment to bacteria and cell membrane insertion. The models in discussion are: the barrel-stove model, the toroidal pore model, both pore-inducing models, the aggregate channel model, and the carpet model, the lipid charge clustering model, and the detergent model, described as non-pore models [4].

The pore-inducing models describe an initial lateral peptide-membrane interaction. Past this first contact, in the barrel-stove model, peptides oligomerize and the monomers are internalized into the membrane. Then, within the lipid bilayer, these minor subunits expose their hydrophobic regions aligning them perpendicularly with the lipid membrane content, forming small pores from where intracellular content is expelled. In the toroidal pore model, hydrophilic regions of peptides and lipid head groups interacts together forming a pore structures, which is generally larger than barrel stave type pores [4, 108].

In the aggregate channel mode, may be seen as a variation of the pore induced models, by the similar channel formation where intracellular content leakage occurs. The initial action of the peptide involves competitive displacement of lipopolysaccharide (LPS) associated with divalent cations (Mg^{2+} and Ca^{2+}), where the peptides destabilize this supramolecular assembly and gain access to both external and internal membranes. Then, aggregation of peptides forms channels allowing diffusion of ions through the membrane [4].

The non-pore models assume that the peptide aggregation and accumulation onto the membrane surface causes several membrane disruptions, which can ultimately lead to disintegration and leakage. In the carpet model, the accumulation of peptides parallel to the membrane past a threshold level disrupts membrane curvature and causes the formation of transient holes. The presence of cationic peptides on membranes can also induce the clustering of anionic lipids, as illustrated by the lipid clustering models. Ultimate consequences to the AMP-membrane interaction can be more dramatic, as suggested by the detergent model. High concentrations of peptide can cause total membrane dissolution, resulting in micelle formation and complete bilayer breakdown [108].

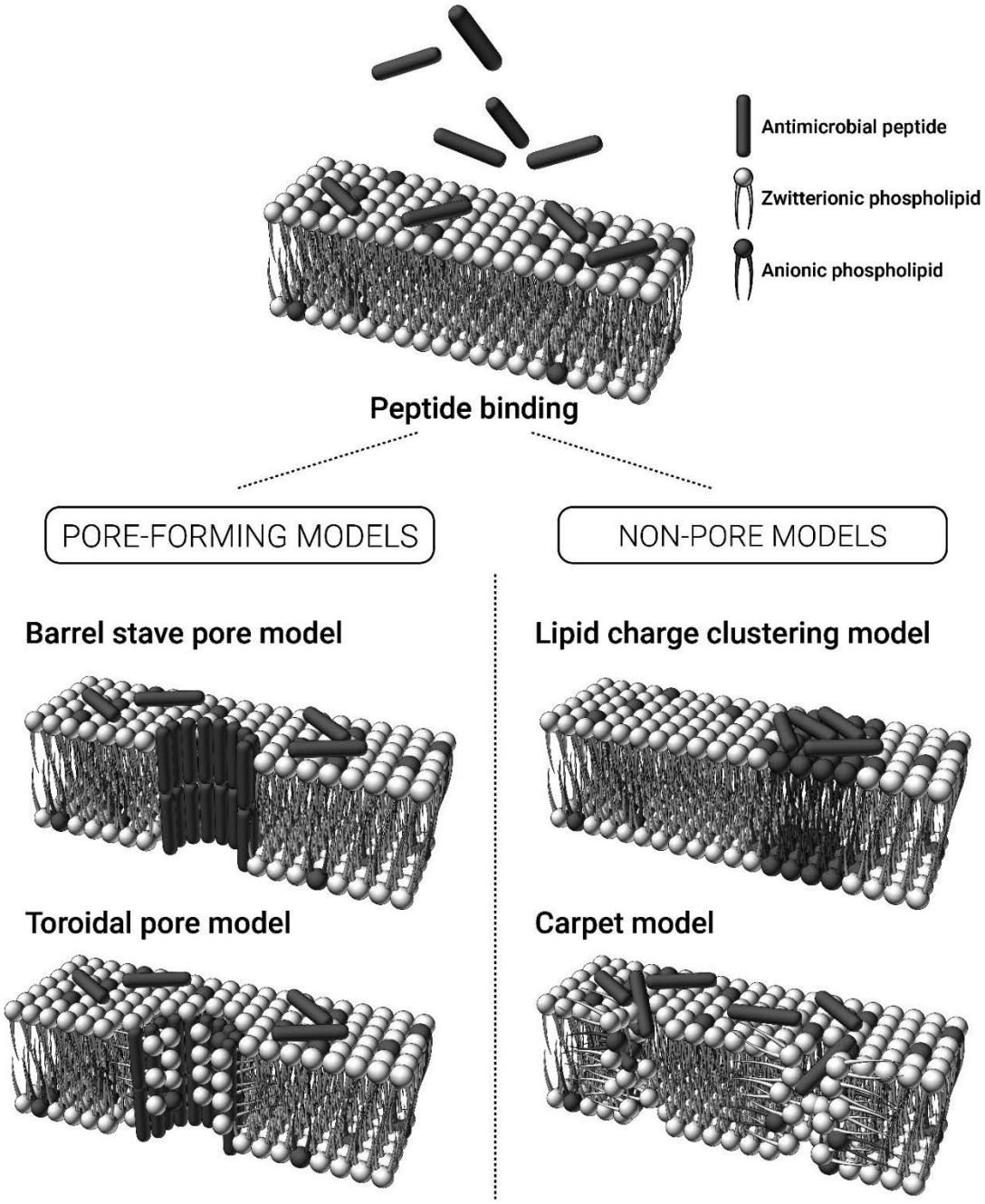

Figure 7 – Membrane permeabilization models. As a consequence of AMP interaction with the membranes of their target microorganisms, their integrity is disrupted by mechanisms that may or may not involve pore formation. The figure illustrates the most recognized models. (adapted from Mok and Li [108]).

3.2.2. Intracellular activity

Beyond disrupting the integrity of the cellular membrane, AMPs can undergo cellular uptake, mainly by endocytosis. The internalization of AMP is the first step to initiate a series of intracellular activities that can compromise cell viability. For instance, APMs can interfere both in DNA and RNA synthesis, inhibiting signaling pathways and enzymatic mechanisms. They can

also inhibit microbe proteases and may interact with certain specific metabolic pathways during bacterial growth [44, 108].

3.2.3. Inducing immune response

In addition to the previous described mechanisms, some AMPs, as host-defensive peptides, have also some intrinsic immunomodulatory properties. The use of immunosuppressive or immunostimulatory agents alone for the treatment of infections may be counterproductive, as immunosuppressive compounds may increase the risk of infections, while immunostimulatory ones can trigger inflammation, autoimmunity, and even fatal cytokine storms. Thus, combination with antimicrobial activity is an important advantage to consider to their therapeutic potential. These peptides have been demonstrated to target various aspects of the host immune response. They include the recruitment of monocytes, neutrophils, mast cells, and other immune cells by chemoattraction, regulation of cytokines, chemokines, and histamine release stimulation of fibroblast growth, vascular endothelium proliferation, angiogenesis, and wound repair, promotion of apoptosis and clearance of infected cells neutralization of bacterial LPS, LTA, and endotoxins and the expression of genes related to cell proliferation and adhesion in cells in a macrophage cell line [108].

3.3. Future perspectives

AMPs are promising molecules with unique characteristics that show enormous potential as a new add on the therapeutic arsenal for infectious disease treatment and for overcoming antimicrobial resistance. However, studies on these same molecules are relatively recent and their knowledge has been growing. The future perspectives on AMPs may be assured in two fronts: the identification of new sources and new molecules, and the establishment of therapeutic use by designing safe and effective formulations.

The study of human synthetized AMPs is very promising, with the intent to isolate and identify specific tissue molecules in order to fight same tissue related infections. For instance the oral cavity and saliva has been considered a very promising source of potent AMPs [106], and they have shown good activity against oral cavity pathogens and infections [112]. Great advances have also been made on designing AMPs therapeutic formulations. These AMPs may be considered to be a new class of antimicrobial therapeutics, which are particularly promising because of unlikely peptide resistance [107]. In 2014 there were 20 AMP and AMP derivatives formulations in clinical or pre-clinical trials, mainly in phase II [4]. Despite the earlier stage, it seems to be just a matter of time in the establishment of therapeutic use and commercialization of AMPs as new class of antimicrobial drugs.

Table 8- Commercial development of AMPs. (adapted from Narayana and Chen [107]).

Company	Drug	Stage	Treatment
Par Advance	113D D2A21	License	Oral candidiasis
Technologies, Inc.		Preclinical	Anti-infective
Genaera	LocilexTM	Completion	Infections of diabetic foot ulcers
Migenix, Inc.	MBI-594 MX-226	Phase IIb Phase IIIb	Acne catheter-related infections
Xoma	Neuprex	Phase III	Infectious complications of trauma and surgery
Agennix	Talactoferrin	Phase II	Advanced non-small cell lung cancer Topical treatment in diabetic ulcers
Zengen	CZEN-002	Phase II	Vulvo-vaginal candidiasis
AM-Pharma	hLF-1-11	Clinical	Anti-endotoxin and fungal infections
Helix Biomedix, Inc.	HB-50 HB-107	Preclinical	Anti-infective, wound healing
Novozymes A/S	Plectasin	Preclinical	Systemic anti- pneumococcal infections

CHAPTER IV

Polymeric nanostructures for AMPs delivery

Different biodegradable polymeric nanosystems have been explored as carriers for antimicrobial agents that exhibit a high bactericidal activity. The efficacy of this strategy is well proven, with reports that polymeric nanosystems can effectively improve the cellular penetration, intracellular retention and specific subcellular distribution of antimicrobial agents, and even evade intracellular inactivation of antimicrobial agents [113].

Controlled drug release using biocompatible and biodegradable polymers further emerged in the 1980s [114]. after the first polymer-based delivery of macromolecules using poly[ethylene vinyl acetate] polymer was demonstrated in 1976 [115, 116], Antimicrobial drug delivery using polymeric NPs offers several advantages: i) structural stability in biological fluids and under harsh and various conditions for preparation; ii) precisely tunable properties, such as size, zeta-potentials, and drug release profiles, by manipulating polymer lengths, surfactants, and organic solvents used for NP preparation [95], and iii) facile and versatile surface functionalization for conjugating drugs and targeting ligands [117].

There are two major types of polymeric for antimicrobial drug delivery NPs have been explored: linear polymers (e.g., polyalkyl acrylates and polymethyl methacrylate) and amphiphilic block copolymers. The majority of polymeric NPs prepared with linear polymers are nanocapsules or solid nanospheres [118]. In polymeric nanocapsules, a polymeric

membrane that controls the release rate surrounds the drugs that are solubilized in aqueous or oily solvents. In solid nanospheres drugs are homogeneously distributed in the polymeric matrices of variable porosities [119, 120]. Amphiphilic block copolymers form self-assemble micellar NPs with the drug being encapsulated in the hydrophobic core and surrounded by a hydrophilic shield. This shied allows the core to be protected from degradation [121]. Several biodegradable polymers, including poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactide-co-glycolide) (PLGA), poly (carprolactone) (PCL), and poly (cyanoacrylate) (PCA), have been used as the hydrophobic core of the amphilphilic copolymers, whereas PEG has been the most commonly used hydrophilic segment [95]. Targeting ligands can also be conjugated on the termini of PEG for targeted and selective delivery [94, 122].

Polymeric NPs have been explored to deliver various antimicrobial agents and greatly enhanced therapeutic efficacy in treating many types of infectious diseases has been reported. For instance, encapsulated ampicillin im polymeric NPs was effective for*S. typhimurium* infection treatment [123] and intracellular *L. monocytogenes* infection in mouse peritoneal macrophages [124]. The use of polymeric NPs can overcome the limited oral administration of unstable or inadequately absorbed drugs, [125].and, in addition, PEGylation of NPs, can increase drug half-life in serum, and improve mucoadhesive capabilities by reducing phagocytosis [126] Thus, among nanoparticle platforms, polymer NPs may be the most suitable system that can be used for antimicrobial drug delivery.

Biodegradable polymers ang bioorganic polymers are also promising materials in the delivery of peptide based drugs due to their compatibility, degradation behavior, and nontoxic nature of administration [127]. The development of polymeric therapeutic nanostructures for AMP delivery may offer an excellent technological strategy to improve drug bioavailability and safety. CS-based NPs (CSNPs) are particularly interesting as the broad spectrum of antibacterial activity of CS is well known and documented, offering the possibility of sinegistic effects with antimicrobial molecules. Moreover, due to its biocompatibility properties, CS nanostructures have been extensively studied for drug delivery and that is no different for AMPs delivery. In fact, tha majority of the studies revised in the present work involves CS nanostructures. Therefore, in addition to the review of the recent advances in polymeric nanodelivery of AMPs, section 4.1 of this chapter is especially dedicated to the recognized properties of this biopolymer.

4.1. Chitosan: a highly recognized biopolymer

CS, a versatile biopolymer of the aminoglucopyran family is being extensively explored for various biomedical and pharmaceutical applications such as drug delivery. CS is a natural, cationic aminopolysaccharide (pKa 6.5) copolymer of glucosamine and N-acetylglucosamine obtained by the alkaline, partial deacetylation of chitin. It is the second most abundant natural polysaccharide and originates from shells of crustaceans. CS is a biodegradable, biocompatible, positively charged nontoxic mucoadhesive biopolymer. The structure of CS is very similar to that of cellulose [made up of b (1-4)-linked D-glucose units], in which there are hydroxyl groups at C-2 positions of glucose rings. CS is a linear copolymer polysaccharide consisting of b (1-4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) units [127].

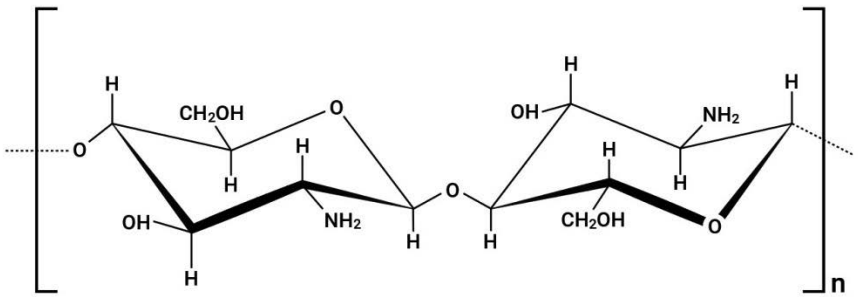

Figure 6 – Chemical structure of Chitosan (CS) polymeric unit.

CS can be considered mostly hydrophilic, but the percentage of acetylated monomers and their distribution in the chains has a critical effect on its solubility and conformation in aqueous media. Because of these molecular features, CS exhibits in solution a pH-dependent behavior and interesting biopharmaceutical properties such as mucoadhesiveness and ability to open epithelial tight junctions. As a result of the research undertaken over the last two decades, there is nowadays an acceptable understanding of the biocompatibility and general safety features of CS. For example, it is currently known that CS is biodegraded by a number of enzymes, such as lysozyme, di-Nacetylchitobiase,N-acetyl-beta-D-glucosaminidase and chitiotriosidase, which are present in human mucosas and other physiological fluids [128]. From a regulatory perspective, CS has a FDA GRAS [129] status and it is currently being used as a common dietary supplement for preventing fat absorption and also in the form of wound dressings. Examples of current marketed CS-based medical devices and oral nutraceuticals are described in Table 4 [130].

Table 9 - Examples of commercial medical devices and oral nutraceuticals with CS (adapted from Szymanska and Winnika [130]).

Product	Material	Usage/Application	Manufacturer
Wound-healing and hemostatic products			
Chitodine®	CS powder with adsorbed elementary iodine	Disinfection of wounded skin, surgical dressing	International Medical Services
ChitoPack C®	Cotton-like CS	Regeneration and reconstruction of body tissue, subcutaneous tissue and skin	Eisai Co.
CeloxTM	Gauze and granules with CS		MedTrade
ChitoFlex®	CS acetate sponge		
HemCon® Bandage Pro		Control of bleeding from non-cavitary grain wounds	HemCon Medical Technologies INC.
HemCon® Strip	Freeze-dried CS acetate salt		
First Aid PosiSep®	*N,O*-carboxymethyl CS sponge	Intranasal hemostatic splint for patients undergoing nasal/sinus surgery	Hemostatis LLC.
Syvek Excel™	Lyophilized three-dimensional CS fibers	Rapid control of bleeding for anticoagulated patients	Marine Polymer Technologies Inc.
Clo-Sur® PAD	Non-woven seal with a soluble CS		Scion Cardio-vascular
ChitoSeal®	Soluble CS salt		Abbott Vascular Devices
TraumaStat®	Porous polyethylene fibers filled with silica, coated with CS (ChitoClear®)	Control of moderate to severe bleeding	Ore-Medix
Tegasorb®	CS particles		Tesla-Pharma
Vulnosorb®	Composition of microcrystalline CS with fibrinogenic tissue glue		3M
Nutraceutical products			
Slim Med™	Non-animal CS	Prevention and treatment of overweight	KitoZyme S.A.
KiOcardio™	Non-animal CS	Maintenance of normal blood cholesterol level	KitoZyme S.A.
LipoSan Ultra®	Composition of CS (ChitoClear®) and succinic acid	Binding dietary fat and reducing its absorption in the intestine	Primex
Liposorb™	CS extracted from squid	Preventing irritable bowel syndrome; Binding dietary fat and reducing its absorption in the intestine	Good Health

4.1.1. Production of Chitosan and Chitosan Derivatives

The starter material for the production of CS is chitin. The main sources of chitin are the shells of crustaceans, mainly crabs and shrimps. Shells of the same size and species are grouped, then cleaned, dried and ground into small shell pieces. There are no standard purification methods as different chitin sources require different treatments due to the diversity in their structures. Conventionally, the protocol is divided into demineralization, deproteinization and decolorization steps which can be carried out using chemical or biological (enzymatic treatment or fermentation) treatments. The end-products need to be highly purified if they are to be used for biomedical or pharmaceutical purposes, as residual proteins, minerals or pigments can cause serious side effects. Conversion of chitin to CS can be achieved by enzymatic or chemical deacetylation. Chemical deacetylation is more commonly used for commercial preparation because of economic issues and feasibility for mass production. The degree of deacetylation influences directly the physicochemical properties of the final polymer [131, 132].

Subsequently, CS molecules can be modified by chemical processes, in order to engineer the optimal physicochemical properties for the purposes required. For instance, chemical modification is a powerful tool in the design of CS based drug delivery systems, controlling the interaction between polymer and drug and thus enhancing the loading capacity and controlling drug release [127]. In the book chapter authored by Sonia *et al.* the following types of modification on CS molecules are revised: hydrophobic modification, thiolation, quaternized forms and chemical grafting. The hydrophobic character of CS can be increased by the covalent attachment of hydrophobic excipients. Hydrophobic interactions are believed to enhance the stability of substituted CS by reducing the hydration of the matrix and thereby increasing resistance to degradation by gastric enzymes.

Thiolated polymers or thiomers are hydrophilic macromolecules exhibiting free thiol groups on the polymeric backbone and represent new promise in the field of mucoadhesive polymers. Quaternized derivatives of CS are obtained by introducing various alkyl groups to the amino groups of CS molecule structure. These derivatives are drastically more soluble in neutral and alkaline environments of the intestine and, hence, are more efficient than CS for drug delivery and absorption across the gastrointestinal tract. Chemical grafting of CS is an important field of study for the functionalization and practical use of CS is an attractive technique for modifying the chemical and physical properties of CS. A graft copolymer is a macromolecular chain with one or more species of block connected to the main chain as side chain(s). The properties of the resulting graft copolymers are broadly controlled by the characteristics of the side chains, including molecular structure, length, and number [127].

Luo *et al.* 2014 review, describes yet another type of CS derivatives by the formation of polyelectrolyte complexes between CS molecules and other natural polysaccharides. Polyelectrolyte complexes [133] are formed simultaneously by mixing oppositely charged polyelectrolytes in solution without any chemical covalent cross-linker, thus biocompatibility of CS is maintained in the final complex, overcoming toxicity issues associated with chemical modification. The major interactions between two polyelectrolyte polymers include the strong but reversible electrostatic and dipole–dipole association, as well as hydrogen and hydrophobic bonds. Due to the protonation of amino groups on the backbone, CS becomes a cationic polyelectrolyte in acidic medium, which could form PEC with negatively charged polyelectrolytes, resulting in various applications based on different preparation methods. CS-based PEC has been proved to possess various applications in biomedical and pharmaceutical areas, such as drug delivery for nutrients with delayed digestibility and controlled release, non-viral vector for gene delivery system, three-dimensional scaffold to mimic tumor microenvironment and for bone tissue engineering [133].

4.1.2. Chitosan recognized properties

Bernkop-Schnürch *et al.* 2012 revised the main recognized physicochemical properties of CS that offer advantage in the design of CS-based drug delivery systems. Considering chemical structure, CS primary amino groups are responsible for properties such as controlled drug release, mucoadhesion, permeation enhancement, and self-assembling. By chemical modifications, most of these properties can even be further improved. When sustained drug release cannot be provided by making use of a simple drug dissolution process, by diffusion, by erosion, by membrane control, or by osmotic systems, retardation mediated by ionic interactions is often the ultimate ratio. The cationic character of CS molecules influences controlled release CS-based systems design and can be achieved by conjugation with anionic polymeric excipients such as polyacrylates, sodium carboxymethylcellulose, or alginate. The mucoadhesive properties are likely also based on its cationic character. The mucus gel layer exhibits anionic substructures in the form of sialic acid and sulfonic acid substructures. Ionic interactions between the cationic primary amino groups of CS and these anionic substructures of the mucus, in addition, with minor hydrophobic interactions mucoadhesion can be achieved. The mechanism being responsible for the permeation enhancing effect of CS is also based on the positive charges of the polymer, which seems to interact with the cell membrane resulting in a structural reorganization of tight junction-associated proteins, promoting membrane opening and solute permeation [134].

Self-assembly is a spontaneous process by which organized structures with particular functions and properties could be obtained without additional complicated processing or modification steps, leading to easier and less onerous production methods for therapeutic vehicles [135]. Self-assembly of CS is mainly possible through electrostatic interaction of ionic polysaccharides of opposite charges. Ionic complexes are attractive for their simplicity and because their self-assembly is responsive to pH and ionic strength. Polysaccharide drug delivery systems can also be made responsive to other stimuli, including heat, magnetism, and light. In many cases, this response to environmental change is often reversible, thus, a "switching" effect can be built into the polymer nanostructure that will respond to stimulation *in vivo*. Some CS derivatives have proven *in situ* gellification properties in response to biological fluids pH alterations, and local stimulation by heat or light [136].

CS and its derivatives have also diverse biological activities, including antioxidant, anti-hypertensive, anticoagulant, antidiabetic, anti-obesity, anti-allergic, anti-inflammatory, antimicrobial, anticancer, neuroprotective and matrix metalloproteinases inhibitory effects. [137].

4.2. Recent advances on the development of polymeric based nanostructures for AMPs delivery

4.2.1. Recent advances in the development of chitosan-based nanostructures

In this subsection a review of current advances in the development of CS-based nanostructures for AMPs deliveryis offered (Table 10). Research has been covering, the technological development of new carrier systems and their full characterization, and the evaluation of their efficacy as drug delivery improvers.

CS micro- and NPs are still the most studied types of CS-based drug delivery systems. In fact, new structures or new production methods are recent study objects. Rivera *et al.*, developed biodegradable nanocapsules, as carriers for two bioactive compounds, 5-aminosalycilic acid and glycomacropeptide. Nanocapsules were produced through layer-by-layer deposition of CS and alginate layers on polystyrene NPs. The bioactive compounds were incorporated on the third layer of the nanocapsules being its encapsulation efficiency and release behavior evaluated. Final results demonstrated that the synthesized nanocapsules presented spherical morphology and a good capacity to encapsulate different bioactive compounds [138]. Developments on the antimicrobial delivery by CS particles are still emerging, with target delivery being one promising field. Cerchiara *et al.* prepared CS-based

particulate formulations for colon delivery of vancomycin. CS particles were prepared by ionic gelation and freeze-drying or spray-drying as recovery methods. Antibacterial activity against *S. aureus*, a Gram-positive model strain, was evaluated [139]. CS particles are also considered for AMPs delivery. Considering peptide character, Piras *et al.*, in 2014, developed a nanoparticle model with commercially available CS loaded with lysozyme [140] as antimicrobial protein drug model. Beyond the intrinsic antibacterial activity of CS and LZ, the LZ-NPs evidenced a sustained antibacterial activity that resulted in about 2 log reduction of the number of viable *S. epidermidis* compared to plain CS NPs. In addition to these potential antimicrobial applications, LZ-NPs demonstrated good biocompatibility, indicating that this study could serve as an optimal model for development of CS NPs carrying antimicrobial peptides for biomedical applications [140]. In 2015, studies for specific encapsulation of AMPs with CS-based particulate systems have been reported. Sun *et al.* have prepared and characterized CS and poly-gamma-glutamic acid [141] composite micro particles as carriers for antimicrobial peptides [141] and nitric oxide (NO) delivery systems using ionic complexation method. The results indicated that both LL-37 and NO were co-loaded successfully in micro particles, and the composite particles could sustain LL-37 and NO release at physiological pH, *in vitro* [141]. Rishi *et al.* encapsulated cryptdin-2 [123] in CS tripolyphosphate (CS-TPP) NPs by ionotropic gelation. The formulation was then characterized on the basis of particle size, zeta potential and polydispersity, and antimicrobial *in vivo* assays were performed. Infected mice when treated against *Salmonella enterica* infection, with the encapsulated peptide showed 83% survivability and approximately 2 log unit reductions in the bacterial load in the tissues versus 100% mortality observed with the free peptide. The study is a first pre-clinical report on the oral effectiveness of cryptdin-2 by its suitable encapsulation and has potential for future clinical applications [123]. Piras *et al*, the same authors who developed the previous described model, tested their hypothesis by encapsulating frog-skin derived AMP temporin B [142] into CS NPs (CS-NPs) and evaluated possible increase in antibacterial activity, while reducing its toxic potential. Temporin B-loaded CS-NPs were prepared based on the ionotropic gelation between CS and sodium tripolyphosphate (NaTPP). The nanocarrier evidenced a sustained antibacterial action against

Table 10 – Recent studies on CS based nanostrucutures for AMP delivery

System	AMP	Study description	Authors
NPs			
CS-alginate nanocapsule	---	Development of nanocapsules carriers for bioactive compounds, produced through LbL technique using, 5-aminosalycilic acid and glycomacropeptide model.	Rivera *et al.* [138]
CS-based nanoparticle	Vancomycin	CS particles were prepared by ionic gelation and freeze-drying or spray-drying as recovery methods. Antibacterial activity against *S. aureus.*	Cerchiara *et al.*[139]
CS-based nanoparticle	Lysozyme as model	Development of a nanoparticle model with commercially available CS loaded with lysozyme as antimicrobial protein drug model.	Piras *et al.* [140]
CS and poly-gamma-glutamic acid composite	LL-37	The results indicated that both LL-37 and NO were co-loaded successfully in micro particles, and the composite particles could sustain LL-37 and NO release at physiological pH, *in vitro.*	Sun *et al.* [141]
CS tripolyphosphate (CS-TPP) NPs	Cryptdin-2	Preparation of CS tripolyphosphate (CS-TPP) NPs by ionotropic gelation. The formulation was then characterized on the basis of particle size, zeta potential and polydispersity, and antimicrobial *in vivo* assays against *Salmonella enterica* were performed.	Rishi *et al.* [123]
CS-based nanoparticle	Temporin B	CS-NPs were prepared based on the ionotropic gelation between CS and sodium tripolyphosphate. The nanocarrier evidenced a sustained antibacterial action against various strains of *S. epidermidis.*	Piras *et al.* [142]
Nanogels			
Nanogel composite	---	Prepation of AgNPs embedded in a biocompatible nanogel comprising degradable, natural polymers. In this study, hybrid nanogels were prepared with varying polymer content and their potential by determining their antibacterial properties against *E. coliand S. aureus* strains.	Coll Ferrer *et al.*, [143]
Glycol- CS nanogel	---	Study of the biocompatibility of a glycol CS nanogel by evaluation of effects on metabolic activity, cell cycles blood compatibility. Overall, the results demonstrated the safety of the use of the GC nanogel as drug delivery system.	Pereira *et al.* [144]
Nanofibers and films			
CS thin films	hLF1-11	Immobilization performed onto CS thin films as a model for an implant coating due to its reported osteogenic and antibacterial properties. CS thin films were produced by spin-coating on Au surfaces. Activity against methicillin-resistant *S. aureus* (MRSA).	Costa *et al.* [145]
Nanofibers	Defensin-1, Dermaseptin LL-37 Magainin 1	Alternate deposition of polycation (CS) and polyanion over cotton gauzes. Antimicrobial assays were performed with two strains: *S. aureus* and *K. pneumonia.*	Gomes *et al.* [145]
Food packaging systems			
CS films	Nisin	Study of the efficiency as antimicrobial carriers of hydroxypropyl methylcellulose [146], chitosan (CS), sodium caseinate (SC) and polylactic acid [146] films, in the release rates of fluorescently labeled nisin Z, evaluating their potential as food packaging polymers.	Imran *et al.* [146]

various strains of *Staphylococcus epidermidis* for at least 4 days, with up to 4-log reduction in the number of viable bacteria compared to plain CS-NPs. The antimicrobial evaluation tests also demonstrated that while the intrinsic antimicrobial activity of CS ensured a "burst" effect, the gradual release of TB further reduced the viable bacterial count, preventing the regrowth of the residual cells and ensuring a long-lasting antibacterial effect [142].

Nanogel carriers are also promising nanobased antimicrobial delivery systems. Coll Ferrer *et al.*, in 2014, have developed a novel and simple synthesis route to create nanosized Ag NPs [143] embedded in a biocompatible nanogel (NG) comprising degradable, natural polymers. In this study, hybrid nanogels were prepared with varying polymer content and their potential by determining their antibacterial properties against *E. coli* and *S. aureus* strains [143]. The research of CS-based nanogels for biomedical applications has also grown exponentially in the last years, however its biocompatibility is still insufficiently reported. Therefore, Pereira *et al.* studied the biocompatibility of a glycol CS [144] nanogel effects on metabolic activity, cell cycles blood compatibility. Overall, the results demonstrated the safety of the use of the GC nanogel as drug delivery system [144].

Studies of AMP functionalized surfaces and fibers have been characterized and evaluated. Costa *et al.* studied the antimicrobial activity effect of hLF1-11, an antimicrobial peptide with high activity against MRSA, immobilization onto a polymeric surface. Immobilization was performed onto CS thin films as a model for an implant coating due to its reported osteogenic and antibacterial properties. CS thin films were produced by spin-coating on Au surfaces. hLF1-11 was immobilized onto these films by its C-terminal cysteine in an orientation that exposes the antimicrobial activity-related arginine-rich portion of the peptide. Surface antimicrobial activity was assessed through surface adhesion and viability assays using an MRSA, and the incorporation of hLF1-11 decreased significantly bacterial adhesion to CS films [147]. Gomes *et al.* developed a new strategy to obtain antimicrobial wound-dressings based on the incorporation of four different antimicrobial peptides into polyelectrolyte multilayer films built by the alternate deposition of polycation (CS) and polyanion over cotton gauzes[145]. Antimicrobial assays were performed with two strains: *S. aureus* and *K. pneumonia*. Results showed that all antimicrobial peptides used in this work have provided a higher antimicrobial effect (in the range of 4 log - 6 log reduction) for both microorganisms, in comparison with the controls, and are non-cytotoxic to normal human dermal fibroblasts at the concentrations tested [145].

In a different concept of application, Imran *et al.* studied the efficiency as antimicrobial carriers of hydroxypropyl methylcellulose (HPMC), chitosan (CS), sodium caseinate (SC) and

polylactic acidfilms, in the release rates of fluorescently labeled nisin Z, evaluating their potential as food packaging polymers [146].Nisin is a small cationic peptide composed of 34 amino acid residues. It exhibits a wide spectrumantimicrobial activity against Gram-positive bacteria and is suitable for food preservation [148]. HPMC, CS, SC packaging films showed ability to progressively release nisin to sustain an anti-bacterial against food borne pathogens effect and can be favorably used for prolonging shelf life of packed food [146].

4.2.2. Recent advances in other polymeric nanostructures

In addition to CS-based nanostructures, recent studies on other polymeric nanostructures for AMPs delivery have been developed. These studies are presented in this subsection (Table 11).

D'Angelo *et al.* designed and developed a system of nano-embedded microparticles (NEM) for sustained delivery of cationic AMPs (CAMPs) [149] in the lung, studying its effect on *P. aeruginosa*, a known lung infection pathogen. To this purpose, PLGA NPs containing a model CAMP, colistin (Col), were produced by the emulsion/solvent diffusion technique, and then spray-dried in different carriers (lactose or mannitol), thus producing NEM. The most promising NEM formulations were selected on the basis of bulk and flow properties, distribution of NPs in the carrier and aerosolization performance upon delivery through a breath-actuated dry powder inhaler. Col–loaded NEM were found to kill *P. aeruginosa* biofilms and to display a prolonged efficacy compared to the free Col.[149]. Another CAMP, plectasin, was encapsulated into PLGA NPs using the double emulsion solvent evaporation method, in the work of Water *et al.* [110] The plectasin-loaded NPs displayed a high encapsulation efficiency (71–90%) and mediated release of the peptide over 24 h. The antimicrobial efficacy was investigated using bronchial epithelial Calu-3 cell monolayers infected with *S. aureus*, and encapsulated plectasin displayed improved efficacy as compared to nonencapsulated plectasin. The author also assessed the subcellular localization of the prepared NPs in different relevant cell lines: Calu-3 epithelial cells, THP-1 macrophages and A549 epithelial cells. Here the results have shown good patterns of penetration on Calu-3 epithelial cell lines, as well as in THP-1 macrophages [110].

Hydrogels and nanogels are an important class of biomaterials that have been widely utilized for a variety of biomedical/medical applications. The biological performance of these systems, particularly those used as wound dressing; can be complemented with antimicrobial activity capable of preventing colonization of the wound site by opportunistic bacterial pathogens [143, 150, 151]. These types of structures have also been studied recentely for AMPs

delivery.Continously to their study of the antimicrobial activity of multi-domain CAMPs (MD-CAMPs) in solution, Jiang *et al.* investigated the same effect of self-assembled 3-D hydrogels supramolecular nanostructures and its rheological properties [152]. Among the studied MD-CAMPs solutions, the bactericidal activity of peptide hydrogels was found to be improved. The improved antimicrobial activity of the self-assembled peptide hydrogels was found to be related to the combined effect of supramolecular surface chemistry and storage modulus of the bulk materials, rather than the ability of individual peptides/peptide assemblies to penetrate bacterial cell membrane as observed in solution. Thus, the structure–property–activity relationship developed through this study may provide important knowldge for designing biocompatible peptide hydrogels with built-in antimicrobial activity for various biomedical applications [152]. The Water *et al.* group also designed novel nanogel-based novicidin delivery system. The peptide novicidin was self-assembled with ananoctenyl succinic anhydride-modified analogue of hyaluronic acid, and this formulation was optimized using a microfluidics-based quality-by-design approach. The encapsulation efficiency of novicidin (15% to 71%) and the zetapotential (−24 to −57 mV) of the nanogels could be tailored bychanging the preparation process parameters, with a maximum peptideloading of 36±4%. The nanogels exhibited good colloidal stabilityunder different ionic strength conditions and allowed complete release of the peptide over 14 days. Furthermore, self-assembly ofnovicidin with hyaluronic acid into nanogels significantly improved the safety profile at least five-fold and six-fold when tested in HUVECsand NIH 3T3 cells, respectively, whilst showing no loss of antimicrobial activity against *E. coli* and *S. aureus*[153].Li *et al.* explored the potential application of AMPs in wound healing, by developing a biodegradable poly(L-lactic acid)-Pluronic L35-poly(L-lactic acid) (PLLA-L35-PLLA) in situ gel-forming system [154]. An injectable formulation composed of human AMPs 57 (AP-57) loaded NPs and thermosensitive hydrogel was prepared. AP-57 peptides were enclosed with biocompatible NPs (AP-57-NPs) with high drug loading and encapsulation efficiency. AP-57-NPs were further encapsulated in a thermosensitive hydrogel (AP-57-NPs-H) to facilitate its application in cutaneous wound repair. As a result, AP-57-NPs-H released AP-57 in an extended period and exhibited quite low cytotoxicity and high anti-oxidant activity *in vitro*. The *in vivo* wound healing assay using full-thickness dermal defect model of SD rats indicated that AP-57-NPs-H could significantly promote wound healing. At day 14 after operation, the treated group showed nearly complete wound closure of 96,78 ±3,12% [154].

Other studies of nisin nanoencapsulation were performed, with the purpose to protect to ensure the stability of this AMP during food processing and storage period. Nisin-loaded

pectin NPs (NLP-NPs) were prepared and analysed by Krivirotova *et al.* by a simple complexation method [155]. Three types of pectin biopolymer were tested and found that the methoxylation degree of pectin influenced on the nisin loading efficiency and particle size. For the complex formation, both electrostatic and hydrophobic interactions were important. NLP-NPs exhibited antimicrobial activity also dependent on the type of biopolymer. Overall, the results indicated that NLP-NPs may be a suitable antimicrobial system to be used in food industry [155].

Table 11 – Recent studies on other polymeric nanostrucutures for AMP delivery

System	AMP	Study description	Authors
NPs			
PLGA nanoparticle	Colistin	Development of a system of nano-embedded microparticles (NEM) for sustained delivery of CAMPs in the lung	D'Angelo *et al.* [149]
PLGA nanoparticle	Plectasin	Intracellullar antibacterial activity against *S. aureus* in epitheial cells.	Water *et al.* [110]
Hydro- and Nanogels			
Self-assembled 3-D hydrogels	MD-CAMPs	Study of bactericidal activity compared to MD-CAMPs in solution, and rheological properties.	Jiang *et al.* [152]
Hyaluronic acid nanogel	Novicdin	Quality-by-design novel nanogel-based novicidin delivery system.	Water *et al.* [153]
PLLA-L35-PLLAin situ gel	AP-57	Potential application of AMPs in wound healing, by developing a biodegradable poly (L-lactic acid)-Pluronic L35-poly (L-lactic acid) (PLLA-L35-PLLA) in situ gel-forming system	Li *et al.* [154]
-			
Food preservation systems			
Pectin nanoparticle	Nisin	Study of a safe suitable antimicrobial system to be used in food industry. Influence of pectin degree of acetilation on NP properties.	Krivorotova *et al.* [155]

CHAPTER V

Inorganic or metallic nanostructures for AMPs delivery

In recent years, an increasing number of papers reporting on a new generation of antimicrobial metallic NPs has been published. Consequently, many of the information on the application of nanotechnology in the infectious disease field regards the use of silver (Ag) and gold (Au) NPs. Recently, derivatives of other metals have been studied for antimicrobial applications, and the antibacterial effects of zero-valent bismuth NPs and uncoated Au, nickel (Ni) and silicon (Si) NPs were reported [156, 157]

Despite the demonstrated intrinsic antimicrobial properties, dispersed metallic NPs tend to aggregate and separate in solution, resulting in a decrease in their antimicrobial efficiency. With the aim of improving antibacterial properties, functionalization of NPs has been attempted with surfactants, polymers, or antibiotics resulting in more stable, less aggregated NPs suspension and innovative synergistic antibacterial agents. For instance, silver NPs stabilized by polymers (polyvinylpyrrolidone) and surfactants (SDS and Tween 80) exhibit enhanced antibacterial activities [158]. NPs can also act as drug-carriers able to pass through cell membranes [159, 160]. Widely used antibiotics such as ciprofloxacin may benefit from the association with NPs, and conjugation may result in an antibacterial effect also against micro-organisms resistant to the same molecule in the naturally occurring form [161]. When the antimicrobial agents are covalently linked to, or contained within, NPs, a higher drug

concentration is attained in the area of interest, resulting in better efficacy at comparable doses and/or in slower release over time that may be exploited for preventing bacterial colonization [162, 163]. Moreover, specific biological sites can be attacked after modification of NPs with target molecules [164, 165]. As the NPs themselves may have antibacterial properties, the combination of NPs and loaded drugs exerts a synergistic action [166].

Current advances in the use of inorganic nanostructures for AMPs delivery, involve essentially the development of Ag and AuNPs, as well as silicon derivates nanosystems. The studies revised in this chapter are summarized in table 12.

5.1. Gold nanoparticles

Currently, two studies have been reported exploring the use of Au nanostructures for AMPs delivery. Photoluminescent Au nanodots (AuNDs) were prepared by Chen *et al.* [167] These AuNDs were functionalized with hybridized ligands, an antimicrobial peptide (surfactin; SFT), and 1-dodecanethiol (DT), on AuNPs. Ultrasmall SFT/DT–Au NDs (size ≈2.5 nm) were achieved and exhibited highly efficient antimicrobial activity. The photoluminescence properties and stability as well as theantimicrobial activity of SFT/DT–Au NDs were also studied, and it was shown that these characteristics are highly dependent on the density of SFT on Au NDs. Relative to SFT, SFT/DT–AuNDs exhibited greater antimicrobial activity, not only to non multidrug-resistant bacteria but also to the multidrug-resistant bacteria. The minimal inhibitory concentration values of SFT/DT–AuNDs were much lower (>80-fold) than that of SFT. The authors considered that the antimicrobial activity of SFT/DT–AuNDs was mainly achieved by the synergistic effect of SFT and DT–AuNDs on the disruption of the bacterial membrane. *In vitro* cytotoxicity and hemolysis analyses were also performed and have revealed superior biocompatibility of SFT/DT–AuNDs than that of SFT. Moreover, *in vivo* methicillin-resistant *S. aureus* infected wound healing studies in rats showed faster healing, better epithelialization. This study suggested that the SFT/DT–AuNDs system may be a promising antimicrobialcandidate for preclinical applications in treating wounds and skin infections [167]. Rai *et al.* also reported a one-step methodology to generate AMP-conjugated (AuNPs) [168]. The AMP-conjugated AuNPs prepared showed controlled size (14 nm) and low polydispersity, and allowed the inclusion of high concentration of AMPs. Also these

Table 12 – Recent studies on inorganic nanostrucutures for AMP delivery

System	AMP	Study description	Authors
Au nanostructures			
Photoluminescent gold nanodots (AuNDs)	Surfactin	AuNDs functionalized with hybridized ligands, an antimicrobial peptide (surfactin; SFT), and 1-dodecanethiol (DT), on Au NPs, for application in wound infection treatment.	Chen *et al.* [167]
AuNPs	Cecropin melittin Magainin-I Tet-20	Development of a one-step synthetic route form functionalization of AuNPs with AMPs	Rai *et al.* [168]
Ag nanostructures			
AgNPs nanofiber	PAs	Study of metallized organic nanofibers for application in wound infection treatment	Pazos *et al.* [169]
AgNPs	MBP-I	Study of the synergistic antibacterial effect of plant peptide MBP-I and AgNPs on infected wounds caused by S. aureus	Salouti *et al.* [170]
Si nanostructures			
Anionic mesoporous SiNPs	LL-37	Study of membrane interaction with AMPs in different types of mesoporous particles.	Braun *et al.* [171]
Nanospheres	Trichogin GA IV Ampullosporin A	Study of AMPs properties adsorbed on silica based surfaces for potential applications in intracellular drug delivery	Syryamina *et al.* [172]
Nanoclays	Nisin Pediocin	Study of nanoclays as nanocarriers for nisin and pediocin adsorption, for applications in food industry.	Meira *et al.* [173]

systems demonstrated higher antimicrobial activity and stability in serum and in the presence of non-physiological concentrations of proteolytic enzymes than soluble AMP, as well as lowcytotoxicity against human cells [168].

5.2. Silver nanoparticles

Recent advances in AMPs delivery by Ag nanostructures, Pazos *et al.* [169] reported on supramolecular assemblies of novel peptide amphiphiles (PAs) containing aldehyde functionality in order to reduce Ag ions and subsequently nucleate Ag metal NPs in water. This proposed system spontaneously generates monodisperse Ag particles at fairly regular distances along the length of the filamentous organic assemblies. The metal−organic hybrid structures exhibited antimicrobial activity and significantly less toxicity toward eukaryotic cells. Metallized organic nanofibers of the type described offer the possibility to create other structures, for instance, hydrogels, that can be potentially applied in wound dressing development [169]. Also addressing the wound infection problem, Salouti *et al.* investigated the synergistic antibacterial effect of plant peptide MBP-1 and Ag NPs on infected wounds caused by *S. aureus* [170].The MIC and MBC of MBP-1 and Ag NPs both on their own and in combination form were determined against *S. aureus* via macrodilution and microdilution methods. The MIC and MBC of MBP-1 were found to be 0.6 and 0.7 mg/mL, respectively. MIC and MBC of AgNPs were determined to be 6.25 and 12.5 mg/L, respectively. MIC and MBC of the AgNPs and MBP-1 combination were found to be 3,125 mg/mL, 0.5 mg/L; and 6.25 mg/mL, 0.6 mg/L, respectively. The synergistic antibacterial effect of Ag NPs and MBP-1 was investigated on infected wounds caused by *S. aureus* in a mouse model and the infected wound healed properly after the combined use of MBP-1 and AgNPs [170].

5.3. Silicon nanostructures

As delivery systems for AMPs, silicon and silicon derivates nanostructures have also been investigated recently. Membrane interactions are critical for the successful use of mesoporous SiNPs. In order to elucidate these, Braun *et al.* have studied the effects of NPcharge and porosity on AMP loading and release, as well as consequences of this for membrane interactions and antimicrobial effects [171]. Anionic mesoporous SiNPs were found to incorporate considerable amounts of the CAMP LL-37, whereas loading was found to be much lower for non-porous or positively charged SiNPs. The results also demonstrated that due to preferential pore localization, anionic mesoporous particles, but not the other particles, protects LL-37 from degradation byinfection-related proteases. For anionic SiNPs, membrane disruption is mediated almost exclusively by peptide release. In contrast, non-porous SiNPs

builded up a resilient LL-37 surface coating due to their higher negative surface charge, and display largely particle-mediated membrane interactions and antimicrobial effects. For positively charged mesoporous SiNPs, LL-37 incorporationpromoted the membrane binding and disruption displayed by the particles in the absence of peptide, but also caused toxicity against human erythrocytes. Thus, the use of mesoporous SiNPsas AMPs delivery systems requires consideration of membrane interactions and selectivity of both free peptide and the peptide-loaded NPs [171]. The properties of AMPs adsorbed on inorganic or organic surfaces are of interest for their potential applications in intracellular drug delivery. In the work of Syryamina *et al.* continuous-wave (CW) electron paramagnetic resonance (EPR) and pulsed electron-electron double resonance (PELDOR) techniques were applied to study adsorption of the AMPs trichogin GA IV and ampullosporin A on monodisperse colloidal silica nanospheres (SiNS) of 20 nm diameter [172]. The results obtained by CW EPR supported the view that the adsorbed peptides form close-packed clusters. PELDOR data show that both trichogin and ampullosporin adsorbed on the silica surface possess a more disordered conformation as compared to that in solution. Forampullosporin, disordering is much more pronounced than for trichogin. Afterdesorption, the peptides restored their conformations; upon adsorption the peptidesi n some cases may lose partly their biradical character [172]. This results may be of interest as antimicrobial activity is often related to peptide conformation.

Nanoclays or layered silicates are an interesting nanostructure that have been used for remediation of environmental contaminants, delivery of drugs and various active molecules, and to enhance polymer mechanical and barrier properties in packaging films. They typically present a stacked arrangement of silicate layers with a nanometric thickness [174]. Meira *et al.* studied three different nanoclays (bentonite, octadecylamine-modified montmorillonite and halloysite) as potential carriers for the AMPs nisin and pediocin, known bacteriocins, the first referred above as having application as a food preservative. Higher adsorption at room temperature of nisin and pediocinwas obtained on bentonite. The antimicrobial activity of the resultant bacteriocin-nanoclay systems was analyzed using skimmed milk agar as food simulant and the largest inhibition zones were observed against Gram-positive bacteria for halloysite samples. Bacteriocins were intercalated into the interlayerspace of montmorillonites as deduced from the increase of the basal spacing measured by X-ray diffraction (XRD) assay. These results indicate that nanoclays, especially halloysite, are suitable nanocarriers for nisin and pediocin adsorption, and the results may be consider interesting for the food industry [173].

CHAPTER VI

Conclusions and future prospects

Microbial infections are still one of the major public health concerns in humans, in particular, in patients suffering from immunosuppressive therapies and/or diseases (e.g. chemotherapy, AIDS). The classical therapeutic strategies that suppress the immune system of compromised patients are costly and associated to severe adverse side effects. In addition, several yeast and fungi organisms, and other pathogenic microorganisms, are responsible for continuous growth of infections and drug resistance against antimicrobial molecules. The current antimicrobial resistance rate is impelling in the urgency to research and develop new strategies to fight drug resistant superbugs. Efforts in new medicines discover have not accompanied the development of drug resistance, thus a technological approach on improving existent ones is gaining special interest.

Therapeutic nanostructures may overcome several of the current therapeutic limitations by a selective delivery of the drug in the site of action, reducing the adverse side effects and microbial drug resistance by the reduced administered dose. Nanomedical research provides easy access to innovative nanodevices and nanosytems, which ultimately enable us to design and fabricate targeted delivery systems of most efficient drugs with increased efficacy and reduced toxicity. Based on good performance, successful experiments, and considerable market prospects, nanotechnology will undoubtedly lead a revolution in medical markets also

for infectious diseases, as it was proven in Chapter II that are several nanotechnological approaches the exhibit important roles in the restoration of antibiotic activity for resistant bacteria.

AMPs are promising antimicrobial compounds that constitute the most promising drug candidates, in a foreseeable future, in the fight against infections and to overcome the alarming rise in microbial drug resistance. However, they also display some limitations in terms of bioavailability and safety, and may also possess additional biological activities and functions. These biological activities and functions include signaling molecules, tissue regeneration, biomarkers and even tumoricidal agents, as the current level of research on AMPs reveals additional roles for these versatile molecules. Nonetheless, no matter the possible fields of applications, AMPs constitute the paradigm of the current view on translational medicine: a collaborative, two-way road from bench to bedside. Even so, after years of promising data, the main question lingers: can we effectively use primitive molecules as the basis for new drug?

The development of AMPs delivery nanostructures seems to offer a very appealing and effective manner to overcome these issues, and several systems have been designed and studied with this purpose, as it was showed in Chapters IV and V. However, several questions in how to regulate the distribution of nanocarriers in the body or specific organs are also needed to be answered. Nano-drugs are foreign substances to the body and may produce inflammation. Therefore, safety data for long-term therapy or repeated dosage are needed to circumvent the potential risk. To date, few studies have investigated the toxicologicaland environmental effects of direct and indirect exposure to nanomaterials and no clear guidelines exist to quantify these effects. Therefore, there is an urgent need for developing guideline, which can assure the safer use of nanomaterials. Moreover, more powerful *ex vivo* models or animal models are needed to assess the safety issues and to comply with government regulations. How to extend the shelf life of nano-drugs is also a problem due to their agglomeration is also a problem. The production methods for nanostructures should also be improved, and scalable studies for industrial production are also of great importance, in order to promote cost effectiveness of these new formulations. The cost and production of nanomaterials at large scale is one of the hurdles in effective implementation of these products. Hence, scientific community should also pay attention to develop affordable methodologies so that nanotechnology can reach to patients.

In conclusion, it seems that, although the promising research results in this area are rising, it is also urgent to start directing efforts in making these new drug formulations a reality as therapeutic agents.

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