Unraveling the potential of nucleic acid mimics to target *Helicobacter pylori* infections – the effect of biological barriers

Joint PhD

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by

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"Temos, sobretudo, de aprender duas coisas: aprender o extraordinário que é o mundo e aprender a ser bastante largo por dentro, para o mundo todo poder entrar."

Agostinho da Silva

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Rita S, Santos., Céu Figueiredo, Nuno F. Azevedo, Kevin Braeckmans, Stefaan C. De Smedt. Nanomaterials and molecular transporters to overcome the bacterial envelope barrier: towards advanced delivery of antibiotics. (submitted to Advanced Drug Delivery Reviews) **(**part of **Chapter 2)**

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Rita S. Santos, George R. Dakwar, Katrien Forier, Elisa Zagato, Katrien Remaut, Céu Figueiredo, Kevin Braeckmans, Nuno F. Azevedo and Stefaan De Smedt. Development of liposomes to target *Helicobacter pylori* in the stomach. Knowledge for Growth 2014. Ghent, Belgium. May 2014.

Rita S. Santos, Katrien Forier, Elisa Zagato, George R. Dakwar, Katrien Remaut, Stephan Stremersch, Sílvia Fontenete, Jesper Wengel, Céu Figueiredo, Stefaan De Smedt, Nuno F. Azevedo and Kevin Braeckmans. LNA/2'OMe Delivery to *Helicobacter pylori* within gastric mucus. Controlled Release Society meeting 2015. Edinburgh, UK. July, 2015.

Rita S. Santos, Diana Guimarães, George R. Dakwar, Céu Figueiredo, Stefaan De Smedt, Nuno F. Azevedo and Kevin Braeckmans. PEGylated liposomes improve the delivery of nucleic acid mimics through native gastric mucus for treatment of *Helicobacter pylori* infections. Crossing Biological Barriers 2015. Dresden, Germany. October, 2015

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Rita S. Santos, Sílvia Fontenete, Jesper Wengel, Marina Leite, Céu Figueiredo, Stefaan De Smedt, Kevin Braeckmans and Nuno F. Azevedo. Fluorescent *in vivo* hybridization as a tool for unraveling the human microbiome. Biofilms 7. Porto, Portugal. June, 2016.

ABSTRACT

The increasing bacterial resistance to antibiotics threatens the global public health. To address this problem, novel therapeutic strategies are needed. In addition, novel diagnostic techniques that are able to quickly identify the bacterial pathogens and their resistance to antibiotics are required. Fast and correct identification of the infection allows to prescribe the most optimal antibiotic, which in turn contributes to prolonging the utility of still active antibiotics. Nucleic acid mimics (NAMs) are very promising in this respect. They can be designed to hybridize with complementary bacterial RNA sequences of choice with high affinity and are not prone to endonucleases degradation. Therefore, through hybridization, NAMs can be used to silence bacterial genes, acting as antibacterial drugs (if the target is an essential bacterial gene), or antibiotic adjuvants (if the target is a gene of resistance to an antibiotic). NAMs coupled to a fluorophore can also serve as diagnostic agents, as hybridization retains the fluorescent NAMs in the bacterial cytosol, 'lighting up' the bacterial cell.

Infectious bacteria locate often in the mucus layer that covers the epithelium in the gastrointestinal, respiratory, urinary and reproductive tract. Mucus offers a first line of defense against foreign entities. Therefore, in order for NAMs to reach the target cells they first need to cross the mucus layer. Having reached the target cells, NAMs have to cross the bacterial envelope for hybridization in the cytosolic compartment. This work aimed to reveal the ability of NAMs to overcome these barriers in relation to *Helicobacter pylori* (*H. pylori*), which is the most common and prevalent chronic infection in the world. In particular, the NAMs used were composed of locked nucleic acids (LNA) and 2'-OMethyl RNA (2'OMe), with either phosphodiester (PO) or phosphorothioate (PS) internucleotide linkages, designed to specifically target *H. pylori*.

It was found that the NAMs, although being able to diffuse fast through the gastric mucus of pigs without degradation, bind to soluble mucus components. This hindered the ability of the NAMs to hybridize in *H. pylori*. However, this problem could be overcome by incorporating NAMs into protective PEGylated DOTAP/DOPE liposomes (DSPE Lpx). The presented results show for the first time that liposomes can deliver active NAMs into *H. pylori*, not only in suspension, but also in the presence of gastric mucus. DSPE Lpx thus hold great potential for the use of NAMs to detect and treat *H. pylori* infections. As a complementary strategy to liposomes, the ability of cell-penetrating

peptides (CPPs) to associate to the NAMs for the intracellular transport into *H. pylori* was tested. It was shown that the peptides PF14 and PF15 can form complexes with the NAMs, but they are too large to hold a chance of directly translocating the bacterial envelope and penetrate into the gastric mucus. Therefore, future research using LNA/2'OMe NAMs should preferably focus on further *in vivo* investigations of the promising DSPE Lpx against *H. pylori* and other important gram-negative pathogens.

Keywords: Bacterial cell envelope, mucus, *Helicobacter pylori*, NAM, DSPE Lpx, CPP, diagnostic, therapy

RESUMO

A crescente resistência das bactérias a antibióticos é uma ameaça para a saúde pública global. Para responder a este problema, novas estratégias terapêuticas são fundamentais. Adicionalmente, novas técnicas de diagnóstico, capazes de identificar rapidamente o agente patogénico e a sua resistência a antibióticos, são necessárias. Uma deteção rápida da infeção é essencial para prolongar a vida útil dos antibióticos. Mímicos de ácidos nucleicos (NAMs) constituem uma solução promissora. Os NAMs podem ser desenhados para hibridar com sequências complementares de ARN, na bactéria. Desta forma, através da sua hibridação, os NAMs podem ser usados para silenciar genes bacterianos, atuando como drogas antibacterianas (caso o gene alvo seja essencial para a bactéria), ou adjuvantes de antibióticos (caso o gene alvo seja um gene de resistência a antibióticos). NAMs, conjugados com um fluoróforo, podem ainda servir de agentes de diagnóstico, uma vez que a sua hibridação retém os NAMs fluorescentes no citosol da bactéria, tornando-a assim fluorescente.

As bactérias patogénicas encontram-se, frequentemente, na camada de muco que cobre o epitélio do trato gastrointestinal, respiratório, urinário e reprodutivo. O muco oferece uma primeira linha de defesa contra entidades exteriores. Assim, para os NAMs poderem chegar à bactéria alvo, é essencial que primeiro ultrapassem a barreira do muco. De seguida, os NAMs têm de transpor o envelope celular da bactéria, para a hibridação ocorrer no citosol. Este trabalho teve por objetivo revelar a capacidade dos NAMs de ultrapassar estas barreiras e assim hibridar com *Helicobacter pylori* (*H. pylori*) – a mais comum e prevalente infeção crónica no mundo. Em particular, foram usados NAMs compostos por 'locked nucleic acids' (LNA) and '2'-OMethyl RNA' (2'OMe), desenhados para reconhecer especificamente *H. pylori*.

Foi descoberto que, apesar dos NAMs conseguirem difundir-se através do muco gástrico de porco sem serem degradados, os NAMs ligam-se a componentes solúveis do muco, o que, por seu turno, os impede de hibridar na *H. pylori.* Esta limitação pode ser obviada pela incorporação dos NAMs em lipossomas PEGylados, compostos por DOTAP/DOPE (DSPE Lpx). Foi possível mostrar, pela primeira vez, que lipossomas são capazes de entregar NAMs, na sua forma ativa, em células de *H. pylori*. Isto foi verificado não só com a bactéria em suspensão, mas também na presença de muco gástrico. DSPE Lpx apresentam-se, assim, como veículos bastante promissores para a aplicação de

NAMs para o diagnóstico e tratamento de infeções por *H. pylori*. Numa estratégia complementar aos lipossomas, ‘cell-penetrating peptides’ (CPPs) foram testados enquanto transportadores dos NAMs na *H. pylori*. Foi mostrado que os péptidos PF14 e PF15 podem formar complexos com os NAMs, mas estes são demasiado grandes para poderem transpor o muco gástrico e o envelope bacteriano. Assim, investigação futura deve preferencialmente incidir sobre o teste *in vivo* dos promissores DSPE Lpx, contra *H. pylori* e outras bactérias patogénicas gram-negativas relevantes.

Palavras-chave: Envelope celular bacteriano, muco, *Helicobacter pylori*, NAM, DSPE Lpx, CPP, diagnóstico, terapia

SAMENVATTING

Bacteriële resistentie tegen antibiotica vormt een wereldwijd probleem. Om dit aan te pakken zijn er nieuwe therapieën nodig. Tevens zou het een sterke vooruitgang zijn indien men resistentie van bacteriële pathogenen tegen antibiotica via geavanceerde diagnostische technieken snel zou kunnen opsporen. Dit zou kunnen leiden tot een optimaal gebruik van antibiotica, wat op zijn beurt zou kunnen bijdragen tot het langer kunnen inzetten van momenteel werkende antibiotica. 'Nucleic acid mimics' (NAMs) zijn hiervoor beloftevol. NAMs kunnen zodanig ontwikkeld worden dat ze (i) niet afgebroken worden door endonucleasen en (ii) met hoge affiniteit hybridiseren met complementaire bacteriële mRNA sequenties waardoor essentiële bacteriële genen kunnen uitgeschakeld worden. NAMs hebben bijgevolg het potentieel om als antibioticum gebruikt te worden; in geval NAMs zouden kunnen ontwikkeld worden die bacteriële genen onderdrukken die een rol spelen in antibiotica-resistentie van bacteriën zouden deze tevens als 'antibioticum adjuvant' kunnen ingezet worden. Fluorescent gemerkte NAMs zouden daarenboven in diagnostische testen kunnen aangewend worden aangezien bij hydridisatie de bacteriën fluorescent licht uitstralen.

Infecterende bacteriën bevinden zich vaak in de mucuslaag die het gastro-intestinaal-, respiratoir- en urinair-epitheel bedekt. Deze mucuslaag vormt een dense laag waarin bacteriën gevangen worden. Bijgevolg dienen NAMs doorheen dergelijke mucuslagen te penetreren om hun target (i.e. de bacteriën) te bereiken. Vervolgens dienen de NAMs zich een weg te banen doorheen de wand van de bacteriën teneinde hun target in het cytosol van de bacteriën te bereiken. Het voorliggend doctoraal proefschrift heeft al doelstelling op te helderen in welke mate NAMs moeilijkheden ondervinden om te hybridiseren in *H. pylori*, een bacterie die de meest voorkomende infectie ter wereld veroorzaakt. Hiertoe werd gebruik gemaakt van NAMs (specifiek gericht op *H. pylori*) opgebouwd uit 'locked nucleic acids' (LNA) en '2'-OMethyl RNA' (2'OMe) met hetzij een 'phosphodiester' (PO) hetzij een 'phosphorothioate' (PS) als internucleotide binding. We stelden vast dat, niettegenstaande NAMs vrij vlug diffunderen doorheen maagmucus van varkens, deze binden aan componenten opgelost in het mucus. Dit verhinderde de hybridisatie van NAMs in *H. pylori*. Dit probleem konden we echter oplossen door NAMs te encapsuleren in PEGylated DOTAP/DOPE liposomen. Voor het eerst toonden we aan dat dergelijke liposomen in staat zijn NAMs in *H. pylori* af te geven, zelfs wanneer mucus aanwezig is.

Tevens testten we het potentieel van cel-penetrerende petiden (CPPs) voor de aflevering van NAMs in *H. pylori*. We stelden vast dat CPP-PF14 en CPP-PF15 inderdaad NAMs konden complexeren doch er niet in slaagden doorheen het mucus te penetreren en de NAMS over de enveloppe van *H. pylori* te tillen. Toekomstig onderzoek zal dienen uit te wijzen in welke mate gepegyleerde liposomen ook *in vivo* in staat zijn NAMs af te leveren in *H. pylori* en andere gram-negatieve pathogenen.

Trefwoorden: Bacteriële envelops, mucus, *Helicobacter pylori*, NAM, DSPE Lpx, CPP, diagnostisch, therapie

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NOMENCLATURE

OM	Outer membrane
LPS	Lipopolysaccharide
OMP	Outer membrane protein
LP	Lipoprotein
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
WTA	Wall teichoic acids
LTA	Lipoteichoic acids
CD	Cyclodextrin
TBDT	TonB-dependent transporter
pmf	Proton motive force
ABC	ATP-binding cassette
NAM	Nucleic acid mimic
PNA	Peptide nucleic acid
PMO	Phosphorodiamidate morpholino oligomer
LNA	Locked nucleic acid
2’OMe	2’-OMethyl RNA
PO	Phosphodiester internucleotide linkage
PS	Phosphorothioate internucleotide linkage
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
NP	Nanoparticle
DOPE	Dioleoylphosphatidylethanolamine
DMPG	Dimiristoylphosphatidylglycerol
DPPC	Dipalmitoylphosphatidylcholine
DSPC	Distearoylglycerophosphocholine
DOTAP	Dioleoyltrimethylammoniumpropane
Chol	Cholesterol
AMP	Antimicrobial peptide
CPP	Cell-penetrating peptide
a.a.	Amino acid

X	6-aminohexanoic acid
B	β-alanine
K	Lysine
F	Phenylalanine
R	Arginine
Q	Glutamine
I	Isoleucine
W	Tryptophan
N	Asparagine
O	ornithine
QN	Trifluoromethylquinoline
GI	Gastrointestinal
PCR	Polymerase chain reaction
H. pylori	*Helicobacter pylori*
PPI	Proton pump inhibitor
PGM	Porcine gastric mucin
sGJ	Simulated gastric juice
FISH	Fluorescence *in situ* hybridization
CLSM	Confocal laser scanning microscope
FRAP	Fluorescence recovery after photobleaching
SPT	Single particle tracking
PAGE	Polyacrylamide gel electrophoresis
DLS	Dynamic light scattering
RP-HLPC	Reversed-phase high-performance liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
ETH	Ethanol
PAR	Paraformaldehyde
Neg	Negative control
Lpx	Lipoplexes
PEG	Polyethylene glycol
Cer Lpx	Lpx modified with CerC8-PEG
CerC8-PEG	N-octanoyl-sphingosine- 1- succinyl[methoxy(polyethylene glycol)2000]

DSPE Lpx	Lpx modified with DSPE-PEG
DSPE-PEG	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethyleneglycol)-2000)
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
EDC	N-(3-Dimethylaminopropyl)-N0-ethylcarbodiimide hydrochloride
sulfo-NHS	N-Hydroxysulfosuccinimide sodium salt
mPEGa	2 kDa methoxy-polyethylene glycol-amine
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
EDTA	Ethylenediaminetetraacetic acid
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
NaCl	Sodium chloride
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
PF	PepFect
Npys	3-nitro-2-pyridinesulphenyl
DIC	Diisopropylcarbodiimide
Oxyma	Ethyl 2-cyano-2-(hydroxyimino)acetate
DCM	Dichloromethane
TFA	Trifluoroacetic acid
DMF	Dimethylformamide
TIS	Triisopropylsilane
DIPEA	Diisopropylethylamine
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
ANOVA	One-Way analysis of variance
SD	Standard deviation
D	Diffusion coefficient
D_w	Diffusion coefficient in water
D_M	Diffusion coefficient in mucus
f	Mobile fraction
t	time
L	thickness

1.

1 DESCRIPTION OF THE OBJECTIVES

The main objective of this work was to investigate the potential of nucleic acid mimics (NAMs) to cross major biological barriers in the body, in order to serve as a novel platform to manage bacterial infections, addressing the antibiotics crisis. In particular, it was aimed to reveal the effect of the gastric mucus and the bacterial envelope as barriers for NAMs directed at *Helicobacter pylori* (*H. pylori*).

This thesis is structured into seven chapters. In this initial **Chapter 1**, the objectives of the work and the thesis organization are presented.

In **Chapter 2** the state-of-the art is reviewed and discussed, first focusing on the bacterial cell envelope barrier, then in the mucus barrier and finally on the *Helicobacter pylori* infection. Given its complexity, a more extensive section is dedicated to the bacterial cell envelope, where the structure of the gram-negative and gram-positive envelopes is presented, followed by the envelope permeability, determined by the cell wall and its internalization routes. Subsequently, it is discussed how the envelopes can be overcome by the use of carriers – liposomes and cell-penetrating peptides (CPPs). The mucus is then described, with a focus on the gastric mucus, a barrier for oral delivery and a potential barrier to target *H. pylori*. In view of the mucus barrier properties, it is discussed how nanocarriers can overcome the mucus. Lastly, the *H. pylori* infection is referred, focusing on the mechanisms that enable the bacterium to establish the infection and the clinical outcomes, as well as the available methods to detect and treat the infection, including the investigated nanocarriers.

In **Chapter 3**, it was intended to reveal at what extent the gastric mucus poses a barrier to the ability of the NAMs to target *H. pylori* in the stomach. This could be attained by i) testing the diffusion of the NAMs through mucus from the stomach of pigs, using fluorescence recovery after photobleaching (FRAP), and ii) testing the hybridization

efficiency of the NAMs in *H. pylori* covered by mucus from the stomach of pigs, using fluorescence *in situ* hybridization (FISH). It was found that binding interactions between mucus and the NAMs prevented efficient hybridization in *H. pylori*.

Therefore, in **Chapter 4** a liposomal nanocarrier was developed aiming to safely transport the NAMs across the porcine gastric mucus, while being able to deliver the NAMs directly into *H. pylori* cells in suspension and within the mucus. In order to accomplish this goal, the porosity of the gastric mucus mesh was first characterized, studying the diffusion of inert model nanoparticles through the porcine gastric mucus, by single particle tracking (SPT). After formulating the liposomal nanocarrier accordingly, the ability of the liposomes to intracellularly deliver the NAMs into *H. pylori*, initially in solution and after within mucus, was tested by FISH.

It was then intended to investigate how the performance of the developed liposomal nanocarrier would compare against that of CPPs. Hence, in **Chapter 5** it was verified if CPPs, normally used to carry charge neutral oligonucleotides into bacterial cells, could be associated to the negatively charged NAMs directed at *H. pylori*. Peptides could be synthesized by solid-phase peptide synthesis and purified by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization (MALDI). After association of the peptides to the NAMs, it was aimed to study the colloidal stability of the constructs in gastric environment, by dynamic light scattering (DLS).

Chapter 6 presents the general discussion and conclusions of the work, with suggestion for future work in the context of the strategies used in the previous chapters.

In **Chapter 7**, the broader international context and relevance of the performed work are presented. In addition, future perspectives are discussed, where strategies different from the ones used in this thesis are suggested for future exploration. The market of the NAMs and carriers applied to bacterial infections is discussed, as well as the challenges for their clinical translation.

This thesis reports the work performed at the Laboratory for General Biochemistry and Physical Pharmacy, from the Faculty of Pharmaceutical Sciences, Ghent University, as well as at the BEL Group at LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy), Faculty of Engineering, University of Porto, and IPATIMUP (Institute of Molecular Pathology and Immunology of the University of Porto), i3S (Instituto de Investigação e Inovação em Saúde).

2.

2 GENERAL INTRODUCTION

Part of this chapter is in preparation for publication:

Santos, R.S., Figueiredo, C., Azevedo, N.F, Braeckmans, K., De Smedt, S.C. Nanomaterials and molecular transporters to overcome the bacterial envelope barrier: towards advanced delivery of antibiotics. (submitted to Advanced Drug Delivery Reviews).

In this work, oligonucleotides, in particular nucleic acid mimics (NAMs), were evaluated as a novel platform to manage – treat and diagnose – bacterial infections, as explained in the first section (2.1) of this chapter. In the following two sections, an overview is given on the two major permeability barriers for the success of NAMs, the bacterial cell envelope (2.2) and the mucus (2.3).

Considering the bacterial cell envelope (section 2.2), its structure (2.2.1) and the possible internalization routes (2.2.2.1/2) are described, focusing in particular in the cell wall that is the outermost and most stringent layer of the envelope of gram-negative and gram-positive bacteria. Subsequently, it was defined at what extent the described envelopes can pose a barrier for the internalization of compounds of interest to manage bacterial infections, starting with the traditional small molecule antibiotics (2.2.2.3) and then comparing with NAMs (2.2.2.4). As a solution to overcome the bacterial cell envelope, carriers (i.e. nanoparticles and molecular transporters) can be used to transport the antibiotics and NAMs. In view of the reviewed cell envelope permeability, the possible mechanisms by which carriers may overcome the bacterial envelope are presented for liposomes and 'membrane-active' peptides.

In the third section of this chapter (2.3), mucus, that most antimicrobial drugs and formulations need to cross to reach the target bacteria, is reviewed. A special focus is

given to the gastric mucus, since i) it harbors the most common and prevalent chronic infection in the world, *Helicobacter pylori* (*H. pylori*), and ii) independently of the infection, every orally delivered antibacterial needs to first cross the gastric mucus to reach its target bacterium. This section ends with a description on how carriers can overcome the gastric mucus barrier.

In this thesis, the investigation of the bacterial envelope and mucus as barriers for the NAMs is directed at the *H. pylori* infection. Therefore, on the fourth and final section of this chapter (2.4), an overview of the *H. pylori* infection is provided, considering the available solutions to detect and treat the infection and the use of carriers to manage the infection.

2.1 Managing bacterial infections – NAMs as a novel platform

The ability to control infections with antibiotics allowed the most important quality of life improvement over the last century [1]. However, the antibiotic era is threatened by bacterial resistance to antibiotics and a very scarce pharmaceutical pipeline of new antibiotics [2]. Infections by resistant bacterial account already for nearly 50 000 deaths annual, in Europe and the United States together [3, 4]. Resistance of bacteria to antibiotics can be intrinsinc (bacteria that are naturally impermeable to antibiotics) or be newly acquired (through expression of genes that render bacteria resistance to antibiotics) [5, 6]. Therefore, improved internalization of antibiotics would solve resistance caused by bacteria impermeability to antibiotics. Most importantly, the development of alternative antibacterial strategies that can fully combat all forms of bacterial resistance are highly required.

Oligonucleotides can be designed to act as antisense antimicrobials, by hybridizing and consequently inhibiting the expression of selected genes [7-9]. In this sense, oligonucleotides, and in particular nucleic acid mimics (NAMs) offer great potential. They act by specifically hybridizing *in situ* with complementary bacterial RNA and consequently inhibiting the expression of selected genes [7-9]. These can be essential bacterial genes, thus preventing bacterial growth, or genes involved in the resistance to antibiotics, thus restoring bacteria susceptibility to antibiotics. This strategy provides a potentially endless source of active therapeutic agents; even if the bacterial target undergoes a point mutation, the oligonucleotide can be easily redesigned to become effective again.

In addition, NAMs conjugated with a contrast agent could serve as detection probes for fast and specific *in vivo* diagnostic, able to inform about the bacterial resistance to traditional antibiotics. These are essential features to extend the life-time of still active antibiotics [6]. The lack of techniques to rapidly identify the specific infecting pathogen and its resistance to antibiotics has often hampered the prescription of narrow spectrum antibiotics and the overuse of broad spectrum antibiotics favors the development of bacterial resistance to antibiotics [6].

Compared to the current diagnostic techniques, labelled NAMs could overcome the traditional time-consuming culture methods as well as the need of bacteria isolation and extraction of target genetic material associated with other molecular methods, like PCR [1, 10, 11]. In addition to their value for therapy and clinical diagnosis, the opportunity to directly localize bacteria *in vivo* is also of interest for research purposes. The host-microbial and microbial-microbial interactions, which can have an impact on the immune system and disease state, are mostly missed by the lack of a technique to visualize live bacteria within their *in vivo* environment [12-14]. NAMs hold the potential to respond to this need.

For NAMs to fulfil their promise as a flexible platform for diagnosis and treatment of bacterial infections, they need to be able to cross the biological barriers in the body. It is pivotal that the NAMs cross the bacterial cell envelope, in particular the cell wall that poses an outer stringent barrier. In addition, the infecting pathogens often locate deep in the mucus that covers the epithelium of all organs exposed to the external environment [15, 16]. Therefore, NAMs need to be able to pass through the mucus, without losing their activity, to reach the target bacteria. Hence, the bacterial cell envelope and the mucus are barriers of major importance to determine the success of NAMs to manage bacterial infections. As a solution to overcome these barriers, the use of carriers (i.e. nanoparticles and molecular transporters) is a promising strategy. Nonetheless, its application in bacterial cells is still on its infancy and a lot is unknown in regard to their interaction at the bacterial cell wall and the chances to overcome it.

2.2 Bacterial cell envelope barrier

In this section, the bacterial cell envelope of gram-negative and gram-positive bacteria is described, considering its architecture (section 2.2.1) and permeability properties (section 2.2.2). Concerning gram-positive bacteria, we focus on the general structure of the typical Firmicutes phylum; Actinobacteria with a different cell wall (such as that of the genus *Mycobacterium, Nocardia, Rhodococcus* and *Corynebacterium*) are beyond the scope of this work.

2.2.1 Cell envelope structure

Bacteria, like mammalian cells, have their cytosol surrounded by a symmetric bilayer composed of amphiphilic phospholipids – the cytoplasmic membrane [17]. However, being unicellular organisms that often inhabit hostile environments, bacteria evolved an extra cell wall that surrounds and protects the cytoplasmic membrane [18]. The cell wall provides protection against osmotic pressure and mechanical damage and maintains the cellular shape, while allowing permeation of key substrates for bacterial metabolism and communication with the environment [19, 20]. Together, the cell wall and the cytoplasmic membrane compose the bacterial cell envelope. The cell wall, in turn, is subdivided into different layers. There is often lack of precision in the field on the reference to the different bacterial envelope layers. In this section, these will be described for gram-negative and gram-positive bacteria (Firmicutes).

2.2.1.1 Gram-negative bacteria

Considering the gram-negative bacterial envelope, the cell wall will be first presented with its sub-layers, before mentioning the cytoplasmic membrane.

The **cell wall** of gram-negative bacteria comprises the outer membrane (OM) and the periplasmic space containing a thin layer of peptidoglycan, Figure 2.1 [21].

The **OM** is not a common phospholipid bilayer, but rather an asymmetric lipid bilayer – it is composed of phospholipids only in the inner leaflet and mostly lipopolysaccharide (LPS) in the outer leaflet [19, 22, 23]. In addition, the OM contains embedded proteins [19].

The **phospholipids** contribute for the outer membrane structure and flexibility. Their composition in the OM is similar to that of the cytoplasmic membrane [24, 25]. In gram-

negative bacteria the major membrane phospholipid is phosphatidylethanolamine (PE), folowed by phosphatidylglycerol (PG) and smaller amounts of cardiolipin [24, 25].

The **LPS** can extend outwards as much as 3 nm; it is a nonfluidic amphiphilic structure, with a net negative charge higher than the usual negatively-charged phospholipids, and it is held in position at the OM surface by divalent cations [18, 19, 22]. LPS is composed of three covalently-linked regions; from the cytoplasmic to the outermost region they are: the lipid A, the core polyssacharide and the O-antigen [18]. The hydrophobic lipid A has a large number of saturated fatty acids that confer a low fluidity to LPS [17, 22, 25]. The lipid A is linked to the hydrophilic core polyssacaride which is a complex anionic oligosaccharide where the metal cations bind, also regulating LPS rigidity [25]. The O-antigen is a specific O-polysaccharide that differs between and within bacterial species [22]. It is highly antigenic, acting as an important virulence factor of pathogenic bacteria [19, 22, 25].

The most abundant **proteins** are the outer membrane proteins (OMPs) [22]. The OMPs are integral proteins, spanning all the OM, tipically with a β-sheet structure that forms aqueous channels [18]. The OM also contains lipoproteins (LPs) which, differently from the transmembranar OMPs, are only embeded in the cytoplasmic leaflet of the OM via their lipid moiety and have an unclear function [18, 25].

The **periplasm** is a gel-like space of about 13-25 nm that is located in between the outer and cytoplasmic membrane [22, 25]. It possesses a much higher viscosity than the cytoplasm, as a result of the high concentration of small molecules, such as amino acids, mono- and oligosaccharides and biosynthetic precursors and degradation products of peptidoglycan [19]. It is crucial for cell structure maintenance, nutrition and protection against potentially harmful compounds; as such it is densely populated with proteins including i) chaperons or binding proteins involved in the transport of sugars, amino acids, vitamins and inorganic ions, and ii) enzymes, such as phosphatases, nucleases, proteases and β-lactamases, to degrade potentially harmful compounds and participate in envelope biogenesis [18, 19, 22, 25].

The periplasmic space comprises a thin layer of **peptidoglycan**. Peptidoglycan is a rigid polymer that provides structure, mechanical protection and osmoregulation [18, 19, 22]. It is structurally similar in gram-negative and -positive bacteria – a disaccharide composed of alternating units of N-acetyl glucosamine and N-acetyl-muramic acid cross-linked by short peptide chains with variable composition [18, 22]. The peptidoglycan is

anchored to the OM via a lipoprotein named Braun´s lipoprotein [18, 19, 22]. The 3D architecture of the peptidoglycan is a matter of debate; it is depicted in Figure 2.1 according to the classical view [19].

The periplasm covers the **cytoplasmic membrane**. Bacteria lack intracellular organelles, thus all the functions performed at the eukaryotic organelles membrane occur in bacteria (gram-negative and positive) at the cytoplasmic membrane [18]. These include energy production, lipid biosynthesis, protein secretion, and transport [18]. Differently from the OM, the cytoplasmic membrane is a symmetric phospholipid bilayer. In gram-negative bacteria, it is composed mostly of PE, followed by PG and smaller amounts of phosphatidylserine, and cardiolipin can also be present [18]. In *Escherichia coli* PE covers around 75% of the phospholipids, while PG around 25%; in *Pseudomonas aeruginosa* PE accounts for 60%, PG 21% and cardiolipin 11% [26]. Besides phospholipids, integral and peripheral proteins are also present in the cytoplasmic membrane [2]. These proteins are either structural or (passive/active) transport proteins [2].

2.2.1.2 Gram-positive bacteria

Covering the cytoplasmic membrane, gram-positive bacteria have a **cell wall** that is markedly different from that of gram-negative bacteria, Figure 2.1. The gram-positive cell wall is composed of teichoic acids and peptidoglycan, being proteins also present.

The **peptidoglycan** of gram-positive bacteria is significantly thicker (about 20-100 nm thick) than that of gram-negative bacteria (few nm), to compensate the absent of an OM [18]. Therefore, the peptidoglycan in gram-positive bacteria provides a much more resistant barrier to mechanical stress [18, 19, 25]. Its structure is in essence the same as in gram-negative bacteria [18, 19, 25].

Considerable amounts of anionic polysaccharides, the **teichoic acids**, contact with the outer environment [18, 25]. Teichoic acids somehow relate to LPS in gram-negative bacteria. Due to their anionic charge they bind metal cations (mainly Mg^{2+}, but also Ca^{2+} and K^{+}), regulating the envelope rigidity and porosity [18, 25]. Teichoic acids are divided in wall teichoic acids (WTA) that covalently attach to the peptidoglycan, expanding the entire peptidoglycan and beyond [18, 27], and lipoteichoic acids (LTA) that anchor to the head groups of the cytoplasmic membrane, protruding through the peptidoglycan to reach

the outer surface [19, 25, 28]. Although much less than LPS, LTA can also bind to host cells triggering immune reactions [25].

Besides teichoic acids**,** the cell wall is also decorated with **proteins**, bound to the teichoic acids, the peptidoglycan, or the cytoplasmic membrane via lipid moieties [18, 25, 29]. These proteins can be similar to the ones found in the periplasm of gram-negative bacteria, being involved in defense, transport, synthesis and turnover of peptidoglycan, adhesion to other bacteria and adhesion to their host for infection [18, 25, 29].

The **cytoplasmic membrane** of gram-positive bacteria is similar to that of gram-negative bacteria, differing only in the relative phospholipid composition. Differently from the gram-negative bacteria, the major phospholipid in the gram-positive bacteria is PG, followed by PE and smaller amounts of cardiolipin and eventually phosphatidylserine [18]. In *Staphylococcus aureus* PG accounts for 57% of the phospholipids and cardiolipin for 5%, while in *Bacillus subtillis* PG accounts for 70%, PE for 12% and cardiolipin for 4% [26]. Like in gram-negative bacteria, integral and peripheral proteins (structural and transport proteins) are also embedded in the phospholipids of the cytoplasmic membrane [2].

2.2.2 Permeability of the bacterial envelope

Having reviewed the basic cell envelope structure of gram-negative and -positive bacteria, it will now be described how these envelopes affect the transport of compounds from the outer environment into the bacteria. Although there are some generally accepted concepts, the transport across bacterial cell envelope is incompletely understood [2, 30, 31]. Below an overview is given of the current knowledge with a focus on the cell wall, as it is the determinant intake barrier that sets bacteria singularity from other microbial and animal cells. Firstly, the general routes for penetration the through the gram-negative (2.2.2.1) and gram-positive (2.2.2.2) envelope are detailed. More research has been devoted to the barrier properties of gram-negative bacteria than gram-positive bacteria, which is necessarily reflected on this literature review. It is then considered how these internalization routes regulate the penetration of antibiotics (2.2.2.3) and NAMs (2.2.2.4) to manage bacterial infections.

2.2.2.1 Internalization routes in gram-negative bacteria

In this section, the general routes used by gram-negative bacteria to internalize common nutrients are detailed. The permeability of the OM is especially highlighted, as it harbors the crucial OPMs.

The gram-negative bacterial envelope is decorated with the nonfluidic negatively charged layer of LPS which regulates the permeability to **hydrophobic compounds** [18, 22, 23, 25, 32]. LPS retards the simple diffusion through the OM by 50-100 times when compared to the diffusion through a regular phospholipid bilayer [18, 22, 23, 25, 32]. Hydrophobic substrates that can still cross LPS will be normally internalized by diffusion via the lipid bilayers of the outer and cytoplasmic membranes.

The hydrophobic OM lipid bilayer is traversed by the OMPs that form aqueous channels that regulate the permeability to **hydrophilic compounds** [18]. The OMPs can be divided into passive channels (general porins and specific channels) and active transporters (Figure 2.1).

General porins (or simply porins) are the most abundant proteins of the OM [25, 33]. Porins allow the internalization of small hydrophilic substrates (including small sugars, amino acids and ions) that are available in high concentrations and thus passively diffuse through porins, moving down the concentration gradient [32-36]. Porins do not bind the transported compounds; porins are relatively unspecific and mostly discriminate their substrates by size, although charge may also play a role (Figure 2.1) [23]. The better known porins are the ones identified in *Escherichia coli* (*E. coli*): OmpF, OmpC and PhoE [22, 25, 35]. OmpF and OmpC pores possess an excess of negatively charged residues; while **OmpC** is slightly cation selective, OmpF allows the diffusion of both cationic and uncharged molecules [25, 30, 37]. **OmpF** is the largest porin, so its exclusion limit is traditionally considered as the size limit for diffusion through passive OMPs (Figure 2.1). The most constricted region of the OmpF pore (also called the “eyelet”) was found by crystallography to be around **0.7 nm** in diameter, having an exclusion limit of around **600 Da** [23, 25, 34]. **PhoE** is slightly anion selective due to the accumulation of positively charged residues on the pore surface; it is involved in the uptake of ions as phosphate [25, 37]. Besides these three classical porins, another general porin that can take up unspecific oligosaccharides was later found in *E. coli* (and other bacteria), named OmpG [25, 38]. Differently from the classical trimers, OmpG is a monomeric β-barrel of 14 strands and it seems to be exceptionally large but exist only in rather small amounts [38, 39]. Its pore

diameter is report to be 1.5 nm but it can be constricted to **0.8 nm** [38]. All the gram-negative species investigated possess general porins with permeability similar to that of *E. coli*, with the exception of *P. aeruginosa* which major OMP is OprF, a porin that allows only slow diffusion [17, 23].

Hydrophilic substrates needed for bacterial growth are often not available in sufficiently high concentrations to allow a fast diffusion through general porins [25]. Thus, **specific passive OMP channels** exist that can bind substrates, with low affinities, in order to preferentially facilitate their passive diffusion [32, 40]. Like general porins, transport is still driven by the concentration gradient and is thus energy-independent [32]. Differently from porins, the expression of these specific OMPs is frequently induced by their substrates, as is the case of the major *E. coli* specific transporters: LamB and Tsx [33, 34].

LamB is dedicated to the specific transport of maltose and larger malto-oligosaccharides [18, 23, 33]. According to its crystal structure, the most constricted region of the LamB pore has a diameter of **0.5-0.6 nm**, therefore smaller than OmpF [23, 33]. Malto-oligosaccharides in their bulk form are bigger than this pore size, but they are converted in a linear form to pass through the LamB channel [41]. Other hydrophilic substrates, such as amino acids and carbohydrates like glucose, lactose, arabinose and even glycerol, can also unspecifically flow through LamB, especially if they are scarce in the environment [23, 25, 33]. In addition, a channel homologous to LamB, named ScrY (≈ **0.85 nm** pore diameter), is encoded by a plasmid and allows the specific transport of several sugars as sucrose, glucose, fructose, arabinose, maltose, raffinose and maltodextrins, in some *E. coli* and *Salmonella* strains [23, 25, 42].

Tsx is involved in the specific transport of nucleosides and deoxynucleosides, which can be used as carbon and nitrogen sources as well as precursors of nucleic acid synthesis [42]. Free bases or nucleoside monophosphates (nucleotide) are not internalized [25]. Tsx is the smallest known OMP that functions as a channel [42]. However, it has several distinct binding sites in the channel; the part that binds the base moiety of nucleosides is only 0.3-0.5 nm wide, but the part that binds the sugar moiety is 0.7-0.8 nm wide [42].

In *P. aeruginosa* the main specific OMPs transporters are OprB, involved in glucose uptake, OprD, involved in basic amino acids and peptides uptake, OprP, dedicated to phosphate transport and OprO, dedicated to pyrophosphate diffusion [23, 25].

In addition to these typical channels, recently it was found that special specific passive channels exist that only open upon the presence of their substrate, without the need of energy, named **ligand-gated channels**. This way, the penetration of bulky substrates, without the need of previous linearization (as needed for malto-oligosaccharides to cross LamB), was found possible via the **CymA** channel in *Klebsiella oxytoca* (a species closely related to *Klebsiella pneumoniae*) [41, 43]. Orthologs of CymA are present in the Enterobacteriaceae and Vibrionaceae, but only the protein from *K. oxytoca* has been studied [41]. Bulky substrates were previously assumed to require active transport for internalization [41]. In particular, α-cyclodextrin (α-CD), a cyclic oligosaccharide with a cylindrical bulky structure of **973 Da** and outer diameter of 1.37 nm (too big to pass through the classical OMP channels), was internalized via the CymA, which forms a large pore with a diameter of around **1.1-1.4 nm** (Figure 2.1) [41]. However, this channel is normally occluded from the periplasm site by a mobile segment that is only displaced to generate a transiently open channel upon substrate income into the channel [41]. This way, the barrier properties of the OM are preserved, while permitting passive diffusion of bulky molecules without energy expense [41]. The authors reflect that the size may not be the determinant factor for the need of active transport, but probably the need to bind with high affinity scarce nutrients as they are too valuable [41].

Another ligand-gated channel found is dedicated to the transport of **hydrophobic** long fatty acids, contradicting the general understanding that only molecules with a hydrophilic surface can diffuse through OMP channels [40, 44]. In particular, oleic acid, 283 Da, could be internalized by **FadL** channels, which are widespread among gram-negative bacteria, but better studied for *E. coli* [40, 44]. As CymA, FadL from *E. coli* was found to contain a plug that blocks the lumen of the channel and is displaced by the presence of the substrate [40]. The major difference compared to the transport of hydrophilic compounds is that the hydrophobic fatty acids are not transported across the membrane via the polar central part of the channels [36, 40]. Instead, the displacement of the plug unblocks a specialized lateral opening (of **0.8 nm** in diameter) in the FadL channel from where the fatty acids diffuse laterally in the OM (Figure 2.1) [36, 40]. The authors envision that this lateral diffusion is a way to overcome the LPS barrier to the diffusion of valuable hydrophobic compounds [36].

Valuable hydrophilic substrates that are available only in extremely low amounts (such as micronutrients) need to be bound with high affinity by **active OMPs** to be internalized.

This transport occurs against their concentration gradient, thus requiring energy expense [32]. Active OMPs, named TonB-dependent transporters (**TBDTs**), form large channels normally used for the uptake of iron complexes (normally up to 1000 Da) and vitamin B12 (around **1355 Da**) [32, 36]. TBDTs are, however, present in much lower amounts than passive channels and they are not permanently open [18, 23, 41]. Similarly, to the ligand-gated passive channels, the active transporters at the OM have a plug that blocks the lumen of the barrel and only substrate binding prompts channel opening [18, 23, 41]. However, differently from ligand-gated diffusion channels, the substrate interaction *per se* is not enough to generate the conformational changes in TBDT needed to open the channel; rather, energy from the proton motive force (pmf) of the cytoplasmic membrane (since the OM is not energized) has to be transmitted to TBDT [41]. Thus, the TBDT in the OM is part of a complex system that spans the envelope (Figure 2.1), composed of: i) a specific TBDT at the OM that binds the substrate, ii) the TonB complex (comprising the TonB protein at the periplasmic space and ExbB and ExbD proteins at the cytoplasmic membrane) that transduces the pmf of the cytoplasmic membrane for the conformational change of TBDT, iii) a protein in the periplasm that captures the substrate that flows from the open TBDT channel and iv) an ATP-binding cassette (ABC) transporter that transports the substrate across the cytoplasmic membrane using ATP [23, 45].

Iron is actively transported in the form of iron complexes, most commonly formed by iron chelating siderophores [45]. In *E. coli* the TBDTs FhuA, FecA and FepA recognize respectively ferrichrome, diferric-dicitrate and ferric enterobactin; while in *P. aeruginosa* the TBDTs FpvA and FptA recognize ferric pyoverdin and ferric pyochelin [45]. The siderophores are transported as a whole via the OM transporters into the periplasm where they bind a periplasmic binding protein and only in the cytoplasm the iron is released from the complex [45, 46]. Differently, some pathogens can use iron from their mammalian host normally associated to transferrin and lactoferrin [45, 46]. Because transferrin and lactoferrin are too big (> 80 kDa) they cannot cross the OM intact, so upon transferrin and lactoferrin recognition by specific TBDTs iron is dissociated and transported alone [45, 46]. Vitamin B12, containing a Co^{2+} ion in a corrin ring, is transported by bacteria as a whole upon recognition by BtuB in *E. coli* [45].

Recently, it was found that besides iron and vitamin B12, the TonB-dependent transport can also be used to take up other substrates, such as nickel and carbohydrates, but with much lower affinity (probably because they are not so scarce) and by a transport mechanism potentially different [45]. For instance, the gastric pathogen *Helicobacter*

pylori actively takes up nickel, which is a cofactor of urease that in turn mediates bacteria resistance to the gastric acidic pH [45]. Also colicins (proteinaceous toxins) can be taken up by TBDTs, as studied for colicin M via FhuA in *E. coli*, being the only example of a protein import by *E. coli* [47]. The **FhuA** pore diameter is around **2.5 nm** in the fully open state (Figure 2.1) and colicin M is 3 to 4 nm of diameter in the folded state, so they need to first unfold to be (fully or partially) imported [47, 48].

Substrates that crossed the OM will encounter the gel-like periplasm containing a thin layer of peptidoglycan [17, 18]. The coarse **peptidoglycan** network in the periplasm will normally offer little resistance to further diffusion [17, 25]. The peptidoglycan mesh pore size is thought to be around **2-3 nm**, as estimated for *E. coli* and *B. subtillus* and *B. megaterium* (Figure 2.1) [26, 49, 50]. It was calculated, using these values, that peptidloglycan, relaxed (isolated from bacteria) and stretched (resembling live bacterial cells), should be permeable to globular uncharged hydrophilic proteins up to 22-24 kDa and **50 kDa**, respectively [49]. Nevertheless, there are still doubts about the peptidoglycan permeability and it is also known that it can differ with the bacterial growth rate, as part of peptidoglycan synthesis regulation [51], and from bacterium to bacterium. For example, *Helicobacter pylori* was hypothesized to have larger pores than *E. coli* [52].

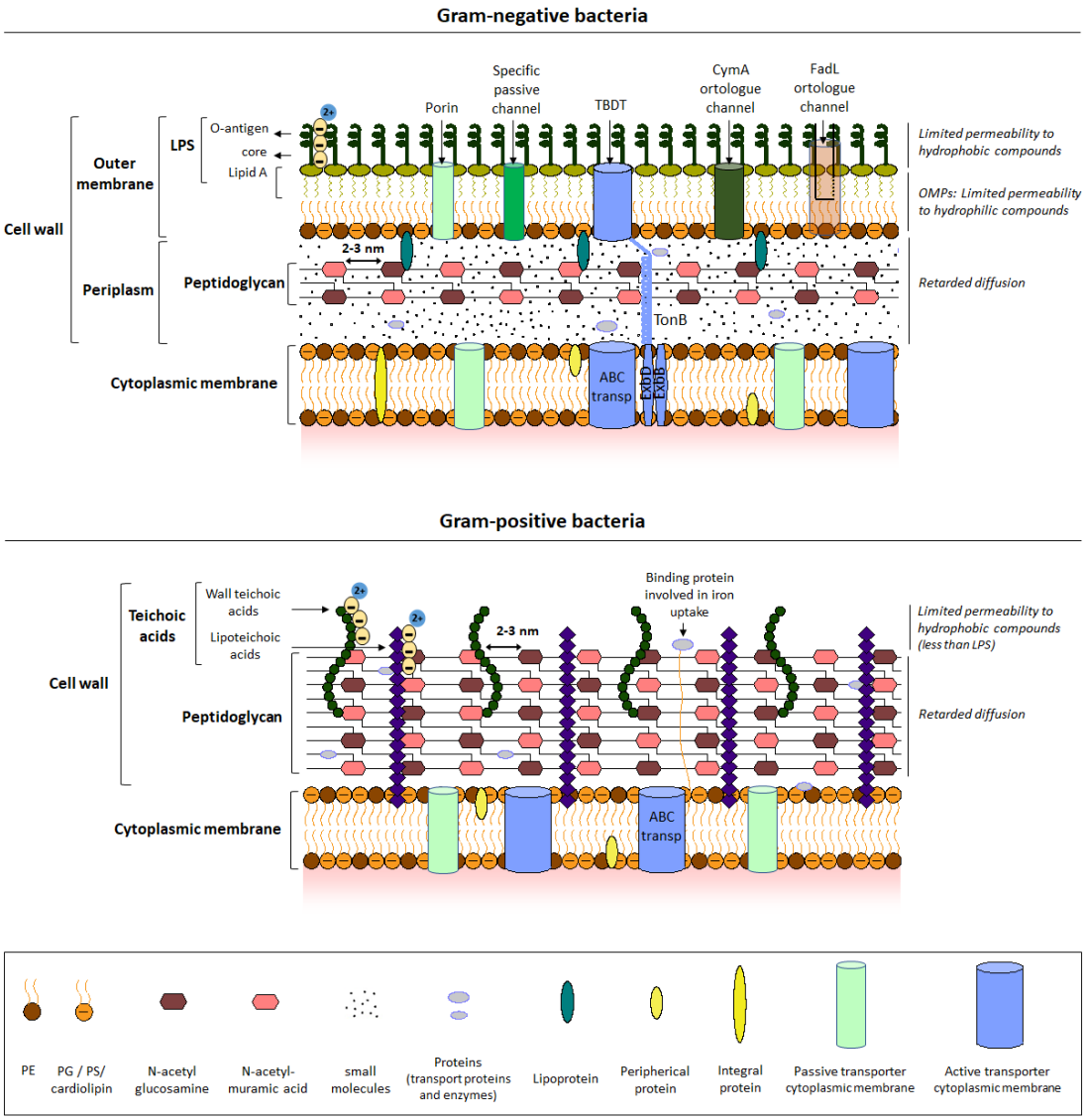
The gel-like periplasmic space can considerably retard diffusion; protein diffusion was slowed down up to 3.5 times when compared to diffusion in the cytosol [53]. Diffusion across the periplasm can occur either unassisted or mediated by periplasmic proteins [18].

Once at the outer leaflet of the cytoplasmic membrane, protein channels allow the passive or active transport of hydrophilic substrates into the cytosol [2]. For instance, most sugars such as maltose are carried by periplasmic proteins to ATP-binding cassette (ABC) transporters in the cytoplasmic membrane [42]. For nucleosides, no periplasmic binding proteins are known and they are transported across the cytoplasmic membrane via the transporters NupC and NupG in *E. coli* mainly energized by the pmf [42, 54]. Differently, hydrophobic substrates will pass through the hydrophobic lipid bilayer of cytoplasmic membrane by simple diffusion.

2.2.2.2 Internalization routes in gram-positive bacteria

The outermost side of gram-positive envelope comprises a negatively charged layer of teichoic acids anchored to a thick layer of peptidoglycan (Figure 2.1). This protects the fluidic cytoplasmic membrane where, as in gram-negative bacteria, passive and active protein transporters are present [2]. The hydrophilic WTA, similarly to the LPS in gram-negative bacteria, can limit the permeability to hydrophobic compounds, although far less than LPS (Figure 2.1) [27]. In agreement, It was shown that WTA-intact bacteria are more resistant to human antibacterial fatty acids than WTA-deficient bacteria [27].

The peptidoglycan has a similar porosity to that of gram-negative bacteria, with a mesh pore diameter around 2-3 nm and a theorized size exclusion of 50 kDa [26, 49]. Reaching the cytoplasmic membrane, passive and active transporter proteins mediate the internalization into the cytosol [2]. For instance, similarly to gram-negative bacteria, iron is associated to siderophores, transferrin or lactoferrin and actively transported [46]. The iron transport involves a protein anchored on the cytoplasmic membrane (resembling the gram-negative periplasmic binding protein) that binds the extracellular iron-siderophores, and an ABC transporter that brings the complex into the cytosol using ATP [46].



OMPs			
Passive diffusion			Active transport
General Porins Do not bind substrates	Specific channels Bind substrates with low affinity		TBDTs Bind substrates with high affinity
		Ligand-gated channels FadL orthologue channel / CymA orthologue channel	
≤ 0.7-0.8 nm (*OmpF*) ≤ 600 Da	≤ 0.5-0.9 nm (*LamB/Tsx*) ≤ 850 Da	0.8 nm ≤ 300 Da	1.1-1.4 nm ≤ 980 Da	≤ 2.5 nm (*FhuA*) ≤ 1360 Da

Figure 2.1 Bacterial envelope of gram-negative and gram-positive bacteria and its porosity to general substrates (according to the channel's characterization studies). LPS and teichoic acids are represented with their net negative charge (yellow circles) and the divalent cations attached (blue circles). Note that the number of circles depicted, as well as the number of OMPs are not representative. The porosity of OMPs is schematically detailed on the table with the respective

molecular weight exclusion, based on the size of the substrates known to penetrate the respective referred channels (it should be considered only as a reference, as no strict values are known). LPS – lipopolysaccharide; OMP – outer membrane protein; ABC transp – ATP-binding cassette transporter involved in the active iron uptake; PE – Phosphatidylethanolamine; PG – Phosphatidylglycerol; PS – Phosphatidylserine; TBDT – TonB-dependent transporter.

2.2.2.3 Internalization of antibiotics

Since their discovery before the 50's [55], antibiotics became the gold standard against bacterial infections. In this section, antibiotics are briefly introduced and how the bacterial envelope allows or restricts their access to their target site.

Antibiotics may act at the peptidoglycan, the cytoplasmic membrane or in the bacterial cytosol [56]. The peptidoglycan cross-linking and/or polymerization can be inhibited, thus affecting the cell wall synthesis, by the glycopeptides (vancomycin being the most common one) and β-lactams, including penicillins, monobactams, cephalosporins and carbapenems [57-59]. Intracellularly, antibiotics can target i) DNA/RNA synthesis, the case of quinolones (as ciprofloxacin), ansamycins (as rifamycin), actinomycins, novobiocin and albicidin, ii) protein synthesis, the case of tetracyclines, nitrofurans, macrolides (as clarithromycin and erythromycin), aminoglycosides (as tobramycin, streptomycin, kanamycin, gentamicin and amikacin), or chloramphenicol, and iii) folate synthesis, the case of sulfonamides and trimethoprim [56, 60]. Thus, in order to reach their targets, all antibiotics need to first cross the gram-negative OM. For antibiotics acting in the cytosol, the complete gram-negative and -positive cell envelope needs to be crossed.

Teichoic acids, in gram-positive bacteria, and LPS, in gram-negative bacteria, limit the permeation of antibiotics (Figure 2.2) [22, 25, 27]. Particularly, LPS is associated to the intrinsic resistance of gram-negative bacteria to several hydrophobic antibiotics [23]. In agreement, the expression of a shortened LPS core polysaccharide can result in increased susceptibility to aminoglycosides, macrolides or rifamycins [35]. Also, gram-positive bacteria deficient in WTA are more susceptible to hydrophobic antibacterial fatty acids which can presumably easily penetrate the less hydrophilic cell wall [27]. The peptidoglycan is believed not to be a significant barrier for antibiotics permeation [17]. Some antibiotics as aminoglycosides may also "force" their penetration into bacteria by disturbing the bacterial cell wall for "selfpromoted uptake" [23]. Studies on

gram-negative bacteria suggest that these antibiotics may compete with divalent cations for binding to LPS, thus destabilizing LPS and forcing its own penetration [23].

Most of the antibiotics that can then reach the outer/cytoplasmic membrane are able to traverse by passive diffusion (Figure 2.2) [33]. Some antibiotics can also be actively transported into the bacterial cytosol (Figure 2.2) [33].

From the antibiotics that passively diffuse, most of them have some degree of hydrophobicity to passively diffuse across the lipid bilayers, in gram-negative and -positive bacteria [17]. This way novobiocin, macrolides, tetracyclines and quinolones may penetrate into the bacterial cytosol [33, 34]. Diffusion of small hydrophilic antibiotics can also occur across the gram-negative OM through the general porins and specific passive channels [23, 33, 35]. This is the case of chloramphenicol, carbapenems and the protonated form of quinolones and tetracyclines [23, 33, 35]. Quinolones and tetracyclines, depending on the pH, may exist in an uncharged or charged form, crossing the OM respectively via the lipid mediated diffusion and porins [23, 35, 61]. Penicillins with a size similar to the OmpF pore may depend on their ability to interact with OmpF for translocation; zwitterionic ampicillin and amoxicillin can complementary interact with charged residues in the pore facilitating their penetration, while dianionic carbenicillin does not find an efficient binding site in the pore and is poorly translocated [35, 62]. The carbapenem imipenem can also penetrate *E. coli* via the general OmpF and *P. aeruginosa* via the specific OprD channel which uptakes basic amino acids and peptides structurally similar to this carbapenem molecule [30]. The gram-negative Tsx specific channel also allows the passive diffusion of albicidin, a relatively high molecular weight (~850 Da) antibiotic [33, 42]. Nevertheless, the size filtering effect of porins hinders or severally retards the diffusion of bigger hydrophilic antibiotics, as is the case of glycopeptides (for instance vancomycin is 1450-1500 Da) which are thus only active against gram-positive bacteria [17, 58]. Therefore, gram-negative bacteria are naturally resistant to several antibiotics and among gram-negative bacteria *P. aeruginosa* is particularly resistant since its OM is left with a slow porin (OprF) [17, 23].

Some antibiotics such as tobramycin are able to actively cross the OM through the expense of energy by bacteria; hence, their active transport contributes to increased antimicrobial effect [33, 63]. In addition, 0.8 kDa rifamycin and 1 kDa albomycin (albomycin is comprised by an antibiotic moiety naturally covalently linked to Fe^{3+}-siderophores) are known to use the FhuA TBDT channel [33, 64].

Natural resistance to antibiotics due to its restricted penetration especially into gram-negative bacteria greatly limits antibiotics efficiency. In addition, bacteria have also acquired resistance by elevated mutation rates that result in further limited permeability to antibiotics, in an altered antibiotic's target or direct inactivation of the antibiotic [56]. Therefore, novel antibacterials are of utmost need.

2.2.2.4 Internalization of NAMs

To respond to the antibiotic crisis, oligonucleotides, in particular nucleic acid mimics (NAMs), can provide valuable alternative solutions (as referred in 2.1). In this section, NAMs are first introduced and how the bacteria envelope properties may pose a barrier for the penetration of NAMs is subsequently discussed.

NAMs are promising alternative antibacterials by hybridizing and consequently inhibiting the expression of specific bacterial sequences. In addition, NAMs hybridization can be used to *in vivo* identify bacteria and their genes of resistance to antibiotics. Both as therapeutic or diagnostic agents, oligonucleotides act by specifically hybridizing *in situ* with complementary bacterial sequences, typically of RNA [7-9]. Among oligonucleotides, NAMs are especially interesting, as, contrary to traditional DNA oligonucleotides, NAMs are composed of modified DNA or RNA sugars that make them resistant to endonuclease degradation and improve their affinity towards RNA targets [65-68]. In particular, charge neutral peptide nucleic acids (PNA) and phosphorodiamidate morpholino oligomers (PMO), and the negatively charged locked nucleic acids (LNA) and 2'-OMethyl RNA (2'OMe) are promising to target bacteria [69-77]. These can be further modified on the backbone by including phosphorothioate internucleotide linkages (PS), instead of the normal phosphodiester linkages (PO), leading to further improved stability and affinity [78, 79].

The improved affinity towards target RNA sequences allows the design of shorter NAMs [78, 80] which in turn may decrease penetration impairment into bacteria. Actually, 11 mers bactericidal PMO could reduce the growth of *E. coli* in pure culture and in infected mice, although growth recovery was observed after 4h *in vitro* and 12h *in vivo*, with multiple doses necessary to sustain the growth reduction [75]. Also, 17 mers LNA/DNA were found to penetrate *E. coli*, but a very long incubation of 18h was performed and still only 14% of the bacteria showed association with the LNA/DNA [71]. Although penetration can happen at a certain extent, depending on the NAM, bacteria

physiology and models used, for the majority of the cases oligonucleotides penetration into bacteria is inexistent/poor and insufficient to eradicate bacteria [8]. Detection *in vitro* is also dependent on the use of permeabilization treatments of the bacterial envelope, to allow penetration of the detection probe; these are not only time-consuming, but also involve toxic/noxious chemicals that are not transferable to an *in vivo* situation [81]. Thus, although NAMs solve the stability and affinity issues of natural oligonucleotides, penetration into bacteria is still the major bottleneck for their use to manage infections [8, 74, 82, 83].

In agreement, studies using PNA in *E. coli* showed that the OM is the rate limiting layer in the kinetics of PNA penetration [69]. Also, the use of *E. coli* AS19 strain that has an abnormally permeable OM, owing to its severely depleted LPS layer, allowed a significant penetration of antisense 15 mers PNA, compared to the inefficient penetration in *E. coli* wild-type [84]. It was thus suggested that LPS, in particular, is a major barrier for PNA penetration into *E. coli* [84], probably due to the relatively high hydrophobicity of PNA, compared to charged oligonucleotides (Figure 2.2). After the OM, PNA needs to cross the peptidoglycan; Good et al. observed that antibiotics that block peptidoglycan formation did not improve PNA potency against *E. coli* and thus inferred that the peptidoglycan is not a barrier for PNA penetration [84]. However, this might not be the case for all bacteria. Studies on the hybridization of PNA for the detection of bacteria with different peptidoglycan thicknesses suggested that thick peptidoglycan layers of gram-positive bacteria may have a relevant effect on retarding PNA penetration, as seen for *Bacillus cereus* (for which peptidoglycan is 6-7 times thicker than that of *E. coli* [85]) (Figure 2.2) [86, 87]. It was observed that longer time and a higher PNA concentration gradient was required for efficient PNA hybridization in bacteria with thicker peptidoglycan layers [86, 87]. Actually, to detect bacteria *ex vivo* by *in situ* hybridization harsher permeabilization treatments are typically applied in gram-positive than gram-negative bacteria so that a sufficient amount of oligonucleotide can be timely internalized [88, 89].

For negatively charged NAMs to be internalized they would need to first cross the LPS and the OMPs in gram-negative bacteria. The LPS may constitute a barrier by electrostatic repulsion effect between the negatively charged LPS and NAMs. The application of NAMs within a salt solution can, nevertheless, counterbalance this repulsion [76, 77]. OMPs, as previously discussed, constitute a size dependent barrier for

hydrophilic compounds. Most NAMs have a molecular weight of about 2-4 kDa [8, 70]. Therefore, passive diffusion of the NAMs through the OM is highly unlikely, considering the size exclusion of porins of 0.7-0.8 nm in diameter and 600 Da in weight [90]. Even if specific passive channels and active TBDT channels could accommodate such structurally different compounds as the NAMs (compared to their substrates), they would still be most of the times too narrow for NAMs (Figure 2.1 and 2.2). Nevertheless, one report shows that the LPS defective *E. coli* AS19 strain allowed the penetration of an antibacterial LNA/DNA (17 mers) into nearly 50% of the bacterial population, while the *E. coli* wild-type strain was impermeable [82]. This suggests that in these 50% bacteria the OMP channels were not a barrier for NAMs penetration. Wider protein channels (up to 8.8 nm [91]) exist on the OM, which could be able to accommodate the NAMs, but these channels are dedicated to active protein export. It is thus unclear how negatively charged NAMs can penetrate the OM of some bacteria [82].

Far more outstanding is the ability of a 108 bp construct consisting of a DNA nanoparticle carrying a 13-mer PNA (to inhibit the expression of a β-lactamase) to penetrate *E. coli*, as inferred from the potentiation of a β-lactam antibiotic [92]. The DNA nanoparticle alone did not affect the bacteria viability, so the authors conclude that penetration is not dependent on membrane perturbation [92]. Penetration of this big construct should occur by an alternative unknown via that may be driven by the shape, size and sequence of the 3D DNA [92].

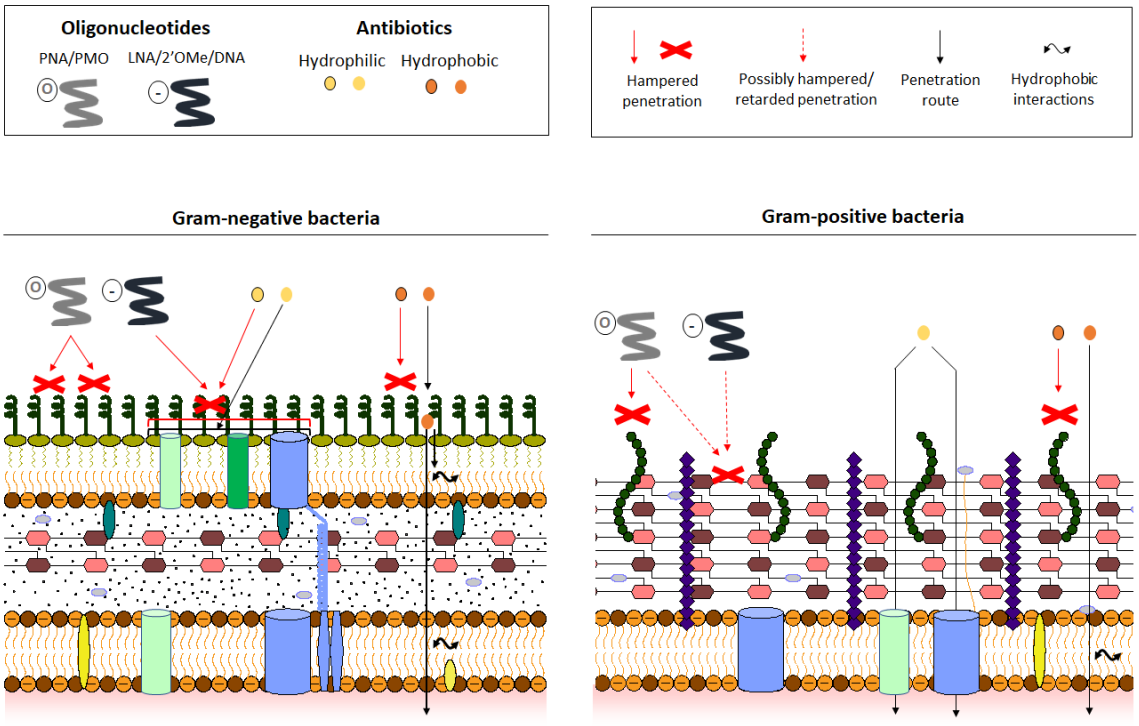


Figure 2.2 Representation of the most common interface interactions of oligonucleotides and antibiotics that determine their (in)ability to overcome the bacterial cell envelope, of gram-negative and -positive bacteria. The circles represent the antibiotics that can be internalized (circles without an outline) and the ones that cannot penetrate (circles with a black outline). The antibiotics identified as hydrophilic are the ones with a degree of hydrophobicity insufficient for diffusion through the lipid bilayers. On the gram-positive bacteria, the cross on top of wall teichoic acids should be considered as hampered penetration by teichoic acids in general (lipoteichoic acids and wall teichoic acids).

2.2.3 Carriers to overcome the bacterial cell envelope – mechanisms of interface interaction

As discussed in the previous two sections, the efficient use of antibiotics and NAMs is several times hampered by the poor ability of antibiotics and even more NAMs to overcome the bacterial envelope. Molecular transporters and nanoparticles (here generically referred as carriers) have been increasingly engineered to mediate intracellular penetration of antibiotics and oligonucleotides into bacteria [93]. Also, carriers may prevent drug efflux from the bacterial cells, as intracellular delivery of a high dosage of drug into bacteria may overwhelm the efflux pumps [94, 95] (the efflux of antibiotics will not be detailed here since it has already been extensive reviewed [34, 96-

98]). However, the application of carriers in bacteria is still on its infancy and has mostly relied on a trial and error approach. In order to better understand the potential of carriers to overcome the bacterial envelope a profound ´talk´ between nanotechnology and microbiology is lacking. Differently from animal cells, which can take up carriers by endocytosis [99], it is believed that bacteria do not endocytose (with the exception of a restricted group belonging to the phyla Planctomycetes [100]). Therefore, carriers can only overcome the bacterial envelope of live bacteria by i) fusing with it and thus delivering their cargo intracellularly into bacteria, which certain liposomes do, or by ii) directly penetrating with the associated cargo into the bacterial cell, which cell-penetrating peptides do. Therefore, these carriers will be the focus of this section, discussed in their mechanistic interaction with the bacterial cell envelope (2.2.3.1 and 2.2.3.2).

2.2.3.1 Liposomes

Liposomes started to be extensively investigated already in the 70's for drug delivery into mammalian cells [101, 102], but only recently their application into bacterial cells have gained interest. Liposomes, together with poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs), are the most popular NPs used in antimicrobial studies. However, they are mainly used to improve antibiotics pharmacokinetics and tolerability; in particular they are used to protect antibiotics (from interactions with biological fluids/barriers in the body that could reduce their activity) and/or increase the local antibiotic concentration (by sustained release of antibiotic from the liposomes), thereby decreasing the dose required and the associated toxicity [103-108].

Far less explored is the ability of some liposomes to directly overcome the bacterial envelope permeability barrier by fusing with bacterial membranes and thus directly delivering antibacterial cargo intracellularly [106]. Besides antibiotics, liposomes may offer an interesting solution to deliver negatively charged NAMs into bacteria, as NAMs can be easily complexed to cationic liposomes by electrostatic interactions. Despite its promise, to our knowledge only 3 reports have shown intracellular deliver of oligonucleotides into bacteria by liposomes so far [109-111]. In this section, the fusion of liposomes with the bacterial envelope is discussed (2.2.3.1.2), starting by their initial contact with the outer surface of the cell wall (2.2.3.1.1).

2.2.3.1.1 Initial interaction at the cell wall

Fusion between liposomes and bacteria can only occur at the level of gram-negative OM and gram-positive cytoplasmic membrane.

For gram-negative bacteria, liposomes first need to overcome LPS to reach the OM lipids. Cationic liposomes will bind electrostatically to the negatively charged bacteria surface [112]; in particular, binding to the gram-negative LPS may lead to chains flattening so that liposomes contact with the negatively charged leaflet of the OM (Figure 2.3). For negatively charged liposomes (such as dipalmitoylphosphatidylcholine (DPPC) /dimiristoylphosphatidylglycerol (DMPG) liposomes), Ca^{2+} is typically added in the liposomes medium which can limit the electrostatic repulsion between anionic liposomes and LPS; in addition, the present Ca^{2+} may also bridge the liposomes-OM interaction and contribute to dehydrate the OM PE thereby improving fusion [113]. Other divalent cations besides Ca^{2+} can mediate fusion, but it was found that the higher the atomic radius the lower the fusion, as it probably increases the liposomes-bacteria distance [113]. Electrostatic attraction of anionic liposomes to the metal cations normally bound to the LPS could also contribute to the initial interaction with the cell wall [114, 115].

In gram-positive bacteria, the interaction of liposomes with teichoic acids may occur the same way liposomes bind LPS in gram-negative bacteria. However, for fusion to occur in gram-positive bacteria, liposomes would need to overcome, not only the teichoic acids, but also the thick peptidoglycan in order to contact and fuse with the cytoplasmic membrane lipids. However, it is not simple to picture how liposomes could overcome the thick peptidoglycan of gram-positive bacteria (Figure 2.3).

2.2.3.1.2 Fusion

Fusion between natural membranes is actually a fundamental process in life occurring when two different bilayers merge into a single bilayer [116]. Fusion critically depends on the lipid composition of the two bilayers involved, which also applies for fusion between liposomal and bacterial bilayers [116]. The PE moiety is the most commonly referred fusogenic lipid [117, 118]. PE has a low hydration of its polar head group, which may decrease the fluid spacing between bilayers and thereby facilitate energetically favorable interactions between lipid bilayers [117-119]. In addition, PE has a cone-shaped molecular shape (small head cross section and large chain cross section) and ability to promote bilayer-to-hexagonal phase transition which may trigger membrane

destabilization [117, 118]. Thus, PE, in the form of the phosphatidylethanolamine (DOPE) lipid, has been incorporated into liposomes to produce fusogenic liposomes. DOPE containing liposomes have been used to successfully improve antibiotics penetration into the gram-negative *P. aeruginosa*, *E. coli*, *Klebsiella spp.* and *A. baumannii* [106, 119, 120]. Fusion with the OM is fast and occurs spontaneously by hydrophobic and van der Waals interactions [94].

Liposomes without DOPE have also been reported to be able to, in some cases, intimately interact and even fuse with bacteria, improving antibacterial drug permeation. A popular formulation is DPPC/DMPG negatively charged liposomes, frequently called "fluidosomes" [113, 120-123]. This designation comes from the ability of DMPG to increase liposomes fluidity (as it is a phospholipid with short acyl chains and increased number of unsaturated bonds, resulting in a relatively low gel-liquid crystalline transition temperature, Tc) [124, 125]. Studies report efficient interaction of DPPC/DMPG liposomes with bacteria, improving the permeation of antibiotics [121-123] and even of one antisense PS DNA oligonucleotide [109]. However, it has also been reported that antibiotic encapsulation into DPPC/DMPG liposomes even decreased the antimicrobial efficiency over the free antibiotic [106]. It was latter clarified that DPPC/DMPG are not fusogenic *per se* and that their improved antibiotic permeation depends on the presence of divalent cations as Ca^{2+} in the media to reduce the electrostatic repulsion between the negatively charged liposomes and bacteria surface (as tested for the gram-negative *P. aeruginosa*, *E. coli*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia* and the gram-positive *Streptococcus agalactiae* and *S. aureus*) [113, 120].

In addition, intimate interactions were observed between the more rigid liposomal formulations, such as distearoylglycerophosphocholine (DSPC)/cholesterol (Chol), phosphatidylcholine (PC)/Chol/dioleoyltrimethylammoniumpropane (DOTAP) or DPPC/Chol, and gram-negative bacteria like *P. aeruginosa* and *Burkholderia cenocepacia* [112, 126, 127]. Despite the inclusion of lipids with increased Tc and Chol (which should increase liposomes rigidity/stability), fusion/adhesion of these liposomes with the bacterial OM was shown [112, 126, 127]. However, others have seen that the inclusion of only 10% Chol dramatically decreased DPPC/DMPG fusion in *P.aeruginosa* [113, 120].

These studies using DPPC/DMPG and more rigid liposomes (containing Chol and DSPC) show that fusion with bacterial membranes may occur at some extent using non-DOPE liposomes. Although there is no PE on the liposomes, there is a high amount of

PE in the gram-negative OM that may mediate fusion [26, 128]. Indeed DPPC/DMPG liposomes cannot fuse with the more rigid cytoplasmic membrane of human cells (which contain cholesterol and have PC as the major lipid on their membrane surface and a minor amount of PE sheltered in the cytoplasmic membrane inner leaflet) [26, 128]. However, DPPC/DMPG liposomes could fuse with gram-negative bacteria and the higher the PE content of the bacterial OM, the higher the fusion [113, 128]. When PE (in the form of DOPE) is also included in the liposomes, the tendency for fusion between bacteria and liposomes is further increased, as seen by the dramatic improvement of the antibiotics efficacy when encapsulated in DOPE-containing liposomes in comparison to non-DOPE liposomes [112, 119, 120].

Although liposomes composition affects their fusion ability, no clear effect has been observed for liposome's size ranging from 100-800 nm on *P. aeruginosa* and *E. coli* [109, 120]. Besides PE content, the effect of bacterial features on fusion is not completely understood, but it can depend on fine details. For instance, different *P. aeruginosa* strains showed different degrees of fusion to PC/Chol/DOTAP liposomes and the expression of one 18-kDa OMP was found to be positively correlated with fusion [112].

Upon fusion with the gram-negative OM the carried molecules will be delivered into the periplasm (Figure 2.3). From here, the molecules will cross the viscous periplasmic space and contact with the peptidoglycan, where antibiotics that disrupt the peptidoglycan synthesis can act. Molecules acting on the cytosol (other antibiotics and NAMs) will cross the peptidoglycan and then the cytoplasmic membrane, as discussed in sections 2.2.2.3 and 2.2.2.4, respectively [33].

If fusion indeed occurs with the gram-positive cytoplasmic membrane, the drugs would be directly delivered into the bacterial cytosol. In fact, whether fusion can occur in gram-positive bacteria or the reported results actually derive only from "proximal" release in the bacteria close vicinity is a matter of debate. The studies considering fusion with gram-positive bacteria are indeed few; they were performed with the bacteria *S. aureus*, *Enterococcus faecalis* and *Streptococcus agalactiae* and liposomes composed of DPPC/DMPG liposomes (in the presence of Ca^{2+}), egg PC/DMPG/DSPE-PEG, DPPC/DOTAP/DSPE-PEG (with and without wheat germ agglutinin as a targeting moiety), dimyristoylglycerophosphocholine monohydrate (MC)/Chol/dihexadecyl hydrogen phosphate and MC/Chol/dipalmitoyl phosphatidylserine [110, 113, 129]. These studies mention possible fusion to explain the improved efficiency of the encapsulated

compared to free antimicrobial drug [110, 113, 129]. Also, one study in *S. aureus*, resistant to β-lactam antibiotics, showed that the bacteria susceptibility to antibiotics could be improved by the use of anionic egg PC/DMPG/DSPE-PEG liposomes, carrying a PS DNA to downregulate a gene of resistance to β-lactams [110]. The PS DNA was first complexed with the cationic polymer polyethyleneimine (PEI), resulting in an 80 nm complex that was only then encapsulated into the liposomes [110]. The observed downregulation was interpreted as a result of liposomes fusion and intracellular delivery of the PS DNA [110], but the authors did not test the effect of the complex alone nor the interaction of liposomes with *S. aureus*. Actually only one of the studies on gram-positive bacteria show interaction between liposomes and bacteria, using flow cytometry and fluorescence microscopy [130], and none of them show experimental evidence of direct intracellular delivery by liposomes, as far as I am concerned. Therefore, it may be possible that liposomes only contact with gram-positive bacteria cell wall and along the time allow "proximal" drug release [129]. This drug release close to the bacteria surface can increase the drug concentration gradient in the cell vicinity and thus lead to a higher drug diffusion across the peptidoglycan and cytoplasmic membrane [129]. "Proximal" release may also contribute to the delivery into gram-negative bacteria, especially when non-fusogenic liposomes (liposomes without DOPE) are employed.

Even upon liposomal interaction at the OM of gram-negative bacteria, it is very challenging to experimentally distinguish from adhesion on the OM from fusion with intracellular delivery and few studies address this question. Electron microscopy typically shows generic interaction/adhesion at the bacterial envelope. Immunohistochemistry combined with TEM (transmission electron microscopy) can be useful to find intracellularly delivered drugs, but it depends on the availability of specific antibodies, besides being a costly and time-consuming technique. Regular fluorescence microscopy and flow cytometry based on fluorescent constructs (liposomes carrying fluorescent molecules) can hardly distinguish between surface adhered fluorescent constructs and internalized fluorescent molecules, since their optical resolution limit is rather close to the bacteria size (resolution limit typically not higher than ~0.25 μm and width of most bacteria ~0.5-1 μm). Fillion et al. used fluorescence-activated cell sorting (FACS) at 37 °C and 4 °C to distinguish from liposomal fusion and only agglutination on the *E. coli* OM, respectively (as a way to explain the downregulation levels of antisense PS DNA encapsulated into DPPC/DPMG liposomes) [109]. However, at 4 °C the level of agglutination may be an underestimation of the agglutination that may occur at 37 °C.

In summary, liposomes, especially DOPE-containing fusogenic liposomes, are promising carriers to directly overcome the OM of gram-negative bacteria.

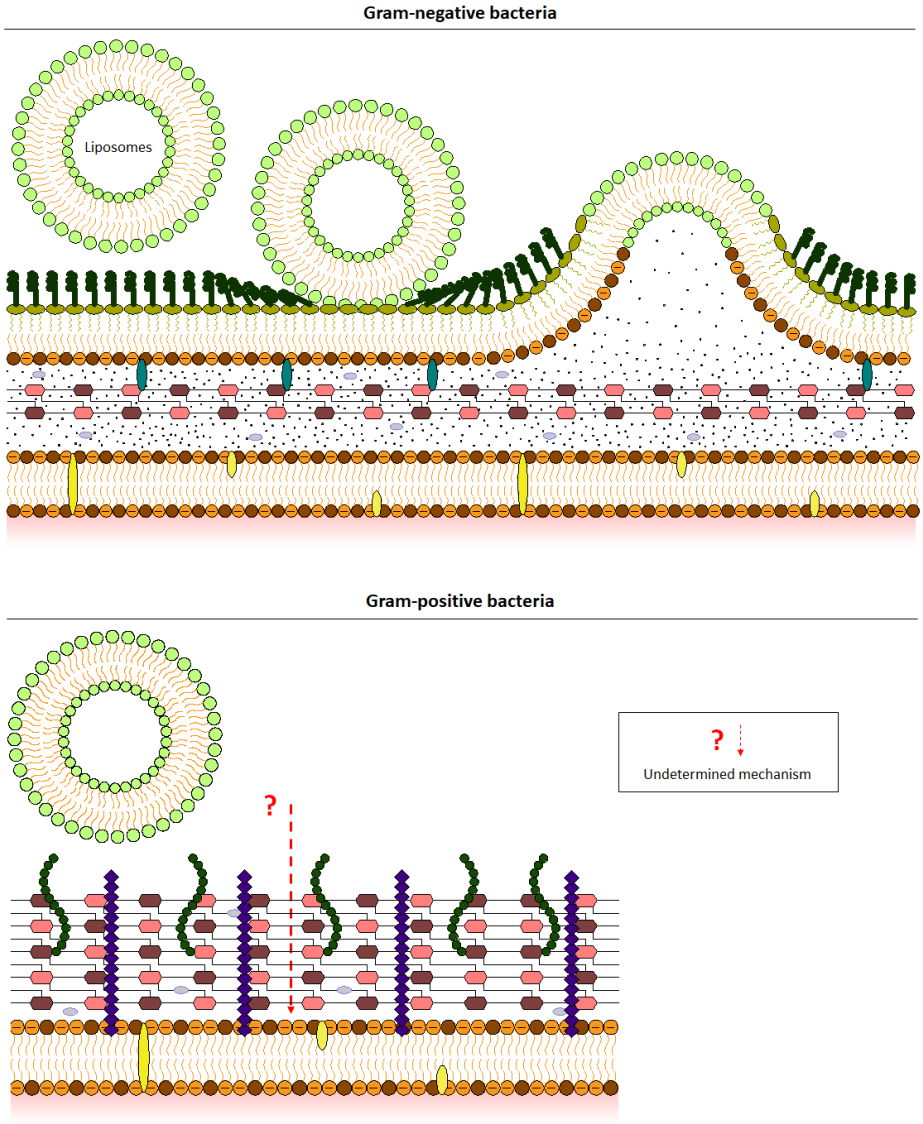


Figure 2.3 Representation of fusion between the liposomes (able to fuse with bacteria) and the bacterial membranes. In gram-negative bacteria, liposomes fuse with the outer membrane (Adapted from Wang et al. [113]). At the gram-positive envelope, liposomes would have to cross the thick peptidoglycan layer to fuse with the cytoplasmic membrane, via an undetermined mechanism.

2.2.3.2 Membrane active peptides

In alternative to liposomes, cell-penetrating peptides (CPPs) can be used to directly penetrate the bacterial envelope with their associated cargo. They have been used to do so using covalently conjugated charge neutral NAMs; very few reports focus on the conjugation to negatively charged NAMs and such conjugates have never been tested in bacteria. In alternative, recently some CPPs showed to have the ability to electrostatically complex negatively charged NAMs, forming NPs of variable sizes, and allow significantly improved uptake of NAMs in mammalian cells [80]. It was never tested if these CPP NPs hold a chance to penetrate in bacterial cells. Considering the literature available, this section will focus on the penetration mechanism of CPPs, as peptides and conjugates (without considering CPP NPs).

While CPPs are used as molecular transporters of drugs, structurally similar peptides named antimicrobial peptides (AMPs) have been used to directly kill bacteria *per se* (without any attached cargo). CPPs and AMPs are both 'membrane active' peptides that can translocate the bacterial envelope, only distinguished by the purpose of their use [131-133]. Most AMPs are antimicrobial by disrupting the bacterial cytoplasmic membrane, but some AMPs can also force their translocation across the cytoplasmic membrane (without disruption) and bind intracellular DNA, RNA or proteins, lethally inhibiting their synthesis/activity [134, 135]. The interaction of AMPs with the bacterial envelope is far more explored mechanistically than for CPPs. On the contrary, CPPs have been traditionally designed to carry drugs into mammalian cells, without cell lysis and only more recently they started being explored to carry antimicrobial drugs [136]. Due to their historically different application, AMPs and CPPs have been mostly discussed separately. However, modes of 'membrane activity' proposed in the literature are shared for CPPs translocation into mammalian cells and AMPs disruption of bacterial cells [135, 136]. This is a consequence of their similar features: i) short size, with less than 50 (AMPs) and 30 (CPPs) amino acids, ii) net positive charge and iii) significant amount of hydrophobic amino acids [131-133]. Therefore, the mechanistic interaction of AMPs and CPPs at the bacterial envelope will be following discussed together, as 'membrane active' peptides.

2.2.3.2.1 Initial interaction at the cell wall

The ‘membrane active’ peptides are not active on the cell wall, but only on the cytoplasmic membrane. Nonetheless, in order to reach the cytoplasmic membrane, they first need to interact with the cell wall. Although this interaction is rarely studied, it is believed to be initially mediated mostly by non-specific electrostatic interactions (Figure 2.4a) [134, 135]. In gram-negative bacteria, the peptides (via their cationic residues) will be electrostatically attracted to the anionic LPS, possibly competing with divalent cations for LPS binding, and then traverse the OM via self-promoted uptake (a process that has not been thoroughly investigated) [69, 137-139]. In gram-positive bacteria, the cationic peptides will probably be attracted to the anionic teichoic acids, in the same way as they are to LPS in gram-negative bacteria [26, 138]. In addition, specific interaction at the cell wall surface mediated by structural affinity can promote the initial interaction [21, 134, 140].

Reaching the peptidoglycan, it has been assumed that the ‘membrane-active’ peptides (which are typically below 5 kDa) may be able to diffuse through the peptidoglycan mesh (considering the size exclusion for globular hydrophilic molecules of 50 kDa) [26, 138]. However, this has not been studied systematically for different peptidoglycan layers and the role of peptidoglycan in the interaction with the peptides is not clear [26]. Differently, the interaction of anionic lipoteichoic acids with the peptides may reduce their concentration at the cytoplasmic membrane and thus their antimicrobial effect [26]. Still, the ‘membrane-active’ peptides, in particular AMPs, are expected to cross the cell wall quickly, as dissipation of the electrochemical gradient across the cytoplasmic membranes is seen within a few seconds of bacteria exposure to AMPs [140, 141].

2.2.3.2.2 Interaction at the cytoplasmic membrane

Reaching the cytoplasmic membrane, the peptides first bind to the lipid membrane by electrostatic interactions (between the cationic amino acids and the anionic phosphate heads of the phospholipid bilayer) (Figure 2.4a) [21, 63, 135, 142]. When the peptides reach a threshold concentration on the membrane (which for AMPs is about full membrane coverage), the peptides insert into the membrane via hydrophobic interactions (between the hydrophobic amino acids and the hydrocarbon bilayer core) (Figure 2.4a) [21, 63, 135, 142].

The modes of membrane insertion/translocation depend on the peptide and the membrane [21]. For mammalian cells, CPPs have been proposed to translocate the mammalian membrane by endocytosis and direct penetration [136, 143]. While endocytosis is not applicable in bacteria, three different modes have been proposed for direct penetration of CPPs in mammalian cells which are the same proposed for AMPs in bacterial cells [135, 136]. Thus, carrier CPPs and antimicrobial AMPs should force their insertion/translocation into the bacterial cytoplasmic membrane using the same mechanisms [144]. The three typically proposed models are: the barrel-stave pore, the toroidal pore and the carpet mechanism (Figure 2.4b) [131, 134, 136, 140]. In the **barrel-stave pore** the peptides insert perpendicularly to the membrane surface and pack together parallel to the hydrocarbon chains, forming an aqueous pore (Figure 2.4b) [131, 134, 140]. The cytoplasmic diameter of the barrel-stave pore formed by the AMP alamethicin has been estimated by structural studies to be around 1.8 nm [145]. In the **toroidal pore** model, the peptides also insert perpendicularly in the bilayer but they induce a toroid-like curvature in the membrane, so that lipid inner and outer leaflets are forced to bend towards one another stablishing a continuity [131, 134, 135, 140]. The pore is thus formed by both the inserted peptides and the phospholipid head groups (Figure 2.4b) [131, 134, 135, 140]. The AMPs melittin and magainin form toroidal pores in lipid vesicles of 2.5-3 nm and 3.0-5 nm, respectively [146, 147]. The (barrel-stave/toroidal) pores may allow the passage of molecules as big as 40 kDa, according to studies performed using the AMP maculatin that forms pores of 1.4 and 4.5 nm in diameter on lipid vesicles mimicking *S. aureus* [148]. The formation of barrel-stave/toroidal pores requires that the peptide is long enough to span the hydrophobic core of the bilayer [148]. Differently, this is not needed in the **carpet model**, since the peptides absorb parallel to the bilayer surface and produce a detergent-like effect that eventually results in membrane disintegration into micelles (Figure 2.4b) [131, 132, 134, 135, 140]. Therefore, smaller peptides, can act via this model [149]. This is the case for the AMPs aurein and cecropin, the later shown to form pores on *E. coli* of 4.2 nm in diameter [150].

Instead of (or in addition to) self-assembling to form pores, peptides may adsorb onto the membrane and lead to dissipation of the transmembrane potential, pH gradient and osmotic balance, as well as to membrane retreating [132, 138, 148]. Different modes of action can be related, depending on the peptide concentration. For instance, at low concentrations cecropin was bactericidal to *E. coli* by dissipation of transmembrane electrochemical ion gradients (as judged from ion gradients dissipation in lipid vesicles),

while higher concentrations were needed to release cytoplasmic contents [151]. The extent and duration of the membrane action of the peptides will dictate the viability of the cytoplasmic membrane. It may depend on the specific bacteria and peptide's concentration, besides specific peptide properties (charge, hydrophobicity, sequence, structure and size) [134, 135, 144, 152].

For CPPs, and AMPs with intracellular targets, translocation of the cytoplasmic membrane without disruption can occur. How exactly the peptides, after inserting into the membrane, translocate into the cytosol is still a matter of debate. For AMPs (with intracellular targets), the peptides that translocate can actually be the ones forming the pore; the disintegration of this transient pore prompts the pore forming peptides to be stochastically internalized into the cytosol [144, 153]. This view is supported by the fact that the concentration of membrane-bound AMPs is typically several orders of magnitude higher than that found in solution; this indeed suggests that most AMPs are involved in pore formation rather than free in solution to freely translocate into the bacterial cytosol [140, 144]. Therefore, it is reasonable to consider that AMPs at high concentrations or/and with very high affinity to the bacteria will remain massively inserted in the membrane, hindering peptide translocation and pore recovering, thus resulting in bacteria death – explaining why most AMPs are antibacterial by membrane disruption.

CPPs have a lower affinity than AMPs to the bacterial membrane; hence, in comparison to AMPs, a lower relative amount of peptide will probably be inserted into the bacterial cytoplasmic membrane, forming the pore. Therefore, more CPPs will be free in solution and thus available to diffuse through the formed pore into the bacterial cytosol. The pores created by 'membrane active' peptides could be large enough to allow CPPs to be internalized into the bacterial cytosol (if it is considered that the pores created by CPPs can be as large as the ones described for AMPs – 2 to 5 nm in diameter, penetration of molecules up to 40 kDa [145-147, 154]). In addition, it can be reasoned that with less peptide bound to the membrane, pore recovery will happen more easily for CPPs than AMPs, explaining the lower membrane disruptive potential of CPPs. When the concentration is sufficiently high, CPPs can also form permanent pores and lead to bacterial membrane disruption, as several studies show that the carrier CPP can contribute to the antibacterial effect of their transported cargo [63, 69].

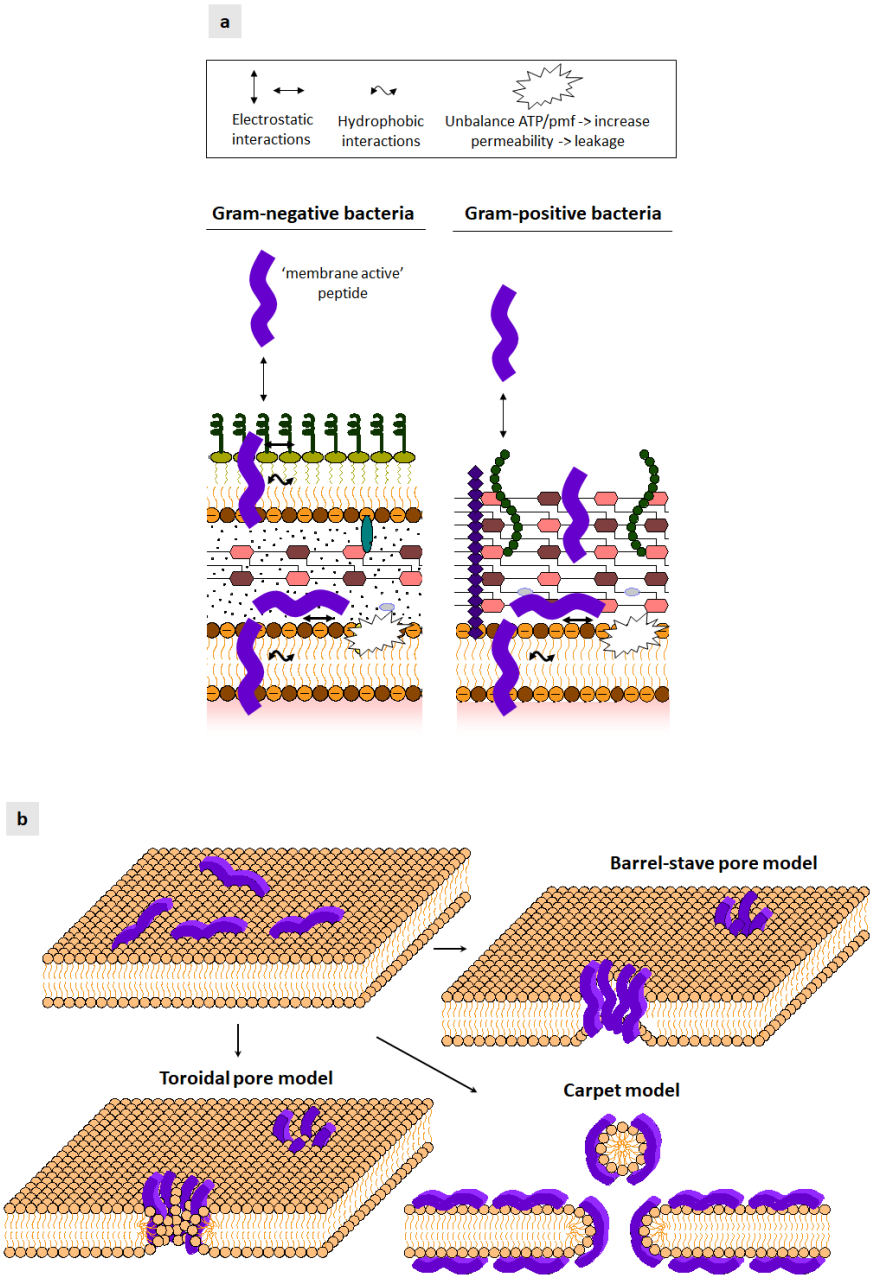


Figure 2.4 Representation of a) the most relevant initial interactions of 'membrane active' peptides at the bacterial cell envelope of gram-negative and gram-positive bacteria [26, 139, 154], and b) the subsequent peptide insertion into the bacterial cytoplasmic membrane, according to the possible different models proposed [21, 131], irrespectively of the gram type (thus the represented membrane phospholipids are not differentiated).

2.2.3.2.3 CPPs conjugated to antimicrobial drugs

Applications of CPPs to manage bacterial infections have focused on their covalent conjugation to antibiotics and, mostly, to antibacterial antisense oligonucleotides, in order to transport them directly into the bacterial cytosol.

The highly cationic 12 amino acids (a.a.) Pen peptide (RQIKIWFQNRRW) was designed based on the 16 a.a penetratin (a well-known CPP for mammalian cells) and conjugated to the antibiotic tobramycin (lethal by ribosome inhibition), in order to increase the antibiotic uptake in persister *E. coli* and *S. aureus* [63]. Persister bacteria have decreased active transport and thus do not take up tobramycin. The conjugated (Pentobra) killed more 4-6 logs of persister bacteria than free tobramycin [63]. This was a result of the combined bactericidal effect of the antibiotic and also the peptide [63]. Pen could *per se* lyse bacteria due to induced strong permeabilization, higher than Pentobra [63]. Nevertheless, Pentobra was more bactericidal than Pen towards *E. coli*, showing the importance of the tobramycin effect combined to that of Pen permeabilization [63].

An earlier report prepared the cationic peptide $(KFF)_3K$ to permeabilize the gram-negative OM to hydrophobic antibiotics (which penetrate intact OM very poorly) [155]. Pre-treatment of bacteria with this peptide was found to sensitize enteric bacteria, such as *E. coli*, *E. cloacae*, *K. pneumoniae*, and *Salmonella* Typhimurium, to the hydrophobic antibiotic rifampin [155].

This same peptide $(KFF)_3K$, conjugated to PNA, was also found to permeabilize the OM of *E. coli* to antisense PNA [69, 72, 84]. This peptide has been widely used since then for the transport of neutral antisense oligonucleotides PNA and PMO [69, 70, 156, 157], being the covalent conjugation of negatively charged oligonucleotides technically more difficult [80]. Besides *E. coli*, $(KFF)_3K$ was also shown to improve the potency of PNAs and PMOs into the gram-negative *Salmonella* Typhimurium, *Klebsiella pneumoniae* and gram-positive *Staphylococcus aureus* and *Bacillus subtilis* [158-160]. This improved potency was ascribed to $(KFF)_3K$ mediated improved penetration [69]. Depending on the concentration, the $(KFF)_3K$ could also contribute to the killing efficacy of the antisense PNA by causing bactericidal cell leakage [69]. $(KFF)_3K$ could act not only against the cytoplasmic membrane, as normally considered for 'membrane active' peptides, but also disrupted the OM of *E. coli* [69, 139].

Other peptides have also been tested. It was observed that $(RXR)_4XB$ conjugated to an antisense PNA provided more potent bactericidal effects than $(KFF)_3K$ against the

tested gram-negative *Salmonella enterica, Klebsiella pneumoniae, Escherichia coli*, and *Shigella flexneri in vitro* (the last two also *in vivo*), without peptide mediated killing [157]. Also, the CPP Tat (having the sequence: GRKKRRQRRRPQ which is derived from the transactivator of transcription (TAT) of HIV) provided more efficient antisense PNA killing of the gram-positive *Streptococcus pyogenes* than $(KFF)_3K$, while inducing less unspecific CPP toxicity [142, 161, 162]. The same was found for the peptides $(RXR)_4XB$ and $(RFR)_4XB$ on the gram-positive *Listeria monocytogenes* [83].

Adding to the peptide sequence, the extent of CPP permeabilization has been observed to depend on the bacteria. Vaara et al. showed that $(KFF)_3K$ *per se* was bactericidal against the gram-positive Micrococcus tested, while it did not affect the viability of the tested gram-negative (*E. coli, E. cloacae, K. pneumoniae*, and *Salmonella* Typhimurium) [155]. Hatamoto et al. also found that the gram-positive bacteria *Bacillus subtilis* and *Corynebacterium efficiens* exhibited increased susceptibility to $(KFF)_3K$ conjugated to antibacterial PNA than the gram-negative *E. coli*; however, the gram-negative bacterium *Ralstonia eutropha* was not affected by the conjugate [160]. Thus, it is not evident that a gram dependent susceptibility exists.

The cargo can also affect the peptide mediated translocation of the conjugate in different ways. For instance, as already mentioned, the conjugation of the cationic tobramycin to the Pen peptide decreased the permeabilization of *E. coli* and *S. aureus* compared to the Pen peptide alone [63]. In contrast, the conjugation of uncharged PNA to the $(KFF)_3K$ peptide made it more membrane-active towards *E. coli* than the $(KFF)_3K$ peptide alone [69]. The authors hypothesize that PNA, being charge neutral, could benefit membrane interaction by increasing the conjugate amphipathic character [69].

In summary, CPPs conjugates have shown to be promise to directly penetrate through the bacterial envelope.

2.3 Mucus barrier

Besides the critical bacterial envelope barrier, mucus is also a stringent barrier that needs to be overcome for the success of NAMs as diagnostic and therapeutic agents.

Mucus is a complex secretion that protects all organs that are exposed to the external environment from foreign entities, including bacteria, drugs, macromolecules and nanoparticles [15]. However, pathogenic bacteria have evolved strategies to bind to mucus and also overcome it to adhere to the epithelia underneath and proliferate at their surface [16, 163]. This is the case in the gastrointestinal (GI), urinary, reproductive or respiratory tract [16, 164]. The mucus in the stomach is particularly important, not only because it is especially thick and viscous [165], but also because oral administration is the preferred route of administration of antibacterials [166]. Therefore, it is critical that antibacterials can diffuse through the gastric mucus and, importantly, without losing their activity [166].

2.3.1 Gastric mucus

Irrespectively of its location, mucus is a complex, viscoelastic gel, mainly composed of water and mucins, but also lipids, proteins, peptides, DNA, ions, bacteria and cellular debris [164, 167]. Mucus has a bulk viscosity from 1000–10000 times that of water [168]. Viscoelasticity of the mucus is greatly determined by mucins concentration and mucus hydration, but also affected by the protein, lipid and ion content, and pH [169].

Mucins form packed, entangled fibers of 0.5-40 MDa in size, composed of glycoprotein monomers cross-linked by disulfide bridges [15, 164, 166]. They are composed of a protein core, formed by repeating units of serine, threonine and proline, that is linked to oligosaccharides (Figure 2.5) [166]. These oligosaccharides include N-acetylgalactosamine, N-acetylglucosamine, fucose, galactose, sialic acid, mannose and sulfate [15]. These glycosylated regions are highly hydrophilic, with a strong negative charged surface given by the sialic acid and sulfate content (Figure 2.5) [164, 167]. The oligosaccharides vary in length and branching, according to the individual and body location; in the stomach, long, branched oligosaccharides may have a relevant protecting role from proteolytic degradation by gastric proteases [170]. The glycosylated regions are separated by regions devoid of glycans and rich in cysteine that are relatively hydrophobic

(Figure 2.5) [167]. These cysteine residues are involved in disulfide bonds that connect two adjacent monomers (Figure 2.5) [15, 167].

Mucins can be divided into cell membrane-bound mucins and secreted mucins; in the stomach the major mucins are MUC1 (membrane-bound mucin), MUC5AC and MUC6 (both secreted mucins) [171, 172]. These two types of mucins are involved in forming the two mucus layers: the cell-bound mucins form the cell-adherent (or firmly adherent) mucus layer, while the secreted mucins form the outer loosely adherent mucus layer [164]. The loosely adherent layer binds undigested material and is continually removed by shearing forces, while the unstirred adherent layer is maintained firmly adhering to the epithelium, protecting it from peristalsis-induced damage [164, 166, 173]. The balance between mucins secretion, to lubricate the epithelium, and mucus shedding and degradation, to remove undigested food and potentially damaging particles, determines the mucus thickness [167]. In the human stomach, the mucus thickness can vary from 50-450 μm, with an average value of 180 μm [174, 175]. The gastric mucus should be the thickest in the body, followed by the colonic mucus (110-160 μm) [166, 176], although reported values vary considerably (affected by confusion regarding total mucus and the particular firmly and loosely adherent layers) [177]. In addition, the mucus thickness and viscoelasticity varies between individuals, their fed state and also their disease state [166, 175]. For instance, in response to *Helicobacter pylori* the bulk viscoelasticity of gastric mucus might increase [178], similarly to what happens in cystic fibrosis [164, 179].

Also, the pore size of the human mucus network is poorly characterized for most mucosal locations [166]. Actually, the mucus porosity is heterogenous at the micro and nano scale [164]. The gastric mucus has not been characterized, but in porcine intestinal mucus, pores from 100 nm to several μm were found by cryo-SEM (scanning electron microscopy) [180].

In summary, mucus forms a flexible structure with mucins possessing alternating hydrophobic and hydrophilic regions with a net negative charge [167]. Also the lipid constituents of mucus contribute to the hydrophobic barrier properties of mucus [166]. Thus, mucus can entrap foreign particles by several low-affinity hydrophobic bonds and/or hydrophilic interactions, including electrostatic interactions and H-bonds [167]. In addition, the mucus mesh can sterically obstruct particles smaller than its pores [181]. Together, these properties turn mucus into a very efficient interactive and steric permeation barrier [181].

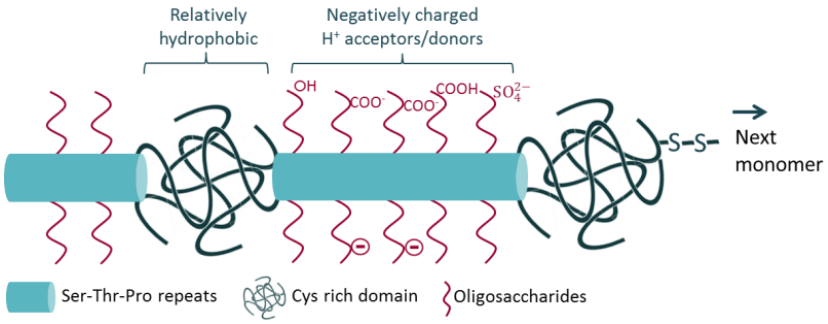


Figure 2.5 Illustration of mucin glycoproteins, formed by the protein core, composed of serine (Ser) - threonine (Thr) - proline (Pro) repeats, linked to oligosaccharides that provide a net negative charge to mucins and several proton acceptors and donors. These building blocks are flanked by cysteine (Cys)-rich domains, relatively hydrophobic, involved in dimerization via disulfide bridges. (Adapted from Bansil et al. [15])

2.3.2 Carriers to overcome the gastric mucus

For orally delivered NAMs to exert their role as diagnostic or therapeutic agents against bacterial infections NAMs need to cross the gastric mucus while keeping their activity. As NAMs are relatively small, steric obstruction by mucus might not be a problem, although this has never been studied. Similarly, steric obstruction may not be an issue for the penetration through the gastric mucus of CPPs covalently conjugated to NAMs. If CPPs are not conjugated to the NAMs but rather complexed to the NAMs, NPs are formed. In this case, size becomes a potentially significant issue, as it also the case when using liposomes to carry the NAMs. Besides steric obstruction, NPs also need to avoid chemical interactions with the gastric mucus in order to overcome this barrier.

Generally, for NPs to penetrate deep into the mucus and therefore avoid rapid clearance in the loosely adherent mucus, it is necessary that i) the NPs are small enough to hold a chance to diffuse through the mucus mesh pores, and ii) hydrophobic and/or charged domains of the NPs do not significantly interact with the mucus [164]. The most used strategy to avoid these interactions is the modification of NPs surface with PEG (polyethylene glycol) chains, a strategy typically referred as PEGylation [166, 182]. PEGylation is inspired on the surface properties of capsid viruses adapted to diffuse through mucus and infect mucosal surfaces [167]. Viruses that infect mucosal surfaces are approximately 30-200 nm in diameter, and include polio of 38 nm in diameter,

hepatitis B of 43 nm, adenovirus of 60-90 nm, HIV (human immunodeficiency virus) of 120 nm and HSV (Herpes simplex virus) of 180 nm [167]. When comparing the diffusion in cervical mucus of capsid viruses and polystyrene spheres of similar size it was found that the viruses can freely diffuse, opposed to the polystyrene NPs that bind to the mucin fibers [183, 184]. The surface of the polystyrene NPs is hydrophobic, thus prone to hydrophobic interactions with mucus. Differently, the surface of capsid virus (as found for of Norwalk and rhinoviruses that rapidly cross gastric and nasal mucus) is i) hydrophilic [184], thus preventing hydrophobic interactions with mucus, and ii) densely coated with equal densities of positive and negative charges, resulting in a net neutral charge and thus avoiding electrostatic interactions with the negatively charged glycan domains of mucus [167]. Based on this, covering the NPs with PEG, a polymer Generally Regarded as Safe (GRAS) by the FDA and with a long history of use in humans, has been widely used to improve NPs penetration in different types of mucus [185-188]. PEG forms a hydrophilic shield that protects the hydrophobic and charged NP's surfaces from binding interactions with mucus [166].

The shielding effect of PEG depends on the PEG molecular weight and density of PEG on the NP's surface [185]. The molecular weight is proportional to the polymer chain length and it should, therefore, be sufficiently high [189, 190]. PEG of 2 kDa or 5 kDa provides good shielding; however, 10 kDa PEG seems to be too heavy, since NPs modified with 10 kDa PEG diffused in human cervicovaginal mucus 1000-fold slower than the same NPs modified with 2 kDa PEG [191]. In addition, a sufficiently high density of PEG coverage is important for optimal shielding of the NPs [192], with 10 mol% PEGylated lipids normally providing sufficient shielding of liposomes [193]. The density of PEG coverage affects the PEG chains conformation on the surface of the NPs. With a low density, the distance between adjacent PEG chains is high enough to allow them to adopt a "mushroom" configuration; differently, with a high density, the distance between the PEG chains is small and, hence, they stretch away from the NP surface, forming a "brush" layer that provides optimal protection [194]. In this sense, PEGylation showed to increase the mobility of negatively charged carboxylate polystyrene NPs in several types of mucus, including murine colonic mucus [190], murine intestinal mucus [188], murine airways mucus [195], human cystic fibrosis sputum [186], and bovine vitreous humor [187], using NPs of sizes from 40-500 nm in diameter. Using charged polystyrene NPs, positively charged NPs show higher immobilization than negatively charged ones, as cationic surfaces will be strongly attracted to the negatively charged mucus glycans [186,

187]. In addition, the shielding effect provided by PEG also improves the colloidal stability of NPs, which is important to prevent NPs aggregation in biological media [185, 193].

For penetration in mucus, an inert NP's surface needs to be combined with suitable NP's size. To the best of our knowledge, the size filtering effect of mucus has never been tested in mucus from the stomach, only from the intestine. Polystyrene NPs modified with PEG were used to serve as model inert NPs in intestinal mucus, so that the size effect can be evaluated without the influence of binding interactions with mucus. In porcine and murine intestinal mucus, it was reported that NPs of 500 nm in diameter could not diffuse through mucus, while the 200 nm ones were able to penetrate murine small intestinal mucus, but not colonic mucus [190, 196]. Accordingly, 200 nm and 500 nm inert NPs distributed throughout the colorectal epithelium of mice *in vivo* less uniformly than 100 nm and 40 nm NPs [188].

Instead of designing particles to penetrate the mucus, some authors use mucolytics to affect the mucus' structure and this way facilitate the NPs penetration [166, 197, 198]. The mostly used mucolytic is N-acetylcysteine (NAC), which cleaves the mucins by reducing the disulfide bonds [199]. However, its effect on the penetration of mucus by NPs is not completely clear; NAC treatment of cystic fibrosis sputum improved the penetration of PEGylated 200 nm NPs, but resulted only in small improvement for unmodified NPs [200]. More importantly, mucolytics disrupt the mucus and compromise the protective barrier properties of mucus, permitting increased access of pathogens to the underlying epithelium [199, 201].

In order to evaluate the ability of molecules/carriers to safely cross the gastric mucus it is critical to choose a relevant model that is representative of the human gastric mucus. As mucins are the main component of mucus, studies have used mucin suspensions or mixture thereof to mimic the mucus barrier [202-206]. However, these models are too simplistic, as the complex mixture composing mucus affects its properties and reactivity against molecules [207, 208]. This was confirmed by rheology studies showing that a suspension of dried porcine gastric mucin (commercial mucins typically used to mimic GI mucus) does not compare with gastric mucus isolated from pigs [208, 209]. Instead of mucin suspensions, native mucus extracted from animals is the suited model for gastric mucus [207]. While cystic fibrosis sputum or cervical mucus is easily obtained from humans, gastric mucus is not. Therefore, an animal having a stomach that resembles the human stomach should be chosen, since important variations are found between different

animals [166]. Varum et al. compared in the same study the thickness of gastric mucus from pig, rabbit, and rat and found that it was higher for the pig (190.7 ± 80.7 mm), than in the rabbit (155.1 ± 85.8 mm) and much lower in the rat (31.3 ± 11.4 mm) [210]. In addition, considering the size, anatomy and physiology, the human stomach is considerably more similar to the stomach of pigs than it is to those of rabbits or mice/rats [211]. Therefore, porcine gastric mucus is generally considered an appropriate model for human gastric mucus and gastric infections [210, 211] and it was thus used in this work.

2.4 *Helicobacter pylori* infection

In this work, we focused on the barriers of gastric mucus and the bacterial envelope to manage *Helicobacter pylori* (*H. pylori*) infection, the most common and prevalent chronic infection in the world [212, 213]. It infects the stomach of more than 50% of the world population and it is associated with gastric and duodenal ulcers (also called peptic ulcers) and gastric cancer [212, 213]. *H. pylori* is mostly located deep in the gastric mucus and also adhering to the surface of the underlying gastric epithelial cells [214, 215]. Most recently, confocal microscopy coupled 3-dimensional reconstruction of entire gastric glands showed that *H. pylori* can also be found in deeper regions of the gastric glands [216].

H. pylori is a gram-negative microaerophilic bacterium, with a spiral shape, size about 2-4 µm x 0.5-1.0 µm, and having 2 to 6 sheathed unipolar flagella of ≈ 3 µm in length [211, 212, 217]. *H. pylori* is a fastidious microorganism that requires complex media for *in vitro* growth and still grows slowly [211, 212, 217]. Besides its spiral shape, *H. pylori* can also adopt a coccoid form, after prolonged *in vitro* culture or antibiotic treatment [211]. It is believed that coccoids are viable but nonculturable cells [211]. Evidence exists that transformation from spiral to coccoid form is a protection strategy used by *H. pylori* for extended survival under adverse environments [218]. The exact mechanism how *H. pylori* is acquired is unknown, but it has been mostly considered that the infection is acquired during childhood, via direct person-to-person transmission by an oral-oral or fecal-oral route [211]. However, since coccoids can persist in less optimal environments such as drinking water distribution systems and are able to infect mice, alternative routes of infection related to poor sanitary conditions have been considered [218-220].

2.4.1 *H. pylori* penetration of the gastric mucus

H. pylori is remarkably adapted to the harsh gastric conditions, in order to establish the infection and persist within the host for many years [221]. *H. pylori* is able to withstand the acidic pH of the stomach, cross the gastric mucus layer and adhere to the surface of the gastric epithelial cells [221].

In the stomach, the pH is 1-2 in the gastric lumen and increases along the mucus layer depth towards a neutral pH in the surface of the gastric epithelium [222]. Although *H. pylori* can survive the acidic pH of the gastric lumen, *H. pylori* is not an acidophile but rather a neutralophile (it grows at pH from 5.5 to 8) [211]. Therefore, in order to protect itself from the acidic pH, *H. pylori* uses two strategies: the local increase of the gastric pH in its surroundings and the ability to diffuse through the mucus towards the near-neutral pH at the gastric epithelium's surface [222, 223]. The ability to increase the local pH is given by the production of urease. Urease hydrolyses urea into ammonia and carbon dioxide; ammonia elevates the pH in the bacteria microenvironment, providing temporary protection against the gastric pH [212]. Urease is expressed in all *H. pylori* strains and has a much higher affinity for urea than the urease of other bacteria, for optimal use by *H. pylori* of the low urea amounts presents in the stomach [222].

To move towards higher pH at the epithelium's surface *H. pylori* crosses the thick gastric mucus [224]. Considering the micrometer size of bacteria, it would be expected that *H. pylori* would be sterically entrapped in the mucus mesh, as it happens with most NPs. The helical shape of the bacteria facilitates the polar flagella to propel *H. pylori* through the mucus [212, 224]. However, the flagella are insufficient for muco-penetration; most importantly, *H. pylori* uses the same urease-induced pH elevation (described above) to diffuse through the mucus and reach the epithelium surface [222]. The local pH elevation increases the mucus fluidity, allowing *H. pylori* to swim towards the epithelium [225]. This was experimentally showed adding *H. pylori* to a suspension of porcine gastric mucin (PGM) (obtained and purified from pig stomachs), at different pHs [225]. At pH 6 and 7, the PGM suspension was a viscous solution and *H. pylori* was able to move significant distances in it; when the pH of PGM suspension was decreased to pH 4 and 2 (in the absence of urea), the PGM suspension formed a gel and *H. pylori* was mostly immobile in it [225]. However, when urea was added to PGM at pH 2, after 30 minutes *H. pylori* became as mobile as in pH 6 PGM, showing the effect of the *H.*

pylori's urease on the fluidization of mucus [225]. Also, in gerbils it was found that *H. pylori* is mobile at gastric pH 6 for more than 15 min, while at pH 4 it is only mobile for 2 min and at pH 2-3 it becomes immobilized in less than 1 min [226]. The fact that *H. pylori* loses motility in the lumen pH so fast, implies that *H. pylori* needs to penetrate into the deeper layers of higher pH in mucus very fast, so that it can establish the infection [222].

In addition to the *H. pylori* effects on mucus rheology, *H. pylori* can affect mucins in different ways, in order to facilitate the bacteria penetration. *H. pylori* may decrease mucin production, reduce levels of MUC1, and decrease mucin turnover, as observed in *H. pylori* infected mice [227]. In addition, *H. pylori* can degrade mucins, via thioredoxin reductase that breaks the disulfide bonds between the mucin monomers, via proteases (that cleave the proteinaceous mucus structure) or sulfomucins (that release mucus sulfate) [228].

Conversely, *H. pylori* also needs to bind to mucins to prevent being 'washed away' by peristalsis and gastric emptying [229]. Binding to gastric mucins is mediated by *H. pylori* outer membrane protein (OMP) adhesins, being the blood group antigen binding adhesin A (BabA) and the sialic-acid binding adhesin A (SabA) the best well-known adhesins [221, 222]. BabA binds to fucosylated structures on mucin glycans, including the blood group antigen structure Lewis^{b}, while the SabA binds sialylated structures, mainly the sialyl-Lewis^{x} and sialyl-Lewis^{a} [230, 231]. The mucosal inflammation caused by *H. pylori* infection is thought to increase the amount of sialylated structures, thus enabling enhanced SabA-mediated *H. pylori* binding [232, 233]. Other unknown adhesins should also be involved in binding since *H. pylori* strains not expressing BabA nor SabA were able to bind mucins (from different animals) as efficient as the ones that do express BabA or SabA [222].

On the one hand, binding of *H. pylori* to mucins allows the bacteria to colonize the gastric mucus and create a reservoir of bacteria for infection of the underlying epithelial cells, needed for the development of disease [222]. On the other hand, binding of *H. pylori* to mucins is also a protective host mechanism, preventing most bacteria to adhere to the gastric epithelial cells underneath [221, 234]. The minority of bacteria that can contact with the epithelium use their adhesins to bind to the epithelial cells, in particular the BabA and SabA adhesins to bind to respectively fucosylated and sialylated receptors on the cell's surface [230, 235]. Upon binding, the CagA oncoprotein, encoded by the cytotoxin-associated gene A (*cagA*), is injected directly into the host cell cytoplasm [212]. CagA

interacts with the host cell signaling mechanisms, leading to deregulation of host cell proliferation as well as inflammation [222]. In addition, CagA can also cause cytoskeletal rearrangements and affect the host cell tight junctions, diminishing the adhesion between adjacent cells and causing loss of cell polarity [212, 236]. Therefore, CagA-positive strains are associated with higher risk to develop gastric ulcers and cancer [212]. Another important virulence factor is the vacuolating toxin A (VacA) which, as the same suggests, can induce the formation of large vacuoles in the host cell [237]. VacA is endocytosed by the host cell (without the need of bacteria contact) and is able to form anion-selective channels on the membrane of the late endosomes, leading to the accumulation of chloride and eventual osmotic swelling and cell vacuolation [237]. VacA can also allow the leakage of iron, nickel and amino acids from the host cell to be used as nutrients for *H. pylori* [237]. Therefore, VacA is believed to facilitate *H. pylori* persistence and contribute to the development of peptic ulcer disease and gastric cancer [238].

2.4.2 Clinical outcomes

H. pylori colonization leads to chronic gastritis and increases the risk to develop ulcers and gastric cancer [211, 215]. *H. pylori* is responsible for 70-85% of the gastric ulcers and 90-95% of duodenal ulcers [211, 239]. It is estimated that the *H. pylori* infection increases 10-fold the risk of gastric cancer; *H. pylori* is thus classified as a group I carcinogen by the WHO (World Health Organization) [240]. Gastric cancer is the third most mortal cancer [241]. In addition, *H. pylori* is associated to more than 90% of the cases of gastric MALT (mucosa-associated lymphoid tissue) lymphoma, a relatively rare disease [211, 212].

Persistent *H. pylori* colonization, leads to chronic gastritis, affecting the normal acid secretion and recruiting neutrophils and mononuclear cells to the gastric mucosa [211, 212]. Chronic gastritis is typically antrum-predominant, since *H. pylori* preferentially colonizes the antrum region of the stomach (also called pyloric antrum) which is less acidic than the corpus [211, 212]. The corpus region contains more acid-secretory parietal cells; thus, additional *H. pylori* colonization of the corpus typically occurs only in individuals with a deficient acid secretory capacity or that underwent acid-suppressive therapies [211, 212]. In these cases, *H. pylori* can be found both in the antrum and in the corpus; the corpus bacteria are in close contact with the mucosa, leading to pangastritis (gastritis that occurs both in the antrum and in the corpus) [211, 212]. Antrum-

predominant gastritis favors the development of duodenal ulcers, while pangastritis is associated with the development of gastric ulcers and gastric cancer [211, 212]. Peptic (gastric and duodenal) ulcers are mucosal defects bigger than 0.5 cm in diameter [211]. *H. pylori* contributes to gastric cancer by inducing inflammation, which increases gastric cell turnover and creates a microenvironment rich in reactive oxygen species, and by inducing genetic and epigenetic changes, which increase the likelihood of cell transformation [242]. Although *H. pylori* is the strongest known risk factor for the development of gastric cancer, other factors also contribute to gastric cancer such as the virulence of the infecting *H. pylori* strain, the host genetic make-up and environmental factors like smoking and high-salt diets [242-244].

Despite the severe pathologies that can originate from *H. pylori* infection, most infected individuals do not show apparent clinical signs of the infection. It has been thus debated if some *H. pylori* strains are harmless and even beneficial [212]. However, it is premature to conclude that any *H. pylori* strain is a commensal, as reflected by the increasing list of diseases found to be associated *H. pylori* over the recent years [212]. In addition, the clinical studies show with no doubt that eradication of *H. pylori* clearly reduces the occurrence of peptic ulcers and appears to decrease gastric cancer incidence [245, 246].

2.4.3 Detection and treatment of the infection

H. pylori infection can be **detected** by non-invasive methods, but severe pathology requires an upper endoscopic examination in order to collect gastric biopsy specimens that can be later analyzed for the presence of *H. pylori* [247]. It is critical that the technique later applied allows the detection of bacterial resistance to antibiotics, so that a proper therapeutic routine can be chosen. Therefore, the biopsy can be analyzed by culture methods, polymerase chain reaction (PCR), or fluorescence *in situ* hybridization (FISH) [248]. Culture methods allow the detection of *H. pylori* and its resistance to antibiotics with high specificity [248]. However, the sensitivity of culture methods is low and the result is only obtained after several days which can be one week for the fastidious *H. pylori* [248]. PCR allows the detection of *H. pylori* and its resistance to antibiotics with a high sensitivity and specificity [249]. However, PCR requires the extraction of *H. pylori* DNA, which increases the complexity and time-to-result [249]. Differently, FISH allows

sensitive and specific detection of bacteria and their resistance to antibiotics without the need for nucleic acids extraction [250]. FISH is based on the hybridization by Watson-Crick base pairing of oligonucleotides designed to be complementary to bacterial RNA (typically rRNA); as the oligonucleotides are fluorescently labelled, positive samples can be detected by fluorescence [81]. In this way, FISH can not only allow the detection of bacteria, but also point mutations known to be responsible for resistance to antibiotics. FISH could already be used for *in vitro* detection of clarithromycin resistance in *H. pylori* [251]. In addition, FISH also holds promise to be used *in vivo* at the time of endoscopic examination, obviating the need to isolate a biopsy and wait for further *in vitro* detection. Already existing endomicroscopic probes would be used for the *in vivo* detection of fluorescent bacteria within the stomach during upper endoscopy [252, 253]. Nevertheless, FISH hybridization typically requires elevated temperatures, controlled conditions (including pH) and the use of toxic/noxious compounds to render the bacterial envelope permeable to the bacterial envelope to the oligonucleotides. All these requirements can hamper the *in vivo* application of FISH. NAMs composed of LNA/2'OMe, PS and PO, were already developed to target *H. pylori* at 37 °C [254] and proved to successfully target *H. pylori* even at a gastric pH [77]. However, improved internalization of NAMs into *H. pylori* is still required to stablish NAMs as a novel platform to detect and treat *H. pylori* infections.

The recommended **treatment** for *H. pylori* infection is a triple therapy based on antibiotics [255]. It is clear that its success depends on the ability of antibiotics to resist the acidic gastric pH and pepsin degradation, penetrate the gastric mucus while keeping its activity, and crossing the bacterial cell wall (and also the cytoplasmic membrane for antibiotics acting in the cytosol) [211, 255, 256]. In particular, the triple therapy employs a proton pump inhibitor (PPI) together with clarithromycin plus amoxicillin or metronidazole [213]. Metronidazole is very stable in gastric juice and pH from 2 to 7, amoxicillin is relatively unstable at low pH but still has a half-life of 15h at pH 2, and clarithromycin is very sensitive to acid degradation with a half-life of less than 1h [257, 258]. Therefore, a PPI (usually omeprazole) is needed to protect clarithromycin from acidic degradation, by decreasing the acid production by gastric parietal cells [256, 259].

Even with this triple therapy, eradication rates keep decreasing and already fails in more than 30% of the patients [255]. This is mostly due to *H. pylori* point mutations that set the bacteria resistant to antibiotics. Resistance to clarithromycin is particular relevant,

since clarithromycin is the key antibiotic of the triple therapy and the most active antibiotic against *H. pylori* [260, 261]. Clarithromycin (a macrolide) is able to diffuse across the lipid bilayers [35] into the bacterial cytosol; there, it binds to the bacterial ribosome, leading to premature release of peptidyl-tRNA from the acceptor site and consequent protein synthesis inhibition [260, 262]. Resistance to clarithromycin mostly arises as a consequence of three point mutations in the peptidyl transferase loop encoded by the *23S* rRNA gene that inhibits the binding of clarithromycin to the ribosome [260, 263]. These mutations occur by chance and can remain for many generations [263]. Moreover, these mutations similarly affect the action of all macrolides [263]. Therefore, the prescription of a macrolide to combat another infection contributes to select the resistant *H. pylori* mutants [263]. In Europe, the resistance to clarithromycin has increased from 9% in 1998 [264] to 17.6% in 2008/9 [265]. The resistance prevalence is already > 20% in most European countries, as well as in China, Japan and the United States [261]. The resistance prevalence to clarithromycin is however < 10% in the Northern European countries, as a result of their policy of restricted antibiotic's use [263].

Resistance to metronidazole is also important. Metronidazole, after passively diffusing across the lipid bilayers, needs to be reduced in the bacterial cytosol to become active and lethally interact with DNA [266]. Resistant bacteria, as a result of point mutations, reduce the metronidazole more slowly than susceptible bacteria [262]. The prevalence of metronidazole resistance is around 17% in Europe and 40% in the United States and Asia. It is particularly high in Africa (almost 90%), probably a result of the widespread use of metronidazole against parasitic infections [261].

Differently from clarithromycin and metronidazole, the prevalence of resistance to amoxicillin is lower than 1% in Europe, around 2% in Asia and United States, and about 18% in Africa [261]. Amoxicillin (a β-lactam antibiotic) penetrates the outer membrane of gram-negative bacteria through general porins [35, 62], and inhibits the peptidoglycan synthesis by blocking the penicillin binding protein that catalyzes the cross-linking of peptidoglycan [260]. Resistance occurs by a point mutation in the gene that codes for one of the penicillin binding proteins, hampering amoxicillin binding [260]. Mutations may also occur on the genes that code for porins, leading to reduced permeability to amoxicillin [260].

Upon failure of the triple therapy, second line regiments have been employed, including levofloxacin (instead of clarithromycin) together with amoxicillin and the PPI,

or bismuth salts combined with metronidazole, tetracycline and the PPI (quadruple therapy) [255]. Levofloxacin is a quinolone, and thus, as previously referred, has a pH dependent net charge that determines the penetration route into bacterial cells [23, 35, 61]. At the pH of the gastric mucus (where *H. pylori* is mostly located), levofloxacin should have an overall cationic charge and should, thus, use the general porins for internalization into the *H. pylori* cytosol. There, levofloxacin targets the bacterial gyrases (which control DNA relaxation for replication, recombination and transcription), leading to inhibition of DNA and RNA synthesis [262]. Mutations that set bacterial resistance to levofloxacin also exist, resulting in a resistance prevalence of around 24% and 14%, respectively in Europe and Asia [261, 262]. The route used by tetracycline for internalization in bacteria is also pH dependent [23, 35, 61]. In the deep mucus tetracycline should be zwitterionic and thus use a lipid mediated diffusion to access *H. pylori* cytosol; in the cytosol, tetracycline inhibits protein synthesis by binding to the ribosome and preventing the attachment of new aminoacyl-tRNA [23, 35, 61, 262, 267]. Although resistance to tetracycline is still low, it appears to be increasing due to point mutations that result in changes in the primary tetracycline binding site [260, 262]. The mechanism of action of bismuth, an heavy metal, is unknown; several mechanisms have been suggested including oxidative stress, inhibition of proteins function, and deregulation of nickel homeostasis [268]. No resistance to bismuth has so far been reported [263].

The important and rising resistance to different antibiotics, even to those used as second line approaches, calls for novel therapeutic strategies, as well as for improved diagnose to make a better use of the still valid antibiotics.

2.4.4 Carriers to manage the infection

Nanoparticles have been increasingly investigated as novel strategies to manage *H. pylori* infection, majorly focusing on the treatment of the infection. The few reports on detection mostly use gold NPs functionalized with oligonucleotide probes or antibodies to improve the detection signal. However, *H. pylori* detection based on antibodies [269] does not allow the identification of bacterial resistance, while the oligonucleotide based detection requires the isolation of *H. pylori* DNA [270].

Similarly to what happens with bacterial infections in general, the use of NPs towards *H. pylori* has been almost exclusively devoted to the use of antibiotics to be sustainably released in the bacteria vicinity. These strategies aim to improve the concentration of active antibiotic close to *H. pylori* in the mucus, by protecting the antibiotics from acid degradation and/or improve the retention in the gastric mucus, as well as to decrease antibiotics toxicity. The reported NPs include liposomes [271-273], chitosan NPs [274-276] and polymeric microparticles [277-280].

Mucoadhesive NPs, in particular chitosan, are especially popular as antibiotic carriers against the *H. pylori* infection. The rationale behind its use is that mucoadhesion will allow higher gastric retention and thus improve drug bioavailability [281]. This may be helpful to contend removal of NPs related to gastric peristalsis; however, mucoadhesion will prevent the penetration of the NPs into the deep layers of mucus where *H. pylori* is mostly located [188]. Therefore, the NPs adhered on the loosely adherent mucus layer will suffer from clearance by the frequent shedding of this mucus layer, before reaching the infection spot [282]. Actually, most studies on the *in vitro* efficacy of the NPs do not include the mucus barrier [274, 283-286]. Nevertheless *in vivo* studies, performed in mice, showed improved anti-*H. pylori* effect compared to free antibiotics [275-277]. One of these studied used gastroretentive polymethacrylate (Eudragit® RS) microparticles to encapsulate metronidazole, but the therapeutic advantage over the free antibiotic was only slight [277]. More interesting results were obtained with chitosan NPs. Positively charged chitosan/heparin nanoemulsion loaded with amoxicillin (of 270 nm in diameter) resulted in approximately a 4-fold reduction in the *H. pylori* counts over free amoxicillin, although total *H. pylori* clearance was not obtained [275]. Also, chitosan/heparin conjugated with fucose via genipin cross-linking allowed nearly a 4-fold improved therapeutic effect compared to free amoxicillin, without full *H. pylori* clearance [276]. Fucose was included to target the NPs towards *H. pylori* adhesins (as previously mentioned, adhesin BabA binds to fucosylated structures on mucins and epithelial cells [236]).

The binding of particles to *H. pylori* may also prevent *H. pylori* from binding to epithelial cells, and this has been explored as an alternative therapeutic strategy. For this purpose, naked chitosan microspheres [285] and gliadin nanoparticles conjugated with fucose- and mannose-specific lectins [287] showed to decrease the *H. pylori* adherence to a gastric cell line, in solution. Also, a peptide isolated from *Pisum sativum* was found to be anti-adhesive by binding to BabA adhesin in *H. pylori* and preventing bacterial

adhesion to AGS cells [288]. Other AMPs active against *H. pylori* have been tested, in solution [288-293], including broad spectrum peptides isolated from amphibians [289, 294], peptides synthesized based on tomato's AMPs [291], or peptides synthesized *de novo* [293]. To the best of our knowledge the use of 'membrane active' peptides on *H. pylori* has been restricted to AMPs, with no CPP used for the intracellular transport of antibacterials into *H. pylori*.

In summary, the described therapeutic strategies against *H. pylori* majorly focus on the delivery of antibiotics, but these do not provide a solution for bacteria already resistant to the carried antibiotics. In addition, they rely on controlled drug release of the antibiotic from the NP in the bacteria vicinity and thus do not transport the drug across the bacterial cell wall; the later would increase the therapeutic efficacy of the still valid antibiotics and help reduce the necessary dose, important to prevent the development of antibiotic resistance [295]. On top, we are not aware of any study aiming to target *H. pylori* that considers the critical native gastric mucus [166].

This thesis addresses the identified needs, focusing on NAMs with potential to overcome the resistance to antibiotics and treat *H. pylori* infections, within gastric mucus isolated from pigs. The porcine gastric mucus and the *H. pylori* envelope were studied as potential barriers for the NAMs. On top, ways to overcome these barriers were revealed, for the success of NAMs as a novel platform to manage *H. pylori* infections.

2.5 References

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3.

3 EFFECT OF GASTRIC MUCUS ON *IN VIVO* HYBRIDIZATION THERAPIES AND DIAGNOSIS DIRECTED AT *HELICOBACTER PYLORI*

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Abstract

The rising resistance of bacteria to antibiotics poses a threat to the public health. Therefore, alternative antimicrobial drugs are of utmost need. In addition, diagnostic techniques able to rapidly detect the specific infecting bacteria and their resistance to antibiotics is required, so that the life-time of the still valid antibiotics can be extended. *Helicobacter pylori* (*H. pylori*) infects more than 50% of the worldwide population. It is mostly found deep in the gastric mucus lining of the stomach, being a major cause of peptic ulcers and gastric adenocarcinoma. To face the increasing resistance of *H. pylori* to antibiotics, nucleic acid mimics (NAMs) are promising as alternative antimicrobials, as well as diagnostic probes. In particular, locked nucleic acids (LNA)/2'-OMethyl RNA (2'OMe) have shown to specifically target *H. pylori*, as evidenced by *in situ* hybridization. The success of *in vivo* hybridization depends on the ability of these NAMs to penetrate the major physical barriers – the highly viscoelastic gastric mucus and the bacterial cell envelope. It was found that the tested NAMs are capable of diffusing rapidly

through native, undiluted, gastric mucus isolated from porcine stomachs, without degradation. Moreover, although the NAMs hybridization was still successful without permeabilization and fixation of the bacteria, which is normally part of *in vitro* studies, the ability of the NAMs to efficiently hybridize with *H. pylori* was hampered by the presence of mucus. Future research should focus on developing nanocarriers that shield the NAMs from components in the gastric mucus, while remaining capable of diffusing through the mucus and delivering these NAMs directly into the bacteria.

Keywords: Gastric mucus, *Helicobacter pylori*, Locked Nucleic Acid, 2'-OMethyl RNA, FISH

3.1 Introduction

The ability to fight infections is threatened by bacterial resistance to antibiotics [1]. Infections by resistant bacteria account already for nearly 50 000 annual deaths, in Europe and the United States together [2, 3]. The most common and prevalent chronic infection in the world is caused by *H. pylori* [4]. *H. pylori* is a stomach-colonizing gram-negative bacterium that infects more than half of the world's population [5, 6]. Because it increases the susceptibility to gastric cancer, associated with one of the highest mortality rates [7], it is classified as a Group 1 carcinogen by the World Health Organization [8]. Moreover, it is associated with 95% of duodenal ulcers and 80% of gastric ulcers [9]. *H. pylori* colonizes almost exclusively the stomach, lying deep in the mucus layer that covers the gastric epithelium [10], and also adhering to the surface of gastric epithelial cells and in deeper regions of the gastric glands [11-13] (Figure 3.1a).

The prevailing treatment for *H. pylori* eradication relies on a standard triple therapy, involving two antibiotics (clarithromycin plus amoxicillin or metronidazole) combined with a proton pump inhibitor [14, 15]. Even with this triple therapy, full eradication fails in nearly 25% of the patients [16, 17]. This is caused by (i) the highly acidic gastric pH the drugs need to endure, (ii) the very viscous mucus the drugs have to permeate [18] and (iii) the rising resistance of *H. pylori* to antibiotics [19]. Therefore, alternative treatments are of utmost importance. To fulfil this need, antisense therapy, through the use of oligonucleotides to inhibit the expression of specific bacterial genes, is a promising strategy [20-22]. Essential bacterial genes can be targeted, so that bacterial growth is

prevented, as well as genes of resistance to antibiotics, so that the bacterial susceptibility to antibiotics is reestablished. An attractive aspect of this approach is its flexibility. In case microbial resistance emerges, commonly by point mutations [23], the oligonucleotides can be easily redesigned to target a new mutation, becoming an effective antibacterial again. In addition, oligonucleotides conjugated with a contrast agent could use used as diagnostic tools, to rapidly identify the presence of the bacterium and its resistance to antibiotics *in vivo*, during the upper endoscopic exam (avoiding the need to collect a gastric biopsy for later *ex vivo* detection of *H. pylori*, in a time-consuming process) [24, 25].

Conventional DNA oligonucleotides have relatively low affinity towards RNA and DNA complementary sequences and are susceptible to degradation by endonucleases [26, 27]. To overcome these drawbacks nucleic acid mimics (NAMs) have emerged as alternatives [26, 28]. Among them, locked nucleic acids (LNA), 2'-OMethyl RNA (2'OMe) and phosphorothioate modified oligonucleotides (Figure 3.1b) have shown the most promising antisense *in vivo* [29-33]. While nucleic acid therapy of bacterial infections is a relatively unexplored field [22], delivery of oligonucleotides in bacteria is well-studied to detect microorganisms *in vitro* based on fluorescence *in situ* hybridization (FISH). FISH (Figure 3.1c) is based on specific base pairing, at high temperature, of an oligonucleotide with rRNA or mRNA target sequences, obeying to Watson-Crick hydrogen-bonding [34-37]. Recently, Fontenete et al. have proven that NAMs composed of both LNA and 2'OMe (which we term LNA/2'OMe), with either phosphodiester (PO) or phosphorothioate (PS) as backbone linkages, allow specific detection of *H. pylori* at 37 °C [38]. In addition, *H. pylori* detection by LNA/2'OMe (PS) oligonucleotides was successful even at a gastric pH [39]. However, the presence of gastric mucus was not addressed in these hybridization experiments and the hybridization in solution was dependent on the pre-treatment of *H. pylori* cells with ethanol to render them permeable to the NAMs [39].

Clearly, the success of NAMs to detect and treat *H. pylori* infection *in vivo* depends on the ability of the NAMs to overcome two main barriers: the gastric mucus and the bacterial cell envelope (Figure 3.1a). Mucus is a highly complex and viscoelastic mixture that forms a major protective barrier for the penetration of particles [40]. The rheological properties of the mucus are mostly determined by mucins, the main component of dry mucus, but also affected by the lipids, DNA, cells and cellular debris, proteins and ions present [40-43]. Having crossed the mucus layer, the next barrier encountered by the

NAMs will be the bacterial cell envelope. Unlike eukaryotic cells, which possess only a cell membrane, bacteria are protected from the outer environment by a multi-layered cell envelope [44]. Traditional FISH employs fixation/permeabilization agents (as paraformaldehyde and ethanol), allowing oligonucleotides to reach the cytoplasm (Figure 3.1c) [36]. As these agents are typically toxic or noxious [45, 46], this step should be circumvented in future *in vivo* hybridization therapies.

Considering the potential of NAMs, it was investigated to which extent gastric mucus may be a barrier for the delivery of oligonucleotides directed at *H. pylori* infection. More specifically, it was studied (i) the (chemical) stability of LNA/2'OMe oligonucleotides in native gastric mucus scrapped off from porcine stomachs, (ii) the diffusion of LNA/2'OMe oligonucleotides, PO and PS, through the mucus and (iii) the effect of gastric mucus on the efficiency of the fluorescence *in situ* hybridization in *H. pylori*. On top, it was tested to which extent the bacterial cell envelope poses a barrier for the internalization of LNA/2'OMe oligonucleotides. The experiments of this work disclose important insights which will be of interest for the further development of *in vivo* hybridization therapies and diagnosis of bacterial infections.

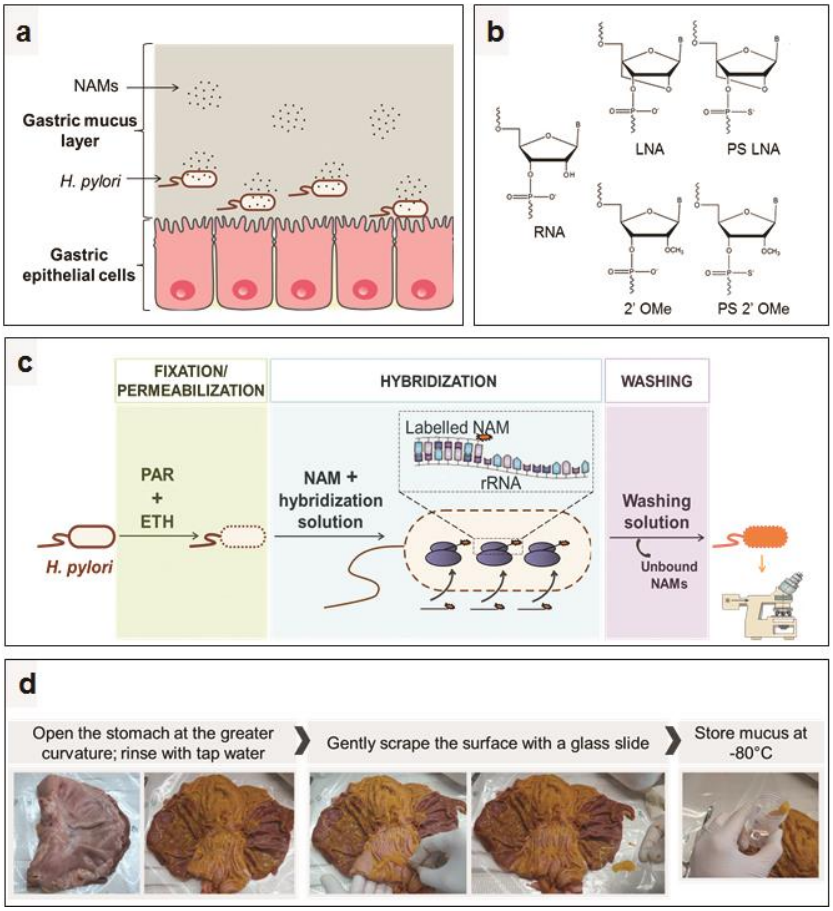

Figure 3.1 Illustration of NAMs hybridization to *H. pylori* and the different components of the implemented model. (a) Schematic representation of NAMs in gastric mucus, on their way to target *H. pylori*. (b) Monomers of the NAMs used in FISH, compared to RNA (adapted from Fontenete et al. [38]). (c) Illustration of the standard FISH procedure. PAR and ETH being paraformaldehyde and ethanol, respectively (adapted from Amann et al. [36]). (d) Procedure followed for the collection of mucus from the stomach of pigs obtained from the slaughterhouse.

3.2 Materials and methods

3.2.1 Isolation of gastric mucus

Native gastric mucus from the stomach of pigs was used in this study, since porcine gastric mucus is considered a suitable model for human gastric mucus [47, 48]. Mucus from three different pigs was included in each assay. Stomachs were collected from a slaughterhouse and opened at the major curvature and rinsed with tap water [49]. The mucus from was then gently scrapped off using a glass slide and placed in a sterile tube

(Figure 3.1d). Each tube was then aliquoted, placing 200 µL into sterile Eppendorf vials with a sterile plastic syringe, and stored at -80°C.

3.2.2 Synthesis of NAMs

Two oligonucleotides complementary to a sequence of the *H. pylori* 16S rRNA gene, were used in this study [38]. These are composed of LNA and 2'OMe, possess the same sequence and differ only in the internucleotide bonds. One possesses normal phosphodiester oligonucleotides (PO), while the other has one of the two non-bridging oxygen atoms replaced by a sulphur atom at each internucleotide linkage (PS) (Figure 3.1b). To simplify, these oligonucleotides will be herein designated as PO and PS, respectively. The sequence of PO is 5'-lGmeAmeClTmeAmeAlGmeCmeClC-3', while the sequence of PS is 5'-lG*meA*meC*lT*meA*meA*lG*meC*meC*lC*-3', where "l" represents the LNA monomers, "me" the 2'OMe monomers and * the phosphorothioate linkages. Oligonucleotides labelled at 5' with fluorescein phosphoramidite (FAM, Glen Research, VA, USA) were synthetized and purified according to [38]. The same oligonucleotides labelled at the 5'-terminal with Cy3 were acquired from Eurogentec (Ougrée, Belgium).

3.2.3 Measuring the diffusion of NAMs in gastric mucus by FRAP

Approximately 30 µL of native (undiluted) gastric mucus was taken with a plastic syringe and mixed with 4 µL of 100 µM FAM-labelled PO or PS. The mixture was then placed on a microscopy glass slide sealed with an adhesive spacer (S24735, Secure-Seal™, Life Technologies, Paisley, UK) and a cover slip. The sample was placed in a stage top incubation chamber (Tokai Hit, Shizuoka, Japan), to perform measurements at 37 ºC. Mucus from three different pigs was tested and for each mucus-NAM sample the diffusion measurements were done in triplicate. Measurements in HEPES buffer were performed as control.

Fluorescence recovery after photobleaching (FRAP) was used to measure the diffusion of the fluorescently labelled oligonucleotides [50, 51]. For a detailed description of the FRAP experiments the reader is referred to a previous publication from our group [50]. The measurements were performed using a Nikon C1si confocal laser scanning microscope (CLSM), equipped with a Plan Apo 10x NA 1.4 objective lens. The 488 nm

argon ion laser (CVI Melles Griot, CA, USA) was used for photobleaching and imaging. In brief, a FRAP experiment occurs as follows. Using a strong laser beam the fluorescence in a rectangular area (30 x 30 µm) of the mucus-NAM sample is bleached. Next, using an attenuated laser beam, a time-lapse image series is recorded to visualize the fluorescence recovery in the bleached area which is due to diffusion of the fluorescent NAMs from the (non-bleached) surrounding into the bleached zone (see Figure 3.3a). The local diffusion coefficient (D) and the fraction of mobile (f) and immobile oligonucleotides can be calculated by fitting the fluorescence recovery data to a theoretical model, as developed in our group by Deschout et al. [51].

3.2.4 Measuring the stability of NAMs in gastric mucus by PAGE

Two microliters of 20 µM PS or PO (labelled with FAM) were gently mixed with approximately 5 µL of porcine gastric mucus and incubated at 37 ºC for 10 min (the approximate time needed for a FRAP measurement). Loading buffer (2 µL of Ambion® gel loading solution, Life Technologies, Paisley, UK) was then mixed with the mucus-NAM sample. FAM-labelled oligonucleotides alone and mucus alone were run as controls on a PAGE gel. In addition, the stability of PS and PO was tested in a suspension of commercial mucin from porcine stomach (type II, Sigma-Aldrich, Bornem, Belgium). Therefore, PS and PO were incubated in a 3% (w/v) mucin dispersion (in 0.9% (w/v) NaCl solution) [52, 53].

To investigate the degradation of the oligonucleotides by nucleases, PO was incubated for 2 hours at 37 °C with DNase (TURBO™ DNase, Life Technologies, Paisley, UK) and RNase (RNase A, Qiagen Benelux B.V., Venlo, The Netherlands). The reaction mixture contained 4 µL of a 5 µM PO, 1 µL of DNase (2U) or RNase (7U) and 13 µL of the respective supplied reaction buffer.

All the samples were loaded onto 20% non-denaturing polyacrylamide gels prepared in 5xTBE buffer. Electrophoresis was performed at 110 V, during 75 min. Thereafter, the fluorescently labelled oligonucleotides on the gels were visualized by UV transillumination and gel photography.

3.2.5 *H. pylori* FISH – NAMs uptake and hybridization in gastric mucus

Helicobacter pylori 26695 (ATCC 700392) was grown in trypticase soy agar (TSA) supplemented with 5% sheep blood (Becton Dickinson GmbH, Germany) for 48 hours,

at 37 °C, under microaerobic conditions. The biomass was recovered from the plates using sterile saline (0.9% (w/v) NaCl) and diluted to nearly $1x10^6$ CFU/mL. Smears were prepared on glass slides (20 µL per slide well) by drying at 37 °C. Fluorescence *in situ* hybridization (FISH) on slide was performed according to Fontenete et al. [38, 54], with slight modifications. The main steps of standard FISH are schematically presented in Figure 3.1c. For standard fixation/permeabilization, the smears were first immersed in 4% (w/v) paraformaldehyde (Fluka - Sigma-Aldrich, Bornem, Belgium) for 10 min, followed by 15 min in 50% (v/v) ethanol (Fluka - Sigma-Aldrich, Bornem, Belgium) and allowed to dry. To test the role of fixation/permeabilization on hybridization efficiency, samples treated only with ethanol, and samples not fixed/permeabilized at all were included. The smears were then covered with 20 µL hybridization solution containing 500 mM urea (Vel - VWR, Haasrode, Belgium), 500 mM Tris-HCl, pH 7 (Sigma-Aldrich, Bornem, Belgium), 900 mM NaCl (Sigma-Aldrich, Bornem, Belgium) and 400 nM of PS or PO labelled with Cy3. To test the effect of native gastric mucus on the hybridization efficiency, approximately 30 µL of gastric mucus was first mixed with 20 µL of hybridization solution, and then applied on the bacteria smear (note that the final concentration of NAMs and hybridization solution applied on the bacteria were kept the same as in standard FISH). Samples were covered with a coverslip, placed in dark moist chambers and allowed to hybridize during 30 min, at 37 °C. The coverslips were then removed, together with the mucus mixture (when present). To avoid non-fixed *H. pylori* to be removed from the slide together with the mucus mixture, slides coated with 0.1% (w/v) Poly-L-lysine (Sigma-Aldrich, Bornem, Belgium) were used for better adhesion of *H. pylori*. The slides were then washed during 15 min using warm (37 °C) water at pH 7, allowed to dry and visualized on the microscope. The experiments were performed using gastric mucus from three different pigs, in triplicate. The effect of native gastric mucus was compared with that of a mucin dispersion, by performing hybridization in the presence of a 3% (w/v) suspension of commercial mucin (from porcine stomach), instead of mucus. In addition, the influence of acidic washing with simulated gastric juice without pepsin (0.2% w/v NaCl, 80 mM HCl, pH 1.8, [55]) was evaluated, after hybridization at the same average pH found in the collected porcine gastric mucus (pH 5.8). Negative controls using hybridization solution without oligonucleotides, and maintaining the remaining FISH conditions, were performed in all experiments.

Microscopy images were acquired using a Nikon TE2000 microscope equipped with a Nikon DS-Qi 1Mc epifluorescence camera and a Plan Apo VC 100× 1.4 NA oil

immersion objective lens (Nikon). Cy3 fluorescence was visualized using a TRITC filter (excitation: 530-560 nm; emission: 590-650 nm, Nikon). The same exposure time and the same intensity of the fluorescent lamp were used for all the samples. For fluorescence quantification, 10 to 15 images per well were taken randomly, covering all the areas of the slide well.

3.2.6 Quantification of *H. pylori* hybridization

The hybridization efficiency in the different conditions was compared through quantification of the fluorescence intensity. This was done using image processing routines available in Matlab (The MathWorks, Natick, MA, USA). First, bacteria were identified in each image based on an automatically determined intensity threshold. After removal of remaining noise (by selecting areas with a minimum number of pixels), a binary mask was generated that corresponds to the location of bacteria in the image. For the regions within the mask, the fluorescence intensity in the original image was quantified as

$$Intensity = \sum [(int_b - BG) \times pix_b]$$

where int_b corresponds to the fluorescence intensity of each bacterium, BG the background value and pix_b the number of pixels of the bacterium.

Per condition, 10 to 15 images were analysed in this fashion from which the average fluorescence intensity was calculated. These absolute values were normalized to the respective positive control of PS or PO, hybridized in hybridization solution and washed with water or simulated gastric juice without pepsin.

3.2.7 Statistical analysis

Statistical analysis was performed by GraphPad Prism6 software (GraphPad Software, San Diego, USA). FISH results were analysed using two-way analysis of variance (ANOVA) and Tukey's multiple comparison test, comparing the conditions within the same NAM. FRAP results were analysed by one-way ANOVA and Sidak's multiple comparisons test. Significance was set at $P \leq 0.05$ (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$).

3.3 Results

3.3.1 NAMs uptake by *H. pylori* without prior permeabilization

For future *H. pylori* therapy and *in vivo* detection, fixation and permeabilization of bacteria should be avoided as the reagents used are toxic or noxious. Therefore, the hybridization efficiency of LNA/2'OMe oligonucleotides without fixation/permeabilization of the bacteria on slide was investigated (Figure 3.2). The hybridization was rather efficient, as nearly 50% (PS) and 70% (PO) of the fluorescence of the fully fixed/permeabilized bacteria (treatment with both paraformaldehyde and ethanol) was achieved (Figure 3.2b).

In Figure 3.2b, the hybridization and washing steps were performed at pH 7. In an *in vivo* setting, however, the hybridization will have to happen within the gastric mucus; note that the mucus samples isolated from pigs showed an average pH of 5.8. In addition, highly acidic gastric juice in the gastric lumen (upwards the gastric mucus) might contribute to wash away free NAMs. Therefore, hybridization experiments at pH 5.8 were performed, followed by washing using simulated gastric juice (pH 1.8), instead of water (Figure 3.2c). It was verified that uptake of the NAMs occurred without fixation/permeabilization at neutral pH 7 (Figure 3.2b), as well as at conditions at pH close to the *in vivo* (Figure 3.2c).

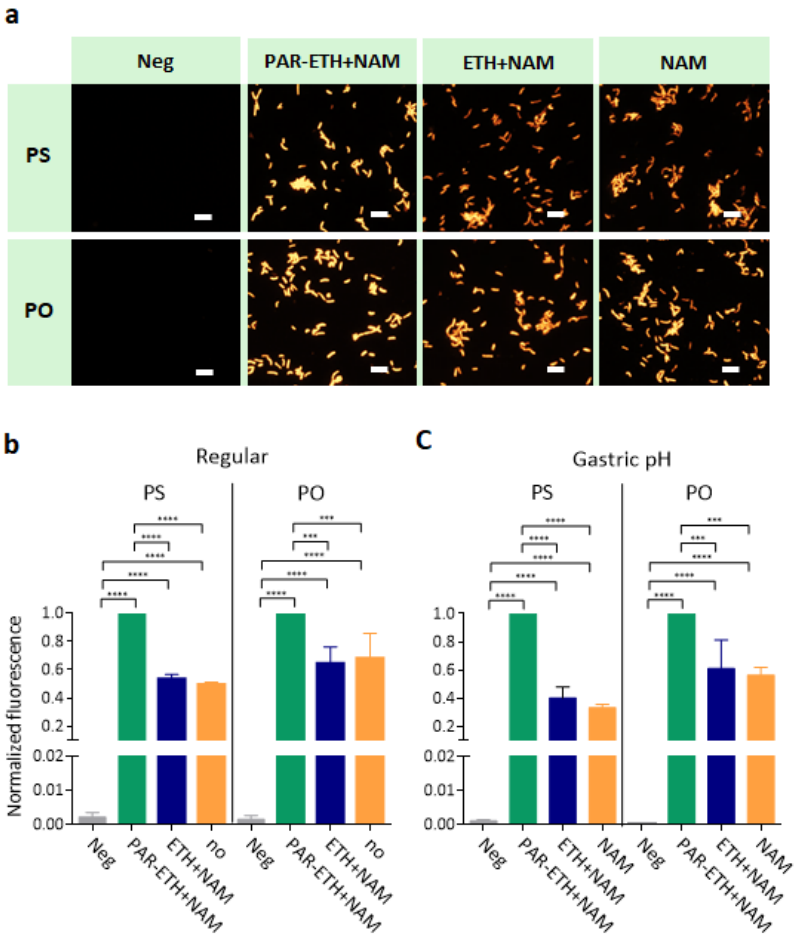

Figure 3.2 Effect of fixation/permeabilization of *H. pylori* on the hybridization efficiency by the NAM PS and PO. The combined use of paraformaldehyde and ethanol (PAR-ETH+NAM) was compared to respectively the use of only ethanol (ETH+NAM) and no pretreatment of bacteria (NAM). Normalized fluorescence being the fluorescence of the bacteria normalized to their fluorescence as measured in PAR-ETH+NAM. Results are presented as mean ± SD, n=3. Negative controls (i.e. without the use of oligonucleotides) (Neg) were included as well. (a) Representative epifluorescence microscopy images obtained with regular hybridization and washing at pH 7 and acquired at equal light exposure conditions. Scale bars represent 10 µm. (b) Regular hybridization and washing at pH 7. (c) Hybridization at the average pH found in the collected porcine gastric mucus (pH 5.8), followed by washing with simulated gastric juice without pepsin (pH 1.8).

3.3.2 Diffusion of NAMs in gastric mucus

FRAP was used to measure the diffusion of both NAMs in native porcine gastric mucus (Figure 3.3). As Figure 3.3b shows, when compared to HEPES buffer, the diffusion in gastric mucus was slowed down, especially for the PS NAM. As the f values reveal in Figure 3.3b, all PO NAMs are mobile in mucus, while a small fraction of the PS NAMs becomes immobilized by the gastric mucus. Visual inspection of the microscopy images also showed a more heterogeneous distribution in mucus of PS, when compared to PO (Figure S3.1).

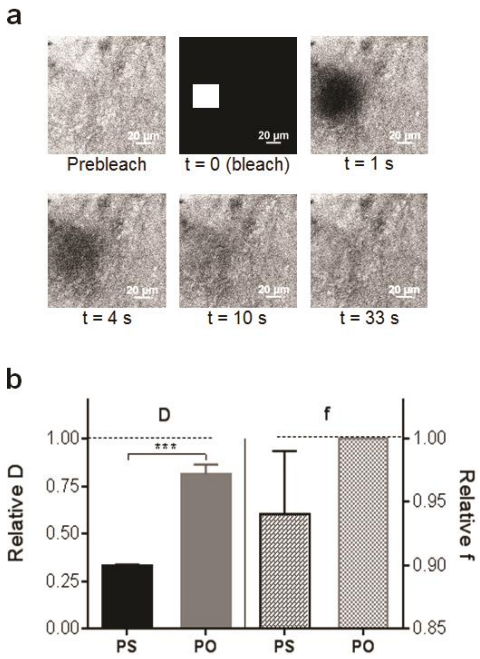

Figure 3.3 Diffusion of NAMs in native gastric mucus. (a) A representative FRAP measurement on PO in porcine gastric mucus. The first frame shows the prebleach image. Next, a square region (30 x 30 µm) is bleached (at t=0), followed by a time-lapse recording of the subsequent fluorescence recovery. (b) Average diffusion coefficient (D) and mobile fraction (f) of both NAMs in gastric mucus normalized to their values in HEPES buffer. Results are presented as mean ± SD, n=3.

3.3.3 Stability of NAMs in gastric mucus

Polyacrylamide gel electrophoresis (PAGE) experiments revealed that both PO and PS NAMs are stable in gastric mucus (Figure 3.4b). The faster diffusion of the PO in mucus, compared to the PS oligonucleotides (as seen in Figure 3.4b), cannot be attributed to the enzymatic degradation of PO, since it is stable and DNase and RNase resistant (Figure 3.4a).

Comparing each NAM in water and in mucus, it was observed that gastric mucus 'smeared' the NAMs over the gel, thereby reducing the intensity of the NAM band (Figure 3.4b); this smearing was more pronounced in case of the PS NAMs. Similar results were obtained using mucus from two other pigs. When incubating the oligonucleotides in a (commercial) mucin dispersion, the same trend was noticed (Figure 3.4c), although the smearing of the oligonucleotides was less pronounced. The PAGE results suggest that the NAMs interact with mucus components, likely mucins. In agreement with the outcome of the FRAP experiments (f values in Figure 3.3b), the PS NAMs seem to adhere more strongly to gastric mucus than the PO NAMs.

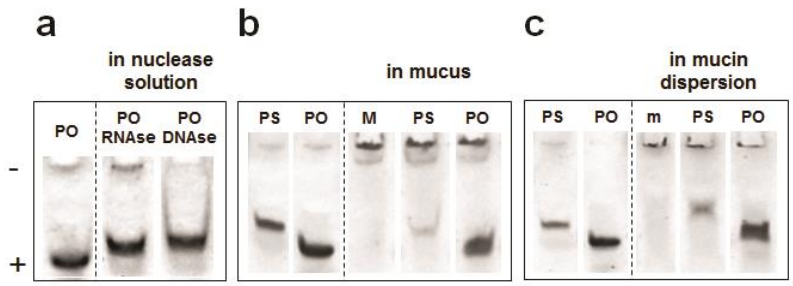

Figure 3.4 Stability of NAMs in native gastric mucus. (a) PAGE on PO incubated with RNase and DNase; PO in water was taken as a control. (b) PS and PO incubated in porcine gastric mucus; mucus alone (M) was taken as a control. (c) PS and PO incubated in a commercial mucin dispersion; mucin dispersion alone (m) was run as a control. The lanes at the left of the dotted lines show PS and PO in water.

3.3.4 *H. pylori* hybridization in gastric mucus

To find out whether the interactions between NAMs and mucus, as observed above, could affect the ability of NAMs to hybridize in *H. pylori*, FISH was performed with and without mucus in the hybridization step. Additionally, for each of these conditions, we

tested both PAR-ETH treated and untreated bacteria. As shown in Figure 3.5, the presence of native gastric mucus significantly decreased the hybridization efficiency of both NAMs, in fixed/permeabilized and untreated *H. pylori*. When using fixed/permeabilized *H. pylori*, a lower efficiency was noticed for PS than PO, in line with the previously observed stronger interaction of PS to mucus. This was not, however, reflected in untreated *H. pylori* (Figure 3.5b, orange bars). Also, the mucin dispersion reduced the hybridization efficiency, but much less than native mucus (PS presented 55% and PO 50% of the positive control's fluorescence), suggesting that mucin molecules alone do not fully explain the decreased hybridization in gastric mucus.

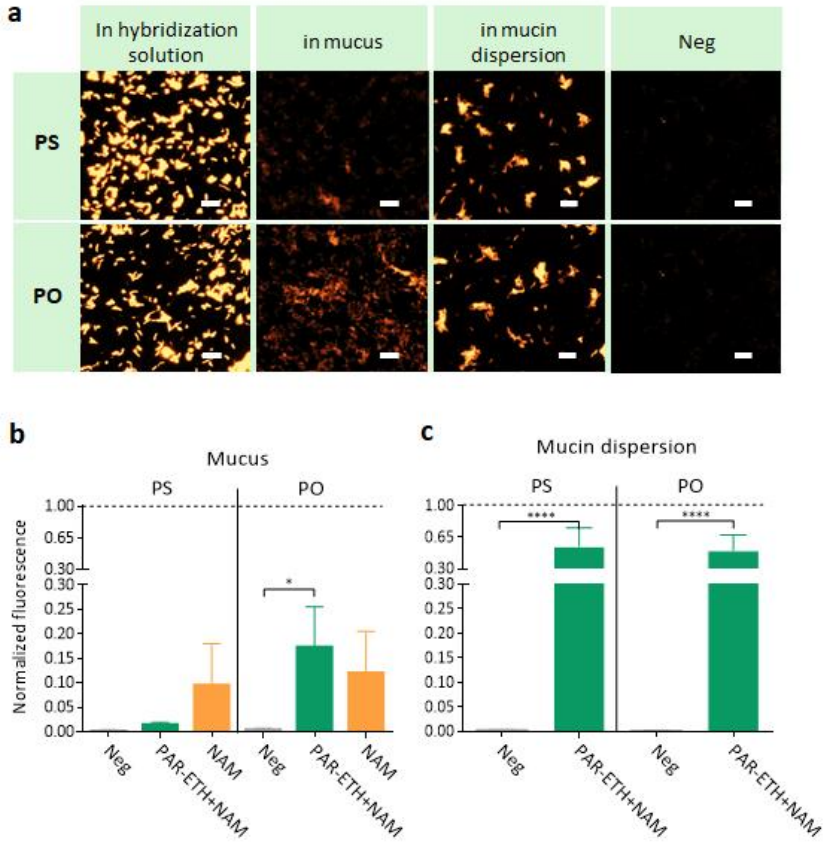

Figure 3.5 Effect of native gastric mucus, compared to a mucin dispersion, on the hybridization efficiency by PS and PO. *H. pylori* was fixed/permeabilized with paraformaldehyde and ethanol (images and green bars; PAR-ETH+NAM) or hybridized without any pretreatment (orange bars; NAM). Negative controls (in which no NAMs were used; Neg) were included. (a) Representative epifluorescence microscopy images, acquired at equal light exposure conditions. Scale bars represent 10 µm. (b) and (c) FISH fluorescence normalized to that measured when using the standard hybridization solution, without mucus or mucin dispersion. Results are presented as mean ± SD, n=3.

3.4 Discussion

LNA and 2'OMe oligonucleotides are promising NAMs for novel gene therapies and *in vivo* diagnosis, due to their biological stability and improved target affinity, compared to unmodified variants [31]. Following the discovery of two LNA/2'OMe oligonucleotides, one further modified with PS internucleotide linkages and another possessing normal PO linkages, able to specifically target *H. pylori* at human body temperature [38], the present work aimed to reveal whether the LNA/2'OMe oligonucleotides hold promise to be used *in vivo* to detect and treat *H. pylori* infections. Therefore, it was investigated if LNA/2'OMe oligonucleotides can hybridize in *H. pylori* in the presence of native gastric mucus, without prior permeabilization and fixation of the bacteria.

First, it was investigated if LNA/2'OMe can penetrate the bacterial cell envelope without prior permeabilization and fixation, employing FISH without mucus. Paraformaldehyde and ethanol are among the most common permeabilization and fixation agents [5, 27, 36, 37, 56-58]. While paraformaldehyde is a fixative, ethanol acts both as a fixative and permeabilizer of the bacterial membrane lipids [59, 60]. Fixation and permeabilization agents cannot, however, be used for *in vivo* applications due to their toxicity [59]. The obtained results showed successful hybridization of LNA/2'OMe oligonucleotides in untreated *H. pylori*, not only when hybridization and washing were performed at pH 7 (Figure 3.2b), but also under pH conditions representative for the *in vivo* gastric environment (Figure 3.2c). These findings are in line with a previous report, where it was shown that FISH in untreated bacteria was sufficiently efficient to detect bacteria in sludge samples [61]. The penetration of our NAMs into *H. pylori* cytosol was certainly favored by the relatively small size of the NAMs (10 mers). Also, one cannot exclude that having bacteria adhered on a glass slide (needed for the addition of mucus) may facilitate NAMs penetration; it is generally acknowledged that adhered cells are easier to permeate than cells in suspension [62]. In addition, it is of note that the combined use of paraformaldehyde and ethanol did result in a stronger fluorescence of the bacteria (Figures 3.2b, c).

Next, the mobility and stability of the NAMs in native porcine gastric mucus was investigated. So far, the presence of mucus has been neglected in relation to the delivery of macromolecules and antibiotics to *H. pylori* [16, 63-65]. On the other hand, several

studies considering macromolecules/particles diffusion in mucus use mimic models based on commercial mucins or mixtures thereof [48, 66-68], which are still far from the real native gastric mucus [69]. By PAGE it could be confirmed the integrity of both oligonucleotides in native gastric mucus. In addition, by FRAP it was found that gastric mucus is not a major diffusion barrier for the oligonucleotides, although the diffusion of PS in mucus was 3 times slower than in HEPES buffer, while for PO it was 1.2 times slower (D_{PS}=58.1 ± 8.1 μm^2/s and D_{PO}=161.6 ± 8.0 μm^2/s). This difference should not be attributed to the oligos' size (since they differ only in 0.15 kDa), but to the presence of the thioate group (PS). This might lead to more or stronger interactions of oligo PS with mucus components, likely mucins, since a similar interaction trend was observed in a suspension of commercial mucins, by PAGE. In any case, assuming an average thickness of the gastric mucus layer of 180 μm [70], according to Eq. 1 this means that crossing this layer would take only 2 min for PO and less than 5 min for PS.

$$t = \frac{L^2}{2D} \qquad \text{Eq.1}$$

t being the time, L the thickness of mucus layer and D the diffusion coefficient.

Therefore, it can be concluded that the oligonucleotides are sufficiently mobile in gastric mucus and can easily reach the bacteria which are present in the deeper layers of the mucus, adhered to the gastric epithelium.

In the presence of gastric mucus, the hybridization in *H. pylori* was significantly hampered for both types of oligonucleotides (Figures 3.5a, b), even at longer hybridization time (90 min instead of 30 min; results not shown), ruling out the possibility of diffusion being the limiting factor – even though that is not expected from the FRAP diffusion measurements. It is clear that the mucus effect on hybridization was not masked by the dilution upon mixing with the oligonucleotides solution; actually, a relevant dilution may also occur *in vivo*, especially if a common administration by oral gavage is used. Hybridization within mucus must be hindered by interactions of the NAMs with mucus. Mucus is a complex mixture, composed mainly of water and mucins, but also cellular debris, lipids, DNA, proteins, ions [43, 70]. Mechanisms of NAMs mucoadhesion may include electrostatic interactions and multiple low-affinity intermolecular bonds, as hydrophobic interactions and hydrogen bonds, given the increased amount of proton donors and acceptors in mucus [71]. Adhesion of oligo PS was more evident than PO, likely due to the increased hydrophobicity of PS [72] and possible establishment of

disulfide bonds with mucins (mucins are formed by glycoprotein monomers cross-linked by disulfide bonds [36, 37, 55]), thereby explaining the slower diffusion of PS in mucus compared to PO. Interestingly, replacing mucus by a simple mucin suspension did partially restore hybridization (Figure 3.5c). This shows that hybridization in mucus is not simply hindered due to interaction of the oligonucleotides with mucins, but rather with other mucus constituents, as well. Although the particular role/identity of mucus components in the interaction with NAMs remains unclear at this time, soluble factors must account for it, as the FRAP experiments did not show substantial immobilization (i.e. f >0.9). A corollary of the mucin experiment is that clearly a simple mucin dispersion is not a sufficiently accurate model for gastric mucus and may lead to misleading conclusions when used alone. Another factor that might contribute to decreased hybridization in the presence of mucus is that binding of *H. pylori* to mucins [73, 74], may decrease the *H. pylori* envelope surface area available for the NAMs penetration.

3.5 Conclusions

This study points out that *situ* hybridization of LNA/2'OMe NAMs does not require permeabilization of the cell envelope of adherent bacteria, which is beneficial towards potential future *in vivo* hybridization. On the other hand, while neither NAMs stability is a concern nor their diffusion through native gastric mucus is a major obstacle, binding interactions with mucus components other than mucins reduced the hybridization efficiency. This work revealed the importance of considering the effect of a representative, native, undiluted, gastric mucus on NAMs delivery to mucosal sites. Future work should focus on developing strategies that protect the NAMs from interacting with mucus components while traveling through the mucus layer. One interesting approach could be the incorporation of these NAMs into suitable nanocarriers, such as fusogenic liposomes, that shield the NAMs from the environment, while being able to deliver them efficiently into the bacterial cells.

3.6 References

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3.7 Supplementary materials

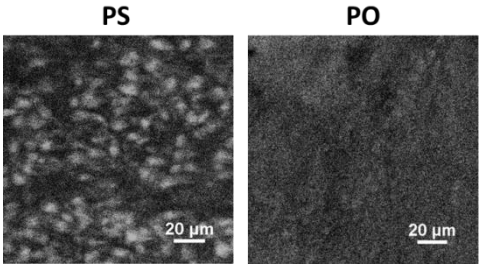

Figure S3.1. Confocal images of porcine gastric mucus after mixing with respectively PS and PO oligonucleotides. Images from FRAP experiments prior to bleaching.

4.

4 INTRACELLULAR DELIVERY OF NAMs IN *HELICOBACTER PYLORI* BY FUSOGENIC LIPOSOMES IN THE PRESENCE OF GASTRIC MUCUS

This chapter is included in the publication:

Rita S. Santos, George R. Dakwar, Elisa Zagato, Toon Brans, Céu Figueiredo, Koen Raemdonck, Nuno F. Azevedo, Stefaan C. De Smedt and Kevin Braeckmans. Intracellular delivery of oligonucleotides in *Helicobacter pylori* by fusogenic liposomes in the presence of gastric mucus. Biomaterials, (2017). **138**: p. 1-12.

Abstract

The rising antimicrobial resistance contributes to 25000 annual deaths in Europe. This threat to the public health can only be tackled if novel antimicrobials are developed, combined with a more precise use of the currently available antibiotics through the implementation of fast, specific, diagnostic methods. Nucleic acid mimics (NAMs) that are able to hybridize intracellular bacterial RNA have the potential to become such a new class of antimicrobials and additionally could serve as specific detection probes. An essential requirement is that these NAMs should be delivered across the mucus, where bacteria are often located, and into the bacterial cytoplasm.

These delivery challenges were considered in relation to the gastric pathogen *Helicobacter pylori*, the most frequent chronic infection worldwide. In particular, we evaluate if cationic fusogenic liposomes are suitable carriers to deliver NAMs across the gastric mucus barrier and the bacterial envelope. This study shows that DOTAP-DOPE liposomes post-PEGylated with DSPE-PEG (DSPE Lpx) can indeed successfully deliver NAMs into *Helicobacter pylori*, while offering protection to the NAMs from binding and

inactivation in native porcine gastric mucus. DSPE Lpx thus offer exciting new possibilities for *in vivo* diagnosis and treatment of *Helicobacter pylori* infections.

Keywords: Infections, Lipoplexes, Native Mucus, Nucleic Acid Mimics, *Helicobacter pylori*

4.1 Introduction

Infectious diseases are responsible for 14 million annual deaths, representing around 90% of the health problems worldwide [1]. Infections that could be treated for decades by classic antibiotics have become a serious threat to human health due to the advent of antimicrobial resistance [1, 2]. However, nucleic acid mimics (NAMs) designed to specifically hybridize *in situ* with complementary bacterial RNA, hold promise both for treatment and diagnosis of infections. Contrary to traditional oligonucleotides, NAMs are composed of modified DNA or RNA sugars that make them resistant to endonuclease degradation and improve their affinity towards RNA targets [3-6].

NAMs can be designed to act as antisense antimicrobials, by hybridizing and consequently inhibiting the expression of selected genes [7-9]. These can be essential bacterial genes, thus preventing bacteria growth, or genes involved in the resistance to antibiotics, thus restoring bacteria susceptibility to antibiotics. This strategy provides a potentially endless source of active antibacterials. Even if the bacterial target undergoes a point mutation, which is the most common form of resistance to antibiotics [10], the oligonucleotide can be easily redesigned to become effective again.

In addition, NAMs conjugated with a contrast agent could serve as detection probes for rapid and comprehensive *in vivo* diagnosis. They could detect not only the presence of specific bacteria, but also the presence of bacterial genes responsible for resistance to antibiotics, so that an effective antibacterial drug can be prescribed in time [3, 11-13]. Compared to the current diagnostic techniques, labelled NAMs have the potential to overcome the traditional time-consuming culture methods as well as the need of bacteria isolation and extraction of target genetic material associated with other molecular methods, like PCR [2, 12, 14].

In addition to their value for therapy and clinical diagnosis, the opportunity to directly localize bacteria *in vivo* is also of interest for research purposes. NAMs hold the potential

to help unravelling the host-microbial and microbial-microbial interactions that shape the immune system and determine the disease state [13, 15, 16].

For NAMs to fulfil their promise as a flexible platform for diagnosis and treatment of bacterial infections, they should be safely delivered across biological barriers in the body. Mucus in the gastrointestinal, respiratory, reproductive and urinary tracts presents a first barrier for NAMs [17-19]. Mucus is a highly complex and viscoelastic network able to bind foreign entities (through electrostatic, hydrophobic or hydrogen bonds) and/or sterically obstruct particles which are larger than the size of the pores in mucus [18, 20, 21]. Therefore, NAMs need to be able to pass through mucus to reach the target bacteria [22]. Importantly, they should do so without losing their activity. Indeed, it was shown in the previous chapter that interactions with gastric mucus can significantly compromise the ability of NAMs to hybridize with *Helicobacter pylori* (*H. pylori)*.

Apart from crossing the mucus layer, it is pivotal that the NAMs overcome the multilayered bacterial cell envelope. It was showed in the previous chapter that small NAMs could be internalized into adherent *H. pylori* cells. However, insufficient penetration of oligonucleotides into bacterial cells in suspension is recognized as a major bottleneck that limits the success of antisense therapy against bacteria [8, 23]. Therefore, a strategy to overcome the bacterial envelope would be valuable to maximize the potential of NAMs to manage bacterial infections. Strategies to permeabilize the bacterial envelope *in vitro* include electroporation, enzymatic (e.g. lysozyme) or chemical (e.g. ethanol) treatment. None of those are, however, easily transferable to infections *in vivo*.

In this work both delivery challenges were considered, i.e. crossing mucus and the cell envelope, in the context of gastric *H. pylori* infection (Figure 4.1a), which is the most frequently occurring chronic bacterial infection worldwide [24]. *H. pylori* reside within the gastric mucus layer as well as in close proximity with the epithelial cells underlying the gastric mucus layer [25]. Here we investigated if fusogenic stealth liposomes are suitable nanocarriers for NAMs to treat and diagnose *H. pylori* infections. Liposomes have been extensively studied to deliver nucleic acids into eukaryotic cells [26]. Until now their application to treat bacterial infections is almost exclusively limited to the delivery of traditional small molecule antibiotics [27-30]. So far, only two papers report on the use of liposomes to deliver phosphorothioate DNA in *Escherichia coli* and in *Staphylococcus aureus* [31, 32]. In spite of its promise it shows that liposomal delivery of nucleic acids into bacteria is a virtually unexplored area.

In the current study, fusogenic cationic liposomes were used, made from the lipids DOTAP and DOPE, which are well-characterized carriers for nucleic acids in eukaryotic cells [33-35]. It was herein reasoned that DOTAP-DOPE liposomes may be of interest for delivery in gram-negative bacteria due to the presence of the fusogenic lipid DOPE which may promote fusion of the liposomes with the outer membrane, thus allowing diffusion through the periplasm into the bacterial cytoplasm [36, 37]. Based on previous work from the group, LNA and 2'OMe, with either phosphodiester (PO) or phosphorothioate (PS) as backbone linkages (Figure 4.1b), were used as NAMs to hybridize to *H. pylori* rRNA [38, 39]. Electrostatic complexes between (anionic) NAMs and (cationic) DOTAP-DOPE liposomes were formed, which were further modified by post-insertion of PEG-lipids with the aim to improve the stability and mobility of the complexes in the harsh gastric environment [40, 41]. We tested two PEGylation strategies: DSPE-PEG that stably incorporates into liposomes and Cer-PEG that can diffuse out of the liposomes over time [42, 43]. First, it was evaluated if those PEGylated lipoplexes have good diffusional mobility in porcine gastric mucus isolated from pigs, being a clear prerequisite to be able to reach the bacteria dispersed in the mucus. Next, it was investigated whether the PEGylated lipoplexes could deliver NAMs into *H. pylori* in suspension by fluorescence *in situ* hybridization (FISH). Finally, it was assessed if the PEGylated lipoplexes could still successfully deliver functional NAMs in *H. pylori* in the presence of gastric mucus.

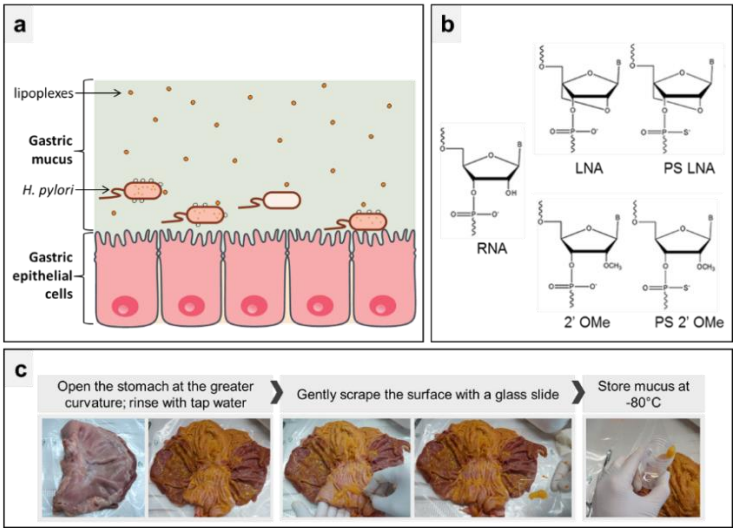

Figure 4.1 (a) Illustration of the study concept: representation of liposomal delivery of NAMs across the gastric mucus to target *H. pylori*. (b) Monomers of the NAMs used in FISH, compared to RNA (Adapted from Fontenete et al. [38]. (c) Procedure followed for the collection of mucus from the stomach of pigs.

4.2 Materials and methods

4.2.1 Materials

(2,3-Dioleoyloxy-propyl)-trimethylammonium-chloride (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethyleneglycol)-2000) (DSPE-PEG), N-octanoyl-sphingosine-1-succinyl[methoxy(polyethylene glycol)2000] (CerC8-PEG) and DOPE-LissamineRhodamineB were purchased from Avanti Polar Lipids (Alabaster, AL). Yellow-green (505/515) fluorescent carboxylate-modified polystyrene FluoSpheres® of 40, 100, 200 and 500 nm of diameter were purchased from Invitrogen Molecular Probes (Eugene, OR). N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) were acquired from Sigma-Aldrich (Bornem, Belgium) and 2 kDa methoxy-polyethylene glycol-amine (mPEGa) from Creative PEGWorks (Winston Salem, USA). Chloroform, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl), sodium chloride (NACl) and triton X-100 were purchased from Sigma-Aldrich (Bornem, Belgium). Paraformaldehyde was acquired from Fluka – Sigma-Aldrich, (Bornem, Belgium). Urea was purchased from Vel – VWR (Haasrode, Belgium). Pepsin (from porcine gastric mucosa) was purchased from Merck Millipore (Madrid, Spain). Trypticase soy agar plates supplemented with 2% (v/v) sheep blood were purchased from Becton Dickinson GmbH (Erembodegem, Belgium). CampyGen sachets to generate microaerobic conditions were acquired from Oxoid – Thermo Scientific (Waltham, MA, USA).

4.2.2 Collection of porcine gastric mucus

Mucus scraped from the stomach of pigs was used in this study, as its bio-relevance is markedly higher than mucus solutions prepared from commercial mucins [44, 45]. Also, pigs have a gastric physiology similar to humans, making them representative animal models for *Helicobacter* infection studies [46, 47].

Stomachs were collected from a local slaughterhouse, opened at the greater curvature and gently rinsed with tap water to remove most of the food debris [48]. The mucus,

loosely and firmly adherent, was then gently scrapped off with a glass slide, aliquoted and stored at -80°C (Figure 4.1c). Mucus from three different pigs was included in each experiment.

4.2.3 NAMs synthesis

Two oligonucleotides complementary to a sequence of *H. pylori* 16S rRNA, were used in this study. These are composed of LNA and 2'OMe, possess the same sequence and differ only in the internucleotide bonds. One possesses normal phosphodiester oligonucleotides (PO), while the other has one of the two non-bridging oxygen atoms replaced by a sulphur atom at each internucleotide linkage (PS) (Figure 4.1b). These oligonucleotides will be herein designated as PO and PS, respectively. The sequence of PO is 5'-lGmeAmeClTmeAmeAlGmeCmeClC-3', while the sequence of PS is 5'-lG*meA*meC*lT*meA*meA*lG*meC*meC*lC*-3', where "l" represents the LNA monomers, "me" the 2'OMe monomers and * the phosphorothioate linkages. These oligonucleotides were fluorescently labelled at 5' with Cy3. They were synthetized and purified according to [38].

4.2.4 Preparation of liposomes and lipoplexes and colloidal stability in simulated gastric juice

Cationic liposomes composed of DOTAP and DOPE (in a 1:1 molar ratio) were prepared as reported before [49]. In brief, the lipids dissolved in chloroform were mixed and a dry lipid film was prepared by rotary evaporation of the chloroform at 40 ºC. The lipid film was hydrated with 20 mM HEPES buffer (pH 7.2) and sonicated using a probe sonicator (Branson Ultrasonics Digital Sonifier®, Danbury, USA), resulting in a final concentration of 5 mM DOTAP and 5 mM DOPE. The average hydrodynamic size and zeta potential of the cationic liposomes was routinely checked by dynamic light scattering (Zetasizer Nano-ZS, Malvern, Worcestershire, UK).

Lipoplexes were prepared through complexation of cationic liposomes with Cy3 labeled NAMs at a +/- charge ratio of 15; the +/- charge ratio is calculated as the molar amount of positive charges on the DOTAP molecules, divided by the molar amount of negative charges on the NAMs (with 1 NAM molecule containing 10 negatively charged phosphate groups). Complexation was performed by addition of a diluted dispersion of

cationic liposomes to a NAM-solution (PS or PO), followed by incubation for 30 min, at room temperature.

PEGylation of the thus obtained lipoplexes was performed by 'post-insertion' of PEG lipids (2 kDa CerC8-PEG or 2 kDa DSPE-PEG) [50]. Therefore, the PEG lipids were first re-dissolved in sterile milli-Q water (after chloroform removal by nitrogen flush), added to the DOTAP/DOPE-NAM complexes and incubated for 1h at 37 ºC. The PEG lipids accounted for 10% of the total lipids in the lipoplexes. The post-PEGylation efficacy was evaluated from the decrease in the (absolute) zeta potential value of the complexes, measured with the Zetasizer Nano-ZS. Throughout the paper, cationic lipoplexes are referred as Lpx, lipoplexes post-PEGylated with DSPE-PEG as DSPE Lpx, and lipoplexes post-PEGylated with Cer-PEG as Cer Lpx.

Upon oral intake, the formulations will come in contact with the gastric juice. Therefore, it was first tested if the liposomes are colloidally stable in simulated gastric juice (sGJ). The sGJ was prepared according to the US pharmacopeia: a solution of 0.2% (w/v) NaCl, 0.32% (w/v) gastric pepsin and 0.7% (v/v) HCl, final pH ≈ 1.2. The liposomes were diluted (10 times) in sGJ and incubated during 4 hours at 37 ˚C. Their hydrodynamic diameter was measured by dynamic light scattering.

4.2.5 PEGylation of polystyrene nanospheres

To assess the effect of the size of nanoparticles on their diffusion through gastric mucus isolated from pigs, polystyrene FluoSpheres® were PEGylated to render them muco-inert. Indeed, PEGylation may avoid the potential binding of the nanospheres to compounds present in mucus [19, 21].

Yellow-green (505/515) fluorescent carboxylate-modified polystyrene FluoSpheres® of 40, 100, 200 and 500 nm nominal diameter were covalently modified with PEG via amine coupling, as described elsewhere [40, 51]. In brief, mPEGa, EDC and sulfo-NHS were dissolved in HEPES Buffered Saline (HBS, pH 8). The nanospheres were added to this mixture so that a final concentration of 4 mg/mL EDC, 1.13 mg/mL sulfo-NHS, 10 mg/mL mPEGa and 1% solids (nanospheres) was obtained. The reaction mixture was left shaking overnight, at 200 rpm. Purification was then performed using a centrifugal filter (Amikon ultra centrifugal filters, 100 K membrane, Millipore, MA, USA) for 12 min at 14000 rpm, followed by two washings in HBS (12 min at 14000 rpm). The filter was then placed upside down in a new vial and the purified PEGylated nanospheres were collected

after centrifugation for 3 min at 1000 rpm. They were resuspended in HBS to a final concentration of 2% solids (nanospheres) and stored at 4 °C, until use. To allow optimal PEG coverage a slightly different protocol was used for the polystyrene nanospheres of 40 nm nominal diameter. The reaction mixture contained 0.5% solids (40 nm nanospheres) and it was left rotating for 5 days, at 30 rpm, using a Hulamixer (Life Technologies, Carlsbad, CA, USA). Purification was done by dialysis for 6h, employing a Slide-A-Lyzer™ dialysis cassette with a 10 kDa molecular weight cut off (Thermo Scientific, Waltham, MA, USA), followed by ultracentrifugation (L8-70 M, Beckman, Coulter, Fullerton, CA, USA). The purified PEGylated 40 nm nanospheres were resuspended in HBS to a final concentration of 1% solids and stored at 4 °C.

PEGylation of the polystyrene nanospheres was confirmed by measuring the zeta potential of the nanospheres in HEPES buffer, before and after reaction, using a Zetasizer Nano-ZS.

4.2.6 Diffusion measurements of polystyrene nanospheres and lipoplexes in gastric mucus by single particle tracking (SPT)

Diffusion measurements on the PEGylated polystyrene nanospheres and the (fluorescent) lipoplexes in mucus from the stomach of pigs were performed by fluorescence single particle tracking (SPT), at 37 °C, as described by our group elsewhere [40, 52]. For this, the PEGylated nanospheres were first sonicated for 5 min and diluted in sterile milli-Q water to a concentration suitable for SPT-measurements. Five µL of the diluted PEGylated nanospheres was gently mixed with ≈ 30 µL of mucus (taken with a plastic syringe, due to its high viscosity). This mixture was placed between a microscope slide and a coverslip with a double-sided silicon spacer of 1 mm thickness and 20 mm diameter in between (Press-to-seal silicone isolators; Molecular Probes, Leiden, The Netherlands). Measurements in sterile milli-Q water were performed as a control. For these, 7 µL of properly diluted PEGylated nanospheres was placed between a microscope slide and a coverslip with a double-sided adhesive spacer of 0.12 mm thickness and 9 mm diameter in between (Secure-Seal spacer; Molecular Probes, Leiden, The Netherlands). Before starting the SPT-measurements, the samples were equilibrated for 10 min at 37 °C in a stage-top incubator (Tokai Hit, Fujinomiya, Japan) mounted on the microscope.

The diffusion measurements on the lipoplexes (Lpx, Cer Lpx and DSPE Lpx) were performed in the same way as described above for the PEGylated nanospheres. For these

measurements, the liposomes were prepared to contain 1 mol% DOPE-LissamineRhodamineB for fluorescence imaging within the mucus.

To study the diffusion of the fluorescent nanospheres and lipoplexes, typically 25 to 35 'SPT-movies' were recorded at different locations in the mucus. Each SPT-movie was 5 s long and consisted of about 155 frames (recorded at a rate of 30.9 frames per second). The SPT-measurements were done on a custom-built laser wide field microscope setup [40, 52]. The videos were analysed using an in-house developed software [53] to translate the nanoparticles trajectories into a distribution of apparent diffusion coefficients.

4.2.7 Fluorescence *in situ* hybridization in *H. pylori* in suspension by free NAMs and lipoplexes

H. pylori 26695 (ATCC 700392) was grown in trypticase soy agar supplemented with 5% sheep blood (Becton Dickinson GmbH, Germany) for 48 hours, at 37 °C, under microaerobic conditions. The bacteria were then harvested from the agar plates, using sterile saline solution, and diluted to nearly $2x10^8$ cells/mL. The FISH procedure was based on a previously reported protocol [54], with some modifications. The bacteria in suspension were either treated with ethanol or mixed directly with the lipoplexes.

For FISH experiments by ethanol based permeabilization of the bacteria, 100 µL of the bacterial suspension was pelleted by centrifugation for 15 min at 10000 x g and resuspended in 500 µL of 50% (v/v) ethanol, for 15 min. Large *H. pylori* aggregates were removed by filtration through a sterile 10 µm pore size filter (CellTrics®, Görliz, Germany). 100 µL of the ethanol treated bacteria was then resuspended in 100 µL of hybridization solution (900 mM NaCl, 500 mM Urea, 50 mM Tris-HCl, pH 7) containing 400 nM of Cy3 labelled NAM PS or PO.

For FISH experiments using lipoplexes, 100 µL of the initial bacterial suspension (diluted to a similar concentration as described above for the ethanol treatment) was resuspended in 100 µL of hybridization solution containing lipoplexes (Lpx, Cer Lpx or DSPE Lpx) instead of free NAM. The amount of lipoplexes added was such that 400 nM of NAM PS or PO was present in the hybridization mixture. To be able to take into account the auto-fluorescence of *H. pylori*, negative control experiments were performed on bacteria resuspended in hybridization solution without NAM, for the conditions tested (with/without ethanol permeabilization and liposomes). As all negative controls looked the same, we only present example images of bacteria without exposure to ethanol nor

liposomes. Hybridization was then performed by incubating the sample at 37 °C, for 30 min. Hybridization was followed by a washing step. For this, the bacteria were pelleted by centrifugation at 10000 x g for 5 min, and resuspended in 500 µL of pre-warmed (37 °C) aqueous solution at pH 7. Instead, some samples were resuspended in 500 µL of a 0.1% (v/v) Triton X-100 solution to remove lipoplexes that remain bound to the bacterial outer membrane. The samples were then incubated at 37 °C, for 15 min. After washing, the bacteria were centrifuged at 10000 x g for 5 min and resuspended in sterile milli-Q water. 20 µL of bacterial suspension was placed on a diagnostic slide (Thermo Scientific, Waltham, MA, USA) and allowed to dry at 37 °C.

The slides were mounted with immersion oil and a coverslip and visualized under the microscope. Microscopy images were acquired using a Nikon TE2000 microscope equipped with a Nikon DS-Qi 1Mc digital camera and a Plan Apo VC 100× 1.4 NA oil immersion objective lens (Nikon). Cy3 fluorescence was visualized using a TRITC filter (excitation: 530-560 nm; emission: 590-650 nm, Nikon). For fluorescence quantification, all samples were analysed using the same exposure time and the same excitation intensity; around 10 images per slide well were taken at different locations of the well.

4.2.8 Fluorescence *in situ* hybridization in *H. pylori* by free NAMs and DSPE Lpx exposed to gastric mucus

The hybridization efficiency of free NAMs and DSPE Lpx (being the most successfully Lpx in delivering the NAMs into *H. pylori* in suspension, see above), mixed with porcine gastric mucus was subsequently tested. As this required the application of mucus on top of the bacteria, it was not feasible to perform FISH on bacteria in suspension; instead FISH was performed on bacteria applied on glass slides, following the method used in the previous chapter. Briefly, *H. pylori* in the exponential growth phase was harvested from the agar plates, using sterile saline solution, and diluted to nearly $1.6x10^6$ cells/mL. Smears were prepared on glass slides (20 µL per slide well) by drying at 37 °C. The bacterial cells were fixed before contact with the mucus by incubation with 40 µL of 4% (w/v) paraformaldehyde, for 10 min. The free NAMs or the DSPE Lpx were 10x diluted in hybridization solution. 20 µL of these samples was added to approximately 30 µL of mucus and gently mixed. The final concentration of NAMs (whether free or in lipoplexes) within this mucus mixture was 400 nM (as is the case in the FISH experiments described above on FISH in suspension). The bacteria smear on

the glass slide was covered by the mucus mixture and closed by a coverslip. The slide was placed in a dark moist chamber, hybridization was allowed for 30 min, at 37 °C. After hybridization, the coverslip was removed together with the mucus mixture and the slide was washed during 15 min, 37 °C, in pre-warmed aqueous solution, at pH 7. The slide was allowed to dry and visualized under the microscope as described above. To be able to take into account the auto-fluorescence of *H. pylori*, negative controls were performed using hybridization solution without free NAMs nor lipoplexes.

4.2.9 Quantification of *H. pylori* hybridization

For both hybridization with and without mucus the quantification was based on fluorescence microscopy and image processing, so that a reliable comparison could be done between all conditions; flow cytometry quantification would not be an option since it is not applicable to hybridization with mucus.

The fluorescence of the bacteria obtained in the FISH experiments was quantified using an in-house developed software based on image processing routines available in Matlab (The MathWorks, Natick, MA, USA). First, bacteria were identified in each image based on an automatically determined intensity threshold. This results in a binary mask that corresponds to the location of bacteria in the image. For the regions within the mask, the average fluorescence intensity in the original image (F_{image}) was quantified as:

$$F_{image} = \frac{\sum_{b=1}^{n}[(f_b - BG_b) \times \#pix_b]}{\sum_{b=1}^{n} \#pix_b},$$

where b refers to each bacterium, n corresponds to the number of bacteria in the image, f_b corresponds to the mean pixel fluorescence of each bacterium, BG_b is the local background value around each bacterium and $\#pix_b$ is the number of pixels comprising each bacterium.

Per sample, around 10 images were analysed in this fashion. Three repeated samples were analysed per treatment, from which the average fluorescence intensity was calculated. These absolute values were normalized to the respective positive control of PS or PO. Three independent experiments were performed and the average normalized fluorescence from the 3 treatment replicates was finally obtained.

4.2.10 Statistical analysis

Statistical analysis was performed by GraphPad Prism6 software (GraphPad Software, San Diego, USA). The FISH results were analysed using two-way analysis of variance (ANOVA) and Sidak's multiple comparison test, comparing the samples with the respective reference (fluorescence 1) within the same NAM. Fluorescence 1 corresponds to ETH+NAM (Figure 4.3 and 4.5), or NAM (Figure 4.6). Significance was set at $P \leq 0.05$ (**** $P \leq 0.0001$, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$).

4.3 Results

4.3.1 Characterization of the liposomes and lipoplexes

Cationic liposomes were prepared from DOTAP and DOPE, in a 1:1 molar ratio, following the lipid film hydration method [49]. The liposomes had an average hydrodynamic diameter of around 80-100 nm and an average zeta potential about +40-55 mV. Cationic lipoplexes were prepared by incubation of DOTAP-DOPE liposomes with NAM PS or PO. The cationic lipoplexes (Lpx) were subsequently post-PEGylated with Cer-PEG (Cer Lpx) or DSPE-PEG (DSPE Lpx). Both types of PEG chains were successfully inserted into the complexes, as was observed from the decrease in zeta potential of the lipoplexes (Figure S4.1a). In all cases the final zeta potential of the PEGylated lipoplexes was around 15 mV, with an average hydrodynamic diameter ranging from 98 nm to 133 nm (Figure S4.1a). Upon oral intake, liposomes will come into contact with the gastric juice. It was, therefore, tested the colloidal stability of the liposomes (both for non-PEGylated and PEGylated liposomes) in simulated gastric juice, and found that their size remains constant for at least 4h (Figure S4.2a). Together with the chemical stability of free NAMs under gastric pH [54], it is a strong indication that the gastric juice will not impose a constraint in a clinical application.

4.3.2 Diffusion measurements of polystyrene nanospheres and lipoplexes in gastric mucus by single particle tracking (SPT)

As gastric mucus represents a challenging diffusional barrier, it was first evaluated how the size of nanoparticles influences their mobility in gastric mucus. To that end, the diffusion of model nanospheres of defined sizes was assessed. It was noticed that the nanospheres of 40 nm nominal size were actually bigger with a hydrodynamic radius of approximately 60 nm, as measured by dynamic light scattering (Figure S4.1b); therefore, in the presented results they are referred as ’60 nm’ nanospheres (instead of ’40 nm’). The nanospheres were first PEGylated to avoid muco-adhesion [20, 40]. The PEG could be conjugated to the polystyrene nanospheres, as judged from the clear zeta potential change towards neutral values (Figure S4.1b).

As expected, due to the size-filtering effect of mucus [18], the larger the PEGylated nanospheres, the lower their diffusional mobility in gastric mucus, as measured by SPT (Figure 4.2a and Table S4.1). Both 60 and 100 nm nanospheres showed a bimodal distribution of diffusion coefficients, indicating that a part of those particles is mobile while another fraction is immobilized. The mobile fraction was clearly smaller for the 200 nm nanospheres and almost non-existing for the 500 nm particles. This shows that the mesh size of gastric mucus is quite heterogeneous, as already observed before for gastrointestinal mucus [17, 18]. Other types of mucus do not seem to show such high level of heterogenicity; for instance in cystic fibrosis sputum PEGylated nanospheres of 100 nm and 200 nm (similar to the ones used here) showed good and uniform mobility with an unimodal distribution of diffusion coefficients [40]. The diffusion results in gastric mucus presented in this study indicate that nanoparticles should preferably be as small as possible for efficient diffusion in gastric mucus, and in any case they should be smaller than 200 nm.

While the lipoplexes under investigation do fulfil this criterion, their diffusion in porcine gastric mucus was experimentally verified by SPT as well. Figure 4.2b show that non-PEGylated Lpx (Lpx) clearly had the lowest mobility, as could be expected due to interactions with mucus constituents. Post-PEGylation (Cer Lpx and DSPE Lpx) increases the mobile fraction slightly, resulting in a 3 to 5-fold higher average diffusion coefficient (Table S4.1). Nevertheless, the improvement of the diffusion by PEGylation is rather moderate and a substantial non-mobile fraction remains present. It confirms that nanoparticle size poses a serious limitation to diffusion in the dense gastric mucus.

In the previous chapter it was found that the type of internucleotide linkage (PS or PO) affected the diffusion and interaction with mucus. In particular, the PS modification (generally used to increase oligonucleotides stability against nucleases and affinity towards the target sequence [55]) led to slower diffusion and increased interaction with mucus components. The results presented in this chapter show that the use of liposomes as carriers for the NAMs removes these differences as they diffuse at a similar rate irrespective of the type of NAM used.

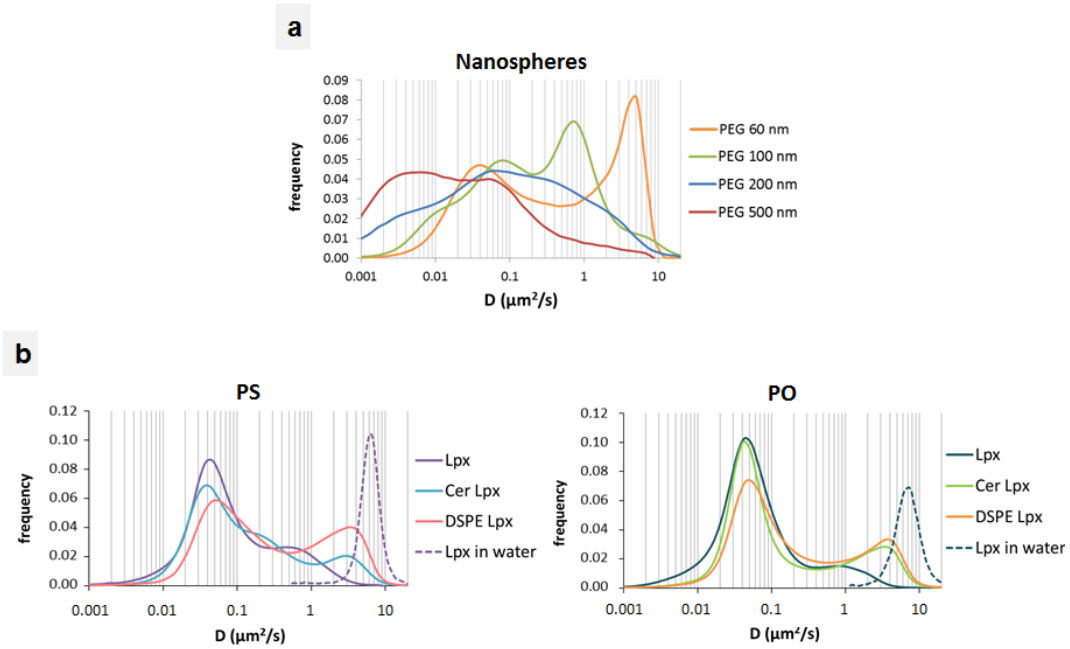

Figure 4.2 Distribution of diffusion coefficients of polystyrene nanospheres and lipoplexes in gastric mucus isolated from pigs. Results obtained on gastric mucus from 3 different porcine mucus samples were pooled. (a) PEGylated nanospheres of around 60 nm (PEG 60 nm), 100 nm (PEG 100 nm), 200 nm (PEG 200 nm) and 500 nm (PEG 500 nm) in diameter. (b) Cationic lipoplexes (Lpx) compared with Cer Lpx and DSPE Lpx. The dotted line shows the diffusion of Lpx in water.

4.3.3 Fluorescence *in situ* hybridization in *H. pylori* in suspension by free NAMs and lipoplexes

After crossing the gastric mucus, lipoplexes should be able to deliver the NAMs across the cell envelope (Figure 4.1a). To test this, we first compared the hybridization efficiency of lipoplexes in a suspension of *H. pylori* to the hybridization efficiency of free NAMs in

a suspension of *H. pylori* permeabilized by typical ethanol treatment [54]. As presented in Figure 4.3, similar results were found for PO and PS NAMs. Free NAMs are only slightly taken up by untreated cells in suspension, which increases 2-3 fold upon permeabilization of the bacteria with ethanol. Interestingly, Lpx and Cer Lpx performed equally well as the standard ethanol treatment. The lipoplexes functionalized with DSPE-PEG performed even better, with a 4-fold higher hybridization efficiency as compared to ethanol treatment. Based on the transfection experience with eukaryotic cells it was rather unexpected that non-PEGylated Lpx did not have the best performance, since PEGylation typically reduces the interactions with the cell membrane [56, 57]. It is reckoned that this is due to the extensive aggregation of non-PEGylated Lpx in the hybridization solution (Figure S4.2b), which likely reduces the liposomal interaction with the bacterial envelope. PEGylated Lpx, on the other hand, showed excellent colloidal stability in the hybridization solution (Figure S4.2b). This improved stability did not prevent effective lipoplexes interactions with *H. pylori* membrane (as shown in Figure 4.3). This is in line with previous reports on antimicrobials delivery into bacteria by PEGylated liposomes [58, 59] which were shown to interact with bacterial cell membranes [60].

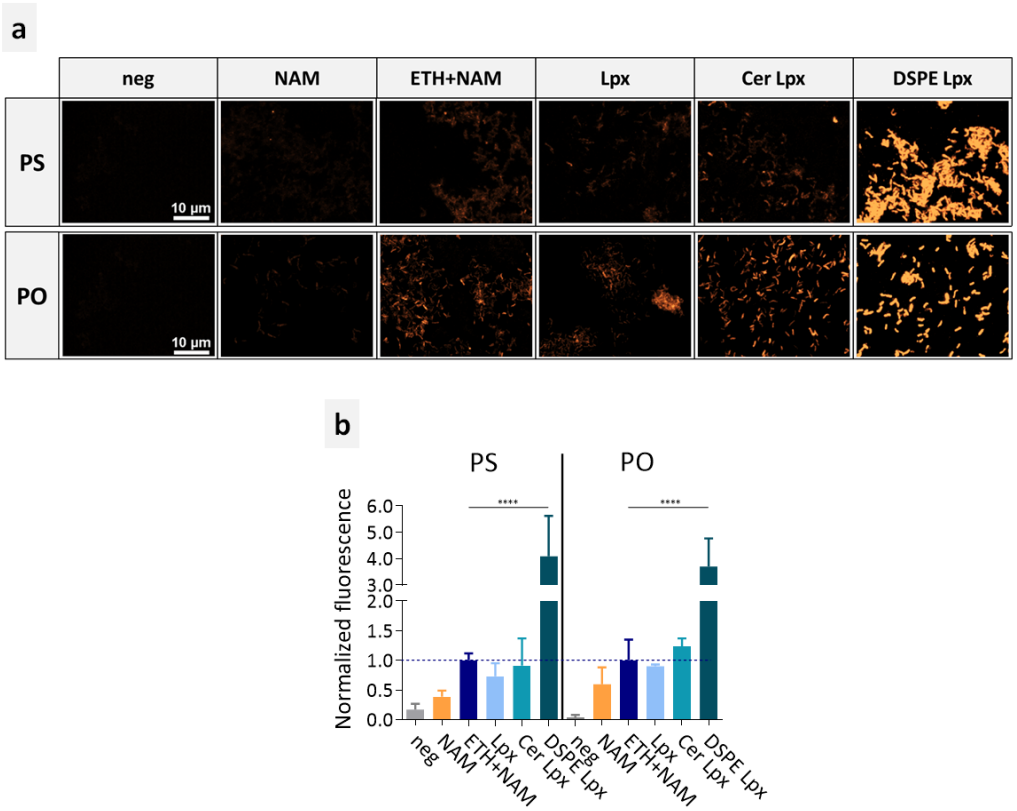

Figure 4.3. FISH in *H. pylori* in suspension. The hybridization efficiency of Lpx, Cer Lpx and DSPE Lpx was compared with the hybridization efficiencies observed with free NAMs in *H.*

pylori respectively not treated (NAM) and pre-treated with ethanol (ETH+NAM), for both PS and PO. Negative controls without NAM (neg) were also included. (a) Representative epifluorescence microscopy images, all taken with the same exposure time. (b) FISH fluorescence normalized to that of free NAM in ethanol permeabilized *H. pylori* (ETH+NAM). Within one experiment, each condition was performed in triplicate. Three independent experiments were performed. Results are presented as mean values and respective standard deviation.

4.3.4 Confirmation of the intracellular delivery of NAMs in *H. pylori* by DSPE Lpx

The FISH results as presented in Figure 4.3 are obtained by fluorescence microscopy. It is to be noted, however, that the width of the bacteria is only 0.5-1 µm, which is rather close to the optical resolution limit (~ 0.25 µm). As such, it was intended to confirm that the fluorescence of the bacteria is coming from intracellular NAMs and not just from DSPE Lpx that remain attached to the outer surface of the bacteria. To this end, DSPE Lpx were applied to *H. salomonis* to which the NAMs should not hybridize [38, 54]. Interestingly, for both PS and PO, DSPE Lpx appeared as fluorescent halos around *H. salomonis* cells, with no fluorescence in the cytosol (Figure 4.4). This is in stark contrast with the uniform fluorescence that is observed throughout the *H. pylori* cells (Figure 4.4). This confirms that the observed fluorescence in *H. pylori* at least in part stems from intracellular NAMs retained by hybridization with the target rRNA.

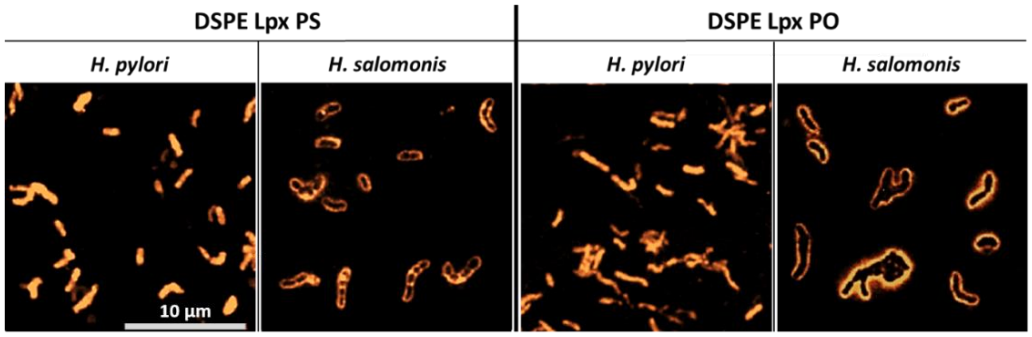

Figure 4.4. FISH using DSPE Lpx PS and DSPE Lpx PO, respectively, in *H. pylori* and *H. salomonis*. Uniform fluorescence is seen in *H. pylori*, while *H. salomonis* shows a hollow interior with an extracellular fluorescent halo.

It cannot be excluded, however, that part of the observed fluorescence in *H. pylori* (Figure 4.4) results from remaining membrane-bound DSPE Lpx. To find this out, a mild triton wash was performed after hybridization. The samples were washed with a 0.1% (v/v) Triton X-100 solution (instead of the regular washing solution), to remove any remaining lipoplexes that are bound to the outer bacterial membrane. As expected, the extracellular fluorescent halos in *H. salomonis* could be almost completely removed by the triton wash (Figure S4.3), which demonstrates that this protocol works as intended. When applied to *H. pylori*, the triton did lower the observed fluorescence intensity, leaving only the fluorescence coming from the intracellular NAMs (Figure 4.5). For PS NAMs, the remaining fluorescence was at the same level as for ethanol permeabilized cells treated with free NAMs. For PO NAMs, the fluorescence remained about 3-fold higher. The reason for this difference is not clear. In conclusion, although a part of the DSPE Lpx PS fluorescence in *H. pylori* did come from membrane bound lipoplexes, these results give clear evidence that NAMs were successfully delivered inside *H. pylori* to an extent that is at least as good as the typical *in vitro* ethanol permeabilization method.

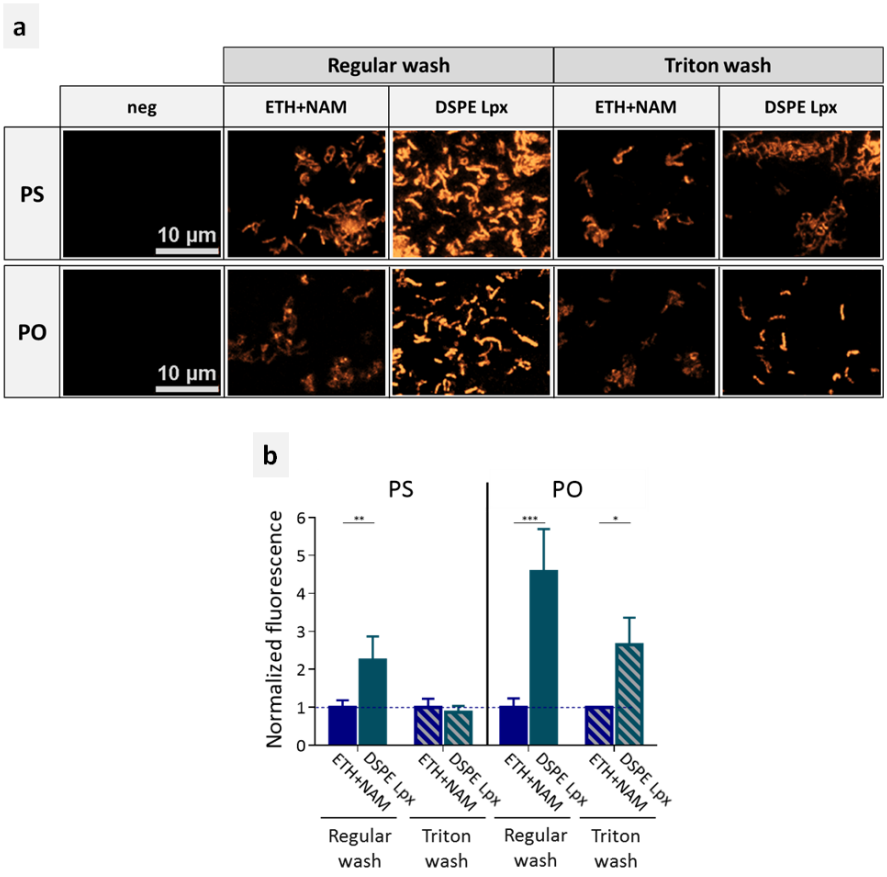

Figure 4.5.Discrimination between extracellular adhesion and intracellular delivery of NAMs into *H. pylori* cells by lipoplexes. The *H. pylori* fluorescence obtained upon regular wash (representing

the total, i.e. intra- plus extracellular, fluorescence) was compared with the one obtained upon mild triton wash (representing the intracellular fluorescence). DSPE Lpx were compared with free NAMs in ethanol treated bacteria (ETH+NAM). Negative controls without NAMs (neg) were also included. (a) Representative epifluorescence microscopy images (all taken with the same exposure time). (b) FISH fluorescence normalized to that of free NAMs in ethanol treated (ETH+NAM) *H. pylori*. Within one experiment, each condition was performed in triplicate. Three independent experiments were performed. Results are presented as mean values and respective standard deviation.

4.3.5 Fluorescence *in situ* hybridization in *H. pylori* by free NAMs and DSPE Lpx exposed to gastric mucus

It was showed above that DSPE Lpx can successfully deliver NAMs in *H. pylori* in suspension (Figure 4.4 and 4.5) while at least a fraction of DSPE Lpx is mobile in gastric mucus isolated from pigs (Figure 4.2b). In a next step, it was investigated whether hybridization in *H. pylori* by DSPE Lpx is still successful in the presence of gastric mucus. To this end, free NAMs or DSPE Lpx dispersed in hybridization solution were mixed with porcine gastric mucus (in a 2:3 ratio) and applied on top of an *H. pylori* bacterial smear on a glass slide. After incubation for 30 min, the mucus mixture was removed, the slide was washed (regular wash) and cells were imaged by fluorescence microscopy. Compared to free NAMs, the DSPE Lpx show a 2-2.7 fold increase in fluorescence intensity of *H. pylori*, both for PO and PS NAMs (Figure 4.6). The question again comes if perhaps part of this fluorescence is coming from membrane bound lipoplexes. When the same experiments were performed with *H. salomonis*, the outer fluorescent rim, as observed in solution, was no longer present (Figure S4.4). This suggests that excessive sticking of lipoplexes to the outer membrane does not happen in the presence of mucus, so that the observed fluorescence in *H. pylori* (Figure 4.6a and 4.6b) can be ascribed to intracellularly delivered and hybridized NAMs. Taken together it can be concluded that DSPE Lpx (both PS and PO) have the capacity to deliver NAMs into *H. pylori* even in the presence of the tough gastric mucus barrier.

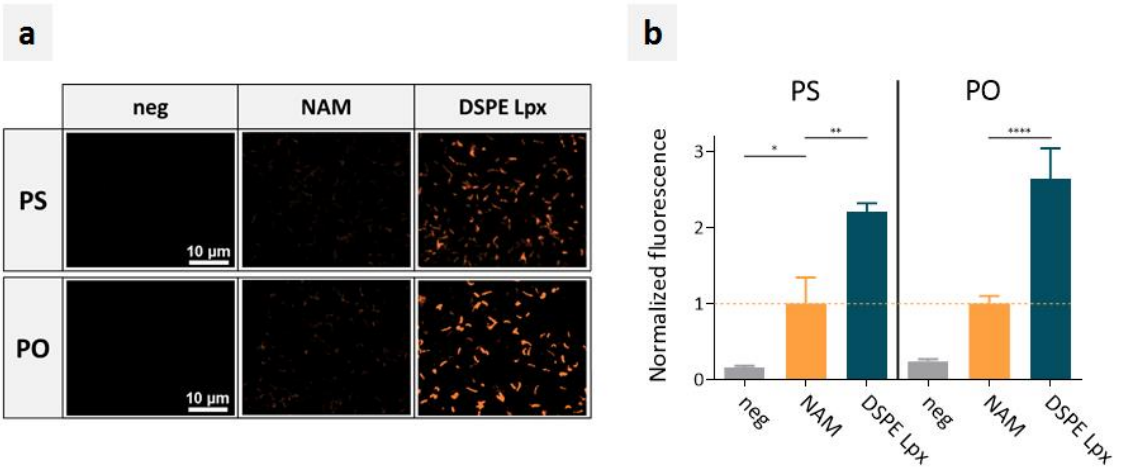

Figure 4.6.FISH in *H. pylori* (on a glass slide) by free NAMs and DSPE Lpx exposed to gastric mucus isolated from pigs. Note that there was no pre-treatment of the bacteria with ethanol. Negative controls without NAMs (neg) were also included. (a) Representative epifluorescence microscopy images, all taken with the same exposure time. (b) FISH fluorescence normalized to that of free NAMs. Within one experiment, each condition was performed in triplicate. Independent experiments were done using mucus from 3 different pigs. Results are presented as mean values and respective standard deviation.

4.4 Discussion

For NAMs to become a novel platform to target infections *in vivo* for diagnosis and therapy, they have to cross biological barriers, like the bacterial cell envelope and the mucus that covers the epithelia, in order to reach their intracellular bacterial targets without loss of activity [22, 61]. It was investigated whether liposomes, composed of the cationic lipid DOTAP and the neutral fusogenic lipid DOPE, would be suitable nanocarriers for delivering NAMs across the gastric mucus barrier in *H. pylori* cells. Cationic and post-PEGylated lipoplexes were compared, anticipating the need of PEG stabilization to safely cross the harsh gastric environment. Based on previous experiments with mammalian cells, PEGylation typically comes at the price of reduced cell interactions. Therefore, two different PEGs were tested: DSPE-PEG, which stably incorporates into liposomes, and Cer-PEG, which can diffuse out of the liposomes over time [42, 43].

Before reaching *H. pylori* in the stomach, lipoplexes will encounter the gastric mucus (Figure 4.1a) which is a tough barrier that limits the diffusion of nanoparticles and

molecules [17]. It was shown in the previous chapter that, while gastric mucus does not hinder the diffusion of the small sized NAMs, binding of mucus components to NAMs does happen which hampers efficient hybridization in *H. pylori*. It was reasoned that a nanocarrier that (i) shields NAMs from such interactions and (ii) can penetrate into the mucus where bacteria reside could be valuable [22, 45]. Nanoparticle diffusion through mucus is determined both by their size and surface properties [62]. PEGylation is the commonly employed strategy to limit the adhesive interactions of nanoparticles with mucus [28, 41]. Furthermore, PEGylation may offer additional protection to the NAMs bound at the surface of liposomes by providing a steric barrier against muco-interactions. As the size of the pores in human gastric mucus is still not well characterized [19], the diffusion of PEGylated nanospheres, varying in size, in mucus isolated from the stomach of pigs was first studied. It is of note that porcine gastric mucus is considered to better resemble human mucus than gastric murine or rabbit mucus [47]. The results presented in this study suggest that nanoparticles should be smaller than 200 nm to maintain a mobile fraction in gastric mucus (Figure 4.2a). Although similar diffusion studies in porcine gastric mucus have not yet been performed to our knowledge, the observations of this study are in line with studies in gastrointestinal mucus. For instance, in intestinal mucus, using porcine and murine models, it was also reported that PEGylated 500 nm nanospheres were hindered, while PEGylated 200 nm nanospheres were able to penetrate murine small intestinal mucus, but not colonic mucus [19, 63]. This is further supported by the observation that PEGylated 200 and 500 nm nanoparticles distributed throughout the colorectal epithelium of mice *in vivo* less uniformly than PEGylated 100 and 40 nm nanospheres [21].

Moving on to the diffusion of NAM lipoplexes, it was found that PEGylation did improve mobility, although a fraction of the lipoplexes remained immobilized (Figure 4.2b). This fraction appeared to be higher than for PEGylated nanospheres of similar size (Figure 4.2a). Although both lipoplexes and nanospheres have a PEGylated surface and thus a substantially reduced surface charge, compared to nanospheres the lipoplexes possess a slightly higher and net positive charge. This may contribute to increased binding, and hence slower diffusion in mucus, as also reported for other cationic nanoparticles in other types of mucus [40, 52, 64].

In future work, one could consider developing even smaller liposomes, which could be prepared by microfluidic mixing [65, 66], to further improve diffusion in dense gastric mucus [67, 68]. DOTAP-DOPE liposomes of 50 to 75 nm could be reproducibly

manufactured in this way [69]. An alternative to enhance diffusion through gastric mucus could be the use of mucolytics. However, the effect of mucolytic agents like N-acetyl-L-cysteine on nanoparticle penetration through mucus is not always clear, while it can irreversibly compromise the functionality of mucus as a defensive barrier against pathogens [67, 70, 71].

Next, it was evaluated if (PEGylated) Lpx have the potential to deliver NAMs across the cell envelope and into the cytoplasm of *H. pylori* cells. The multi-layered envelope of gram-negative bacteria [72], is repeatedly reported as a hurdle for sufficient oligonucleotide penetration in different bacteria [9, 73, 74]. It is believed that oligonucleotides are normally too large to passively diffuse through the outer membrane proteins of the gram-negative outer membrane [8]. Membrane permeabilization with ethanol is, therefore, typically performed *in vitro* to deliver NAMs in gram-negative bacteria [54]. Clearly, ethanol mediated permeabilization is not transferable to *in vivo* conditions which necessitates other methods for the delivery of NAMs into bacterial cells in suspension. In this study, it was evaluated if fusogenic DOTAP-DOPE liposomes could be suitable nanocarriers. It was reasoned that upon DOPE mediated fusion between the liposomes and the gram-negative outer membrane, the NAMs should diffuse through the periplasmic space and the thin and coarse peptidoglycan layer, into the bacterial cytosol [75, 76]. As the fluorescent NAMs in the hybridization solution were designed to specifically hybridize in *H. pylori* [38], FISH could be used to evaluate successful intracellular delivery. It was found that while the cationic and Cer Lpx hybridization performance was nearly similar to free NAMs in ethanol treated cells, DSPE Lpx performed even markedly better (Figure 4.3). After removing extracellular outer membrane bound DSPE Lpx, still a 1-3-fold increase in hybridization efficiency was retained as compared to the standard ethanol protocol (Figure 4.5). This demonstrates the capacity of DSPE Lpx to successfully deliver NAMs in the cytosol of *H. pylori*.

Having found that DSPE Lpx have partial mobility in gastric mucus and can deliver NAMs in the cytosol of *H. pylori*, it was finally tested the hybridization efficacy of DSPE Lpx in the presence of gastric mucus. A more than 2-fold improvement in hybridization efficiency was found with DSPE Lpx when compared to free NAMs (Figure 4.6). This enhancement shows the ability of DSPE Lpx to protect NAMs from interacting with mucus constituents, thus retaining their hybridization activity. It was noticed that the remaining adhesion of DSPE Lpx to the bacterial outer membrane, as observed in solution, was almost entirely invisible in the presence of mucus. This may be due to the

presence of mucins that compete with lipoplexes for interaction with the bacterial envelope, thereby reducing the sticking of DSPE Lpx to the outer membrane [77, 78]. When the aim is to specifically detect *H. pylori* by FISH in a diagnostic setting, this could be an advantage as it may avoid unspecific fluorescence due to the binding of the DSPE Lpx to non-target bacteria. However, when the aim is to treat the infection, one could argue that reduced interaction with the cell envelope may reduce the delivery efficiency. In that sense active targeting of the lipoplexes towards *H. pylori* membranes, e.g. by conjugation of mannose-specific or fucose-specific lectins [79, 80], could be evaluated to improve interactions with the bacterial cell envelope and increase the intracellular delivery efficiency.

Based on the obtained encouraging *in vitro* results, future investigation should focus on the *in vivo* performance of DSPE Lpx PS and PO. The necessary dose to diagnose/treat the infection needs to be investigated, considering the potential dose dependent toxicity to the animal. For the diagnosis of the *H. pylori* infection, the *in vivo* detection of the fluorescence signal, its sensitivity and specificity need to be considered. Detection in the stomach could be performed using an existing confocal endomicroscopic probe [81, 82]. Although accessibility may be a limitation, a porcine model would be the indicated *in vivo* model, as it resembles the human gastric environment and mucus barrier much better than mice models [46, 47]. Moreover, it would be feasible for the detection by the endomicroscopic probe, as the small size of mice may limit a proper assessment of the endomicroscopy's capability to visualize the fluorescence signal in the animal stomach.

4.5 Conclusions

This study shows, for the first time, successful delivery of NAMs into bacterial cells by DSPE-PEGylated liposomes in which also the mucus associated to the bacterial infection is considered as a potential barrier. It shows that such PEGylated liposomes represent a valuable opportunity in the post-antibiotic era to deliver NAMs as a novel class of therapeutic antimicrobials and diagnostic agents. Future research should focus on evaluating DSPE Lpx in other important pathogens and testing their *in vivo* performance.

4.6 References

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4.7 Supplementary materials

Table S4.1. Average apparent diffusion coefficients in porcine gastric mucus (D_M) of the PEGylated nanospheres, different diameters, and the lipoplexes, cationic (Lpx) and functionalized with Cer-PEG (Cer Lpx) and DSPE-PEG (DSPE Lpx). The results from 3 different porcine mucus samples were pooled. Their average apparent diffusion coefficients in water (D_w) was measured as a control. Respective standard deviation is presented as SD. The retardation in mucus is shown as the ratio between D_w and D_M (D_w/D_M).

			D_M ($\mu m^2/s$)	SD ($\mu m^2/s$)	**D_w ($\mu m^2/s$)**	SD ($\mu m^2/s$)	**D_w/D_M**
Nanospheres	PEG 60 nm		1.65	2.18	8.49	8.60	5.16
	PEG 100 nm		0.86	2.09	5.28	1.89	6.17
	PEG 200 nm		0.57	1.40	2.76	0.39	4.82
	PEG 500 nm		0.17	0.63	1.16	0.32	6.65
Lipoplexes	PS	Lpx	0.26	0.57	6.42	2.69	24.23
		Cer Lpx	0.65	1.49	6.13	3.82	9.44
		DSPE Lpx	1.15	1.94	10.52	3.14	9.18
	PO	Lpx	0.22	0.60	7.60	4.01	33.80
		Cer Lpx	0.85	1.86	7.24	3.47	8.48
		DSPE Lpx	1.04	1.98	8.82	3.81	8.51

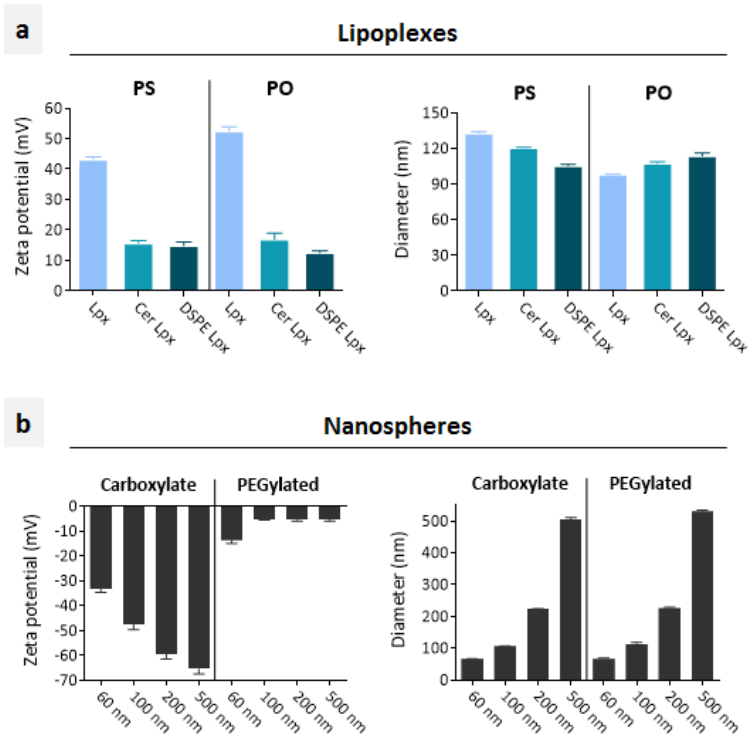

Figure S4.1. The average zeta-potential and hydrodynamic diameter of the nanoparticles used in this study. (a) Lipoplexes of NAM PS or NAM PO – cationic (Lpx) and post-PEGylated with Cer-PEG (Cer Lpx) or DSPE-PEG (DSPE Lpx); b) nanospheres of different nominal sizes before (carboxylate) and after PEG conjugation (PEGylated).

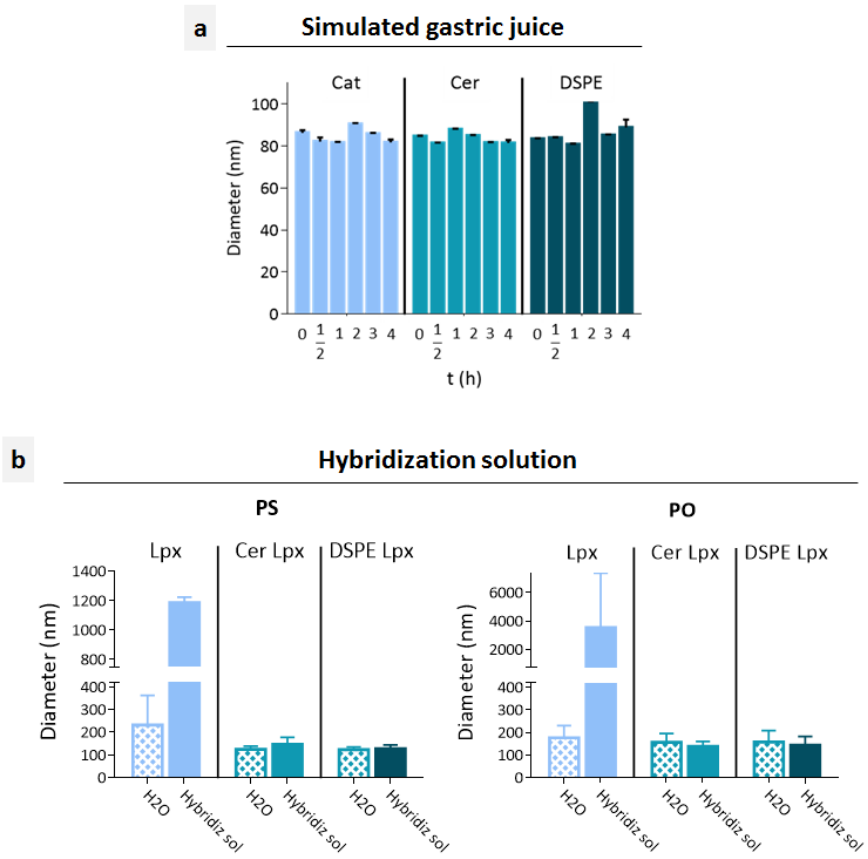

Figure S4.2. Colloidal stability of liposomal formulations. (a) Average hydrodynamic diameter of liposomes (cationic (Cat) and post-PEGylated with Cer-PEG (Cer) or DSPE-PEG (DSPE)) in simulated gastric juice (prepared according to the US pharmacopeia), measured by dynamic light scattering. The diameter was fairly constant over 4h, confirming the colloidal stability in simulated gastric juice. (b) Average diameter of lipoplexes PS and PO, diluted in hybridization solution according to the conditions used in FISH assays (solid bars). The size was measured after 30 min incubation, by SPT. Dilutions in water were taken as controls (punctuated bars). The increase of Lpx size in hybridization solution, compared to water, shows that Lpx majorly aggregates, contrary to Cer Lpx and DSPE Lpx that are colloidally stable in hybridization solution.

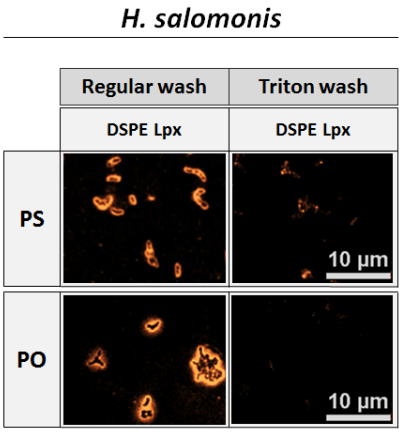

Figure S4.3. Use of a mild triton wash to remove extracellular adhered lipoplexes (DSPE Lpx). The extracellular fluorescent halos in *H. salomonis* almost completely disappeared when the regular wash was replaced by the triton wash.

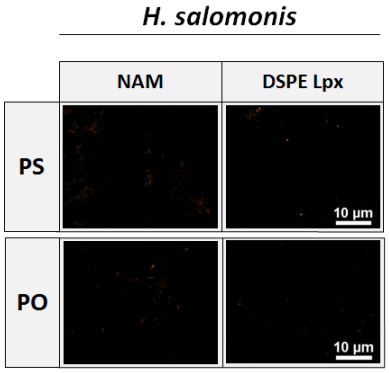

Figure S4.4. Representative epifluorescence microscopy images of FISH in *H. salomonis* within native gastric mucus, on slide. The use of lipoplexes post-PEGylated with DSPE-PEG (DSPE Lpx) was compared with the use of free NAM in untreated bacteria (NAM), for both PS and PO. Negative controls without NAM (neg) were also included.

5.

5 DEVELOPMENT OF CELL PENETRATING PEPTIDES TOWARDS THE TRANSPORT OF NAMs INTO *HELICOBACTER PYLORI*

Abstract

Nucleic acid mimics (NAMs) hold great potential as new therapeutic drugs against bacterial infections in an era where the bacterial resistance to antibiotics poses a serious threat to the public health. In addition, they can serve as diagnostic probes of infections, to address the need of fast diagnostic methods for an informed prescription of still valid antibiotics. NAMs act by hybridization to complementary bacterial RNA, to either inhibit the expression of genes of interest (for therapy), or to detect specific bacteria via a NAM coupled to a contrast agent (for diagnosis). Therefore, for NAMs to fulfill their promise, they need to penetrate into the bacterial cells. Cell-penetrating peptides (CPPs) are the most used vehicles to intracellularly transport oligonucleotides into bacterial cells. Nevertheless, their application is still rather recent and limited to covalent conjugation to charge-neutral NAMs. Certain CPPs have the ability to transport negatively charged oligonucleotides by complexation, as shown by studies in mammalian cells. In here, it was investigated the ability of two of these CPPs, PF14 and PF15, to associate with 10 mers LNA/2'OMe NAMs, containing either phosphodiester (PO) or phosphorothioate (PS) internucleotide linkages. These NAMs specifically target *Helicobacter pylori*, typically found deep in the gastric mucus in the stomach of more than 50% of the world population. The complexation of PF14 and PF15 with NAM PO and PS was first tested and the colloidal stability of the complexes in gastric and hybridization conditions was

characterized. It was found that both peptides can efficiently complex with NAM PS and PO. However, although the dilution in simulated gastric juice allows size reduction of the complexes without peptide degradation, the complexes are in the micrometer range size when incubated in hybridization conditions. Therefore, the complexes are too large to hold promise of penetrating through the gastric mucus and the bacterial envelope. Therefore, it was intended to test if the peptides could be conjugated to the NAMs by disulfide linkage, in order to obtain smaller and more stable constructs. This required initial modification and further purification of the peptides which was successful for PF14. However, when the conjugation was tested with NAM PO no evidence of conjugate formation was found. This study confirms the challenges of CPPs association to negatively charged oligonucleotides and suggests that future research on CPPs as molecular transporters into bacterial cells should preferentially focus on the conjugation to charge neutral PNAs.

Keywords: Cell-penetrating peptides, PF14, PF15, NAM, LNA/2'OMe

5.1 Introduction

Infections are a major and growing healthcare problem due to the advent of bacterial resistance to antibiotics [1]. Antimicrobial resistant infections are estimated to contribute to around 25000 annual deaths, only in Europe [2]. Nucleic acid mimics (NAMs) designed to specifically hybridize *in situ* with complementary bacterial RNA, with improved affinity and resistance to nucleases when compared to parent unmodified DNA and RNA [3, 4], hold promise as novel antisense antibacterials. NAMs can be used to hybridize with and consequently inhibit the expression of essential bacterial genes, thus preventing bacteria growth, or genes involved in the resistance to antibiotics, thus restoring susceptibility to antibiotics. Even if bacteria undergo a mutation that renders the particular NAM inactive, the NAM can be easily redesigned to become an effective drug again. Using the same hybridization principle, NAMs conjugated with a contrast agent could also serve to detect the presence of bacteria and their genes of resistance to antibiotics. Therefore, they could be used as detection probes for comprehensive *in vivo* diagnosis. This would allow a fast and informed prescription of valid and specific

antibiotics, avoiding the frequent overuse of large spectrum antibiotics that favors the development of bacterial resistance [5].

For NAMs to fulfil their promise as a flexible platform for diagnosis and treatment of bacterial infections it is critical that they reach the bacterial cytosol. NAMs penetrate very poorly the cell envelope of bacteria in suspension if the bacteria are not treated to become permeable with, for instance, ethanol [6-8]. These treatments are, however, toxic/noxious and thus not transferable to *in vivo* settings. Therefore, the NAMs should be intracellularly transported using biocompatible carriers. Currently, the best positioned vehicles to do so are fusogenic liposomes and cell-penetrating peptides (CPPs).

In the previous chapter, it was showed that fusogenic liposomes composed of DOTAP-DOPE liposomes complexed with NAMs and post-PEGylated with DSPE-PEG (DSPE Lpx), could intracellularly deliver NAMs into gram-negative bacteria [7]. In particular, 10 mers NAM composed of locked nucleic acids (LNA) and 2'-OMethyl RNA (2´OMe), with either phosphodiester (PO) or phosphorothioate (PS) as backbone linkages, were used to specifically target *Helicobacter pylori* (*H. pylori*). *H. pylori* infects the stomach of more than half or the world's population, being the most common and prevalent chronic infection in the world [9]. In this chapter, it was intended to investigate how CPPs would compare to the DSPE Lpx, as alternative intracellular transporters of the NAMs into *H. pylori*.

CPPs are typically positively charged amphipathic peptides with less than 30 amino acids (a.a.) and have the ability to translocate cytoplasmic membranes [10]. They have been widely investigated as molecular transporters of nucleic acids into mammalian cells for gene therapy [11, 12]. Their application in bacteria is still on its infancy, but they hold the potential to directly penetrate into the bacterial cytosol with its attached cargo by electrostatic and hydrophobic interactions and the formation of transient pores into the bacterial cytoplasmic membrane [13].

Generally, CPPs can be associated to nucleic acids by covalent conjugation or complexation [14]. Covalent conjugation is typically performed by disulfide or thioester linkages and results in small and well-defined constructs [15]. However, conjugation has been almost limited to charge-neutral NAMs (PNA and PMO) [15]. Internalization of PNA and PMO into several bacteria could be mediated by conjugation to the peptide $(KFF)_3K$ [16-20]. However, this peptide showed hemolytic activity and has thus raised toxicity issues [8, 21]. In addition, covalent conjugation of cationic CPPs to negatively charged nucleic acids has been restricted by technical difficulties in synthesis and

purification of the conjugates [14]. In alternative, some CPPs are able to complex with negatively charged nucleic acids by electrostatic interactions, using a simple and fast procedure [22-25]. One of these classes of peptides is the PepFect (PF) family which have a N-terminal stearic acid modification that enhances the complexation and (mammalian) cell internalization [24, 26]. In particular, PF15 showed to be able to complex with a 13 mers LNA/2'OMe NAM (targeting the telomerase activity in human cervix epithelioid carcinoma HeLa cells) [22] and PF14 showed to be promising for oral delivery, as the incubation in simulated gastric juice (sGJ) of PF14-siRNA complexes retained interesting activity (in human embryonic kidney HEK 293 cells) [27]. Therefore, PF15 and PF14 (Figure 5.1) could be good candidates to transport the 10 mers LNA/2'OMe NAMs in the stomach towards *H. pylori* [28]. Nevertheless, CPPs complexation has the disadvantage to result in complexes that are typically polydisperse in size and show variable colloidal stability [15]. Considering the advantages and drawbacks of each association strategy, in this study it was intended to evaluate the potential of PF14 and PF15 to associate with LNA/2'OMe NAMs (PS and PO), comparing complexation and conjugation, in order to pave the way for the use of such constructs in future *H. pylori* studies.

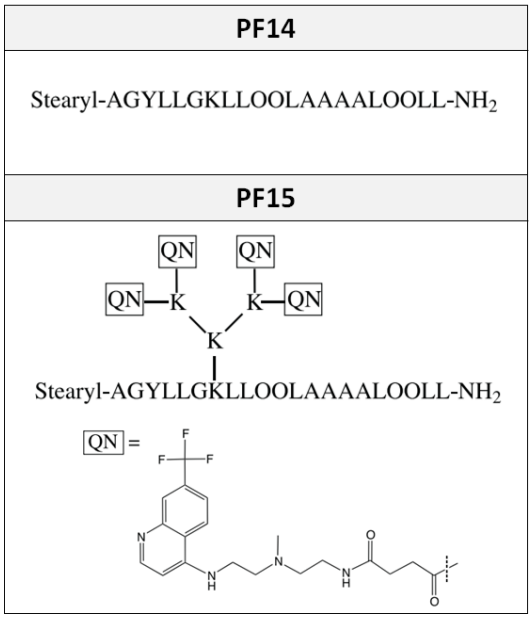

Figure 5.1.Chemical structure of the peptides PF14 and PF15, and the trifluoromethylquinoline (QN). The letters on the peptides denote the amino-acids, with "O" denoting the noncoded amino-acid ornithine. (Adapted from Lindeberg et al. [23]).

5.2 Materials and methods

5.2.1 Synthesis of the NAMs

Two NAMs complementary to a sequence of the *H. pylori* 16S rRNA gene were used in this study. These are composed of LNA and 2'OMe, possess the same sequence and differ only in the internucleotide bonds. One possesses normal phosphodiester oligonucleotides (PO), while the other has one of the two nonbridging oxygen atoms replaced by a Sulphur atom at each internucleotide linkage (PS). These NAMs will be herein designated as PO and PS, respectively. The sequence of PO is 5'-lGmeAmeClTmeAmeAlGmeCmeClC-30, while the sequence of PS is 5'-lG*meA*meC*lT*meA*meA*lG*meC*meC*lC*-30, where "l" represents the LNA monomers, "me" the 2'OMe monomers and *the phosphorothioate linkages. These NAMs were fluorescently labelled at 5' with Cy3 and synthetized and purified according to [28]. These were used in the complexation studies.

For the conjugation studies, the NAM PO was acquired from Exiqon (Vedbaek, Denmark), labelled with TYE563 at the 5' (TYE563 has similar spectral properties as Cy3) and 3' thiol modified (thiol modifier C3 S-S).

5.2.2 Synthesis of the peptides

PF14 and PF15 (a derivative of PF14) were synthesized by standard solid-phase peptide synthesis, based on [24] and [23] respectively. Briefly, PF14 was synthesized on a SYRO multiple peptide synthesizer (MultiSynTech GmbH) using a Chemmatrix Rink amide resin (0.4 mmol/g) and standard Fmoc (9H-fluoren-9-ylmethoxycarbonyl)-a.a.-OH. The amino acids were subsequently coupled using DIC (Diisopropylcarbodiimide) and Oxyma (Ethyl 2-cyano-2-(hydroxyimino)acetate) in dimethylformamide (DMF) as coupling reagents, after deprotection (removal of the Fmoc group) of the previous a.a. using piperidine in DMF. The stearic acid was coupled to the peptide N-terminus (Figure 5.1) using the same reagents. The peptide was cleaved from the resin using 95% (v/v) TFA (trifluoroacetic acid)/ 2.5% (v/v) TIS (triisopropylsilane)/2.5% (v/v) water for 3 h, precipitated in diethylether and vacuum-dried overnight. The obtained crude peptide was purified by RP-HPLC (reversed-phase high-performance liquid chromatography) on a Discovery C18 Supelco preparative column (Sigma-Aldrich, Sweden), using a gradient of acetonitrile/water containing 0.1% TFA. The purified peptide was identified by

MALDI (Matrix-assisted laser desorption/ionization) using an alpha-cyano-4-hydroxy-cinnamic acid as crystallization matrix (Voyager-DE STR, Applied Biosystems).

To obtain PF15, the PF14 backbone was synthesized replacing the standard Fmoc-Lys-OH at position 7 by a Fmoc-Lys(Mtt)-OH. After addition of the steric acid in PF14, the synthesis of PF15 proceeded on the resin-bound PF14. To do so, the Mtt protection group of this Lys7 was first removed by repeated washes with 1% TFA/2.5% TIS in DCM (dichloromethane) and Fmoc-Lys(Fmoc)-OH was coupled. After Fmoc removal by piperidine, repeated coupling and final Fmoc removal resulted in a lysine tree containing four free amino groups. These were treated with succinic anhydride and DIPEA (diisopropylethylamine) in DMF, for 10 min. QN in DMF was coupled overnight to the succinic acid modified lysine tree (Figure 5.1) with PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) and DIPEA. After cleavage of the peptide from the resin, filtration, precipitation and drying (performed the same way as for PF14) the crude PF15 was obtained. The peptide was purified by RP-HPLC and identified by MALDI, as described for PF14.

For conjugation, PF14 and PF15 were synthesized with an extra Cys (Fmoc-Cys(Trt)-OH) at C-terminal. The resin-bound peptide was then cleaved from the resin in the presence of the cysteine protector-activator group Npys (3-nitro-2-pyridinesulphenyl) in the cleavage mixture [29]. The purification and peptide identification was performed as previously referred.

5.2.3 Complexation

The ability of PF14 and PF15 to complex with the NAMs was tested, using different charge ratios of peptide to NAM (calculated as the molar amount of positive charges on the peptide divided by the molar amount of negative charges on the NAM, each NAM having 10 negatively charged phosphate groups). The complexes were prepared by incubating NAM PS or PO with PF14 or PF15 in phosphate buffer (pH 7), for 1h, at room temperature. The complexation efficiency was verified by polyacrylamide gel electrophoresis (PAGE).

5.2.4 Characterization of the complexes

The complexes, at the defined charge ratio, were then characterized by dynamic light scattering and zeta potential determination. The zeta potential and size were first measured in water, using a Zetasizer Nano-ZS (Malvern, Worcestershire, UK). The colloidal stability was then evaluated by size determination after complexes dilution (1:10) in sGJ (prepared according to the US pharmacopeia: a solution of 0.2% (w/v) NaCl, 0.32% (w/v) gastric pepsin and 0.7% (v/v) HCl, final pH 1.2), sGJ without pepsin (a solution of 0.2% (w/v) NaCl and 0.7% (v/v) HCl, final pH 1.2) and pepsin solution (a solution of 0.32% (w/v) gastric pepsin, final pH 3.4 considering the pH dependent pepsin activity). The size of the complexes incubated in sGJ was also measured over 2h, at 37 °C. In addition, as the hybridization of the NAMs in *H. pylori* requires 10x dilution in the hybridization solution (900 mM NaCl, 500 mM Urea, 50 mM Tris-HCl, pH 7 [28, 30]), the complexes were also diluted (1:10) in this hybridization solution and their size was determined.

5.2.5 Conjugation

Conjugation via disulfide bond between the peptide and the NAM was tested using the PF14-Cys(NPys) and the thiol modified NAM PO, according to the strategy depicted in Figure 5.2.

The 3'thiol modified NAM PO protected by an S-S bond was first reduced with DTT in TE buffer, 2h, at room temperature, prior to conjugation. The mixture was then passed through a Sephadex G-25 DNA column (Illustra NAP-25 Columns, GE Healthcare Life Sciences) to remove DTT. The eluted NAM was concentrated by freeze-drying and resuspension in a small volume. The NAM was then diluted in DMF and acetic acid and then incubated with the PF14-Cys(Npys) dissolved in DMF, overnight. The high content of the denaturating DMF in the reaction mixture (75%) should be enough to prevent electrostatic association of the peptide and the NAM that could cause precipitation and prevent covalent coupling [31, 32]. All used solutions and the reaction mixture were deoxygenized by N_2 flow, before incubation. RP-HPLC was then performed to try to identify the product, as well as PAGE gel after desalting and concentration the reaction mixture using a centrifugal filter.

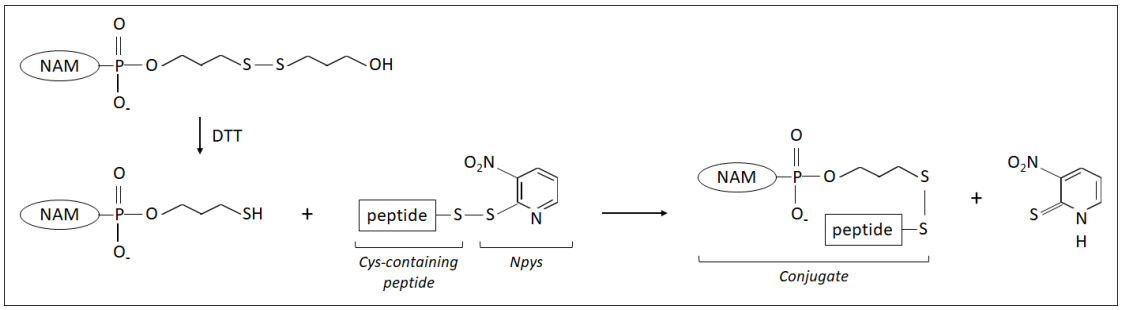

Figure 5.2.Formation of the conjugate by disulfide linkage between the cysteine(Npys)-peptide and the 3'-thiol-modified PO NAM (adapted from Zubin et al.[33]).

5.3 Results

5.3.1 Complexation

The peptides PF14 (Figure S5.1a) and PF15 (Figure S5.1b) were correctly synthesized (Figure S5.1). Nevertheless, some impurities were present in PF14 (Figure S5.1a), suggesting the presence of sodium and potassium associated to the peptide. Therefore, further purification of PF14 could be considered.

In order to determine the ability of the peptides PF14 and PF15 to form complexes with the NAMs, different charge ratios of peptide/NAM were tested (Figure 5.3). Loading the complex mixtures onto a PAGE gel, the decrease in band intensity of the free NAM indicates increased complexation to the peptide. It is seen that from a charge ratio of 6 there is almost no free NAM PS and PO, both using PF14 (Figure 5.3a) and PF15 (Figure 5.3b). Therefore, a charge ratio of 6 was used in the subsequent studies.

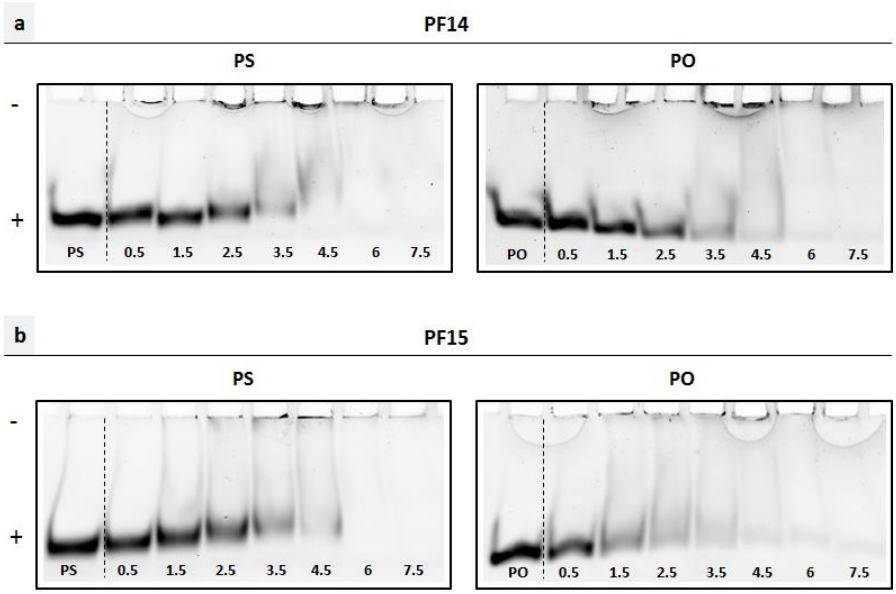

Figure 5.3.PAGE gel showing complexation of peptide PF14 (a) and PF15 (b) with NAM PS and PO at different charge ratios from 0.5 to 7.5. The free NAM PS and PO is separated from the complexation samples by a dashed line.

5.3.2 Characterization of the complexes

After defining the suited charge ratio for complexation, the complexes (at charge ratio 6) size and surface charge were characterized, as well as their colloidal stability in conditions mimicking the gastric lumen and in hybridization conditions.

It was found that the complexes were from 0.9 - 1.6 µm in diameter, with a zeta potential around +19 - 29 mV (Table 5.1).

Table 5.1. The average hydrodynamic diameter and zeta potential of the complexes prepared at a charge ratio 6 and diluted in milli-Q water.

		Diameter (nm)	Zeta potential (mV)
PF14	PS	1396.00 ± 37.37	18.90 ± 0.04
	PO	1275.00 ± 40.22	19.40 ± 0.58
PF15	PS	905.00 ± 44.08	19.80 ± 0.94
	PO	1598.00 ± 53.74	28.40 ± 0.34

In order to target *H. pylori* in the stomach, the complexes would first contact with the gastric juice in the stomach upon oral administration, which may change the packing of

oligonucleotides into the peptide and thus their size. Therefore, it was intended to determine the size of the complexes in sGJ. It was observed that dilution of the complexes in sGJ led to a substantial size decrease, especially for PS complexes, which became smaller than 300 nm when complexed to PF14 (Figure 5.4a1) and smaller than 500 nm when complexed to PF15 (Figure 5.4b1). The size of the PS complexes in pepsin solution is similar to that in water, while it decreases in sGJ without pepsin to about the same as found in sGJ (Figure 5.4a1 and b1). Thus, the sGJ size decrease should be mostly caused by the highly acidic pH (pH 1.2), rather than possible peptide degradation by pepsin. The size changes in PO complexes are not so clear, but both pepsin and the highly acidic pH seem to lead to the size reduction of the complexes (Figure 5.4a1 and b1).

The smaller size of PS complexes in sGJ could be interesting; however, over time their size increases and after 30 min (the duration of hybridization into *H. pylori*), the size is already larger than 900 nm (Figure 5.4a2 and b2).

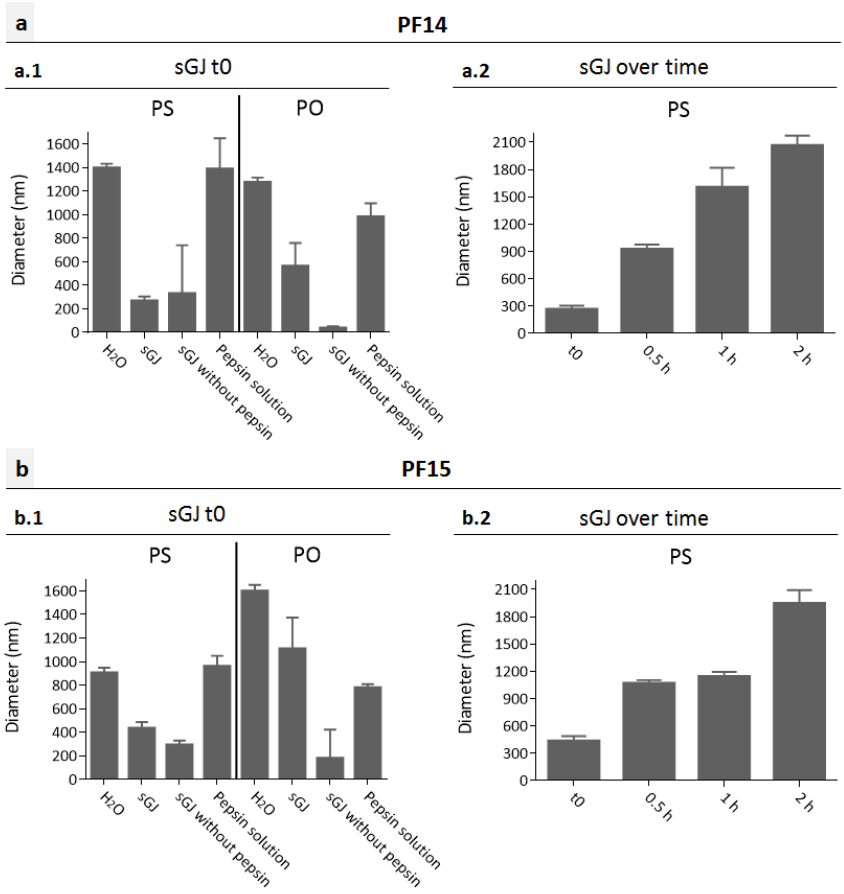

Figure 5.4. Average hydrodynamic diameter of the complexes of PF14 (a) and PF15 (b). The diameter was measured immediately after dilution in sGJ, sGJ without pepsin and pepsin solution (a1 and b1) and over time, during 2h incubation in sGJ (a2 and b2).

In addition, the complexes should be administered in the hybridization solution for hybridization within *H. pylori*. As shown in Figure 5.5, the dilution in hybridization solution further increases the size of the complexes which become bigger than 1.5 µm. These will be too large to cross the gastric mucus to reach *H. pylori*. In addition, considering that *H. pylori* is only about 2-4 µm x 0.5-1.0 µm, it is reasoned that the complexes will also be too large for a feasible penetration (without lysis) into the bacterial cells.

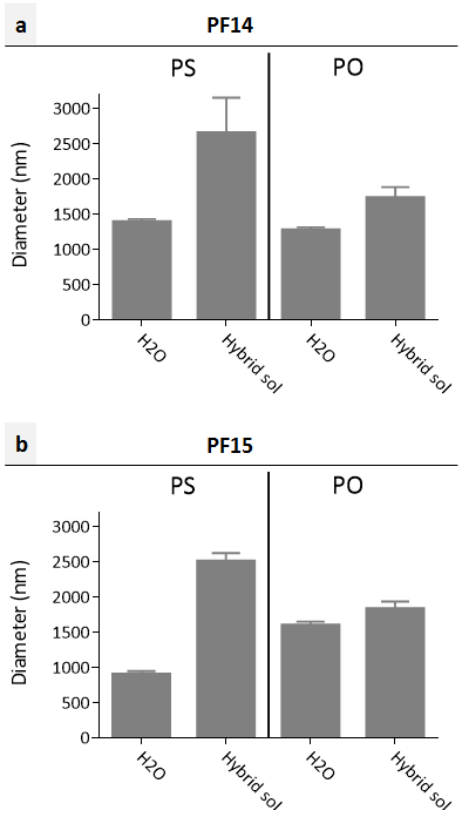

Figure 5.5. Colloidal stability of the complexes of PF14 (a) and PF15 (b), after dilution in hybridization solution (Hybrid sol), compared to water (H20).

5.3.3 Conjugation

Since PF14 and PF15 complexes seem unfeasible for hybridization into the bacteria in the stomach, it was aimed to investigate if smaller constructs could be obtained by conjugation of these peptides with the NAMs. In order to perform conjugation by disulfide linkage, PF14 and PF15 were synthesized with an extra cysteine

protected/activated by Npys [29]. After purification of PF14-Cys(Npys), two major peaks were obtained in the mass profile (Figure S5.2a), corresponding to the PF14-Cys and PF14-Cys(Npys), as MALDI typically breaks part of the disulfide Cys-Npys linkage. Also, the two peaks were obtained for PF15-Cys(Npys), but further purification of the crude modified peptide would be required (Figure S5.2a).

PF14-Cys(Nys) was then tested for conjugation with NAM PO. After the reaction, RP-HPLC was performed but the conjugate could not be separated. It could be that the used HPLC column (available in-house) was not the best suited, as others have also reported failed purification using a reversed phase column and suggested the need of IE (ion exchange)-HPLC (Resource Q column) to separate the conjugate and avoid peptide precipitation [31]. As an alternative to test if the conjugate was formed PAGE gel was used, after desalting and concentration using a centrifugal filter. However, no evidence of conjugate formation was found.

5.4 Discussion

CPPs have been the most used carriers for the intracellular transport of oligonucleotides into bacterial cells. Therefore, it was intended to investigate if CPPs could be valuable carriers of the LNA/2'OMe NAMs directed at *H. pylori* and how they would compare to the promising DSPE Lpx described in the previous chapter. In order to do so, this study focused on peptides from the PepFect family, never tested on bacteria, but previously shown to be able to associate to LNA/2'OMe or withstand the acidic gastric pH [22, 27].

This study showed that PF14 can form complexes with LNA/2'OMe for the first time and confirmed that ability for PF15 reported by Munoz-Alarcon et al.[22]. Studies in mammalian cells have shown that PF14 and PF15 nanocomplexes improve the penetration of nucleic acids in cells [22-25]. CPP nanocomplexes have never been applied into bacterial cells, about 20 times smaller than a mammalian cell. In order to hold promise, they should be as small as possible. The reported PF14 complexes with 18 mers 2'OMe NAMs and PF15 complexes with 18 mers 2'OMe and 13 mers 2'OMe/LNA had a hydrodynamic diameter from 360-500 nm, with a considerably high size dispersion [22-24]. In here, it was showed that the formed complexes between PF14 or PF15 and our 10 mers 2'OMe/LNA were bigger than 900 nm. It should be noted that the charge ratio used

in this study was chosen according to the complexation efficiency found by PAGE gel and it is higher than the charge ratios used in the reported complexation studies. The particular oligonucleotide composition, length and the ratio between the peptide and the oligonucleotide are known to affect the packing and the size of the formed nanocomplexes [15, 23]. When diluted in sGJ, the size of our complexes decreased, but this effect lasted less than 30 min. This differs from observations made for PF14-siRNA complexes which upon incubation in sGJ became bigger [27]. Also, after dilution in the hybridization solution, needed for hybridization into *H. pylori* cells, the complexes became larger than 1.5 µm. Considering that bacteria are few micrometers in size, it seems unrealistic to consider that the obtained large nano/microcomplexes could successfully penetrate live bacteria. The large size will also hamper complexes diffusion through the gastric mucus to come into contact with *H. pylori* in the stomach; as showed in the previous chapter nanoparticles should be smaller than 200 nm to hold a chance to cross the gastric mucus layer.

Aiming at producing smaller and better-defined constructs than the obtained complexes, it was tested if PF14 and PF15 could be conjugated with our NAMs, being the first time the conjugation of PF14 and PF15 with any oligonucleotides is tested. In order to proceed with conjugation via disulfide linkage, PF14 and PF15 were first modified. It was showed that PF14 could be synthesized with a terminal cysteine having its thiol function protected by Npys group, but when disulfide linkage to the NAM PO was tested, no evidence of conjugate formation was found. It could be that conjugation did not occur, for example due to the presence of DTT traces or peptide precipitation in the reaction mixture [31]. In the future, conjugation could be tested for NAM PS and PF15-Cys(Npys) should be further purified to test the conjugation to both NAMs.

Nevertheless, it is known that conjugation of CPPs to negatively charged oligonucleotides poses several challenges in the synthesis and purification, as this study also suggests [14, 31]. Indeed, there has been few attempts to conjugate CPPs with negatively charged oligonucleotides [14, 31]. In addition, conjugation was shown to be limited to certain peptides [14, 31]. Therefore, considering the technical difficulties and the need of small constructs for application in bacteria, future work should consider changing the NAMs to PNA to be conjugated with CPPs better known for conjugation. For instance, $(RXR)_4XB$, $(RFR)_4XB$ and Tat peptides could be used which showed less toxicity than the most common $(KFF)_3K$ and efficient transport of antisense PNA into the gram-negative bacteria *Salmonella enterica*, *Klebsiella pneumoniae*, *Escherichia coli* and

Shigella flexneri, and the gram-positive *Streptococcus pyogenes* and *Listeria monocytogenes* [13, 17, 34].

Besides formulating appropriate conjugates able to penetrate *H. pylori*, the colloidal stability of the conjugates in hybridization solution and sGJ needs to be characterized. In addition, the conjugates should be able to safely diffuse into the gastric mucus where *H. pylori* is mostly located [35]. Actually, the effects of mucus in CPP-mediated transport are poorly studied, as the use of CPPs in oral delivery is still in its infancy [36]. According to the diffusion studies in the previous chapter, particles size is a major determinant of mucus permeation; in that sense, it is not expected that the mucus will impose major steric hindrance to the small conjugates (smaller than 10 kDa). However, surface properties of the particles can also determine binding interactions with mucus [37, 38], restricting the activity of even small compounds as the free NAMs, as found in chapter 3. Particle modification with PEG chains can shield from muco-interactions with the cationic and hydrophobic domains of the CPPs [38] and could thus be an option for conjugates to safely transport the NAMs into the deeper layers of mucus to reach *H. pylori*. This should be addressed in future research, together with the possible effect of PEGylation on the conjugates ability to penetrate into *H. pylori*.

5.5 Conclusions

This study showed that, although small LNA/2'OMe NAMs can be efficiently and easily complexed to CPPs, the formed nanoparticles are too big to hold potential for the diffusion through mucus and penetration into bacterial cells. In addition, conjugation of these negatively charged NAMs to CPPs brings additional technical challenges. Therefore, future work could focus on investigating the use of charge-neutral PNA for easier conjugation to CPPs. This should be followed by testing the stability and diffusion of the formulations in gastric conditions. Finally, the ability of the conjugates to penetrate in *H. pylori*, in suspension and in the presence of porcine gastric mucus, would be tested and compared to that of DSPE Lpx, observed in the last chapter. The best of these strategies should be further validated *in vivo*, preferably in a porcine model, as it resembles the human stomach considerably better than rodents models [39].

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5.7 Supplementary materials

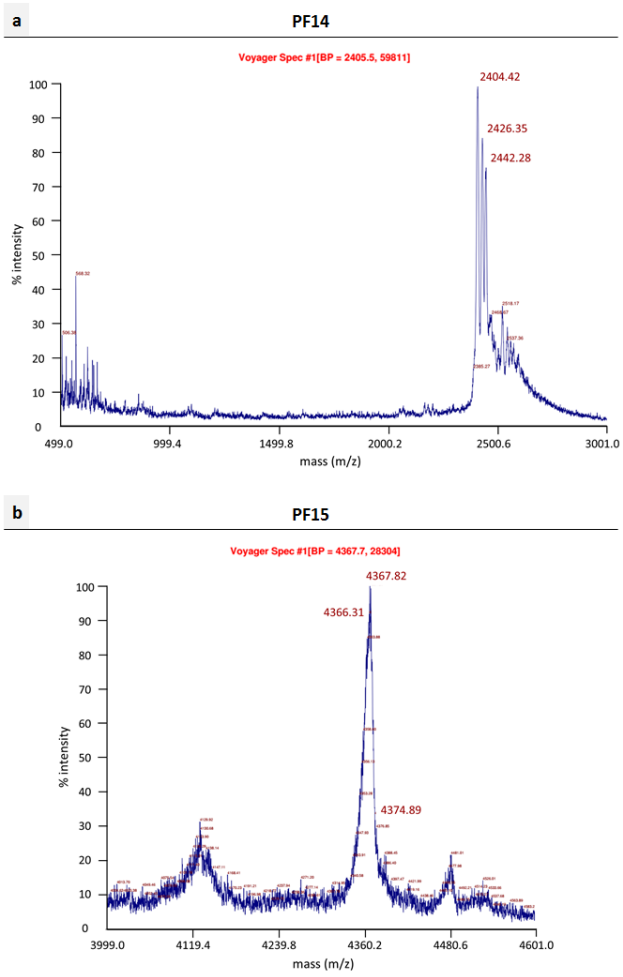

Figure S5.1. Mass spectrum of purified PF14 (a) and PF15 (b).

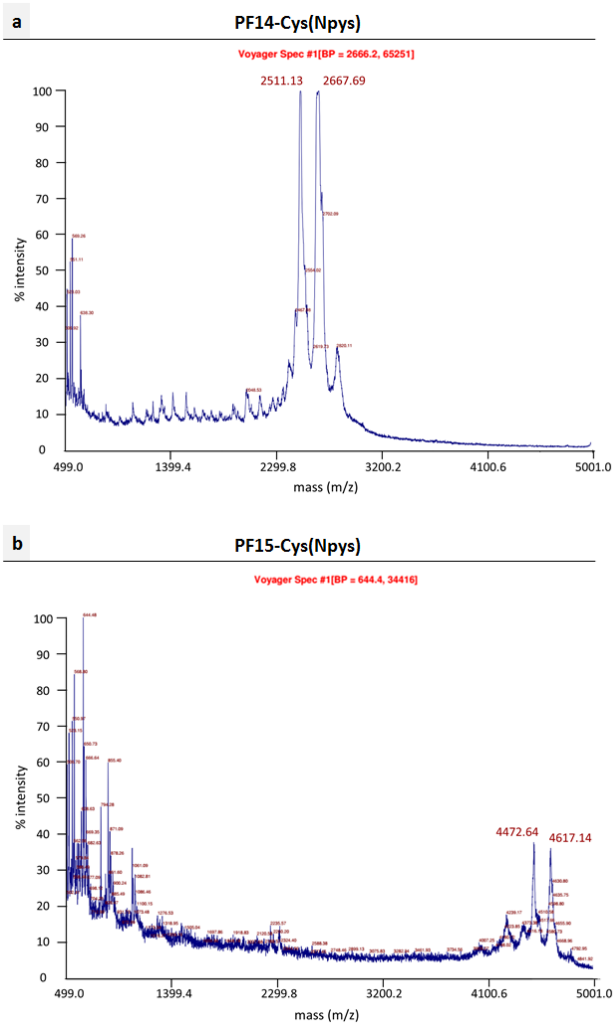

Figure S5.2.Mass spectrum of PF14-Cys(Npys) (a) and PF15-C(Npys) (b) after purification.

6.

6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

The global public health is threatened by the rising bacterial resistance to antibiotics that compromises the ability to fight infections [1]. Resistance of bacterial cells to antibiotics is largely attributed to i) the natural impermeability of some bacteria to antibiotics and ii) bacterial mutations, involving newly acquired resistance to antibiotics by modification (inactivation) of the antibiotic, production of a competitive inhibitor of the antibiotic, production of a different version of the antibiotic's substrate or alteration of the bacterial envelope permeability [2, 3]. Therefore, there is an urgent need for novel therapeutic strategies that can overcome the bacterial cell wall and the bacterial ability to acquire resistance by mutations.

NAMs, designed to hybridize with complementary bacterial RNA sequences, hold great promise as alternative antisense antibacterial drugs or antibiotic adjuvants. They can be designed to target essential bacterial genes, consequently inhibiting their expression and preventing bacterial growth, or target genes of resistance to antibiotics, thereby restoring bacteria susceptibility to antibiotics.

In addition, the life-time of still valid antibiotics should be extended as much as possible, which requires that they are used wisely [4]. However, this has been hampered by a lack of fast diagnostic techniques able to identify the infecting bacteria and their resistance to antibiotics [4]. NAMs coupled to a fluorophore could address this need, serving as probes for a fast and *in vivo* diagnosis, without the need to isolate and process a clinical sample.

It is clear that for NAMs to hybridize with their target sequence in bacteria and fulfill their potential as antibacterial drugs or detection probes, they need to cross the relevant biological barriers in the body to finally reach the bacterial cytosol. Two major barriers are common irrespective of the infection site: the bacterial cell wall, that can hinder/severally retard NAM internalization [5] and the mucus layer in which bacteria often reside (e.g. in the gastrointestinal, urinary, reproductive or respiratory tract) and that poses a barrier for foreign entities by binding them and/or sterically obstructing them [6-9]. Therefore, NAMs need to be able to cross the mucus, without losing their activity, to reach the target bacteria and then cross the bacterial envelope to reach their target in the bacterial cytosol.

This thesis was focused on *H. pylori* infection, the most common and prevalent chronic infection in the world [10]. *H. pylori* is a gram-negative bacterium found in the stomach, mostly deep in the mucus layer that covers the gastric epithelium [11]. In this context, we aimed to reveal the effect of the gastric mucus and the bacterial cell envelope as bio-barriers for intracellular NAM hybridization. Based on that essential knowledge we then intended to provide solutions to overcome these barriers, in order to pave the way for future novel options for treatment and diagnosis of bacterial infections. As NAMs we used 10 mers LNA/2'OMe, with either phosphodiester (PO) or phosphodiester (PS) internucleotide linkages, previously designed to specifically target *H. pylori* and shown to be resistant to the highly acidic gastric pH [12, 13].

In **chapter 3**, using mucus scraped from the stomach of pigs, we tested i) the (chemical) stability of the NAMs in mucus, ii) the diffusion of NAMs through the mucus, and (iii) the effect of gastric mucus on the efficiency of NAM hybridization by FISH in *H. pylori*. We found that the NAMs could quickly diffuse through the porcine gastric mucus (only up to 3-fold slower than in HEPES buffer), without degradation [14]. However, although at least 50% of the small NAMs could penetrate *H. pylori* adhered on a glass slide, mucus hindered the ability of NAMs to hybridize [14]. This was a result of binding interactions of NAMs, especially the PS modified one, with mucus components [14]. Although the exact nature of these interactions was not investigated, it is reasonable to assume that they can be mediated by electrostatic and hydrophobic interactions, as well as hydrogen bonds with the mucins, the main component of mucus (besides water) [15]. However, mucus is a complex mixture also containing lipids, DNA, proteins, ions and cellular debris which may also interact with NAMs [16, 17]. Indeed, it was found that NAMs hybridization in a mucin solution worked to some extent, showing that mucins are

not solely responsible for the NAM inactivation; rather, soluble factors of mucus must be involved, since no substantial immobilization of NAMs in mucus was found [14]. In any case, these experiments highlight the importance of not just using an artificial mucin solution, but instead a bio-relevant mucus model to infer *in vivo* activity. Most importantly, this study pointed to the need of a nanocarrier to safely transport the NAMs across the gastric mucus, so that they are protected from interactions with the mucus and remain capable of hybridizing in *H. pylori*.

Therefore, in **chapter 4** it was aimed to develop a nanocarrier that could shield the NAMs from interactions with the gastric mucus, while allowing the NAMs to directly overcome the cell envelope of *H. pylori.* The latter property is equally important since the typically used permeabilization agents for *in vitro* FISH cannot be transferred to *the in vivo* situation. Fusogenic stealth liposomes, mainly developed for drug delivery into mammalian cells, could offer such a solution. In particular, the DOPE lipid facilitates energetically favorable fusion between lipid bilayers [18]. Thus, DOPE containing liposomes can fuse with the outer membrane of gram-negative bacteria (Figure 2.3) [18-20]. It was found for the first time in this thesis that they can this way intracellularly deliver NAMs into bacteria [21]. Although liposomes have already been explored to treat bacterial infections, it was only for the delivery of traditional small molecule antibiotics [22-24]. In particular, DOTAP/DOPE liposomes complexed to NAM PS or PO and modified by post-insertion of DSPE-PEG (DSPE Lpx) were used. It was observed that DSPE Lpx performed as well (PS) or even 3 times better (PO) than ethanol permeabilization of *H. pylori* [21]. In addition, DSPE Lpx protected the NAMs from binding and inactivation with gastric mucus, retaining the ability of NAMs to hybridize in *H. pylori* [21]. it is reasonable to assume that upon fusion with the *H. pylori* outer membrane, NAMs diffuse through the periplasmic space and the thin and coarse peptidoglycan layer [25, 26] into the bacterial cytosol. DSPE Lpx thus offer exciting new possibilities for *in vivo* diagnosis and treatment of *H. pylori* infections. While the gastric juice did not affect the colloidal stability of the particles, PEGylation showed to be critical to i) maintain the colloidal stability of the Lpx in the hybridization solution and ii) allow a sufficient Lpx fraction to cross the highly viscous porcine gastric mucus to reach *H. pylori* [21]. In addition, the size of the used DSPE Lpx was also determinant for diffusion, as showed (using inert model nanoparticles) that nanoparticles should be as small as possible to diffuse through the heterogenous mucus mesh and in any case smaller than 200 nm [21].

Being cell-penetrating peptides (CPPs) the most used carriers of antisense oligonucleotides into bacterial cells [27-29], in **chapter 5** CPPs were evaluated as a strategy alternative to the Lpx to transport our NAMs targeting *H. pylori*. CPPs have the potential to directly penetrate (with its associated cargo) into the bacterial cytosol. Presumably, their interaction and insertion into the outer and cytoplasmic membrane or gram-negative bacteria is facilitated by initial electrostatic and hydrophobic interactions [28, 30-32] and the formation of transient pores in the cytoplasmic membrane [30, 33] may allow the translocation of the CPP-cargo into the bacterial cytosol, although this is still a matter of debate (Figure 2.4).

Hence, it was intended (chapter 5) to investigate if CPPs could be associated to our NAMs, to target *H. pylori* in the stomach. The use of CPPs in bacteria has focused on the covalent conjugation with charge neutral oligonucleotides (mostly PNA) [27-29]. However, certain CPPs have the ability to complex with negatively charged nucleic acids [34]. Among them PF14 showed promising results for the oral delivery of siRNA [35] and PF15 was able to complex a 13 mers LNA/2'OMe NAM [36]. We investigated the complexation of PF14 and PF15 with our 10 mers LNA/2'OMe NAM and found that both CPPs can form complexes with NAM PS and PO. They seem to resist gastric degradation by pepsin and become smaller upon contact with the very acidic gastric pH. However, this size effect is reversible and in hybridization conditions the complexes are in the micrometer size range. Therefore, the complexes do not fulfil the requirement of being smaller than 200 nm, required for penetration through the gastric mucus (as found in chapter 4). In addition, due to their large size, they hardly stand a chance to be internalized into *H. pylori* [37]. Instead of complexation, covalent conjugation of CPPs produces much smaller and well-defined constructs [34]. However, we were not able to show disulfide conjugation of PF14 and PF15 to the NAMs. This confirms the technical challenges frequently reported in the synthesis and purification of conjugates between cationic CPPs and negatively charged nucleic acids [38]. Therefore, the potential of CPPs to overcome *H. pylori* cell envelope cannot be directly compared to that observed for DSPE Lpx (in chapter 4), using the LNA/2'OMe NAMs. Alternatively, future work could focus on the conjugation of CPPs to charge neutral PNA; after testing their ability to penetrate into *H. pylori,* the colloidal stability of the conjugates in gastric media and ability to diffuse through the gastric mucus need to be addressed.

6.2 General conclusions

Taken together, in this thesis it was revealed that gastric mucus poses a serious barrier to NAMs to hybridize in *H. pylori* in the stomach. Importantly, the obtained results show that the mucus barrier can be overcome by incorporating the NAMs in DSPE Lpx. This is due to the PEG chains that decorate the liposomes and the fact that their size is below 200 nm. In addition, DSPE Lpx can intracellularly deliver active NAMs across the bacterial outer membrane, and thus replaces the chemicals that are normally needed to permeabilize cells *in vitro*. Therefore, DSPE Lpx hold great potential for the use of NAMs as novel agents to manage bacterial infection, addressing the needs posed by the antibiotics crisis.

Based on the encouraging *in vitro* results, future investigation should focus on the *in vivo* performance of DSPE Lpx PS and PO for diagnosis and treatment of *H. pylori* infection. To that end, NAMs designed to silence *H. pylori* genes should be included for the treatment assays. *In vivo* diagnosis of *H. pylori* infections in the stomach could be performed during upper endoscopy examination using confocal endomicroscopy [39, 40]. Although accessibility may be a limitation, a porcine model would be the indicated *in vivo* model, as it resembles the human gastric environment and mucus barrier much better than murine models [41, 42]. Also, the necessary dose to diagnose/treat the infection needs to be investigated, keeping in mind a potential dose dependent toxicity to the animal. In future research, it would be equally interesting to investigate the potential of DSPE Lpx as NAM carriers in other gram-negative bacteria.

In addition, it was found that nanocarriers even smaller than the used Lpx would benefit the penetration in gastric mucus and could thus increase the amount of nanomedicines able to reach the bacteria. CPPs, being small transporters with the potential to directly penetrate into bacteria could be further investigated in the future in conjugation with neutral charged PNA and modified to avoid muco-interactions.

6.3 References

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7.

7 BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES

7.1 Broader international context

Infectious diseases are the main cause of health problems worldwide [1]. Yet, the rising resistance of bacteria to antibiotics, contributing already to nearly 50000 annual deaths in Europe and the USA together, threat our ability to fight infections [2, 3]. This unsettling reality calls for novel therapies against bacterial infections and comprehensive diagnostic methods [4]. With the improved awareness of this dramatic global health problem, infections are gaining increasing attention from the scientific community. In the last decades, cancer has dominated the attention of medical research and the funding agencies. While cancer will remain a field of high relevance, we envision that infections will become an equally important research topic.

Apart from fundamental biological studies, which may lead to new drug targets and therapeutics, the delivery of antimicrobial/diagnostic agents to the site of infection is equally important. For instance, the sophisticated outer membrane of gram-negative bacteria challenges the internalization of therapeutic and diagnostic agents. Similar delivery challenges exist for the treatment of cancer, which has led to the development of carriers – mostly nanoparticles and peptide transporters. Nanoparticles are engineered nanomaterials that have the capacity to protect the therapeutic cargo from inactivation or degradation, and to deliver it close to or even inside the target cells. Peptide transporters (CPPs) are small engineered peptides with the ability to directly translocate cytoplasmic membranes with the associated therapeutic cargo. As such, it does not come as a surprise that the same concept is currently being explored for the treatment of infections as well. In particular, carriers may bring valuable solutions to overcome the bacterial cell wall, as

well as other biological barriers in the body that need to be crossed to reach the target bacteria. While we can profit from many years of research in the cancer field, transferring those concepts to the treatment of infections is not trivial. Indeed, the specific barrier properties of the bacterial envelope need to be considered for the development of carriers in the detection and treatment of bacterial infections. This calls for a close interaction between microbiology and nanotechnology, a research area that is still in its infancy.

This thesis contributes to the needed interaction between the two fields. In particular, the structure of the bacterial cell envelope and its permeability were first detailed, with a special focus on the gram-negative bacteria outer membrane. Understanding the challenges it poses to the permeation of free drugs, carriers were then considered that could bring the drugs directly into the bacterial cytosol. Discussing the mechanisms of interactions at the bacterial envelope, liposomes and CPPs were selected as the best candidate carriers of drugs against gram-negative infections. As before reaching the target bacteria, the carriers commonly need to diffuse through a mucus layer, the dual need of crossing the mucus and the bacterial envelope were considered in the carrier design.

7.2 Relevance

This thesis focused on the use of NAMs as active agents because they offer the potential to address, in different ways, the acquisition of antibiotic resistance genes that limit the efficacy of current antibacterial therapies. They can be used as alternative antibacterial drugs, by silencing essential bacterial genes. If a point mutation would arise that prevents NAMs binding to their target gene in bacteria, the NAMs can be easily redesigning to become an effective adjuvant/drug again. In addition, when conjugated to a detection label, NAMs can also serve as *in vivo* diagnostic probes, directly informing about bacterial resistance to antibiotics.

The great advantage of the NAMs is that, contrary to traditional DNA oligonucleotides, they are composed of modified DNA or RNA sugars that make them resistant to endonuclease degradation and improve their affinity towards their complementary target sequence [5-8]. The improved affinity towards target RNA allows the design of shorter NAMs [9, 10] which may decrease the penetration impairment into bacteria, as we showed in chapter 3 in *H. pylori* adhered on slide. Indeed, there have been reports of some internalization of NAMs, unassisted, in bacteria, but mostly using long

incubation hours, requiring multiple dose applications, or using mutated bacteria with an abnormally permeable OM [11-13]. In agreement, the lack of sufficient penetration of NAMs into the bacterial cells has been considered a major bottleneck of NAMs therapy. For typical *ex vivo* detection of bacteria by fluorescence *in situ* hybridization (FISH), this problem is solved by permeabilization treatments of the bacterial cells. As these use toxic/noxious compounds, these are not an option for *in vivo* detection or treatment of infections. Another issue that could limit the clinical transfer of FISH is the need of high temperatures for the oligonucleotides hybridization to the complementary sequences in bacteria [14]. This limitation could already be solved by the use of small (10 mers) NAMs composed of LNA/2'OMe NAMs, which could detect bacteria at 37 °C [15].

In this thesis, the potential of the NAMs was investigated using the gastric gram-negative bacteria *H. pylori*, as a model infection. This is of particular relevance, since *H. pylori* infects more than half of the world's population [16]. This is the most common and prevalent chronic infection in the world [16]. *H. pylori* is responsible for 70-85% of the gastric ulcers and 90-95% of duodenal ulcers, and increases by 10-fold the risk of gastric cancer, the third most mortal cancer [17-19]. In addition, the fact that the clinical *H. pylori* diagnostic mostly requires an upper endoscopy (where a gastric biopsy is collected for later identification of *H. pylori in vitro*, typically by time-consuming culture methods) [20] brings a great opportunity to perform *in vivo* diagnosis of the infection. Fluorescent NAMs could be used to 'light-up' *H. pylori* and *H. pylori* resistant to clarithromycin (the main cause of treatment failure) during the endomicroscopy procedure [21, 22]. This way, the prescription of the proper therapeutic, including second line regiments with levofloxacin and bismuth [23] if necessary, could be made immediately after the first consultation. A scenario could also be envisioned where, in case of a positive diagnostic result, the therapeutic NAM is already administered during the same examination.

The resistance to of *H. pylori* to clarithromycin could already be detected using FISH, but still *in vitro*, using toxic bacterial envelope permeabilizers [24]. Also for NAMs antisense therapy insufficient permeation of the NAMs into gram-negative bacteria subsists as a general limitation [25]. The gram-negative bacterial cell wall comprises a negatively charged layer of LPS that limits the permeation of hydrophobic compounds [26-30], as the outermost side of the outer membrane which is traversed by OMPs forming aqueous channels that regulate the permeability to hydrophilic compounds (Figure 2.1) [26]. According to protein characterization studies [27, 28, 31], the known pore sizes of these channels would be too narrow to allow diffusion of the NAMs (Figure 2.2). In order

to address this limitation, NAMs can be carried across the bacterial envelope of gram-negative bacteria. CPPs and fusogenic liposomes are the best positioned carriers to do so. For application in bacteria, CPPs have been almost exclusively conjugated to PNA or PMO [13, 32]. In addition, the application of liposomes in bacterial infections have been almost limited to the delivery of antibiotics. Instead, this work was innovative by the use of LNA/2'OMe NAMs as cargo, first naked (**chapter 3**), then complexed to stealth fusogenic liposomes (**chapter 4**) and lastly associated to CPPs (**chapter 5**).

On top, the Lpx study (**chapter 4**) addressed the challenge, rarely considered, of experimentally distinguishing from nanomedicine adhesion on the bacterial envelope from intracellular delivery of the transported cargo. Typically, fluorescence microscopy and flow cytometry are the techniques of choice to visualize the interaction of nanomedicines (made fluorescent by the associated fluorescent cargo) with cells. However, these techniques can hardly distinguish if bacterial fluorescence arises from surface adhered fluorescent constructs or internalized fluorescent cargo. Fillion et al. used fluorescence-activated cell sorting (FACS) at 37 °C and 4 °C to distinguish liposomal fusion with *E. coli* outer membrane and concomitant intracellular delivery of the carried PNA, from liposomal agglutination on the outer membrane, respectively [33]. However, at 4 °C the level of agglutination may be underestimated, compared to that at 37 °C. Differently, we found a way to determine, using fluorescence microscopy, the fraction of NAMs intracellularly delivered by the liposomes into *H. pylori*, by removing the fluorescent Lpx remaining adhered on the outer membrane by a mild triton wash (**chapter 4**).

In addition to the cell envelope, mucus as a barrier for the NAMs (chapter 3) and lipoplexes (chapter 4) to target the bacteria was considered. Mucus covers the underlying epithelia, not only in the gastrointestinal tract, but also in the respiratory, reproductive and urinary tracts, and it is, therefore, a place of pathogenic bacteria colonization [34, 35]. Mucus is a though steric and sticky barrier [36-38]. Therefore, it is crucial that any drug/formulation aiming to target the bacteria is able to cross the mucus without losing its activity. The importance of crossing mucus is increasingly acknowledged in drug delivery studies and in particular in oral delivery [39-41], but mostly neglected in relation to the delivery of macromolecules and antibiotics to bacteria (including *H. pylori*) [42-45]. On the other hand, several studies considering the mucus barrier use mimic models based on commercial mucins or mixtures thereof [46-49] which are very far from the real mucus and thus lack bio-relevance [50]. In addition, gastric mucus from the stomach of

pigs, is far more representative of the human mucus than mucus from mice or rabbit [50]. Therefore, and despite the technical difficulties, porcine gastric mucus was used in this thesis, and from different pigs to account for inter-individual's variability. In particular, the typical FISH protocol was modified to include this crucial barrier in bacteria hybridization studies, for the first time (**chapter 3 and 4**). Representative *ex vivo* models are a requisite to validate any novel proof-of-concept.

Advanced microscopy techniques as FRAP (**chapter 3**) and SPT (**chapter 4**) were used to study the local diffusion of respectively free NAMs and different Lpx formulations in porcine gastric mucus. In addition, the porosity of porcine gastric mucus was characterized for the first time, using model inert nanoparticles and SPT (**chapter 4**). While SPT allows the appreciation of the mucus microrheology effect on the diffusion of individual nanoparticles [51], it could be of future interest to also study the diffusion of nanoparticles over larger distances of mucus. This could be better achieved using a rotating silicone tube test, in which the tube is filled with mucus and a suspension of particles on top and is left rotating; at the end of the experiment the tube is frozen and cut into slices, in order to quantify the particles at defined depths [52]. This way, nanoparticle's penetration through distances representing the range of mucus thickness in the stomach (from 50-450 µm, with an average value of 180 µm [53, 54]) could be tested. In addition, the depth and rate of muco-penetration could be assessed according to the fed and disease state of the individual, which are parameters known to affect mucus thickness, but that have been poorly characterized [36, 54] .

7.3 Future perspectives

It was found that gastric mucus is a great interactive and steric barrier for free NAMs (**chapter 3**) and nanoparticles (**chapter 4**). PEGylation significantly improved diffusion of Lpx in porcine gastric mucus and the diffusive DSPE Lpx were able to intracellularly deliver active NAMs into *H. pylori* in the presence of mucus (**chapter 4**). Still, a considerable fraction of DSPE Lpx was found to be immobilized in gastric mucus. In addition, *H. pylori* is able to bind mucins in the mucus, which helps the bacteria to persist in the stomach [55]. Therefore, mucins may compete with Lpx for interaction with the bacterial envelope which can, in consequence, reduce the amount of NAMs that can be delivered into *H. pylori* (**chapter 4**).

This dual need of safely crossing the mucus and interacting with the bacterial envelope as efficiently as possible to maximize the internalization of the therapeutic/diagnostic cargo is a challenge that generally applies to mucosal infections. While DSPE Lpx seem very promising, it is currently unknown if they would be suitable to intracellularly deliver NAMs in gram-positive bacteria. In that case the Lpx would first come into contact with a 20-80 nm thick peptidoglycan layer, which may hinder effective fusion with the underlying cytoplasmic membrane (Figure 2.3) [56]. In that sense drug-conjugated CPPs could prove beneficial. However, their cationic charge (although lower than the unmodified Lpx used in chapter 4) and hydrophobic domains, may promote restrictive binding interactions with the mucus, as also reported for other cationic nanoparticles in other types of mucus [57-59]. Attachment of PEG chains, as included into the Lpx, may thus be evaluated to shield muco-interactions. These are still quite novel questions, reflecting the fact that CPPs are still poorly used for oral delivery [60].

In terms of efficient nucleic acid delivery to bacteria, perhaps we can learn from bacteriophages (phages), which are sophisticated entities, typically capsid viruses, that protect mucosal surfaces against bacterial infections [61]. They do so by targeting specific bacteria in the mucus and directly introducing their nucleic acids into the bacterial cytosol [62, 63]. Therefore, they are nature's answer to the dual need of crossing the mucus and interacting with bacteria in a specific manner for optimized intracellular delivery of nucleic acids. Capsid viruses, having a size between 30-200 nm in diameter and being densely coated with charged groups with a neutral net surface charge, can penetrate the mucus to encounter their target bacteria [61, 64]. Different bacterial structures can be specifically recognized by phages; for instance, the LPS's O-antigen or lipid A, and OMPs (porins, specific channels Tsx and LamB, or receptors of active TBDT like FhuA) in gram-negative bacteria, and the teichoic acids or specific proteins in gram-positive bacteria [62, 65, 66].

To treat bacterial infections, lytic phages can be used to directly kill bacteria or lysogenic phages can be engineered to deliver therapeutic nucleic acids into bacteria [67, 68]. This last strategy can be preferred, since lytic phages may select for phage-resistant bacteria [68]. Phages could be engineered to deliver diagnostic as well as antibacterial/adjuvant NAMs into bacteria. Indeed, phages have been recently investigated to deliver an antibiotic-sensitive gene to revert resistance to streptomycin, leading to a ten-fold improvement of the streptomycin potency against *E. coli* [69]. Also, insertion of genes to repress the SOS bacterial response (which allow bacteria to better

tolerate antibiotic induced DNA damage) resulted in increased efficacy of a quinolone antibiotic against wild type and resistant *E.coli*, *in vitro* and *in vivo* [70]. Other genes were targeted *in vitro*; for instance, OmpF could presumably increase *E. coli* permeability to a quinolone antibiotic [70]. Nevertheless, the penetration of these engineered phages in mucus needs to be further studied. Despite the theoretical ability of phages to penetrate mucus, this relies on interactions that can be specific for the phage and mucus; it should thus be characterized for each particular 'system' [61, 71]. Therefore, it could be interesting to investigate these questions to clarify the feasibility of phages as NAMs carriers into *H. pylori* and, other bacteria, and how they compare with Lpx. Regulatory issues limit the current use of phages, but only further research on the phage technology can 'push' to solve this constraint; looking at nature, it seems a worthy effort with potential to bring better solutions to manage bacterial infections.

7.4 Clinical translation and challenges

Although during the last decade the big pharma restrained the innovation in microbiology, it is now gaining renewed interest [72]. This comes from acknowledging the relevance of antimicrobial resistance and the impact that novel marketed solutions to tackle this problem can have, together with FDA incentives addressing speedier approvals and longer patent protection periods [72]. Besides, the microbiology market is economically interesting, evaluated at more than $2 billion dollars [72, 73]. This renewed interest has focused mainly in new *in vitro* diagnostics, probably because the economic risk is lower and the technologies are more readily available (often from small or even newly founded companies). One example on the use of NAMs to diagnose bacterial infections is from Merck. Merck will partner with OpGen to invest in technology for the rapid *ex vivo* diagnosis of bacterial resistance to antibiotics [74]. OpGen combines bioinformatics, using a proprietary database of pathogens, with FDA approved laboratorial detection of bacteria in blood samples [75]. The detection is performed by PNA, using FISH on the sample adhered on a glass slide, a technology belonging to AdvanDx® [75]. However, bacteria detection mostly requires fixation/permeabilization treatment, to guarantee PNA penetration into the bacterial cells [75].

Another example is from Roche that acquired a new technology which makes use of the ability of phages to intracellularly deliver nucleic acids into bacteria (Smarticles™)

[76]. This technology, invented at Cornell University, was first developed by GeneWEAVE, a company founded in 2010 and later acquired by Roche in 2015 [73, 77]. This technology is applied to the *ex vivo* detection of bacteria and bacterial resistance to antibiotics, on clinical samples isolated from patients, without the need for bacteria enrichment, isolation or culture, nor the need to permeabilize the bacterial envelope [76]. This technology combines a phage particle that specifically recognizes target bacteria and carries a synthetic plasmid DNA encoding for the luciferase gene [73, 76]. The transcription and translation of the luciferase gene inside the target bacterium generates a positive luminescent signal [73]. As susceptibility to antibiotics generally leads to interrupted bacterial transcription/translation, by including antibiotics in the media susceptible bacteria won´t 'light up' anymore, while resistant bacteria will still do [73, 76]. This technology was first developed to target methicillin-resistant *Staphylococcus aureus* (MRSA) and in 2015 it is was still under clinical trials, waiting U.S Food and Drug Administration (FDA) approval for laboratorial use [78]. Assays for carbapenem- and fluoroquinolone-resistant *Enterobacteriaceae* and vancomycin resistant *Enterococci* are also being developed [73].

However, no solutions are available in the market nor undergoing clinical trials for *in vivo* detection of bacteria and bacterial resistance. Moreover, the clinical translation of (nano)carriers to treat bacterial infections inside the human body is still very scarce, as following described [79, 80].

CPPs are still waiting transition from lab bench to clinical applications [81]. The major challenges are related to low *in vivo* stability of CPP formulations, peptide degradation and strong binding of the cationic CPPs to non-target molecules, leading to drastic reduction of bioavailability [82]. The inclusion of unnatural amino acids together with insertion of stabilization moieties in the CPP structure may tackle these problems [82]. Instead, liposomes are the most synthesized nanoparticles in the drug delivery field and the first FDA approved liposomal nanomedicines dates already from the mid 90's [83]. Nevertheless, their application in microbiology is still rather recent and no liposomal formulations are available on the market yet for bacterial infections (only fungal and viral infections) [79, 84]. Clinical trials have focused on liposomes only for the sustained release of antibiotics, to decrease the necessary antibiotic dose and related toxic effects [79]. One example is Pulmaquin®, a formulation composed of a mixture of liposomes encapsulated and unencapsulated ciprofloxacin to be administered via inhalation for treatment of chronic non-cystic lung infections with *Pseudomonas aeruginosa* [85].

Another example is Arikace®, a charge neutral liposome of 0.2-0.3 μm encapsulating amikacin to treat cystic-fibrosis *Pseudomonas aeruginosa* biofilms, upon inhalation [86, 87].

To the best of our knowledge, there are no clinical trials on the use of nanoparticles or molecular transporters in the body to directly overcome the bacterial envelope, for improved drug internalization into bacterial cells.

Although potentially interesting candidates, phages (besides the given example of *ex vivo* detection) have mostly been applied to disinfect medical devices, since their use directly in the human body has been prevented by the Western countries regulation [88, 89]. Nevertheless, phages have been FDA approved for application as food additive "disinfectants" [69]. Phage therapy dates back already to the 1920s-1930s, but the emergence of antibiotics replaced the use of phage's in Western countries, but not in the former Soviet Union countries and other eastern Europe countries [90, 91]. In these countries, phage therapy is actually approved since almost 80 years ago, and it is has been used against intestinal, urinary tract, dermal, cystic fibrosis and even septic infections with very good success and tolerance rates [89].

The technical and regulatory efforts to bring the much needed solutions in the market to manage bacterial infections should happen together with necessary fundamental research to better understand the bacterial envelope permeability [92]. The current knowledge on the outer membrane permeability of gram-negative bacteria has mostly relied on the static characterization of OMPs by X-ray crystallography [93]. Although very useful, it should be complemented with real-time studies on live bacteria, to reveal interface interactions that may affect the bacterial cell wall permeability to the drugs under investigation [93]. Until today, this has been limited by the resolution of common techniques (such as flow cytometry and fluorescence microscopy) to visualize the bacterial interface in detail. Super-resolution microscopy is starting to be used to elucidate the dynamics of certain bacterial physiological processes [94-96]. We envision that its continuous advance and availability will position super-resolution microscopy as a critical tool, to evaluate nanoparticles interaction with bacterial cells, in the future. This may further boost the use of nanomedicine for novel diagnostic and therapeutic strategies to address the "post-antibiotic era" [97].

7.5 References

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