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# Intracellular delivery of oligonucleotides in *Helicobacter pylori* by fusogenic liposomes in the presence of gastric mucus

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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. However, an essential requirement is that these NAMs should be delivered into the bacterial cytoplasm, which is a particular challenge given the fact that they are charged macromolecules.

We consider these delivery challenges 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. Our 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 gastric mucus isolated from pigs. DSPE Lpx thus offer exciting new possibilities for *in vivo* diagnosis and treatment of *Helicobacter pylori* infections.



## 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

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antibiotics, so that an effective antibacterial drug can be prescribed in time [3,11–14]. 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 [2,12,15].

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 [13,16,17]. 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 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 [18–20]. 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 [19,21,22]. Therefore, NAMs need to be able to pass through mucus to reach the target bacteria [23]. Importantly, they should do so without losing their activity. Indeed, we have recently shown that interactions with gastric mucus can significantly compromise the ability of NAMs to hybridize with *Helicobacter pylori* (*H. pylori*) [24].

Apart from crossing the mucus layer, it is pivotal that the NAMs are delivered into the cytoplasm of bacterial cells. Unlike eukaryotic cells, bacteria possess a multi-layered cell envelope which is recognized as a challenging barrier for oligonucleotide penetration [8,25]. 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 *in vivo*. While cell-penetrating peptides (CPPs) conjugated to antisense NAMs may be promising [14,26–30], conventional CPPs induce cytotoxicity and possess low *in vivo* stability [31–34]. In addition, bacterial resistance to CPP-mediated penetration has been reported [32].

In this work we have considered both delivery challenges, i.e. crossing mucus and the cell envelope, in the context of gastric *H. pylori* infection (Fig. 1a), which is the most frequently occurring chronic bacterial infection worldwide [35]. *H. pylori* reside within the gastric mucus layer as well as in close proximity with the epithelial cells underlying the gastric mucus layer [36]. Here we investigated if fusogenic stealth liposomes are suitable nanocarriers for NAMs to target *H. pylori* infections. Liposomes have been extensively studied to deliver nucleic acids into eukaryotic cells [37]. Until now their application to treat bacterial infections is almost exclusively limited to the delivery of traditional small molecule antibiotics [38–41]. So far, only two papers report on the use of liposomes to deliver phosphorothioate DNA in *Escherichia coli* and in *Staphylococcus aureus* [42,43]. In spite of its promise it shows that liposomal delivery of nucleic acids into bacteria is a virtually unexplored area.

In the current study we use fusogenic cationic liposomes, made from the lipids DOTAP and DOPE, which are well-characterized carriers for nucleic acids in eukaryotic cells [44–46]. We 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 cell envelope, thus delivering its content into the cell's cytoplasm [47,48]. Based on our previous work, locked nucleic acids (LNA) and 2′-OMethylRNA (2′OMe), with either phosphodiester (PO) or phosphorothioate (PS) as backbone linkages (Fig. 1b), were used as NAMs to hybridize to *H. pylori* rRNA [24,49]. 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 gastrointestinal environment [50,51]. We tested two PEGylation strategies: DSPE-PEG that stably incorporates into liposomes and Cer-PEG that can diffuse out of the liposomes over time [52,53]. First, we evaluated if those PEGylated lipoplexes have good diffusional mobility in 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, we assessed if the PEGylated lipoplexes could still successfully deliver functional NAMs in *H. pylori* in the presence of gastric mucus.

## 2. Materials and methods

### 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*′-ethyl-carbodiimide 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).

### 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 [54,55]. Also, pigs have a gastric physiology similar to humans, making them representative animal models for *Helicobacter* infection studies [56,57].

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 [58]. The mucus, loosely bound and adherent, was then gently scrapped off with a glass slide, aliquoted and stored at −80 °C. Mucus from three different pigs was included in each experiment.

### 2.3. NAMs synthesis

Two oligonucleotides complementary to a sequence of the *H.*