Amphiphilic nucleic acid conjugates for transfection of differentiated intestinal cells

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AMPHIPHILIC NUCLEIC ACID CONJUGATES FOR TRANSFECTION OF DIFFERENTIATED INTESTINAL CELLS

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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Abstract

Nucleic acid therapy can be beneficial for the local treatment of gastrointestinal (GI) diseases that currently lack appropriate treatments. The delivery of oligonucleotides (ONs) directly to the GI mucosa allows for high local concentrations with minimal systemic exposure and subsequent side-effects. Indeed, several locally administered ONs are currently progressing through clinical trials as potential treatments for inflammatory bowel diseases. However, due to low uptake of free ONs by mucosal cells, strategies aimed at increasing the potency of orally administered ONs would be highly desirable. The aim of the doctoral work presented herein was to explore the silencing properties of highly resistant ONs modified with a hydrophobic alkyl chain on differentiated intestinal epithelial cells in conditions mimicking those of the GI tract.

A variety of innovative nucleic acid delivery approaches ranging from single-molecule-based conjugates to designer viruses are currently available, some of which are being tested in clinical trials. These strategies are discussed in Chapter 1 of this thesis. The issues related to the oral delivery of biomacromolecules, such as nucleic acids, and the approaches to overcome these hurdles are reviewed and discussed in Chapter 2 with a focus on clinical applications. Chapter 3 presents the synthesis and screening of a series of lipid-ON conjugates for the silencing of a model Bcl-2 mRNA. Among the conjugates tested, the ON modified with 2′-deoxy-2′-fluoroarabinonucleic acid bearing a docosanoyl moiety (L-FANA) was the most potent candidate with the lowest toxicity. The efficacy of L-FANA conjugate was confirmed in two different colon carcinoma cell lines and was preserved in an inverted transfection setup, in contrast to a conventional particulate lipoplex-based delivery carrier. The conjugation of the lipid was essential for efficient target silencing, since FANA without the lipid group failed to significantly downregulate the Bcl-2 mRNA. In Chapter 4, the efficacy of the L-FANA conjugate was tested in simulated intestinal fluids and in an oil-in-water emulsion. Given the resistance of L-FANA to digestion, its silencing efficacy remained intact after its interaction with the oil phase of the oil-in-water emulsion was disrupted with pancreatin-derived lipases. Remarkably, L-FANA conjugate was able to downregulate the target gene expression at both mRNA and protein levels in difficult-to-
transfect polarized epithelial cell monolayers in the absence of complexation, delivery devices, and membrane-disturbing agents. These findings indicate that lipid-ON conjugates are promising therapeutics for the treatment of intestinal diseases, as well as valuable tools for the discovery of new therapeutic targets. **Chapter 5** summarizes the findings of the thesis and presents future perspectives of the work.

Schematic representation of the main questions addressed in this thesis.
Zusammenfassung


Schematische Darstellung der in dieser Dissertation untersuchten zentralen Fragestellungen
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1 Background and Purpose

1.1 Nucleic acid drugs

Nucleic acids are biomacromolecules consisting of tens to millions of nucleotide-based monomer units which perform the most essential functions in living cells, such as carrying the genetic information and regulating gene expression and protein synthesis. Therefore, altering some of these functions by delivering synthetic nucleic acids to cells not only represents an extremely valuable research tool, but also possesses a tremendous therapeutic potential. Nucleic acid drug candidates comprise a wide class of compounds with distinct mechanisms of action capable of targeting genomic DNA, messenger RNA (mRNA), proteins, and endogenous gene regulators such as long non-coding RNA (lncRNA) and microRNA (miRNA) (Scheme 1.1). Nucleic acid therapeutics can be divided in two major classes based on their size: oligonucleotides (ONs) and large nucleic acids. These groups require specific delivery approaches due to differences in stability, size, and cell uptake efficacy.

ONs comprise a large family of molecules including antisense ONs (AONs), RNA interference (RNAi)-based ONs (small interfering RNAs (siRNAs) and miRNAs), miRNA-binding ONs (antimiRs), triplex-forming ONs (TFOs), decoy ONs, DNA/ribozymes, immunostimulatory ONs, and aptamers. They are typically single- or double-stranded sequences of nucleotides (usually 15 – 35 bases long) with relatively high molecular weight (> 5000 Da) and consequently low cell permeability. The key appeal of AON, RNAi, ribozyme, and antimiR therapies is the ability to rationally design them to modulate the expression of any target gene of interest with high specificity based on the Watson-Crick pairing rules. AON's and siRNA's binding to target mRNA can potentially result in splicing correction, endonuclease-mediated degradation, and/or translation repression depending on the chemical structure and degree of complementarity (1–3). Alternatively, antimiRs bind endogenously expressed complementary miRNAs thereby inhibiting their function (4, 5). Ribozymes are ONs with nuclease activity capable of mRNA digestion in a sequence-specific fashion. Notably, DNAzymes and chemically-modified XNAzymes with higher stability than ribozymes have been developed (6).
Scheme 1.1. Types of nucleic acid drugs and their mechanisms of action. mRNA – messenger RNA, pDNA – plasmid DNA, AON – antisense ON, shRNA – short hairpin RNA, TFO – triplex-forming ON, TF – transcription factor, TFD – TF decoy, gRNA – guiding RNA, RISC – RNA-induced silencing complex. (i) A group of nucleic acid drugs acting directly on the genomic DNA. TFD and TFO modulate expression of the target gene by direct binding or sequestration of the corresponding TF. Co-expression of Cas9 and gRNA from a pDNA leads to targeted genome editing. (ii) A group of nucleic acid drugs exerting their effects on mRNA or regulatory RNAs. Ribozymes and their analogs (e.g., DNAzymes) degrade the target mRNA in a sequence-specific manner. shRNA is processed by the endonuclease Dicer to miRNA or siRNA, leading to the target mRNA degradation via RISC and/or translation repression. AONs lead to knockdown of target protein via RNAse H-mediated degradation or inhibition of translation. Additionally, they may be used for splicing correction. AntimiRs can bind and inactivate target miRNAs. (iii) A group of nucleic acids acting on protein level. Transfection of mRNA or pDNA could lead to overexpression of target protein. Aptamers can recognize cell surface receptor leading to its (in)activation or endocytosis. CpG-containing ONs can activate/modulate the immune response.
In addition to RNA-binding ONs, DNA-based ONs containing unmethylated CpG motif stimulate the Toll-like receptor (TLR) 9 expressed primarily by immune cells, leading to modulation of the immune response. For example, DNA-based ON CPG 7909 (Pfizer Inc.) was tested as a vaccine adjuvant for the prevention and treatment of sepsis (7), and BL-7040 (BioLineRx) is being developed for the treatment of inflammatory bowel diseases (IBDs) (8). Moreover, the ability of ONs to form rigid 3D structures can be exploited to obtain specific protein binders, known as aptamers, with remarkable binding affinities comparable to those of antibodies (9, 10). ON therapy constitutes a highly promising approach for the treatment of several genetic, (auto)immune, cancerous, or infectious diseases where conventional therapy is ineffective or unavailable (e.g., Duchenne muscular dystrophy (11), rheumatoid arthritis (12), cancer (13), or hepatitis (14)).

Currently, only a few ONs have reached the market (15): Kynamro®, an AON for the treatment of hypercholesterolemia, Macugen®, an aptamer approved for the treatment of age-related macular degeneration (AMD), and Vitravene™, an antisense drug approved for the treatment of cytomegalovirus retinitis in AIDS patients but discontinued in 2002 due to commercial reasons.

Larger nucleic acid drugs primarily consist in mRNA and plasmid DNA (pDNA) encoding functional protein or short hairpin RNA (shRNA) for target gene silencing. In comparison to pDNA, mRNA has some advantages since there is no risk of integration into the genome and nuclear entry is not required for its activity (16). Currently, several gene therapies based on viral delivery of DNA have been approved: Gendicine™ (Shenzhen Sibiono GeneTech Co.), Glybera® (UniQure), Imlygic® (Amgen Inc.), and Strimvelis™ (GlaxoSmithKline). Gendicine™, the first gene therapy approved for marketing, carries the wild-type p53 gene which aims at restoring the ability of cancer cells to undergo apoptosis, and it received authorization in China in 2003 for the treatment of head and neck squamous cell carcinoma (17). Glybera®, a therapy for familial lipoprotein lipase (LPL) deficiency, relies on the expression of an enhanced variant of LPL upon intramuscular injection (18), and has been approved in Europe in 2012. The first FDA-approved (in 2015) oncolytic virus Imlygic™ was engineered from herpes simplex virus 1 to carry the gene encoding human granulocyte-macrophage colony-stimulating factor for the treatment of inoperable melanoma via intratumoral injection (19). Strimvelis™ involves the autologous transplantation of bone marrow cells transduced ex-vivo with a gene encoding for adenosine deaminase (ADA) (20). It was approved in Europe in 2016 for the treatment of severe combined immunodeficiency due to ADA deficiency. Due to the high costs of product manufacturing and clinical development and low number of patients, currently approved gene therapies are extremely expensive (e.g., Glybera® costs about $1 million), making their marketing even more difficult.
Another application of pDNA and mRNA under investigation is the development of vaccines against cancer and viral or bacterial infections (21, 22). Delivered nucleic acid vaccines express the desired immunogenic protein and do not impose stable integration into the genome. The recent discovery of the bacterial defense system CRISPR-Cas9, capable of permanent gene editing with unprecedented accuracy in a desired location of the genome, has opened the possibility of performing gene therapy not only to transiently alleviate symptoms but rather to 

cure genetic and ageing-related diseases (23).

While the advances in understanding the molecular pathways of nucleic acids are tremendous and promise a plethora of novel therapeutic strategies for the treatment of multiple devastating diseases, achieving their safe and targeted delivery constitutes a bottleneck. Therefore, current research in the field of nucleic acid therapeutics is largely focused on improving their cellular uptake and specific tissue targeting.

1.2 Enhancing the stability of nucleic acids

Nucleases, extra- and intracellular enzymes catalyzing RNA and DNA cleavage, perform crucial functions, such as DNA replication, cell death, RNA maturation, or defense against viral and bacterial infection, and are thus ubiquitously expressed in our body (24). Therefore, to successfully develop nucleic acid-based therapeutics, it is crucial to overcome the stability issues posed by nucleases. Introducing unnatural chemical bonds that cannot be easily recognized and cleaved by these nucleases is one of the strategies employed to address the stability-related limitations of nucleic acid delivery (25) (Scheme 1.2).

First-generation modified ONs include phosphorothioate (PS) substituents on their backbone, resulting in increased nuclease resistance and higher cellular uptake (26). Further improvements in the nuclease resistance, and in some cases potency, have been achieved by combining PS backbone with chemically modified sugar rings. These second-generation ONs most commonly include 2′-O-methyl- (OMe), 2′-O-(2-methoxyethyl)- (MOE), 2′-F-RNA, or locked nucleic acids (LNA) modifications (Scheme 1.2). For certain ONs (e.g., AONs or siRNAs), their mechanism of action involving enzymatic recognition and cleavage of target mRNA imposes certain limitations on the type and location of chemical alterations that can be incorporated. Typically, AONs (short DNA oligomers) rely on RNase H-mediated recognition and cleavage of the formed DNA:mRNA heteroduplex (27); therefore, an unmodified DNA stretch of 8-10 bases should be included in the structure of antisense ONs (28, 29). RNA-like modifications (e.g., OMe,
MOE) generally do not support RNase H binding and therefore can be incorporated only at the termini of the antisense ON (“gapmer” design). A recently discovered 2′-deoxy-2′-fluoro-arabinonucleic acid (FANA) modification was shown to preserve DNA-like conformation upon binding to the complementary mRNA, thereby allowing for RNase H recognition (30). FANA can be incorporated also in the central DNA stretch previously unavailable to 2′-O-modified ribonucleotides, which may further improve resistance to endonucleases (“altimer” design).

Contrary to antisense ONs, the chemical modification of an siRNA generally leads to reduction of its potency despite improving nuclease resistance (31). To retain siRNA-mediated gene silencing, the free 5′ end of its guide strand should be preserved (31). PS backbone modification is the simplest approach to imbue siRNA with nuclease resistance (32), and selective placement of sugar modifications (e.g., OMe) was shown to reduce off-target activation of TLRs and incorporation of the incorrect strand into the RNA-induced silencing complex (RISC) (31).

Complete substitution of the sugar and phosphodiester moieties with an unnatural backbone (e.g., morpholino (PMO) or peptide nucleic acids (PNA)) is the basis for obtaining extremely robust third-generation oligomers which are unrecognizable by natural enzymes. AntimiRs, splicing correcting ONs, steric blocking oligomers, or aptamers do not require enzymatic recognition and can be entirely composed of such heavily modified bases or of the second-generation modifications (33, 34).

Scheme 1.2. The most commonly used chemical modifications of nucleic acid sugar rings (A) and backbone (B).
1.3 Nucleic acids delivery strategies

While the advances in ON chemical modifications have tackled the stability barrier for the development of nucleic acid therapeutics, the pending challenge lies in their high molecular weight and polyanionic nature which limit their permeation across cellular membranes. Only at high concentrations (typically, µM) are ONs able to enter cells via various pathways (e.g., adsorptive endocytosis, receptor-mediated uptake, macropinocytosis; Scheme 1.3), reach the cytoplasm/nucleus compartment, and induce target gene silencing (35–37). When administered systemically, modified ONs are distributed to various tissues within hours and can exhibit prolonged activity (up to several weeks in some cases) (38). The tissue distribution of ONs upon systemic administration is broad, and their highest levels are typically found in the liver and kidney followed by the bone marrow, adipocytes, and lymph nodes (38). PS and MOE modifications have been shown to promote ON binding to serum albumin reducing their glomerular filtration by the kidneys thereby increasing circulation times and tissue bioavailability (39). In contrast, unmodified ONs including siRNAs and non-charged oligomers (PMOs) present negligible binding to serum proteins, and are thus rapidly cleared via renal filtration with predominant uptake by the kidneys (40, 41). Generally, ONs do not cross the blood-brain barrier, but are taken up by neuronal cells and can cause a potent gene silencing when injected into the cerebrospinal fluid (42). This delivery strategy is currently being evaluated in a phase III clinical trial for the treatment of spinal muscular atrophy (NCT02292537).

Despite their preferential deposition in the liver and kidneys upon systemic administration, some of the most potent chemically-modified ONs exhibit strong silencing in multiple organs such as heart, lung, and gall bladder (38). The poor organ-specificity of silencing could potentially lead to adverse effects. Systemic toxicity may be induced when high doses of ONs are administered (40). Lethal doses were estimated to be around 1000 mg·kg⁻¹ in mice (41). Adverse effects may result from off-target gene silencing, unspecific protein binding, induction of immune response via TLRs signaling or complement activation (41, 43). While some deleterious effects such as stimulation of TLRs (44) and off-target gene silencing due to incorporation of the incorrect siRNA strand into RISC (45) can be avoided through careful design of the ONs sequence and modification strategy, most of them are in fact structure-specific and cannot be predicted. For instance, the only FDA-approved antisense ON, mipomersen, has to be administered subcutaneously at a high dose of 200 mg once per week and is often accompanied by injection-site reaction and fever, which could indicate a mild allergic reaction (46). Moreover, mipomersen has been associated to an increased risk of hepatic side effects (e.g., elevated liver transaminase and liver fat levels), which are
probably caused by the drug's mechanism of action relying on the prevention of the hepatic excretion of triglycerides (47). In order to improve the uptake in the targeted tissue and decrease the therapeutic dose required, several physical methods have been tested in preclinical and clinical studies (48). These methods are based on the disruption of the cell membrane and include electroporation, hydrodynamic injection, ultrasound-mediated sonoporation, and gene gun. Despite showing some efficacy for certain diseases, these methods are far from being universally applicable; therefore, several approaches for nucleic acid delivery encompassing particle-based and single-molecule-based strategies have been developed as discussed in detail in the following sections.

**Scheme 1.3.** Cellular uptake and trafficking of ONs (36). TGN – trans-Golgi network; MVBs – multivesicular bodies. ONs can enter cells via several pathways, such as macropinocytosis, caveolin- or clathrin-dependent endocytosis, initially guiding them to early endosomes. After that, ONs can be recycled back to the cell exterior or trafficked further to late endosomes/MVBs. Eventually, ONs can be either degraded in the lysosomes or guided by the retromer complex to the TGN. Even though the intracellular trafficking is highly regulated by multiple proteins, particularly by Rab family of GTPases, a small portion of ON can leak into the cytosol and the nucleus. The formation of exosomes within the MVBs is controlled by the ESCRT-I protein complex, which was recently connected with the efficacy of ONs (49).
1.3.1 Particle-based delivery approaches

One of the most commonly exploited strategies to deliver nucleic acids inside the cells is via nano/microscale delivery vehicles (50). They can accommodate ONs and larger molecules such as pDNA and mRNA, can be decorated with targeting ligands to ensure cell/tissue-specific uptake, and can be imbued with stimuli-responsive properties in order to release their cargo under specific conditions. Ideally, this approach should allow for (i) protection of nucleic acid cargo from extracellular enzymes, (ii) targeting nucleic acids to a desired tissue/cell type with minimal uptake by the other tissues, and (iii) delivery to the intracellular compartment of interest (cytosol or nucleus), bypassing the lysosomal degradation pathway.

Viral and bacterial vectors

Viruses are naturally evolved nucleic acid delivery systems that can be genetically engineered for therapeutic use (51). They are considered to be the most efficient transfection agents, which is supported by the four viral-based gene therapies approved worldwide, as mentioned previously. One of the limitations of viral delivery systems is the maximal size of nucleic acid payload they can carry (up to 5 – 150 kb DNA, depending on the virus type (52)). Additionally, due to the strict safety requirements and relatively complex manufacturing process, their production is expensive (53). Another more important concern is that some viruses (e.g., lentivirus) tend to insert their genes into the host genome, which could lead to the reading frame shift or to the activation of host's oncogenic genes (54). Although safer viruses exist that lack this property (e.g., adenovirus, adeno-associated virus), their capsids still possess immunogenic properties which can lead to serious adverse events or fast clearance of the viral carriers upon repeated administration (54). Genetically engineered invasive bacteria have also been tested as gene delivery systems (55, 56). However, due to the immunogenicity of bacterial products upon systemic exposure, bacteria-derived delivery systems could find their applications primarily for the local therapy of the gastrointestinal (GI) diseases (55, 56). Considering the limitations posed by viral and bacterial gene delivery systems, the development of non-biological vehicles has attracted much attention and raised hopes for bringing safe, efficient, and inexpensive gene therapies on market.
Inorganic particles

As an alternative to biological vectors, inorganic nano/microparticles composed of metal, calcium phosphate, metal oxides, silica, or carbon nanotubes have been developed (57). Some of these materials offer unique electrical, optical, and magnetic properties enabling various novel biomedical applications, such as combination of therapy and diagnostics (theranostics) (57–59). Inorganic delivery vehicles are generally more stable than biological ones, inexpensive, and easy to functionalize. Indeed, nucleic acids can be directly grafted onto the surface of the carrier (e.g., gold nanoparticle via thiol linker (60)), complexed with the cationic-polymer-functionalized particle (e.g., poly(amidoamine) (PAMAM)-modified iron oxide (61)), or incorporated during the particle formation process (e.g., calcium phosphate vehicles (62, 63)). Among the disadvantages of inorganic delivery systems are low loading efficiency, insufficient cargo protection, and poor endosomal escape (64). Another major drawback is often the lack of biodegradation and, therefore, potential toxicity due to accumulation in healthy tissues following repeated dosing (65). While the calcium phosphate nanoparticles are biocompatible and biodegradable, the issue regarding their stability represent an obstacles for their clinical translation. The use of stabilizers (e.g., PEGylation) is an attractive strategy to tackle this problem (63, 66, 67).

Organic complexes (polypelexes and lipoplexes)

Nucleic acids are negatively charged biopolymers that can form nano/microsized complexes with oppositely charged cationic polymers (e.g., poly(ethylene imine) (PEI) (68)) or lipids (e.g., Lipofectamine™ 2000 (LF) (69)), referred to as polypelexes and lipoplexes, respectively. Despite being less efficient in transfecting cells in comparison to viral carriers, organic complexes are advantageous due to tunable chemistries, lower risk of immunogenicity, and the possibility of large-scale production at acceptable costs (70). Generally, the excess of the positive charges on the particles' surface is a prerequisite for their high transfection efficacy in vitro (70). Such particles interact with the negatively charged cell membrane and enter cells via endocytic pathways. In order to release their nucleic acid cargo into the cytoplasm, these carriers must disrupt endosomes via the proton-sponge effect (71), formation of nanopores (72), or membrane fusion/destabilization (73). In case of polypelexes, the inherent polydispersity of polymers potentially results in difficult-to-characterize complexes. The need for the systems with controlled and reproducible structure has led to the development of molecularly-defined cationic dendrimers (e.g., PAMAM (74)) as gene delivery vehicles. Nevertheless, the repeated administration of synthetic non-degradable cationic
polymers such as PEI and PAMAM have raised some safety concerns (75). Incorporation of biodegradable bonds into the polymer backbone (e.g., ester (76) or disulfide (77)) may help to facilitate the polymer's renal clearance after its cleavage in the body. The use of natural biodegradable polycations (e.g., chitosan (78)) or non-charged biodegradable polymers (e.g., poly(lactide-co-glycolide) (PLGA) (79)) are the alternative strategies under investigation. In comparison to synthetic polymers, phospholipids and some lipids have an advantage of higher biocompatibility and biodegradability (80), which was confirmed in multiple clinical trials (81).

In vivo, before exerting their function, positively charged polyplexes and lipoplexes are rapidly coated with serum proteins (i.e., opsonins) followed by their recognition and clearance by mononuclear phagocyte system (MPS). These processes can cause an immune reaction and liver toxicity (68, 82). Conferring particles with the stealth effect by PEGylation may reduce not only the serum protein binding, but also cell uptake of cationic particles (68). Therefore, a combination of PEGylation and attachment of a targeting ligand for the enhanced uptake by the desired cell type may help to overcome these issues (68). For example, CALAA-01, a polyplex system that has entered clinical trial, relies on the uptake of transferrin-modified cationic cyclodextrin/siRNA complex by cancer cells overexpressing the transferrin receptor (83). Unfortunately, despite being PEGylated, CALAA-01 has failed phase Ib clinical trial due to liver toxicity and immunogenicity of the cationic carrier (83). Therefore, potential toxicity of the cationic gene delivery carriers must be carefully evaluated before their clinical translation. To solve this problem, the surface positive charges need to be thoroughly masked in the blood stream and exposed only in the vicinity of or inside the target cell. This could be achieved through the design of responsive polymers and lipids (e.g., pH-, light-, temperature-sensitive) that can release their cargo upon defined stimuli (70). For example, the concept of lipids that become charged only in the acidic environment found in tumor tissues and in the endosomes has been exploited for the liposomal delivery vehicles Smarticles® (Marina Biothech Inc.) (84) and for the stable-nucleic-acid lipid particles (SNALPs) (Tekmira Pharmaceuticals) (85). These pH-sensitive lipid-based nucleic acid delivery vehicles are currently progressing through several clinical trials (e.g., for the treatment of transthyretin-mediated amyloidosis or lung cancer (81, 86)).

While numerous non-viral particle-based delivery systems have been tested, so far none of them has been released on the market. One of the major problems of those delivery systems is their high carrier-to-cargo ratio, resulting in the accumulation of large amount of potentially toxic material in the body (36). Importantly, due to their size, nanocarriers cannot escape from the vascular network and reach parenchyma of most of the underlying organs upon intravenous (i.v.) administration, but they can diffuse primarily into tissues with fenestrated vasculature such as liver,
spleen, solid tumors, or sites of inflammation (Scheme 1.4) (36, 87). Therefore, particle-based gene delivery approaches can be used mainly for the treatment of pathologies of the liver, cancer, and inflammatory diseases (36). Importantly, in case of cancer treatment, distribution of the particles to healthy organs, such as liver and spleen, may raise toxicity issues.

1.3.2 Single-molecule conjugate delivery approaches

Due to the obstacles associated with nano/microsized delivery vehicles, single-molecule systems have received much attention for the development of ON-based therapeutics. They can easily diffuse through the blood vessel endothelium upon i.v. or subcutaneous administration and reach organs inaccessible for nanoparticles (Scheme 1.4) (88). Importantly, the reduction in the amount of administered excipients (e.g., non-biodegradable polymer) may help to avoid some of the toxicity and immunogenicity issues. However, this comes at a certain cost: the need to stabilize unprotected ONs via chemical modifications due to exposure to extracellular nucleases. From an industrial point of view, ON conjugates are attractive because their relatively simple and well-defined structure allows their preparation by solid-phase synthesis, thus reducing the production, characterization, and purification costs. The major drawback of single-molecule ON conjugates is their low potency (typically μM in vitro), which requires administration of higher doses of ONs to achieve the therapeutic effect in comparison to particulate carriers (89, 90).

Conjugation of a receptor-targeting moiety

The conjugation of a receptor-targeting moiety to the ONs is the main strategy employed to facilitate their cell uptake (91). When selecting a suitable receptor for targeting, one must consider its expression levels in target versus non-target tissues, the rate of receptor recycling, and (optionally) the availability of small-molecule ligands (36). In addition, potential activation/inhibition of downstream signaling cascades induced by ligand binding to the selected receptor must be carefully evaluated to avoid potential off-target events especially upon chronic administration (36). Among the receptors often used for targeting are integrins, G protein-coupled receptors, human tyrosine kinases, TLRs, scavenger receptors, the asialoglycoprotein receptor (ASGR), the folate receptor, and some others (36). A range of ligands have been conjugated to ONs for enhancement of their receptor-mediated uptake and tissue selectivity including small-molecule ligands, aptamers, peptides, and even larger molecules such as antibodies. Although it is often difficult to find a suitable small-molecule ligand for a new receptor candidate, biomolecular techniques such as phage
display and SELEX (Systematic Evolution of Ligands by Exponential Enrichment) can be exploited for the search of high-affinity biologics for virtually any target of interest.

The most successful examples of targeted ON delivery systems are those relying on the ASGR-mediated uptake with the help of N-acetylgalactosamine (GalNAc) ligands. The ASGR is predominantly expressed on hepatocytes (92), and is a particularly attractive target due to its presence at a high density on the cell surface, and its rapid internalization, cargo release, and recycling back to the plasma membrane (92). However, recent findings pointed out the involvement of ASGR signaling in platelet homeostasis, which could be disturbed by chronic GalNAc-ON administration (93). Despite this fact, several GalNAc-ON conjugates based on siRNA and antimiR are currently advancing through clinical trials for the treatment of diseases with liver-localized targets (e.g., transthyretin-mediated amyloidosis, hypercholesterolemia, hemophilia, or hepatitis C infection). Integrins constitute another attractive class of targets due to their high expression levels, recycling, and selective expression in certain tissues/disease states (36). For instance, αvβ3 integrin is overexpressed in newly formed blood vessels and invasive cancer cells (94). Cyclic pentapeptide Arg-Gly-Asp-d-Phe-Lys (cRGD), a well-known ligand for the αvβ3 integrin, was conjugated to an siRNA against vascular endothelial growth factor (VEGF), and achieved significant tumor growth retardation in mice upon systemic administration at a relatively low dose of 0.75 mg·kg⁻¹ (94). Some other cancer-specific targets including the prostate-specific membrane antigen (PSMA), B-cell-activating factor receptor (95), and cancer-associated receptor tyrosine kinases (96) were targeted with the help of aptamers (nucleic acid-based binders). The main drawback of aptamer-based targeting ligands — i.e. their rapid degradation in the serum— has been recently addressed by development of polymerases that can operate with chemically modified nucleobases, such as FANA (34). These polymerases have enabled SELEX-based selection of fully modified enzymatically stable aptamers (34). Moreover, another recent optimization of SELEX-based aptamer selection method has helped to identify aptamers that trigger efficient internalization of the aptamer-ON conjugate by the target cells (97).
Scheme 1.4. Schematic biodistribution of nucleic acid delivery systems upon systemic administration. Singe-molecule-based systems can easily diffuse through the blood vessel endothelium (gaps of <5.5 nm) and reach organs inaccessible for nanoparticles (except for the brain). Due to their size, particulate carriers cannot escape from the vascular network and reach parenchyma of most of the underlying organs upon i.v. administration, but they can diffuse primarily into tissues with fenestrated vasculature such as liver, spleen, solid tumors, or sites of inflammation. After reaching the target tissue, nucleic acid delivery carriers can be taken up unspecifically or via receptor-mediated endocytosis. They should be able to escape the endosomes to exert their function.

Due to their size (<20 kDa), ON conjugates with small-molecules, short peptides, or aptamers penetrate well into tissues. It is worth noting that the conjugation of intact antibodies to ONs (>150 kDa) results in drastically reduced tumor extravasation in vivo (98); therefore, strategies involving conjugation of antibody fragments or smaller non-antibody scaffolds (e.g., DARPin 99) may be more promising. However, as mentioned previously, the molecular weight of the ON conjugate should be tuned in the optimal range, since some of the ONs (e.g., siRNA) are rapidly excreted via renal filtration before exerting their function. Therefore, the additional conjugation of macromolecules is a way to achieve long circulation times and significant target binding. Indeed, PEGylated siRNA conjugated to an aptamer against the PSMA showed prolonged target gene silencing in a prostate cancer xenograft model in comparison to its non-PEGylated
Conjugation of targeted ONs to a serum protein (e.g., albumin) is another interesting approach to increase the circulation time, which additionally allows for display of multiple copies of ONs in a single carrier molecule (up to 15) (101). The resulting conjugates are small enough (about 12 nm) for efficient tissue penetration, but too large for renal clearance (101). The potential drawback of the albumin-conjugated ON delivery systems is the absence of endosome-destabilizing properties. The endosomal escape can be achieved by conjugation of a pH-responsive polymer (74). For example, the Dynamic Polyconjugate™ (DPC™) delivery system (Arrowhead Research) relies on the attachment of a targeted (e.g., against tumor-expressed integrins or ASGR of hepatocytes) endosomolytic polymer to siRNA via a disulfide linker (102). The cationic charges of the amphiphilic polymer are masked with pH- and protease-labile groups, which are being cleaved in the endosomal environment thereby promoting the release of siRNA into the cytoplasm (102). Overall, despite having been in focus only for a few years, a strategy of direct conjugation of a targeting moiety to ONs has already achieved significant clinical results at least for liver targeting. The advantages of single-molecule-based systems for clinical development, such as the well-defined structure and their better diffusion in the tissue parenchyma, render them highly competitive with the most clinically advanced lipoplex-based delivery systems.

**Conjugation of non-specific uptake enhancers**

Conjugation of cell-penetrating peptides (CPPs) and lipids to ONs has been shown in vitro to enhance their uptake in multiple cell types, however, they lack cell selectivity (103). Therefore, such systems could find their application primarily for the ON delivery to the cells that are broadly distributed in the body (e.g., muscle cells, virally-infected cells, bacterial cells in the circulation), to the liver, or locally (103–105).

CPPs are typically positively charged peptides containing hydrophobic amino acid residues that interact with cell membranes and trigger uptake (104). They can form insoluble complexes with oppositely charged nucleic acids, similarly to cationic polymers and lipids (103, 104). However, to obtain structurally defined single-molecule-based conjugates, CPPs are preferably attached to non-charged ONs such as PMOs and PNAs. Notably, it was recently shown that relatively hydrophobic CPP-PMO conjugates can self-assemble into nanoparticles and be taken up via scavenger receptors, a type of pathogen-recognition receptors (106). These conjugates have been mainly used for correcting the mis-spliced transcript of dystrophin, the protein involved in the pathology of Duchenne muscular dystrophy (104). Thus, a single i.v. administration of a CPP-
PMO conjugate (12.5 mg·kg⁻¹) to mice resulted in a strong dystrophin expression not only in skeletal muscles, but also in the heart, which is particularly challenging (107). However, the possible toxicity of the cationic CPPs and their interaction with serum proteins should be carefully considered when designing an ON delivery system (103). Another concern related to CPP-based conjugates is their cell-type dependent efficacy (103). In a recent study from our group, a library of 15 different CPPs (previously reported to induce ON-mediated silencing) was conjugated to a single PNA sequence targeting luciferase and screened for gene silencing in a colon cancer cell line (108). While most of the CPP-PNA conjugates were trapped in the endosomes as evidenced by their enhanced activity in the presence of the endosomolytic agent chloroquine, only one CPP was capable of target gene silencing (108). Apart from targeting genetic diseases, CPP-based ON conjugates have been investigated as antiviral and antibacterial agents with some success in vivo (109, 110). However, elimination of the cationic CPP carrier and incorporation of positively charged piperazine groups directly into the backbone of PMOs resulted in less toxic antiviral drug candidates, which were well tolerated in phase I clinical trial for the treatment of Marburg virus infection (111).

Several authors have shown that the conjugation of neutral lipids such as cholesterol and aliphatic fatty acids to ONs improves their uptake and silencing efficacy both in vitro and in vivo (112–114). Following systemic administration, lipid-ON conjugates accumulate mainly in the liver probably due to binding to so-called “in vivo vehicles”, such as low-density lipoproteins (113), chylomicrons (115), or albumin (112, 116). The vehicle/ON complex can bind to the corresponding cell surface receptor on hepatocytes (e.g., lipoprotein receptor) and facilitate the endocytosis of amphiphilic ON conjugates (in case of siRNA conjugates, possibly via a channel for double stranded RNA, SID-1) (113). In the absence of in vivo vehicle, the uptake and silencing efficacy of amphiphilic ONs was found to vary greatly depending on the lipophilic moiety and the cell line tested (112, 117, 118). The cell-line-dependent efficacy of the conjugates could be related to the different expression of the surface receptors involved in the ON’s uptake. For example, an siRNA conjugated with a double-tailed myristic acid derivative was efficiently incorporated into the cell membrane and triggered β₂-integrin-mediated endocytosis of the conjugate in certain cell types with high-level expression of β₂-integrin (119). The association with the cell membrane seems to be the first step in the uptake of amphiphilic conjugates (120), followed by adsorptive endocytosis/pinocytosis or receptor-mediated endocytosis of the ON (117, 119). In the case of amphiphilic bile acid-siRNA derivatives, uptake was shown to be mediated by a hypothetical “needle and thread” transport route (121). Due to the limited number of studies, however, the
mechanism of cellular uptake of amphiphilic ONs remains unclear and further studies are necessary to draw reliable conclusions.

Recently, local administration of carrier-free hydrophobically modified ONs has been exploited for the treatment of several disorders (121–126). For example, intravitreal injection of bile acid-modified siRNA against Sjoögren syndrome type B antigen lead to moderate target knockdown without the signs of inflammation in rats (121). Cholesterol- or docosahexaenoyl-modified chemically-stabilized siRNAs silenced target huntingtin mRNA in mouse brain upon local injection (122, 123). Another cholesterol-modified stabilized siRNA (Accell® siRNA) (127) targeting the mutant keratin 6a mRNA for the treatment of pachyonychia congenital showed enhanced uptake and target silencing in human skin ex vivo and in a mouse model via local injection (124, 125). RXi Pharmaceuticals is developing hydrophobized RNAi compounds for topical and systemic applications (126). The proprietary nucleic acid delivery platform is an OMe- and 2′-F-modified hybrid between siRNA-like short duplex region and single-stranded PS-modified region improving nuclease resistance (126). It is conjugated with lipophilic groups, such as sterol, enhancing the uptake in multiple cell types, i.e., its ‘self-delivery’ (sd-rxRNA®) (126). According to the company, RXI-109, a lead compound currently in phase II clinical trials for reducing skin scarring, has an anti-fibrotic action due to the silencing of connective tissue growth factor. It is additionally being evaluated in AMD patients after intravitreal administration for reduction of retinal fibrosis that contributes to vision loss.

The major drawback of lipid-ON conjugates is their relatively low potency, which requires high dose. Co-administration with small molecules or peptides that enhance ON's uptake and endosomal release is an emerging field of research. Thus, the administration of cholesterol-siRNA conjugate in combination with DPC™ endosomolytic amphiphilic peptide is currently being evaluated for the treatment of hepatitis B infection in phase II clinical trial (105). Recently, co-administration of hydrophobically modified siRNA with FDA-approved small molecule drug guanabenz (an antihypertensive agent) was shown to enhance its silencing effect in vitro (128). In another recent in vitro study, high throughput screening helped to reveal 28 compounds that improved the cholesterol-siRNA silencing by enhancing its cell uptake or release from the endolysosomal compartments (129), opening new directions in the ON delivery strategies.
1.4 Delivery of nucleic acids to the gastrointestinal (GI) tract

Due to the transient nature of mRNA knockdown, ON therapeutics require regular administration, for which oral delivery would be the most convenient route (130). Several attempts to systemically deliver nucleic acids via oral intake have been undertaken in animals and humans. Even when imbued with enzymatic resistance by chemical modification, nucleic acid drugs proved to be minimally absorbed in the GI tract due to their polyanionic nature and high molecular weight (>5000 Da). For instance, in comparison to the highly permeable small molecule drug naproxene, permeability coefficients of 20-mer PS-modified ONs across rat small intestine are approximately two orders of magnitude smaller ($P_{app} = 1-2\times10^{-4}$ cm/s vs. $2-8\times10^{-6}$ cm/s) (131).

Several pathways have been described for ON translocation from the GI tract to the systemic circulation (Scheme 1.5). Those include: (i) internalization of particulate carriers by dendritic cells/M cells of intestinal Peyer's patches and consequently by associated lymphoid macrophages (132, 133), (ii) uptake by the intraepithelial lymphocytes (134), (iii) transcytosis/paracellular transport through the absorptive epithelium (115, 133–135), which in certain cases leads to (iv) binding of hydrophobized ON conjugates to chylomicrons in the lymph duct followed by transport to the systemic circulation (115). These pathways result in the delivery of ONs to MPS cells of peritoneum, liver, spleen, kidneys, lungs, and liver hepatocytes (115, 132–138).

Scheme 1.5. Putative uptake routes for systemic delivery of nucleic acid therapeutics after oral administration: (i) M cell/dendritic cell-mediated uptake, (ii) intraepithelial lymphocyte uptake, (iii) paracellular transport/transcytosis, in certain cases leading to (iv) lymph chylomicron-mediated transport of hydrophobized nucleic acids (115, 132–138).
Employing a permeation enhancer (e.g., sodium caprate) may promote paracellular transport (the passage between epithelial cells) thus improving ON’s absorption (131). However, the systemic bioavailability of ONs was found to be highly variable when co-administered with sodium caprate ranging from 0.7% to 27.5% in humans (139), making this strategy less valuable for clinical applications. The results of clinical trials testing this strategy are discussed in Chapter 2 of the present thesis.

As an alternative delivery approach, the targeting of intrinsic pathways connecting the intestinal mucosa with the systemic compartment could be employed, such as the uptake by the intestinal immune cells and their migration to remote organs or the lipid transport system (Scheme 1.5). Indeed, the pioneer system GeRP (β1,3-D-glucan encapsulated siRNA particles) composed of baker’s yeast-derived shell enveloping PEI/siRNA/tRNA mixed polyplex and the amphiphilic peptide Endoporter was taken up via receptor-mediated endocytosis by macrophages both in vitro and in vivo (132). After gavage of GeRP particles to mice, fluorescently labelled yeast shell and siRNA co-localized with macrophages of peripheral organs such as spleen, liver, lung tissues, and peritoneum (132). These particles suppressed lipopolysaccharide (LPS)-induced systemic inflammation by silencing target Map4k4 mRNA in macrophages, reduced TNF serum levels, and improved survival rate (132). However, the same delivery vehicle loaded with anti-TNF siRNA failed to significantly downregulate the target mRNA and, consequently, to reduce the severity of colitis in a mouse model (140). In general, GeRP represents a highly complex delivery system requiring multi-step preparation, which makes it less attractive for clinical translation.

A simpler particle-based approach relying on the delivery of chitosan/siRNA polyplex showed the presence of intact unmodified siRNA as detected by PCR analysis in the liver, spleen, and kidneys 1 and 5 hours after gavage, but not at the later time point (24 h after gavage) (134). Presumably, this delivery vehicle was either not taken up by the intestinal epithelial and submucosal cells or it was rapidly disassembled within 45 min, as the fluorescently labelled siRNA was detected only at the luminal surface of the intestinal epithelium (134). Unfortunately, the bioactivity of this system remains unknown as it was not further investigated (134).

In subsequent studies, the oral administration of modified chitosan-based polyplexes containing ONs against TNF led to amelioration of LPS-induced systemic inflammation (133, 136, 137). In order to disrupt the intestinal epithelial barrier and reach the underlying MPS cells, a complex multicomponent self-assembling ON delivery system was designed (136). It included oleyl-modified trimethyl chitosan and oleyl-PEG-cysteamine for permeation across intestinal epithelium, oleyl-PEG-mannose for receptor-mediated uptake by mucosal macrophages, cationic
α-helical polypeptide (PVBLG-8) for endosomal escape, and sodium tripolyphosphate (TPP) as a crosslinker (136). The intestinal absorption of these particles was compared in two in vitro models: the non-follicle-associated epithelium (FAE) model based on polarized Caco-2 cell monolayers and the FAE model obtained from co-culture of Caco-2 cell monolayers with Raji B lymphocytes producing M cell phenotype (136). Increased permeability of chitosan/siRNA complex across the non-FAE model was observed in comparison to naked siRNA as a result of the disturbance of tight junctions (TJ), as indicated by the decreased transepithelial electrical resistance (TEER) and the results of immunostaining of TJ proteins (136). Moreover, the permeation coefficient of the chitosan-based delivery system increased 2.9-fold in the FAE model in comparison to that of the non-FAE model, suggesting preferential uptake by M cells (136).

A similar delivery system relying on receptor-mediated uptake by M cells and consequently by underlying mucosal macrophages involved direct functionalization of trimethyl chitosan with mannose and cysteine groups (133). These mannose-modified carriers were shown to enter macrophages via caveolae-mediated endocytosis and micropinocytosis in vitro (133, 136). Alternatively, intracolonic instillation of galactose-modified chitosan complexed with AON against TNF led to its uptake by activated intestinal macrophages overexpressing galactose receptor in dextran sulfate sodium (DSS)-induced ulcerative colitis mouse model (137). Zhang J. et al. reported a similar polyplex system based on galactosylated trimethyl chitosan containing cysteine moieties for the targeted delivery of Map4k4 siRNA to activated intestinal macrophages in a DSS-induced colitis model (138). In a subsequent cancer therapy study by the same group, orally delivered galactosylated cysteine-modified trimethyl chitosan-based polyplexes carrying shRNA and siRNA (targeting survivin and VEGF, respectively) caused target genes silencing in a subcutaneously implanted hepatoma tissue (135). Interestingly, siRNA and shRNA were detected not only in tumor hepatocytes but also in MPS-cells-containing organs, such as liver, spleen, and lungs (135), despite the low level of galactose receptor expression in quiescent macrophages (137, 138). Authors attributed the systemic translocation of the nucleic acids and their hepatocyte uptake to the permeation-enhancing properties of modified trimethyl chitosan (135). In a differentiated intestinal epithelium in vitro model, this delivery system induced a dramatic reduction of TEER, which is an indicator of disturbed TJ (135). These results suggest that the modified chitosan-based delivery vehicle reached systemic circulation directly via paracellular transport through the absorptive epithelium and unspecific uptake by underlying mucosal macrophages.

A newer approach has been reported to target liver hepatocytes taking advantage of the intrinsic lipid transport system, using α-tocopherol-siRNA conjugate (Toc-siRNA) administered rectally in mixed lipid nanoparticles (Scheme 1.5) (115). The main components of lipid
nanoparticles, PEG-60 hydrogenated castor oil (HCO-60) and linoleic acid, served as permeation enhancers. Toc-siRNA conjugate was shown to bind to chylomicrons in the lymph duct and to be transported to the systemic circulation. It was demonstrated that uptake by the liver hepatocytes via remnant receptors caused target apolipoprotein B (ApoB) silencing and decrease in low-density lipoprotein cholesterol and triglycerides serum levels (115). Notably, the size of the nanoparticles and the site of their administration crucially affected the delivery of Toc-siRNA to the liver. While the small uniform nanoparticles (15 nm in diameter) administered rectally were capable of siRNA delivery to the liver, the presence of aggregates in the formulation and/or administration to the small intestine abolished the delivery efficacy (115). Overall, the systemic translocation of nucleic acid drugs after oral administration remains extremely challenging as it often requires complex delivery vehicles and/or co-administration of potentially toxic absorbefacients (141–143). In addition, many of the delivery systems rely on the uptake by M cells, the number of which varies greatly between species (for example, the proportion of M cells in the FAE is 10% in human and mice and 50% in rabbits), making the prediction of absorption in humans based on animal data difficult (144).

Due to the accessibility of the digestive mucosa, local delivery of nucleic acids to the GI tract targets has higher chances for clinical translation in comparison to targeting systemic disorders via the oral route. Several GI ailments with unavailable or ineffective conventional treatments could substantially benefit from nucleic acid therapy, such as GI infections (145), cancer (146), rare genetic diseases (e.g., familial adenomatous polyposis (147)), and IBDs (Crohn's disease, ulcerative colitis, pouchitis (148, 149)). In these cases, local administration would be the most desirable route due to the minimal systemic exposure and potential adverse reactions (e.g., complement activation or gene silencing in non-target organs) (148, 150).

To protect nucleic acids from the acidic gastric pH that leads to their depurination (151), enteric formulations, enema, or complexation with polymers may be employed (150, 152). Chemical modifications, as mentioned previously, can also be included to imbue nucleic acids with resistance to the RNases and DNases found in the intestinal lumen (15). The strategy of delivering high doses of chemically stabilized nucleic acids to the GI tract is currently being tested in clinical trials for the treatment of IBDs (8), which is discussed in more detail in Chapter 2. Importantly, due to their high negative charge density and large molecular weight, nucleic acids are poorly taken up by intestinal cells. Therefore, strategies aimed at enhancing ON's cell uptake may improve their efficacy.
Several particle-based delivery strategies have been developed to deliver nucleic acids targeting cytokines (e.g., TNF), cytokine receptors (e.g., TNFR2), transcription factors (e.g., NFκB), kinases (e.g., Map4k4), metalloproteinases (e.g., MMP-10), and oncogenes (e.g., β-catenin) to the GI mucosa (Table 1.1). Most of the particle-based carriers for local GI therapy target mucosal immune cells capable of disseminating to remote organs (115, 133, 135); therefore, these approaches could potentially cause the same off-target effects as systemically delivered ONs. Generally, particle-based carriers do not require chemical modification of nucleic acids because the vehicle can effectively protect the ON from GI degradation. However, they need to overcome another important barrier constituted by the thick, negatively charged mucus layer in the intestine with fast renewal rates. This mucus layer acts as a sieve for large particulate carriers hampering their diffusion to the epithelium, and additionally entraps nanoparticles via electrostatic and/or hydrophobic interactions leading to their elimination through mucociliary clearance (153). Engineering mucus-penetrating nanoparticles via conjugation of low-molecular-weight PEG chains has shown some success in overcoming this barrier (153).

The first demonstration of local gene knockdown in the GI tract was achieved with lipoplex-based ON delivery systems applied rectally or by intracolonic instillation (154, 155). Further improvement in gene silencing by lipoplex-based vehicles has been attempted using chemically modified siRNAs, given their increased nuclease resistance and reduced immunogenicity (69). While simple and attractive as a proof-of-concept, cationic lipids or polymers have limitations due to their reported toxicity (156, 157) and potentially negative impact on intestinal microbiota (143). Approaches aimed at improving the safety of polycation-based delivery systems have thus been developed, such as including a protective coating with poly(lactic acid) (PLA) and poly(vinyl alcohol) (PVA) (158, 159), employing shorter polymer chains (159, 160), or incorporating biodegradable disulfide bonds (161). Interestingly, a delivery system made of PVA-coated PLA matrix containing siRNA/PEI polyplex was shown to be taken up and silence the target gene not only in macrophages but also in intestinal epithelial cells (159); the mechanism of uptake by polarized epithelium was however not suggested. In another study, some uptake by intestinal epithelial cells was achieved via endocytosis of chitosan/PVA-coated PLGA nanospheres carrying decoy ON (162). Notably, the particles adhered to and were taken up mainly by the inflamed colonic tissue, while the uptake by the non-inflamed tissue was negligible (162). According to the patent (WO 2013138930 A1) and company’s web-site, enGene Inc. has developed a polyplex system based on arginine/gluconic acid-modified chitosan capable of transfecting gut epithelium, and plans to use this system for the treatment of IBDs and diabetes. Another possible approach to enhance polyplex-mediated gene silencing is to modify the cationic polymer with lipophilic groups.
Indeed, polyplex of alkyl-conjugated cyclodextrin and siRNA administered rectally to a colitis mouse model induced target gene silencing in mucosal macrophages and decreased the severity of the disease (165).

Some delivery vehicles use environmental triggers to release their cargo at defined locations within the GI tract. For instance, the incorporation of a polyplex in a pH-sensitive alginate/chitosan hydrogel was shown to release its cargo primarily in the colon at pH values of 5-6 (159). Furthermore, the so-called nanoparticles-in-microsphere oral system (NiMOS) which relies on the encapsulation of gelatin/siRNA nanoparticles in a microshell composed of lipase-labile poly(ε-caprolactone) (PCL), was shown to protect the cargo during gastric transit and enable its release in the small intestine (149, 166). This delivery system has been extensively studied for the local delivery of siRNAs as well as pDNA to the small intestine and colon (166–169). A different system employing thiokektal nanoparticles has been employed to target ON specifically to the inflamed intestinal tissue (140). This system is based on siRNA lipoplex coated with poly(1,4-phenyleneacetone dimethylene thioketal), which is sensitive to reactive oxygen species (ROS) and should allow the thiokektal shells to degrade in the close proximity to the inflammation sites due to the high levels of ROS produced by the mucosal macrophages (140).

Despite the reported efficacy in a colitis model, this delivery system employs potentially toxic cationic lipids, and in addition carcinogenic solvent (i.e., benzene) is used for its preparation, making it less attractive for clinical translation. Generally, the complexity of multicomponent systems often involving several emulsification, complexation, and purification steps represents a major hurdle for their clinical translation.

Another interesting GI delivery system, the transkingdom RNA interference technology platform (tkRNAi), has been developed by Marina Biotech. Based on living attenuated *Escherichia Coli* expressing shRNA for target gene silencing, this delivery system enters mucosal epithelial cells via β1-integrin-mediated endocytosis, escapes the acidic phagosomes with the help of listeriolysin, and releases its nucleic acid cargo in the cytosol (55, 56). One example of tkRNAi is the orally administered CEQ508, aiming at silencing β-catenin to prevent the uncontrolled cell growth in colons of familial adenomatous polyposis patients overexpressing this protein (147). The results from animal models seem promising (55); however, clinical trials will be necessary to evaluate the safety and efficacy of bacteria-based delivery systems.
Table 1.1. Strategies for nucleic acids delivery to the GI tract.

<table>
<thead>
<tr>
<th>Delivery vehicle</th>
<th>Cargo</th>
<th>Target</th>
<th>ON dose/day</th>
<th>Carrier dose/day</th>
<th>Organ/cell type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposome-based lipoplex</td>
<td>AON</td>
<td>CD40</td>
<td>0.0027 mg</td>
<td>0.127 mg*</td>
<td>total colonic tissue</td>
<td>(154)</td>
</tr>
<tr>
<td>LF lipoplex</td>
<td>siRNA</td>
<td>TNF, CCR5</td>
<td>0.0005 mg</td>
<td>0.0004 mg</td>
<td>total rectal tissue</td>
<td>(155)</td>
</tr>
<tr>
<td>DOTAP lipoplex with ROS-labile thioketal polymer</td>
<td>siRNA</td>
<td>NFkB</td>
<td>2 mg</td>
<td>37 mg</td>
<td>colon</td>
<td>(162)</td>
</tr>
<tr>
<td>DOTAP lipoplex in PLGA matrix with chitosan/DNA</td>
<td>siRNA</td>
<td>TNF</td>
<td>0.23 mg·kg⁻¹</td>
<td>164 mg·kg⁻¹*</td>
<td>hepatocytes, kidney, intestine</td>
<td>(115)</td>
</tr>
<tr>
<td>Toc-siRNA conjugate, HCO-60, linoleic acid</td>
<td>siRNA</td>
<td>ApoB</td>
<td>30 mg·kg⁻¹</td>
<td>164 mg·kg⁻¹*</td>
<td>macrophages, IEC</td>
<td>(158, 159)</td>
</tr>
<tr>
<td>PLA-PEI polyplex coated with PVA</td>
<td>siRNA</td>
<td>TNF; CD98</td>
<td>N.A.; 0.5 mg</td>
<td>0.5 mg</td>
<td>MPS organs</td>
<td>(134)</td>
</tr>
<tr>
<td>Chitosan polyplex</td>
<td>siRNA</td>
<td>N.A.; 0.325 mg</td>
<td>3 mg</td>
<td>37 mg</td>
<td>colon</td>
<td>(162)</td>
</tr>
<tr>
<td>Galactosylated chitosan polyplex</td>
<td>AON</td>
<td>TNF</td>
<td>5 mg·kg⁻¹</td>
<td>100 mg·kg⁻¹*</td>
<td>activated macrophages</td>
<td>(138)</td>
</tr>
<tr>
<td>Galactosylated trimethyl chitosan-cysteine, TPP</td>
<td>siRNA</td>
<td>Map4k4</td>
<td>0.25 mg·kg⁻¹</td>
<td>275 mg·kg⁻¹*</td>
<td>MPS organs</td>
<td>(135)</td>
</tr>
<tr>
<td>Galactosylated trimethyl chitosan-cysteine</td>
<td>siRNA</td>
<td>Survivin+VEGF</td>
<td>0.2 mg+1 mg·kg⁻¹</td>
<td>100 mg·kg⁻¹*</td>
<td>hepatoma, liver, MPS organs</td>
<td>(133)</td>
</tr>
<tr>
<td>Mannose-modified trimethyl chitosan-cysteine, TPP</td>
<td>siRNA</td>
<td>TNF</td>
<td>0.05 mg·kg⁻¹</td>
<td>7.7 mg·kg⁻¹*</td>
<td>MPS organs</td>
<td>(136)</td>
</tr>
<tr>
<td>oleyl-PEG-mannose, oleyl-modified trimethyl chitosan, oleyl-PEG-cysteamine, PVBLG-8, TPP</td>
<td>siRNA</td>
<td>TNF</td>
<td>0.2 mg·kg⁻¹</td>
<td>66.5 mg·kg⁻¹*</td>
<td>MPS organs</td>
<td>(132)</td>
</tr>
<tr>
<td>β1,3-D-glucan-encapsulated PEI with Endoporter</td>
<td>siRNA</td>
<td>Map4k4</td>
<td>0.02 mg·kg⁻¹</td>
<td>4·10⁹ GeRPs·kg⁻¹</td>
<td>MPS organs</td>
<td>(166, 169)</td>
</tr>
<tr>
<td>NiMOS (gelatin polyplex in PCL matrix)</td>
<td>siRNA</td>
<td>TNF; CyD1</td>
<td>N.A.; 1.2 mg·kg⁻¹</td>
<td>5·10¹⁰ c.f.u.</td>
<td>small intestine, colon</td>
<td>(167, 168)</td>
</tr>
<tr>
<td>Living E. Coli expressing invasin</td>
<td>shRNA</td>
<td>β-catenin</td>
<td>–</td>
<td>–</td>
<td>IEC</td>
<td>(55)</td>
</tr>
<tr>
<td>None (chemically stabilized ON)</td>
<td>AON</td>
<td>ICAM-1; Smad7</td>
<td>40-160 mg</td>
<td>–</td>
<td>IEC, macrophages</td>
<td>(148, 152)</td>
</tr>
<tr>
<td>Sodium caprate (permeation enhancer)</td>
<td>AON</td>
<td>ApoB; TNF</td>
<td>100 mg</td>
<td>660 mg</td>
<td>N.A.</td>
<td>(139)</td>
</tr>
<tr>
<td>Activatable CPP-PNA conjugate</td>
<td>AON</td>
<td>luciferase</td>
<td>8-20 µM *in vitro</td>
<td>–</td>
<td>proliferating HT-29 cells</td>
<td>(108)</td>
</tr>
<tr>
<td>Amphiphilic docosanoyl-ON conjugate</td>
<td>AON, siRNA</td>
<td>Bcl-2</td>
<td>1-5 µM *in vitro</td>
<td>–</td>
<td>differentiated Caco-2 cells</td>
<td>(170)</td>
</tr>
</tbody>
</table>

* calculated based on components' weight ratios during preparation
‡ not available
Because of the previously discussed advantages of single-molecule-based conjugates over particle-based delivery systems, our group has recently developed an activatable ON conjugate targeting colonic epithelium (108). The conjugate combined neutrally charged PNA with cationic CPP in a single molecule, where the positive charges of CPP were masked by PEG chains via enzyme-labile linker (108). The PEGylated conjugate was designed to be inactive in the upper GI tract and to release the active CPP-PNA only upon arrival to the colon, where bacterial azoreductase cleaves the azobenzene-PEG chains. Despite being a good proof-of-principle example, this system relies on an expensive and protease-labile CPP, which must be additionally stabilized for in vivo applications by incorporating D-amino acids. Moreover, differing activity of colonic bacteria-derived azoreductase between individuals (171) could potentially lead to higher inter-patient variability of the concentration of released active de-PEGylated CPP-PNA conjugate. Additionally, some diseases of the GI tract such as Crohn’s disease are localized not only in the colon but also in the small intestine, where the developed strategy would be inefficient due to the absence of azoreductase-secreting bacterial species (172).

1.5 Scope of this thesis

Intestinal delivery of nucleic acid drugs is a promising therapeutic strategy against a wide range of systemic and intestinal diseases (115, 145–149). However, the complex intestinal environment imposes numerous challenges for the efficient delivery of nucleic acids. Chapter 2 of this thesis discusses in detail the challenges associated with oral delivery of biomacromolecules, such as nucleic acids, and reviews clinically tested formulations aimed at overcoming these hurdles. In this thesis, we seek to identify a simple, potent, and robust nucleic acid delivery platform based on amphiphilic ON conjugates for the target mRNA and protein silencing in intestinal epithelial cells (Scheme 1.6). Such ON conjugates, diffusing freely through the mucus layer to the epithelial cells, could be delivered to the desired location within the GI tract via enteric formulation, endoscopic tube, or enema.

Previously, it was shown by our lab that the conjugate between a single-stranded AON and the docosanoyl (DSA) group is capable of inducing the target mRNA silencing in proliferating prostate cancer cells (112). DSA was significantly more potent than cholesteryl- or docosahexaenyl-group in causing target silencing (112). In Chapter 3, we explore the versatility of DSA moiety as a delivery vehicle for two of the most commonly used types of ONs – i.e. single-stranded AONs
and double-stranded siRNAs. We present the synthesis and screening of a series of amphiphilic nucleic acid conjugates for the silencing of a model Bcl-2 mRNA. The silencing kinetics, cytotoxicity, and contribution of sedimentation to the uptake of DSA-ON conjugates are shown. In Chapter 4, we evaluate the developed single-molecule-based ON delivery system in conditions mimicking the intestinal environment (in the presence of pancreatin or fat-containing media) and using differentiated intestinal cell monolayers for transfection. Lastly, in Chapter 5 we present the main findings of the thesis and discuss the directions for the future development of amphiphilic nucleic acid conjugates as an intestinal delivery platform.

Scheme 1.6. Amphiphilic ON delivery strategy to the intestinal epithelium. Upon arrival to the desired location within the GI tract via enteric formulation, enema, or endoscopic tube, the amphiphilic ON conjugate will be taken up by the epithelial cells leading to the target gene silencing.
2 Oral delivery of biomacromolecules*

2.1 Introduction

The first attempt to deliver insulin orally in humans was undertaken as early as 1922, only one year after the discovery of insulin by Drs. Frederick Banting and Charles Best (173), when increasing doses of insulin were given orally to a single diabetes patient. The results were negative, and already in this first study, the critical challenges of oral protein delivery became apparent: poor and variable absorption, and low efficacy compared with subcutaneous injection. Although the interest and efforts in the oral delivery of biomacromolecules have intensified over the past two decades, safely and effectively delivering high-molecular-weight substrates via the oral route remains highly challenging for formulation scientists (174, 175).

The gastrointestinal (GI) tract is a hostile environment for biomacromolecules because it is evolutionarily optimized to break down nutrients and deactivate pathogens. The highly acidic pH in the stomach results in the protonation of proteins and their unfolding, which exposes more motifs that are recognized by protein-degrading enzymes (176). The enzymes in the stomach (pepsin) and small intestine (e.g., chymotrypsin, amino- and carboxypeptidases, RNases and DNases) cleave proteins and nucleic acids into smaller fragments and single units (176). In the colon, enzymatic fermentation processes further degrade biomacromolecules (176). Because therapeutically active biomacromolecules are equally affected by these processes, the fraction surviving these degradation processes is generally low and variable, especially in the presence of food (177). The macromolecular drug needs to overcome multiple barriers designed to prevent the entry of dietary and bacterial antigens in order to reach the systemic compartment. To access the epithelial cell layer, the biomacromolecule firstly needs to diffuse through the mucus layer covering the intestinal epithelium (177). The latter is another important barrier, as the tight junctions which

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seal the epithelial cells restrict the paracellular transport (i.e., the passage between cells) to small molecules and ions (<600 Da) (178). The transcytotic pathway (i.e., the passage across the cell in an endocytotic vesicle) is mediated by luminally expressed endocytotic receptors (e.g., vitamin B12 receptor, transferrin receptor), and therefore necessitates conjugation to the respective ligands in order to be exploited in drug delivery (179). Another access point to the systemic compartment is the phagocytotic M cells of Peyer’s patches which sample luminal antigens and can take up particular substrates in the low micrometer range (180). However, the proportion of M cells in the gut epithelium is small and varies greatly between species, which complicates predictions of absorption in humans based on animal data (144).

Not surprisingly, only six biomacromolecules have been approved by the Food and Drug Administration (FDA) for oral delivery: two locally and two systemically delivered peptides, one locally delivered non-peptidic macrocycle, and one locally delivered protein mixture. However, several orally applied formulations of proteins, peptides, and nucleic acids are currently under clinical evaluation. Often, these formulations contain at least one of the following excipients (Figure 2.1): an enteric coating and/or protease inhibitors to prevent drug degradation and permeation enhancers to enable paracellular transport of macromolecules (139, 181). Mechanistically, absorption enhancement can be achieved by mechanically disrupting tight junctions or the plasma membrane, lowering mucus viscosity, and modulating tight junction-regulating signaling pathways (174). Additional strategies for the oral delivery of biomacromolecules under clinical development include buccal delivery, utilizing carrier-mediated transcytosis, and local delivery to GI targets.

The overwhelming majority of currently approved oral drugs and clinical candidates exhibit a molecular weight of <1000 Da (182). Above this threshold, low bioavailability, inter- and intraindividual variability, food effects, and long-term safety concerns of bioavailability-enhancing excipients remain important challenges of oral delivery despite clear advances in knowledge after nearly 90 years of trial and error. In this review, we address orally applied biomacromolecular therapeutics (>1000 Da) already marketed or under clinical investigation for local or systemic delivery with an emphasis on the drug formulations and the biopharmaceutical aspects. The oral delivery of vaccines will not be covered in this manuscript, and the readers are referred to other recent reviews for more information on this topic (183–186).
Figure 2.1. Strategies for oral delivery of macromolecular drugs that act on local or systemic targets. In buccal or sublingual delivery, the drug targets the buccal or sublingual mucosa, which avoids degradation pathways in the GI tract. Often, permeation enhancement is necessary to cross the multi-layered buccal epithelium. Mucoadhesive bacteria that secrete the desired protein in situ are a novel means of achieving sustained release in the oral cavity. To enhance stability against degrading enzymes, macromolecular drugs can be chemically modified by polymer conjugation (proteins), backbone and base modifications (nucleic acids), and cyclization as well as by introducing D-amino acids (peptides). Sacrificial proteins, protease inhibitors, and enteric coatings can also be included in the formulation to further improve GI resistance. To achieve meaningful systemic exposure, absorption enhancement by tight junction-disrupting excipients is often needed. A novel approach is the in situ production and secretion of therapeutic biomacromolecules by genetically modified bacteria.

2.2 Local delivery

Macromolecular drugs that act on GI targets increasingly move into focus because local delivery avoids the challenges of reaching the systemic compartments. Advantages of locally delivering high-molecular-weight drugs include fewer restrictions regarding drug size and a potentially more favorable safety profile due to minimal systemic exposure, reduced immunogenicity, and the absence of permeation-enhancing excipients (187, 188). To conserve the therapeutic activity in the GI tract, several formulation strategies have been employed, such as enteric/colon-targeting capsules that protect against the harsh GI environment, supplementation with sacrificial proteins that compete for degradation, and hindering enzymatic access using polymer conjugation or protective antibodies which bind to known cleavage epitopes (176, 187, 189). The ailments that are targeted by locally acting macromolecules include inflammatory diseases.
CHAPTER 2: ORAL DELIVERY OF BIOMACROMOLECULES

(Crohn’s disease, ulcerative colitis), metabolic disorders (e.g., exocrine pancreas insufficiency), constipation, and infections (Table 2.1) (176, 187).

Table 2.1. Macromolecular treatments for local GI therapy in clinical development (as of January 2016, source: Thomson Reuters Integrity®, ClinicalTrials.gov, and company press releases).

<table>
<thead>
<tr>
<th>Type</th>
<th>Generic name, Mw†</th>
<th>Modification/Formulation</th>
<th>Indication</th>
<th>Clinical phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>Vancomycin (Vancocin®), 1449 Da</td>
<td>Cyclic structure, modified amino acid residues; gelatin capsule</td>
<td>Clostridium difficile-associated diarrhea (CDAD) and staphylococcal enterocolitis</td>
<td>Marketed</td>
</tr>
<tr>
<td></td>
<td>Fidaxomycin (Dificid®), 1058 Da</td>
<td>Non-peptidic macrocycle; tablet</td>
<td>CDAD</td>
<td>Marketed</td>
</tr>
<tr>
<td></td>
<td>Surotomycin, 1681 Da</td>
<td>Cyclic structure</td>
<td>CDAD</td>
<td>Phase III (NCT01597505, NCT01598311)</td>
</tr>
<tr>
<td></td>
<td>Ramoplanin, 2554 Da</td>
<td>Cyclic structure</td>
<td>CDAD</td>
<td>Phase IIb (N.A.†)</td>
</tr>
<tr>
<td></td>
<td>LFF-571, 1367 Da</td>
<td>Cyclic structure, thiazolyl peptide</td>
<td>CDAD</td>
<td>Phase II (NCT01232595)</td>
</tr>
<tr>
<td></td>
<td>NVB302, 2115 Da</td>
<td>Cyclic structure, thioether bonds</td>
<td>CDAD</td>
<td>Phase I (N.A.)</td>
</tr>
<tr>
<td></td>
<td>Linaclotide (Linzess®), 1525 Da</td>
<td>Cyclic structure; gelatin capsule</td>
<td>Chronic idiopathic constipation (CIC) and irritable bowel syndrome with constipation (IBS-C)</td>
<td>Marketed</td>
</tr>
<tr>
<td></td>
<td>Plecanatide, 1682 Da</td>
<td>Cyclic structure</td>
<td>CIC and IBS-C</td>
<td>CIC phase III completed in 2015 (NCT01919697) IBS-C phase III (NCT02493452)</td>
</tr>
<tr>
<td></td>
<td>Dolcanatide (SP-333), 1682 Da</td>
<td>Cyclic structure, two D-amino acid substitutions</td>
<td>Opioid-induced constipation (OIC) and ulcerative colitis (UC)</td>
<td>OIC phase II completed in 2014 (NCT01983306) UC phase Ib (N.A.)</td>
</tr>
<tr>
<td>Antibody</td>
<td>AVX-470 (Avaximab™, TNF), broad</td>
<td>Polyclonal antibody; enteric capsule</td>
<td>Pediatric UC</td>
<td>Phase II ongoing (N.A.)</td>
</tr>
<tr>
<td></td>
<td>AG014, 47.8 kDa</td>
<td>Lyophilized Lactococcus lactis secreting certolizumab; enteric capsule</td>
<td>Inflammatory bowel disease</td>
<td>Phase Ia completed in 2014 (N.A.)</td>
</tr>
</tbody>
</table>

† Mw: molecular weight
‡ N.A: unknown or non-existent NCT number
Table 2.1. (Continued) Macromolecular treatments for local GI therapy in clinical development (as of January 2016, source: Thomson Reuters Integrity®, ClinicalTrials.gov, and company press releases).

<table>
<thead>
<tr>
<th>Type</th>
<th>Generic name, Mw§</th>
<th>Modification/Formulation</th>
<th>Indication</th>
<th>Clinical phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>Creon® (porcine pancreatin)</td>
<td>Enteric capsule</td>
<td>Exocrine pancreatic insufficiency</td>
<td>Marketed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral rinsing solution containing 2.0 x 10^{11} CFU/15 mL of Lactococcus lactis secreting human trefoil factor 1</td>
<td>Chemotherapy- or radiation-induced oral mucositis</td>
<td>Phase Ib completed in 2012 (NCT00938080)</td>
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<tr>
<td>Protein-secreting bacteria</td>
<td>AG013, 6.5 kDa</td>
<td>Lactococcus lactis secreting anti-inflammatory cytokine IL-10; enteric capsule with enema</td>
<td>UC</td>
<td>Phase IIa completed in 2009, failed (NCT00729872)</td>
</tr>
<tr>
<td></td>
<td>Mongersen (GED-0301), 6952 Da</td>
<td>Phosphorothioate AON; enteric coating</td>
<td>Moderate to severe Crohn's disease</td>
<td>Phase II completed Phase I (NCT02367183)</td>
</tr>
<tr>
<td></td>
<td>Alicaforsen, 6368 Da</td>
<td>Phosphorothioate AON; nightly enema with hydroxypropyl methylcellulose</td>
<td>UC, pouchitis</td>
<td>Phase III (NCT02525523)</td>
</tr>
<tr>
<td></td>
<td>CEQ508</td>
<td>Suspension of genetically modified E. Coli expressing invasin, listeriolysin, and short hairpin RNA targeting β-catenin</td>
<td>Familial adenomatous polyposis</td>
<td>Phase I/II ongoing since 2011 (N.A.)</td>
</tr>
</tbody>
</table>

2.2.1 Peptides

Vancomycin

Isolated in 1953 from a soil sample in the jungle of the island Borneo, the antibiotic vancomycin is produced by the bacterium *Amycolatopsis orientalis* (190). Vancomycin is a glycosylated tricyclic heptapeptide (1449 Da) which contains modified amino acid residues (*e.g.*, chlorinated tyrosine) (190–192). Due to its highly hydrophilic (log P -3.1) and large cyclic structure, vancomycin is only marginally absorbed and metabolized in the GI tract (193, 194). Intravenously applied

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§ Mw: molecular weight
vancomycin was initially approved by the FDA to treat penicillin-resistant bacterial infections in 1958, and it is still indicated for severe infections caused by susceptible strains of methicillin-resistant staphylococci, *Clostridium difficile*-associated diarrhea, and staphylococcal enterocolitis and for patients who are allergic to beta-lactam antibiotics. Vancomycin is formulated as gelatin capsules containing 125 or 250 mg of active compound (package leaflet). In 2011, a non-peptidic macrocyclic macrolide antibiotic fidaxomicin (1058 Da, Dificid®, Cubist Pharmaceuticals) with minimal systemic absorption and GI metabolism was approved for the treatment of *C. difficile* infections. Fidaxomicin showed lower relapse rates compared with vancomycin. In view of its much higher costs, fidaxomicin treatment may be most suitable for patients who are at highest risk of relapse (195). Further cyclic peptidic compounds in clinical trials for the local treatment of *C. difficile* infections include the lipopeptide surotomycin (1681 Da, phase III, Cubist Pharmaceuticals/Merck), the lipoglycodepsipeptide ramoplanin (2554 Da, NTI-851, phase IIb, Nanotherapeutics), the thiazolyl peptide LFF-571 (1367 Da, phase II but currently removed from pipeline, Novartis), and the lantibiotic NVB302 (2115 Da, phase I, Novacta Biosystems Limited) (196, 197).

**Linaclotide**

Linaclotide (1525 Da) is a truncated derivative of *Escherichia coli* heat-stable (ST) enterotoxin that consists of 14 amino acid residues and three disulfide bonds (198). It acts as a guanylyl cyclase C (GC C) agonist locally in the small intestine (Figure 2.2) (198–200). In 2012, it received FDA approval (Linzess®, Forest Labs LLC) for the treatment of chronic idiopathic constipation and irritable bowel syndrome with constipation. After oral administration, linaclotide is minimally absorbed, and its plasma concentrations are below the limit of quantification (201). Because linaclotide is stable in the stomach, it is formulated as hard gelatin capsules containing 0.145 or 0.290 mg of the active compound (package leaflet). However, it is cleaved in the small intestine to a 13-amino acid active metabolite (201), which is eventually degraded in the intestine (202).
Figure 2.2. Schematic representation of the mechanisms of action, primary structure, and diarrhea incidence of the GC C agonists linaclotide, plecanatide, and dolcanatide. The sequences of these peptides are based on the physiological agonist uroguanylin and the structurally similar heat-stable *Escherichia coli* enterotoxin (ST) but include sequence adaptations such as unnatural D-amino acids (dN = D-asparagine, dL = D-leucine). Lines above and below the peptide sequences denote the disulfide bonds. Upon binding of an activating ligand, GC C converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), increasing the intracellular cGMP concentration and activating the cystic fibrosis trans-membrane conductance regulator (CFTR). The resultant efflux of chloride ions out of the epithelial cell leads to a water influx into the intestinal lumen, which normalizes bowel movements. Extracellular cGMP further inhibits intestinal nociceptors. Thus, GC C agonists improve constipation and alleviate chronic abdominal pain in patients suffering from inflammatory bowel syndrome with constipation. The incidence of diarrhea, a common treatment-emergent adverse event associated with excessive treatment response, is also indicated for the three peptides (dose and regimen in parentheses). The information was collected from (199–204) and the website of Synergy Pharmaceuticals.

**Plecanatide and derivatives**

The GC C agonist plecanatide (1682 Da, Synergy Pharmaceuticals) is a synthetic analog of uroguanylin, a naturally occurring GI regulator of GC C signaling, with one amino acid substitution that results in stronger receptor binding (203). It is a bicyclic peptide consisting of 16 amino acid residues with two disulfide bonds (203). Plecanatide completed the clinical phase III for the treatment of chronic idiopathic constipation, and it is currently being evaluated in phase III for irritable bowel syndrome with constipation. It showed a statistically significant increase in the number of complete spontaneous bowel movements per week compared with placebo (approximately 20% of durable responders at a dose of 3 mg per day *vs.* 12% in the placebo group).
In comparison to linaclotide, plecanatide showed reduced diarrhea incidence (10% vs. 20%) (204). In addition to plecanatide, Synergy Pharmaceuticals is developing a more stable analog, dolcanatide (SP-333), to treat opioid-induced constipation. Dolcanatide has two D-amino acid substitutions at C- and N-terminus in order to improve stability in simulated gastric and intestinal fluids (www.synergypharma.com). Dolcanatide has completed a randomized, double-blind, placebo-controlled phase II clinical trial in patients with constipation who take opioid analgesics for chronic pain (NCT01983306). Despite the increased stability in the intestinal environment, the dose of dolcanatide needed to achieve a therapeutic effect was comparable with that for plecanatide. Furthermore, the dolcanatide-mediated activation of GC C ameliorated inflammation in a colitis mouse model, promoting its development in the treatment of ulcerative colitis (205).

2.2.2 Antibodies

Antibody delivery

The vast majority of clinically tested antibodies with GI luminal targets inactivate undesired molecules, such as bacterial toxins, cytokines, viruses, and virulence factors (187). In the treatment of IBDs, capturing TNF in the intestinal lumen is a promising alternative to systemic treatment with anti-TNF antibodies (e.g., adalimumab, infliximab and certolizumab pegol) with regard to systemic immunosuppression, development of neutralizing antibodies, and needle-free administration (206–208). AVX-470 (Avaximab™-TNF, Avaxia Biologics) is an orally administered polyclonal antibody which targets luminal TNF in the intestine (209). It is produced by purifying total antibodies from the milk of dairy cows that are immunized with human TNF, which results in approximately 0.3-0.9% (m/m) TNF-specific and mainly IgG1 subtype antibodies (www.avaxiabiologics.com). Providing early immunity to neonates, high amounts of colostrum antibodies seem to be partially resistant to GI digestion in adults with fully developed digestive systems (210–212). AVX-470 is developed as an enteric-coated capsule for treating pediatric ulcerative colitis and is currently in phase II of clinical trials. According to the company’s website, a phase Ib trial (NCT01759056) in 36 ulcerative colitis patients showed that the treatment resulted in consistent encouraging trends across multiple disease parameters (colonic TNF levels, endoscopic index of severity, serum C-reactive protein levels) after four weeks, and no allergic reaction or human anti-bovine antibodies in serum were observed. Bovine immunoglobulin was
detected in stools and displayed TNF-binding activity, suggesting that the large excess of decoy antibodies in the formulation (>99%) protected a fraction of the anti-TNF antibodies.

**Genetically modified antibody-secreting bacteria**

Intrexon’s (formerly ActoGeniX) approach for luminal antibody therapy consists of delivering attenuated bacteria to the GI tract, where the antibody is secreted *in situ*. Food-grade *Lactococcus lactis* was genetically modified to secrete certolizumab, an anti-TNF Fab fragment (47.8 kDa). In an open-label clinical trial in healthy volunteers, lyophilized bacteria administered in enteric capsules were recovered in both the small and large intestine by endoscopic sampling, and the antibody was secreted by living bacteria in the colon lumen (www.dna.com). The treatment was safe and well-tolerated. In addition, preclinical studies demonstrated the treatment’s efficacy in multiple colitis models and showed that *Lactococcus lactis* adhere especially to the inflamed mucosa, which increases the concentration of the antibody in the close proximity of target cells (213). However, questions remain regarding the concentration variability and the intestinal stability of the secreted antibody.

### 2.2.3 Oral enzyme therapy

Enzyme replacement therapy *via* the oral route is an important therapeutic option in metabolic disorders in which a certain enzymatic action in the GI tract is absent. Numerous oral enzyme products are on the market for a variety of disorders such as exocrine pancreatic insufficiency due to pancreatitis, cystic fibrosis and other conditions, and lactose, fructose, sucrose and histamine intolerance (*e.g.*, Creon®, Lacteeze®, Sucraid®, DAOSiN®) (176). Whereas the only FDA-approved enzymatic drug is pancreatin (a mixture of bovine or porcine pancreatic amylase, proteases, and lipases), the other oral enzyme products are generally under the legal framework of dietary supplements, which usually do not require thorough efficacy studies.

Because these therapeutic enzymes are inactivated in the stomach by low pH and pepsin, one or a combination of stabilization strategies are generally needed. Creon® (Abbvie), for instance, consists of pancreatin formulated in enteric-coated capsules, whose dissolution is triggered by the pH increase in the small intestine. An uncoated formulation of pancreatin (Viokace®, Aptalis
Pharma) which is used to treat exocrine pancreatic insufficiency is co-administered with proton pump inhibitors to decrease gastric degradation. As an exception, sacrosidase and the acid lactase (tilactase) produced by *Aspergillus oryzae* are intrinsically stable and active in acidic conditions (176), and they do not require sophisticated delivery systems. However, average residence time of lactase in the stomach might be insufficient for complete digestion of high amounts of lactose (214). Therefore, a formulation of a lactase, which is active in neutral to basic pH of the small intestine, was recently developed as a capsule containing enteric-coated pellets (Lactosolv®, Scietec Diagnostic Technologies) (214). The same approach has been successfully applied for other enzyme deficiencies (Xylosolv® for fructose and DAOSiN® for histamine intolerance). As an alternative, the administration of probiotics that secrete β-galactosidase, which is capable of lactose hydrolysis, has been proposed; however, different clinical trials in patients with lactose intolerance gave inconsistent efficacy results (215). Oral formulations of polymer-conjugated enzymes and enzymes in enteric-coated capsules are under preclinical investigation for other pathologies such as celiac disease and phenylketonuria (176, 216–220).

### 2.2.4 Cytoprotective/anti-inflammatory proteins

As a consequence of chemotherapy and radiation, cancer patients often develop a complication called oral mucositis in which the mouth’s mucosal lining is destroyed and ulcers form. AG013 (Intrexon Corporation) is a bacteria-based system which delivers a mucosa-healing protein for the treatment of oral mucositis. The protein, called human trefoil factor 1, is involved in mucosal healing and tissue protection via multiple mechanisms, including inhibiting apoptosis during cell migration and stabilizing the protective mucus layer (221). The formulation consists of an oral rinsing suspension containing attenuated (*i.e.*, unable to replicate due to thymidylate synthase deficiency (221)), genetically engineered *Lactococcus lactis* which adhere to the buccal mucosa and secrete the mucosa-healing factor locally for up to 24 h after administration (222). A phase Ib study in head and neck cancer patients undergoing chemotherapy produced positive results, showing a 35% reduction in the duration of oral ulcers (221).

A similar *in situ* system based on *Lactococcus lactis* which secretes the anti-inflammatory cytokine interleukin-10 (AG011, Intrexon) was proposed for ulcerative colitis. However, a combination of enema and enteric capsules containing interleukin-10-secreting *Lactococcus lactis* did not result in mucosal healing in a phase IIa, double-blind, placebo-controlled study with 60
ulcerative colitis patients (223). Recently, another genetically modified strain of \textit{Lactococcus lactis} was engineered to deliver the endogenous serine protease inhibitor elafin. The expression of this anti-inflammatory peptide is lowered in patients with active celiac disease, and elafin treatment was effective in decreasing inflammation and intestinal permeability in a mouse model of celiac disease (224).

### 2.2.5 Nucleic acids

AONs are short, single-stranded, synthetic sequences of nucleotides which can hybridize with complementary mRNA and consequently decrease the expression of the encoded protein (103). Several GI tract pathologies hold attractive targets for local nucleic acid therapy (e.g., IBD or familial adenomatous polyposis) (149, 225). Because nucleic acids are readily degraded by the RNases and DNases in the GI tract and depurinated in the acidic stomach environment, they require stability-enhancing chemical modifications of the backbone or bases (151). Various modifications of the ribose ring (e.g., 2′-O-(2-methoxyethyl) or 2′-F-ANA) not only exhibit enhanced nuclease resistance but also show higher affinity to complementary mRNAs and thus improved knockdown efficacy (25, 226).

Mongersen (GED-0301, Celgene) is a 21-mer phosphorothioate AON targeting the mRNA of the Smad7 protein, which is overexpressed in IBD mucosal tissues and leads to suppression of anti-inflammatory TGF-β signaling (152). Mongersen acts on epithelial and lamina propria cells, and its bioavailability after oral administration is minimal (227). An enteric-coated oral formulation of mongersen was evaluated in 166 patients with active Crohn’s disease in a phase II trial (152). Participants received 10, 40, or 160 mg of mongersen once daily for 2 weeks. In the mongersen groups that received 40 and 160 mg, significantly more patients reached clinical remission, the primary end point, after four weeks of treatment (55% and 65%, respectively), compared with 10% in the placebo group. According to the company, follow-up data of this trial (e.g., on mucosal healing by endoscopy) will be presented in the future.

Alicaforsen (Atlantic Healthcare) is a 20-mer phosphorothioate AON targeting the mRNA of intercellular adhesion molecule 1 (ICAM-1), which recruits immune cells to the site of inflammation and is overexpressed in the gut epithelium of IBD patients (148). Although a four-week treatment with injectable alicaforsen fell short of inducing clinical remission in two phase II
trials (NCT00048113; NCT00048295), a six-week regimen of nightly 60 mL alicaforsen enemas containing hydroxypropyl methylcellulose as a thickening agent was beneficial in treating moderate to severe ulcerative colitis and pouchitis (i.e., an ileal pouch inflammation in colectomy patients) (148, 150). According to the company’s website (www.atlantichc.com), alicaforsen enema is undergoing phase III clinical trials for the treatment of moderate to severe ulcerative colitis and pouchitis.

Because the cellular absorption of naked ONs is limited, Marina Biotech is developing a novel strategy to enhance ON cell uptake using a transkingdom RNA interference platform (tkRNAi) based on genetically modified Escherichia coli. The most advanced tkRNAi candidate, CEQ508, is designed to express and deliver short hairpin RNA silencing β-catenin, which is overexpressed in gut epithelial cells of familial adenomatous polyposis patients and is responsible for uncontrolled cell growth (147). The genome of CEQ508 has been altered to allow for whole bacterium endocytosis by the epithelial cells and, ultimately, β-catenin silencing (56). In 2010, CEQ508 received orphan drug status from the FDA, and a phase Ib clinical study was initiated; however, due to financial problems, the development program was halted. Recently, the FDA granted Fast Track designation to CEQ508, and clinical trials are expected to begin again (www.marinabio.com). Other approaches for nucleic acid delivery to the mucosa are under investigation, including nano/microparticles that target mucosal macrophages and colon-activatable PNAs (108, 225).

2.3 Systemic delivery

Delivering macromolecules systemically after oral intake is highly challenging because, in addition to the stability issues due to GI degradation, the limited permeability of the GI mucosa generally leads to low and erratic absorption. Therefore, inter- and intraindividual differences in pharmacokinetic parameters are generally high, especially in the presence of food. This chapter provides an overview of oral macromolecular drugs that reached clinical trials or received market approval (Table 2.2).
Table 2.2. Macromolecular treatments for oral administration in clinical development (as of January 2016, source: Thomson Reuters Integrity®, ClinicalTrials.gov, and company press releases).

<table>
<thead>
<tr>
<th>Type</th>
<th>Generic name, Mw(^*)</th>
<th>Formulation</th>
<th>Indication</th>
<th>Clinical phase</th>
</tr>
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<tbody>
<tr>
<td>Peptide</td>
<td>Octeotide (Mycapssa™), 1019 Da</td>
<td>Oily suspension containing sodium caprate</td>
<td>Acromegaly</td>
<td>New Drug Application (NDA) under review</td>
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<td>Desmopressin (DDAVP®), 1128 Da</td>
<td>Tablet, lyophilisate</td>
<td>Central diabetes insipidus, nocturnal enuresis</td>
<td>Approved</td>
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<td>Cyclosporine (Neoral®), 1202 Da</td>
<td>Self-emulsifying system</td>
<td>Organ transplant rejection, autoimmune diseases</td>
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<tr>
<td></td>
<td>Acyline (MER-104), 1533 Da</td>
<td>GIPET tablet (sodium caprate)</td>
<td>Prostate cancer, male oral contraception</td>
<td>Phase I/II completed in 2008 (NCT00603187)</td>
</tr>
<tr>
<td></td>
<td>Calcitonin (TBRIA™), 3432 Da</td>
<td>Citric acid-containing enteric coated tablet</td>
<td>Postmenopausal osteoporosis</td>
<td>NDA under review</td>
</tr>
<tr>
<td></td>
<td>Semaglutide (OG217SC), 4114 Da</td>
<td>Eligen®-based tablet (SNAC)</td>
<td>Diabetes Type II (T2D)</td>
<td>Phase III (N.A.(^††))</td>
</tr>
<tr>
<td>Protein</td>
<td>Human insulin (Capsulin™), 5800 Da</td>
<td>Enteric coated tablet with hydrophilic aromatic alcohol</td>
<td>Diabetes Types I (T1D) and T2D</td>
<td>Phase IIb (N.A.)</td>
</tr>
<tr>
<td></td>
<td>Human insulin (ORMD-0801)</td>
<td>Enteric coated capsule containing several protease inhibitors, permeation enhancers, lipoidal carriers</td>
<td>T1D, T2D</td>
<td>Phase II (NCT02496000)</td>
</tr>
<tr>
<td></td>
<td>Long-acting insulin analogue (OI338GT)</td>
<td>GIPET tablet</td>
<td>T1D, T2D</td>
<td>Phase I/II (NCT02470039)</td>
</tr>
<tr>
<td></td>
<td>Insulin-containing hepatocyte-directed vesicles</td>
<td>Liposomes decorated with biotin</td>
<td>T1D, T2D</td>
<td>Phase III (NCT00814294)</td>
</tr>
<tr>
<td></td>
<td>Insulin-triethylene glycol conjugate (IN-105)</td>
<td>Tablet</td>
<td>T1D, T2D</td>
<td>Phase III (N.A.)</td>
</tr>
</tbody>
</table>

\(^*\) Mw: molecular weight

\(^††\) N.A: unknown or non-existent NCT number
Table 2.2. (Continued) Macromolecular treatments for oral administration in clinical development (as of January 2016, source: Thomson Reuters Integrity®, ClinicalTrials.gov, and company press releases).

<table>
<thead>
<tr>
<th>Type</th>
<th>Generic name, Mw‡‡</th>
<th>Formulation</th>
<th>Indication</th>
<th>Clinical phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin (Oral-lyn™)</td>
<td>Spray for buccal delivery</td>
<td>T1D, T2D</td>
<td>Updating IND</td>
<td></td>
</tr>
<tr>
<td>Monarsen (EN 101, BL-7040), 6139 Da</td>
<td>OMe modifications at the three terminal bases at 3’ end; aqueous solution</td>
<td>Myasthenia gravis (MG), ulcerative colitis (UC)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>ISIS 104838 sodium salt, 7701 Da</td>
<td>MOE modifications at 5 terminal bases of both ends; enteric-coated capsule containing sodium caprate</td>
<td>Rheumatoid arthritis, Crohn's disease</td>
<td>Discontinued</td>
</tr>
<tr>
<td>Mipomersen sodium (ISIS 301012), 7595 Da</td>
<td>MOE modifications at 5 terminal bases of both ends; enteric-coated capsule containing sodium caprate</td>
<td>Homozygous familial hypercholesterolemia</td>
<td>Discontinued</td>
<td></td>
</tr>
</tbody>
</table>

2.3.1 Peptides

Octreotide

Octreotide is a synthetic analogue of the endogenous hormone somatostatin, a regulator of the neuroendocrine system (228). It was approved by the FDA in 1998 as a subcutaneous or intramuscular treatment for acromegaly (usually a result of growth hormone-secreting tumors), carcinoid tumors and vasoactive intestinal peptide-producing tumors. In comparison with its mother compound, the cyclic octapeptide possesses a lower molecular weight (1019 vs. 1638 Da), a higher logP (-1.69 vs. -8.50), and two D-amino acids. Additionally, the terminal carboxyl group is reduced to an alcohol (229). The higher stability of octreotide over somatostatin in pepsin-
containing simulated gastric fluid results in part from the inclusion of a D-amino acid in the pepsin-vulnerable amide bond of octreotide, rendering octreotide non-recognizable by this enzyme (228). Despite these structural improvements, orally administered aqueous octreotide showed an unfavorable pharmacokinetic profile (low bioavailability, highly variable plasma concentrations) in pigs and humans (230, 231). To improve the pharmacokinetics upon oral administration, octreotide (Mycapssa™, formerly Octreolin™, Chiasma) was formulated as an oily suspension with the permeation enhancer sodium caprylate, which transiently increases paracellular permeability for approximately 1.5 h (232). The absorption of octreotide in the oily suspension was dose-dependent and independent of the intestinal region (232). The passage through the stomach, however, led to low bioavailability such that in the subsequent clinical studies, the suspension was formulated in an enteric-coated capsule (232). As reported in a publication of four phase I and phase II studies, a 20-mg dose of Octreolin™ in fasting healthy volunteers led to a similar pharmacokinetic profile as a subcutaneous injection of 0.1 mg (apart from a lag phase of 20 min) (233). A high-fat meal decreased the bioavailability of oral octreotide by 90%, probably due to the premature dissolution of the enteric-coated capsule caused by increased stomach pH (233). Furthermore, the presence of food in the intestine may slow down diffusion to the intestinal mucosa, hindering octreotide absorption (233). A baseline-controlled phase III clinical trial was conducted in acromegaly patients who had previously received somatostatin receptor ligand injections and were switched to a twice-daily regimen of oral octreotide. The intervention met its primary end point in that it resulted in controlled levels of insulin-like growth factor-1 and growth hormone in 65% of the subjects after seven months of the treatment, compared with 89% at baseline (234). In 6.5% of the subjects, adverse GI events such as nausea, diarrhea and abdominal pain led to early termination of the study (234). Hoffmann-La Roche bought the rights for development and commercialization of Octreolin™ but terminated the development in 2014 and transitioned the drug back to Chiasma, which has filed a New Drug Application (NDA) in 2015.

Desmopressin

The cyclic nonapeptide desmopressin (1-desamino-8-D-arginine vasopressin, 1128 Da) is a synthetic derivative of the peptide hormone vasopressin (anti-diuretic hormone, ADH) (228, 235). In comparison to the endogenous vasopressin, desmopressin exhibits desirable features such as a significantly longer antidiuretic effect and strongly diminished vasopressor action (235). Desmopressin was approved by the FDA in 1978 for the treatment of post-hypophysectomy polyuria/polydipsia, central diabetes insipidus, and nocturnal enuresis. The oral formulation
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(DDAVP®, Ferring Pharmaceuticals) was approved in 1992 by the FDA for the latter two indications. A variety of formulations of desmopressin are currently marketed for parenteral, nasal, and oral applications. Desmopressin exhibits exceptionally high stability in human gastric fluid, presumably due to its rigid cyclic structure and the absence of pepsin cleavage motifs (228). In contrast, the stability of desmopressin in intestinal fluids is limited but enhanced compared with that of vasopressin (228). This finding may be due to the replacement of the L-arginine at position 8 with its D-enantiomer in desmopressin, which seems to hinder the recognition by trypsin (228).

Because of its hydrophilicity (logP -1.95), the permeability of desmopressin across a Caco-2 monolayer is low and concentration-dependent, which suggests passive permeation (236). The oral bioavailability of a standard-dose desmopressin acetate tablet (0.2 mg) is only approximately 0.1% (237). In 2005, a lyophilisate tablet for sublingual administration of desmopressin was approved (DDAVP® Melt, Ferring Pharmaceuticals). Despite its circumventing the harsh conditions of the GI tract, the bioavailability of this formulation is only approximately 0.25% (238). With regard to the broad therapeutic window of desmopressin, the antidiuretic action does not seem to be influenced by the low and variable oral bioavailability. Indeed, food intake did not change the efficacy (urine production and osmolarity) or safety (plasma sodium concentrations) of desmopressin tablets despite significantly altering the pharmacokinetic parameters (239). Furthermore, the incidence of water intoxication-induced hyponatremia, a serious adverse drug reaction related to desmopressin overdose, was lower in oral than in nasal application, suggesting a low risk of this adverse drug reaction due to erratic absorption (240).

Cyclosporine

Originally isolated in 1969 from the fungus Tolypocladium inflatum in a Norwegian soil sample, cyclosporine (Cyclosporine A) has become a widely used immune-suppressing peptide approved by the FDA for the prophylaxis of organ transplant rejection and the second-line treatment of the autoimmune diseases rheumatoid arthritis and psoriasis. Cyclosporine is a cyclic peptide composed of 11 amino acid residues (1202 Da). The exceptionally high stability in gastric and intestinal fluids is most probably related to cyclosporine’s high structural rigidity and low potential for H-bonding-mediated interaction with peptidases (228). The high hydrophobicity (logP 3.6) of cyclosporine enables its transcellular passage by passive diffusion, but it also makes it susceptible to p-glycoprotein-mediated expulsion on the apical side of enterocytes (241). Interestingly, structural motifs such as the β-turns of cyclosporine seem to be important features in cell permeability (242). The low solubility in aqueous solutions due to the high hydrophobicity led to
the development of a self-emulsifying formulation in soft gelatin capsules or as an oral solution, approved by the FDA in 1995 (Neoral®, Novartis) (243). Compared with the originally developed oil-in-water emulsion (SandImmun®, Novartis) this formulation is characterized by lower intra- and interindividual variability, a more linear dose-exposure relationship, and a markedly decreased influence of food fat content (243–245). Nonetheless, the narrow therapeutic window generally necessitates monitoring cyclosporine plasma concentrations to determine the optimal dose.

Acyline

The linear decapeptide acyline (MER 104, Merrion Pharmaceuticals, 1533 Da) competitively inhibits the receptor binding of gonadotropin-releasing hormone, thereby inhibiting the secretion of luteinizing and follicle-stimulating hormone and interfering with the production of downstream sex hormones (e.g., testosterone, estrogen). It is a candidate for treating hormone-dependent conditions (breast and prostate cancer, endometriosis) and male oral contraception. Acyline contains a variety of modifications (e.g., five D-amino acids, two acylated 4-aminophenylalanines) enhancing its stability and receptor-binding properties (246). It is formulated with the Gastrointestinal Permeation Enhancement Technology (GIPET, Merrion) as an oral dosage form. GIPET is composed of an enteric-coated matrix tablet that contains the permeation enhancer sodium caprate (181, 247). In a phase I clinical trial, acyline tablets (10, 20, and 40 mg) were administered in eight healthy men after overnight fasting and under continued fasting for 4 h (248). High variability was observed among the subjects, and no dose effect on pharmacokinetic parameters (maximum concentration and time of maximum concentration, half-life, and area under the curve, AUC) was observed (248). After 12 h, all three doses suppressed luteinizing and follicle-stimulating hormone serum concentrations by approximately 70% and 30%, respectively (248). Suppression of the downstream hormone testosterone was also observed, and the effects of the lowest and highest doses could be discriminated (248). One study participant reported a GI-related adverse event (nausea and vomiting) (248). While acyline is still listed as being under active development by the company’s website, no trials have been published since 2009.

Calcitonin

Calcitonin is a natural peptide hormone regulator of calcium homeostasis (249). It was approved by the FDA in 1978 and is indicated for the treatment of hypercalcemia and symptomatic Paget’s disease and as a second-line treatment of postmenopausal osteoporosis. Marketed calcitonin products are administered via the parenteral and nasal routes and contain salmon
calcitonin as the active ingredient because this peptide exhibits forty times higher potency compared with the human version (249). Salmon calcitonin is a 32 amino acid peptide (3432 Da) with a highly negative logP (-28.49) (228). Due to its size and peptidase-recognizable motifs, it is rapidly degraded in gastric and small intestinal fluids in vitro, and it only marginally diffuses across Caco-2 monolayers (228, 250). The bioavailability of salmon calcitonin in a rat model ranged from 0.0011% after intrajejunal to 1% after colonic administration (250). SMC021 (Novartis) is an oral salmon calcitonin formulation which includes the permeation enhancer N-(5-chlorosalicyloyl)-8-aminocaprylic acid (5-CNAC, Eligen® Technology, Emisphere Technologies). In two phase I trials, oral salmon calcitonin tablets were tested in healthy postmenopausal women and patients with osteoarthritis, and it reached the systemic compartment and suppressed bone resorption markers (251, 252). However, the amount of water administered with the tablet and the time of administration with respect to food intake were identified as influencing factors (Figure 2.3) (251, 252). These studies presumably contributed to the decision to terminate SMC021 development.

Another oral calcitonin formulation (TBRIATM, formerly Ostora, Tarsa Therapeutics) that is currently in clinical development consists of an enteric-coated tablet, which releases its content at a pH of 5.5 in the duodenum (253). The tablet contains citric acid to decrease the small intestinal pH and thus diminishes the activity of small intestinal peptidases whose pH optimums are in the neutral to basic range (253). Despite reports that citrate’s calcium chelating properties may increase paracellular absorption by disrupting tight junction complexes by depleting intracellular calcium, permeation enhancement was low in Caco-2 monolayers (254). In a phase III trial, oral or nasal calcitonin or placebo were administered to 565 postmenopausal women who were also receiving vitamin D and calcium supplements (253). The oral salmon calcitonin formulation given at a dose 6 times higher than the nasal one met the primary endpoint of this clinical trial in that it was superior to nasal calcitonin and placebo in increasing lumbar spine bone mineral density after 48 weeks of treatment (253). Adverse events were mainly limited to the GI tract (253). Nausea and dyspepsia were significantly more common in the group receiving the calcitonin tablet compared to the nasal formulation which could have been associated with the administration route and the higher dose in the oral treatment group (253). In a recently reported phase II trial, no food effect on therapeutic efficacy was observed (255). Following the phase III clinical trial, Tarsa has filed an NDA with the FDA (under review). The FDA's benefit-risk assessment of this formulation will likely be influenced by their warning about a higher incidence of cancer in patients who received calcitonin compared with placebo, even though a causal relationship could not be established (256).
Figure 2.3. Influence of water and food on the plasma profile of SMC021 (salmon calcitonin formulated with the permeation enhancer 5-CNAC). (A) The higher amount of water co-administered with the tablet resulted in lower calcitonin plasma concentrations (60 min before a meal). (B) The time span between tablet administration and meal intake further influenced systemic exposure to calcitonin. (C) In a twice-daily dosing scheme, the morning dose resulted in higher calcitonin plasma concentrations than the evening dose, potentially due to the fasting before and after the morning dose in contrast to meals ingested 1.5 h before and 1 h after tablet administration. Figures adapted from (251, 252) with permission.
## Semaglutide

Semaglutide (Novo Nordisk, 4114 Da) is a glucagon-like peptide 1 (GLP-1) analogue consisting of 31 amino acid residues. GLP-1 derivatives are used to treat type 2 diabetes because they improve glucose control by three mechanisms: stimulating insulin release in a glucose-dependent manner, suppressing glucagon activity under hyperglycemia, and delaying gastric emptying leading to slower glucose absorption (257). Semaglutide has more favorable pharmacokinetics than its rapidly inactivated mother compound owing to several structural modifications. An amino acid substitution at position 8 (alanine to \(\alpha\)-aminoisobutyric acid) impairs dipeptidyl peptidase-4-mediated degradation (257). Furthermore, the conjugation of C18 fatty diacid via a spacer to the lysine in position 26 prolongs plasma half-life by increasing binding to albumin and reducing renal clearance, making semaglutide suitable for once weekly subcutaneous dosing (257). Oral semaglutide (OG217SC, formerly NN9924, Novo Nordisk) was formulated as a tablet containing the permeation enhancer \(N\)-(8-[2-hydroxybenzoyl]amino)caprylic acid (SNAC, Eligen\(^\text{®}\) Technology, Emisphere Technologies) (181). In a phase II trial involving 600 type II diabetes patients, OG217SC tablets were administered once daily (2.5 to 40 mg) for 26 weeks (www.novonordisk.com). HbA\(_1c\) improvements were dose-dependent from 0.7 to 1.9\%, making the 40 mg oral dose comparable with the 1 mg subcutaneous dose. Oral semaglutide was well tolerated and adverse events were largely dose-dependent, transient, and confined to the GI tract. According to the company's website, Novo Nordisk will initiate phase IIIa development of OG217SC.

### 2.3.2 Proteins

#### Oral insulin

Human insulin consists of 51 amino acids (5800 Da) organized in two chains (A chain 21 aa, B chain 30 aa) linked with two disulfide bridges (228). Although insulin formulations have only been approved for subcutaneous and pulmonary administration, their application via the oral route remains an attractive and vital field of research. In addition to the greater convenience of orally applying insulin, absorbing insulin from the gut into the portacaval vein and the liver mimics the pancreatic insulin secretion better than the subcutaneous route, and promises to decrease
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Peripheral hyperinsulinemia (258). Multiple insulin formulations for oral administration have entered clinical trials, ranging from enteric-coated capsules and formulations with permeability enhancers, liposomes, and polymer conjugates to buccal sprays (259). Referring the readers to three comprehensive reviews (258–260) on oral insulin delivery, we will present here the most clinically advanced insulin-containing drug delivery systems for oral or buccal administration with published results from clinical trials.

Several oral insulin delivery systems are based on enteric-coated capsules with bioavailability-improving adjuvants. Unfortunately, the exact compositions of the formulations were generally not communicated (258). CapsulinTM (Diabetology) is formulated as an enteric-coated capsule that contains insulin, a non-disclosed “dissolution aid”, and an aromatic alcohol (e.g., phenoxyethanol, benzyl alcohol and phenyl alcohol, according to the patent (261)) for absorption enhancement (262). In a randomized controlled crossover phase IIa study, CapsulinTM was administered 60 min before breakfast and the evening meal in sixteen patients with type II diabetes (262). Although significant hypoglycemic action of CapsulinTM was shown, postprandial blood glucose remained above baseline within 2 h after administration. Moreover, a dose-dependent increase in plasma insulin levels upon CapsulinTM administration was not observed in the reported clinical trials, and the effect of food remained unclear (259). CapsulinTM is supposedly starting phase IIb clinical trials after Diabetology entered a licensing agreement with the Indian pharmaceutical company USV Limited in 2012 (www.diabetology.co.uk) (260).

ORMD-0801 (Oramed) is another insulin-containing enteric capsule (263). According to the filed patent, the undisclosed formulation likely contains one or several protease inhibitors (e.g., soya bean trypsin inhibitor, ethylenediaminetetraacetate, EDTA), permeation enhancers (e.g., EDTA), and lipoidal carriers (e.g., vegetable, fish or synthetic omega-3 fatty acid) (261). In a pilot study involving eight type I diabetes patients, ORMD-0801 capsules were administered 45 min before meals as an add-on to the standard insulin treatment and dietary regimen (264). The add-on significantly decreased the frequency of high blood glucose readings and glucose AUC, but the response varied among the patients, as reported in other early-phase clinical trials of ORMD-0801 (259, 264). The ORMD-0801 is currently in phase II clinical trials for type I and type II diabetes.

An insulin formulation that used the permeation enhancer SNAC was tested in sixteen healthy fasting volunteers (265). Plasma glucose decreased 20 – 40 min after insulin administration in a dose-dependent manner (265). Despite the high amount of administered SNAC (2.1 g), no adverse events were reported (265). In the meantime, the development of this formulation was terminated by Emisphere Technology (260). Furthermore, an oral insulin formulation OI338GT
(formerly NN1953, Novo Nordisk), that is based on GIPET is currently in phase I and II clinical development for diabetes type I and II, respectively, but published results were not available at the time of submission of this review.

IN-105 (Biocon) is an insulin derivative based on conjugating a methoxy-triethylene glycol propionyl moiety to a lysine on the B chain of human insulin which aims at improving stability in the GI tract (266, 267). In a cross-over study, 20 diabetes type II patients received IN-105 as tablets (10 to 30 mg) 20 min before ingesting a standardized high-carbohydrate meal (268). The postprandial blood glucose levels were significantly decreased for all IN-105 doses in a dose-dependent manner, but insulin concentration levels varied, and hypoglycemic events were reported for all doses except the lowest (268). In an unpublished six-month phase III clinical trial, IN-105 failed to meet the primary efficacy endpoint of achieving an HbA\textsubscript{1c} (glycated hemoglobin) reduction of 0.7% after placebo adjustment (259). In 2012, Biocon and Bristol-Myers Squibb signed a license option agreement for worldwide development and marketing of IN-105 (except India), but the exact state of the current development is unclear.

Insulin was further encapsulated in “hepatocyte-directed vesicles” (Diasome Pharmaceuticals). The unilamellar liposomes (25 to 125 nm) are composed of the structural phospholipid distearoyl lecithin, negatively charged dicetyl phosphate, cholesterol, and a liver-targeting agent (biotin-phosphatidylethanolamine), which aims at decreasing peripheral hyperinsulinemia (269). However, scientific publications that demonstrate the uptake and liver-targeting effect are not available. The formulation is dried using a proprietary procedure and formulated as an oral gelatin capsule (270). In a placebo-controlled clinical trial, liposomal insulin was given 30 min before every meal as an add-on therapy to six type II diabetes patients (270). It was significantly more effective in lowering mean postprandial plasma glucose concentrations and AUC than the placebo but no dose-response relationship was observed (270).

**Buccal insulin**

Oral-lyn\textsuperscript{TM} (Generex Biotechnology Corporation) is an oral formulation for buccal delivery that contains recombinant human insulin and non-disclosed absorption enhancers (258). According to the patent, these GRAS status excipients are likely to be sodium alkyl sulfate, EDTA, sodium salicylate, and a lecithin-based or bile salt-derived agent, claimed by the company to form insulin-containing mixed micelles and enhance absorption (261, 271). The insulin liquid formulation is propelled into the mouth as a fine-particle aerosol by the RapidMist\textsuperscript{TM} device (271, 272). In a study with 31 subjects who suffered from impaired glucose tolerance, six puffs of Oral-
lym™ right before and six puffs 30 min after a standard oral glucose tolerance test decreased plasma glucose levels approximately 30% after 2 and 3 h compared with no treatment, and hypoglycemia was not observed in this time frame (272). However, an increase in plasma insulin levels was only observed 30 min after the administration of the first six puffs (272). According to an announcement by Generex, the company has improved the formulation to increase insulin bioavailability and the insulin amount per puff, and is planning to update its IND on file at the FDA.

In conclusion, the goal of reproducibly delivering insulin via the oral route has not yet been achieved. Inter- and intraindividual variability often undermines reliable dosing, especially in the presence of food. Presumably, non-buccal insulin formulations will have to be taken at specific times before food is ingested, and the meals cannot be fully or partially skipped to avoid hypoglycemia. These restrictions in eating habits and the limited reliability counterbalance the advantages of needle-free insulin delivery. The most appropriate role of the currently tested oral insulin formulation may be as an add-on to improve blood glucose control, provided that the risk of hypoglycemic episodes is small.

### 2.3.3 Nucleic acids

Despite the polyanionic nature and high molecular weight (>5000 Da) of AONs (131), several attempts at systemic AON delivery via the oral route have been undertaken. Mipomersen (formerly ISIS 301012, Genzyme/Isis Pharmaceuticals) is a 20-mer phosphorothioate AON stabilized with 2′-O-(2-methoxyethyl) modifications at the five terminal bases at both ends. It is used to treat homozygous familial hypercholesterolemia because it targets the liver apoB mRNA which encodes apolipoprotein B, an essential component of several fat carriers (e.g., chylomicrons, LDL, VLDL). The AON was formulated with the permeability enhancer sodium caprate, and upon its administration once daily for 90 days, the average plasma bioavailability was 6% (273). A maximum reduction of apoB of 12-15% was observed on day 55 of the treatment. The formulation for subcutaneous administration of mipomersen was further developed and finally approved by the FDA in 2013 (Kynamro®, Genzyme). In 1999, Isis Pharmaceuticals and Elan formed Orasense to develop an oral formulation of ISIS 104838, a 20-mer phosphorothioate AON that targeted the TNF mRNA. It contains 2′-O-(2-methoxyethyl) modifications at the five terminal bases at both ends (139). Several enteric-coated capsules with varying durations of sodium caprate release (total 660 mg of sodium caprate and 100-140 mg of AON per capsule) were evaluated in healthy humans.
for the oral delivery of ISIS 104838 (139). Each subject received five capsules per treatment, and all formulations were well-tolerated. However, the inter-individual variability in AON oral bioavailability was high, ranging from 0.7% to 27.5% in ten fasting subjects and with 12% being the average bioavailability of the best formulation (274). In 2005, Isis Pharmaceuticals terminated the development of ISIS 104838.

Monarsen (EN 101, Amarin Corporation) is an AON under investigation for myasthenia gravis (MG), a neuromuscular autoimmune disorder. It downregulates a pathologic mRNA splicing variant of acetylcholinesterase, which constitutes an established target in MG (275). Monarsen is a 20-mer phosphodiester ON with 2′-O-methyl modifications at the three terminal bases of the 3′ end (275). It was given orally for four days as an aqueous solution to 16 patients who suffered from MG (276). Thirteen patients showed improved Quantitative MG scores; however, the study was not placebo-controlled. In the following phase IIa study, oral monarsen given for 7 days at a maximum daily dose of 40 mg decreased Quantitative MG scores by 20.3% compared to baseline (277). Further development of monarsen for MG was discontinued. Recently, monarsen was reported to exert an off-target anti-inflammatory effect through activation of Toll-like receptor 9, which additionally decreased AChE activity. Therefore, the observed therapeutic effect might be attributable to the indirect action of AON rather than the target knockdown (278). This finding led to further development of monarsen for the oral treatment of ulcerative colitis by BioLineRx under the name BL-7040. This drug has completed phase II clinical trial with positive results, showing reduced neutrophil levels and histological improvement (www.biolinerx.com).

2.4 Safety of bioavailability-increasing excipients

To address the challenges posed by the low stability and intestinal absorption of orally applied macromolecular drugs, excipients such as permeation enhancers and/or protease inhibitors are often added to oral drug delivery systems. Given that the body is exposed to the excipients as much as it is exposed to the drug, their safety profiles are essential features of the final product, especially in case of long-term use. This section reviews protease inhibitors and permeation enhancers with a focus on excipient toxicity. Enteric coatings will not be covered due to their widespread use and established safety profiles.
2.4.1 Protease inhibitors

A major obstacle to the local and systemic delivery of peptides and proteins is their susceptibility to enzymatic degradation in the GI tract \(^{(279)}\). Protease inhibitors reversibly or irreversibly inactivate luminal and/or brush border membrane-bound enzymes in the GI tract \(^{(279)}\). A conceptual drawback of protease inhibitors is the impaired digestion of dietary proteins \(^{(279)}\). In rodents, protease inhibitors led to the activation of a feedback loop that resulted in enhanced secretion of proteases, and pancreas hypertrophy and hyperplasia \(^{(280–283)}\). Inhibition of protease activity should therefore be restricted to the area of the liberation and absorption of the macromolecular drug.

Protease inhibitors are grouped into amino acid-based, (poly)peptidic, and non-amino acid-based inhibitors \(^{(279)}\). Those based on amino acids, (poly)peptides, and their derivatives competitively bind to the enzyme active site \(^{(279)}\). However, their efficacy is often limited due to generally low inhibitory activity and rapid dilution, digestion, and absorption in the GI tract. For instance, in a small short-term clinical study, the peptidic serine protease inhibitor aprotinin was co-administered with human calcitonin in the colon, and although no adverse events related to aprotinin were reported \(^{(284, 285)}\), the excipient failed to increase the bioavailability of human calcitonin. In rats, aprotinin and several other protease inhibitors (soybean trypsin inhibitor, sodium glycocholate, camostat mesilate, and bacitracin) fell short in enhancing insulin absorption upon small-intestinal administration \(^{(285)}\). In view of the low potency and presumably high doses needed to generate an effect, concerns regarding the safety profiles of the excipients and their potential systemic availability have been raised. The systemic exposure of peptidic aminopeptidase-inhibitor bacitracin, for instance, was associated with nephrotoxicity \(^{(286)}\). Non-amino acid-based chelating agents inactivate proteases by scavenging enzyme cofactors (calcium, zinc, cobalt, manganese, magnesium). The chelating agent EDTA is widely used in the pharmaceutical and food industries, but its binding capacity for calcium is not sufficiently high to inhibit the activity of the calcium-dependent luminal endoproteases trypsin and chymotrypsin \(^{(279)}\). Due to its considerably higher affinity to zinc, EDTA may be more useful in targeting zinc-dependent exonucleases, provided that it is not excessively diluted. However, the binding to essential trace elements may lead to nutritional deficiencies, especially upon chronic administration. Furthermore, small-molecule protease inhibitors can be associated with local and systemic adverse reactions. The synthetic trypsin inhibitor camostat mesylate, for instance, increased insulin bioavailability upon co-administration in the colon but was associated with pancreas hypertrophy \(^{(281, 285)}\). This serine protease inhibitor further showed cross-reactivity with other proteases, leading to off-target effects.
(e.g., enhanced pulmonary mucociliary clearance due to inhibition of airway channel-activating proteases) (287, 288).

### 2.4.2 Permeation enhancers

A variety of compounds with different mechanisms of action are grouped under the term permeation enhancer (289). They enhance membrane permeability by disrupting the mucus layer on the intestinal epithelium, transiently opening tight junctions, disrupting the membrane bilayer packing, and directly binding to the substrate to form an absorbable complex (289). The most important and clinically advanced permeation enhancers are salts of medium-chain fatty acids (290). Sodium caprate, the salt of decanoic acid, opens tight junctions, and thus enhances permeability via the paracellular pathway (291). Even though cytotoxic effects of sodium caprate were observed in Caco-2 monolayers, repeated oral administrations of sodium caprate did not result in morphological changes in the gastrointestinal mucosa of several animal species (274, 292–294). Orally administered sodium caprate were investigated in several clinical studies and, even upon long-term administration, generally well-tolerated apart from being associated with GI adverse events in some patients (290). Rectal administration of sodium caprate-containing suppositories, however, led to transient mucosal damage in healthy subjects (295). Furthermore, the safety profile of sodium caprate remains insufficiently investigated in certain high-risk populations, notably in patients with increased GI permeability at baseline (e.g., irritable bowel diseases) or subjects exposed to GI-irritant drugs such as nonsteroidal anti-inflammatory drugs and ethanol (290).

Another strategy for enhancing membrane permeation is the formation of a membrane-permeable complex between the substrate and a complexing agent. SNAC and 5-CNAC are claimed to form a non-covalent complex with the substrate, protecting it from intestinal degradation and increasing its hydrophobicity in order to enable passive permeation of the epithelium, after which the complex disassembles (296). However, this mechanism of action has been the subject of controversial discussions (181, 289). Interestingly, SNAC damaged Caco-2 cells in various cytotoxicity assays but the toxicity profile in rats was favorable apart from the appearance of histopathological changes in the stomach which the authors partly related to the application method (gavage) (296–298). It was generally well-tolerated in clinical studies, with occasional reports of adverse GI events (nausea, vomiting) (265, 299–301).

Conceptually, enhancing intestinal permeation entails the risk of increasing the exposure to dietary antigens, which potentially increases the risk of triggering chronic autoimmune diseases
(141). However, currently published clinical trials have not confirmed this hypothesis. Furthermore, surfactant-type permeation enhancers impair the protective properties of the mucus layer, facilitating the diffusion of luminal bacteria to the intestinal epithelium and ultimately disturbing the host-microbiota relationship. For instance, it was recently found that the ubiquitous pharmaceutical excipients and food additives polysorbate-80 and carboxymethyl cellulose induced a microbiome composition shift, inducing low-grade inflammation and obesity in wild-type mice and promoting colitis in a predisposed mouse model (142). While the clinical relevance of these intriguing in vivo findings have yet to be established, alterations in the composition of the gut microbiota were also reported in healthy humans upon long-term administration of the permeation enhancer chitosan (143). These studies show that even widely used excipients with GRAS status may lead to local and systemic pathophysiologic effects, underlining the need for more thorough long-term preclinical and clinical testing.

2.5 Conclusion

In spite of the considerable progress made in pharmaceutical technology since the first oral insulin application in 1922, the administration of biomacromolecules via the oral route remains one of the greatest challenges for formulation scientists. In the systemic delivery of orally administered biomacromolecules, the harsh environment of the GI tract and the intestinal epithelial barrier often result in considerable luminal degradation, low and erratic absorption and bioavailability, and highly variable plasma concentrations. Food effects and drug interactions (e.g., proton pump inhibitors) may further influence the pharmacokinetic profile. Therefore, the oral administration is currently restricted to potent low-molecular-weight peptides with large therapeutic windows or with plasma concentration monitoring. Although novel approaches such as buccal delivery are under clinical investigation, it remains to be seen if the systemic delivery of orally applied macromolecules above 5000 Da, notably insulin, can be achieved sufficiently reproducibly to reach a favorable efficacy and safety profile. In the current trials, permeation enhancers in combination with protease inhibitors are often used to overcome the absorption and enzymatic barriers. However, serious risks might be associated with the long-term application of these excipients, including increased exposure to dietary antigens and pathogens due to the sustained increased permeability of intestinal mucosa and potentially pathogenic changes caused by alterations in the intestinal microbiome. Therefore, thorough long-term toxicity studies of these excipients are needed.
In contrast, the local delivery to GI targets avoids the challenges of crossing the intestinal epithelium. GI resistance is often conferred by chemical stabilization strategies, which are especially straightforward for peptides and short nucleic acids. Furthermore, novel approaches such as genetically modified probiotics that produce and secrete the desired proteins \textit{in situ} are on the horizon. However, luminal degradation, potential immunogenicity, and the risk of inadvertent systemic exposure (\textit{e.g.}, in patients with increased intestinal permeability) remain challenging. Moreover, the therapeutic areas of local delivery are mainly restricted to diseases of the GI tract (GI infections, IBD, and certain metabolic diseases).

In view of these challenges, we believe that a better understanding of the molecular interactions between targets and their biomacromolecular substrates and ligands will eventually yield low-molecular-weight drugs, which are more suitable for oral delivery than their macromolecular parent compounds.
3 Amphiphilic nucleic acid conjugates

3.1 Introduction

Nucleic acids are highly attractive class of therapeutics due to their potential to regulate any selected gene of interest. Given their capacity to modulate conventionally undruggable targets, ONs have been extensively investigated as potential therapeutics to treat cancer, viral infections, genetic diseases, and immunological disorders (302, 303). Despite the clear therapeutic potential of ONs, their poor permeability across cellular membranes (due to their intrinsic polyanionic nature and high molecular weight) and susceptibility to degradation by ubiquitous nucleases hamper their clinical translation (302). Recent advances in the development of various delivery vehicles (e.g., polymer-, lipid-, peptide-, nano/microparticles-, or viral-based) have helped overcoming some of the ON delivery problems; however, issues such as systemic toxicity, low concentration at target sites and pharmaceutical complexity of the delivery systems still represent obstacles to the clinical translation of ON therapeutics (127, 304). The conception of stable ONs with enhanced affinity via various chemical modifications is one of the most remarkable achievements in this field. For example, the combination of PS backbone modification with OMe and MOE moieties in the sugar units or bicyclic ribonucleosides are the most widely used chemical strategies under clinical investigation (15, 305, 306). Another approach is modification with FANA, which upon binding to the target mRNA induces its RNase H-mediated degradation (30, 307). Several chemically stabilized ONs are already marketed (e.g., mipomersen for homozygous familial hypercholesterolemia (306)) or in late-phase clinical trials (15).

Nucleic acid therapy could be especially beneficial for several disorders of the gastrointestinal (GI) system that currently lack appropriate treatments such as IBDs, colon cancer, and familial adenomatous polyposis (8). The delivery of nucleic acids directly to the GI mucosa could achieve
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high local concentrations while minimizing systemic exposure (308) and subsequent side-effects (41). Indeed, a group of carrier-free ONs targeting GI mucosa for IBD therapy is progressing through clinical trials, although high doses of ONs are required to obtain the positive therapeutic effects (8, 227, 308). Therefore, a safe and efficient delivery approach to the GI mucosa would be desirable to facilitate the cellular uptake of ON and to decrease dosing. Two major strategies are currently investigated to improve the ON delivery efficacy to the intestinal tissue: particle-based systems (e.g., polyplex- or lipoplex-based (155, 303)) and single-molecule-based conjugates (e.g., free ON or CPP-ON conjugates (108, 227)). ON incorporated in particles can be taken up primarily by M cells in the Peyer’s patches of the intestine and eventually by mucosal macrophages via phagocytosis (132). Therefore particle-based systems would be suitable for the targeting of disease-related genes expressed mainly in the immune cells, such as TNF. In contrast, single-molecule-based system, such as free ONs, can be taken up not only by lamina propria immune cells but also by epithelial cells (227). Considering that in some intestinal diseases the dysregulated expression of genes is localized in the gut epithelium (147, 309, 310), single-molecule-based systems could open promising therapeutic avenues for previously inaccessible epithelium-specific targets. In addition, these systems possess the advantage of reduced carrier toxicity and immunogenicity (83, 91, 311), as well as simpler characterization processes, compared to particle-based carriers (91, 311).

In a recent investigation from our group, the conjugation of a lipid moiety to chemically modified ONs enabled significant target gene knockdown in a prostate cancer cell line in the absence of transfection reagents (112). ON conjugates with the long-chain DSA group had higher silencing efficacy in comparison to cholesterol or docosahexaenoic acid derivatives. However, only one type of ONs (i.e., single-stranded AONs) with only one type of chemical modifications (i.e., FANA) was investigated. Therefore, the present work aimed at exploring the versatility of DSA as a delivery vehicle for single-stranded AONs and double-stranded siRNAs targeting a model Bcl-2 mRNA. The cytotoxicity and the silencing efficacy of the resulting conjugates were assessed in vitro in an intestinal cell line. The most potent conjugate was further evaluated for its silencing kinetics including the important transfection controls in two intestinal cell lines, and contribution of sedimentation to the uptake of DSA-ON conjugate was investigated.
3.2 Materials and Methods

3.2.1 Materials

DNA, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), N,N-diisopropylethylamine (DIPEA), N,N'-diisopropylcarbodiimide (DIC), trichloroacetic acid (TCA), dichloromethane (DCM), tetrahydrofuran (THF), acetonitrile (ACN), N,N-dimethylformamide (DMF), ethanol (EtOH), pyridine, triethylammonium acetate (TEAA) buffer 1 M, concentrated aqueous ammonia, and methylamine (40% solution in water) were obtained from Sigma-Aldrich (Buchs, Switzerland). Methanol (MeOH), 4-dimethylaminopyridine (DMAP), and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC HCl), were purchased from Acros Organics (Geel, Belgium). DNA and RNA phosphoramidites were obtained from Thermo Fisher Scientific (Waltham, MA). Monomethoxytrityl (MMT)-protected 6-amino-hexanol phosphoramidite and 3% dichloroacetic acid (DCA) in DCM were obtained from Glen Research (Sterling, VA). 5-Ethylthiotetrazole was purchased from ChemGenes (Wilmington, MA). 5-(Benzylthio)-1H-tetrazole was obtained from Carbosynth (Compton, UK). Lithium perchlorate (LiClO₄) was obtained from Alfa Aesar (Haverhill, MA). Parafilm M® was purchased from Bemis Company (Neenah, WI). Hexafluoroisopropanol (HFIP) was obtained from Fluorochem (Hadfield, UK). Duplex annealing buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5) was purchased from Integrated DNA Technologies (IDT, Coralville, IA). HCT-116 cell line was kindly provided by Prof. Azzalin’s group at ETH Zurich, and Caco-2 cells were purchased from ATCC (Manassas, VA). DMEM with GlutaMAX™ medium, Opti-MEM medium, fetal bovine serum (FBS), non-essential amino acids, penicillin-streptomycin solution, trypsin, Lipofectamine® 2000 (LF), phosphate-buffered saline (PBS; 1 mM KH₂PO₄, 3 mM Na₂HPO₄, 155 mM NaCl, pH 7.4), and RNase-free distilled water were obtained from Invitrogen (Carlsbad, CA). MycoAlert™ PLUS Mycoplasma Detection Kit was purchased from Lonza (Basel, Switzerland). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Rockville, MD). Thermanox™ coverslips were purchased from Thermo Fisher Scientific (Waltham, MA). Low-binding microcentrifugation tubes (DNA Lobind®) were purchased from Eppendorf-Vaudaux (Schönenbuch, Switzerland). RNeasy Mini kit and specific primers for α-splicing variant of human Bcl-2 mRNA (Hs_BCL2_1_SG; QT00025011) and human β-actin (Hs_ACTB_2_SG; QT01680476) were obtained from Qiagen (Valencia, CA). High-capacity cDNA reverse transcription kit and Power SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA).
3.2.2 Synthesis of ONs and their derivatives

Unmodified Bcl-2 targeting siRNA (112) and mismatch siRNA (63) were synthesized by Bioneer Co. (Daejeon, South Korea). Unmodified antisense strand of siRNA and L-OMe conjugate were obtained from Microsynth (Balgach, Switzerland). All other ONs were synthesized according to the standard protocol for automated phosphoramidite solid-phase synthesis. DSA (L) was conjugated via an aminohexanol-linker to the 5′ end of ONs in line with previously described method (112). All L-ON conjugates were purified by reverse-phase HPLC, analyzed by liquid chromatography – mass spectrometry (LC-MS), and quantified via UV spectrophotometry (NanoPhotometer P 330, Implen, Germany). Details of synthetic procedure and analytical data are presented in the appendix. The ONs’ molar extinction coefficients at 260 nm were calculated using the software of IDT website (OligoAnalyzer tool, www.idtdna.com/calc/analyzer). FANA and MOE extinction coefficients were calculated using DNA and OMe values, respectively. These modifications were assumed to have negligible effect on the extinction coefficients, as previously described (35, 63). The complementary single strands of siRNAs were combined in the duplex annealing buffer at a concentration of 50 µM each, heated up to 95 °C for 1 min and cooled slowly to 4 °C overnight to ensure proper annealing. Various ONs and L-ON conjugates were dissolved in RNase-free deionized water at a concentration of 100 µM and stored at -20 °C.

3.2.3 Cell culture

HCT-116 cells were maintained in DMEM medium supplemented with GlutaMAX™ containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a 5%-CO₂ humidified atmosphere. The cells were seeded in a 24-multiwell plate at a density of 4 × 10⁴ cells/well. For the inverted transfection, the cells were seeded on Thermanox™ coverslips in a 24-multiwell plate at a density of 1 × 10⁵ cells/well. The cells were incubated for 1 day before experiments. Caco-2 cells were maintained in DMEM medium supplemented with GlutaMAX™ containing 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 1% of non-essential amino acids at 37 °C in a 5%-CO₂ humidified atmosphere. Cells with passage number between 58 and 77 were seeded in a 12-multiwell plate at a density of 7 × 10⁴ cells/well.

All experiments were performed on mycoplasma-free cell lines (regularly checked by MycoAlert™ PLUS Mycoplasma Detection Kit), and only cells in the exponential phase of growth were used for seeding.
3.2.4 Cytotoxicity assay

To compare the cytotoxicities of different L-ON conjugates, HCT-116 cells were seeded in a 96-multiwell plate at a density of 7 × 10^3 cells/well the day before the experiment. The cells were treated with various concentrations of L-ON conjugates or with free PS siRNA at 1 µM in 50 µL of serum-deficient Opti-MEM medium for 15 h. Alternatively, the cells were treated with 50 nM of various siRNAs complexed with LF according to the manufacturer's instructions in 50 µL of serum deficient Opti-MEM medium for 5 or 15 h. The Opti-MEM medium containing no L-ON or siRNAs was used as a control. The medium was exchanged for 100 µL of DMEM supplemented with 10% FBS, and the cells were further incubated for 1 or 2.5 days, after which the cell viability was assessed using tetrazolium-based CCK-8 reagent following the manufacturer's instructions.

3.2.5 Screening of Bcl-2 silencing efficiencies of various L-ONs

To assess the silencing efficiency of various L-ON conjugates, the conjugates at various concentrations (0.25-1 µM) were incubated with HCT-116 and Caco-2 cells. Given the absence of intact serum in the intestinal environment, all transfection experiments were performed in serum deficient Opti-MEM medium. The Opti-MEM medium containing no ONs was used as a control. Following overnight incubation (15 h), the transfection medium was exchanged with fresh DMEM supplemented with 10% FBS. After 1, 2.5, or 3.5 days of further incubation cells were washed with PBS, and total RNA was isolated using RNeasy Mini kit (Abs260/ Abs230 >1.8) according to the previously optimized method (112). The expression levels of Bcl-2 mRNA relative to the internal control β-actin mRNA were quantified by two-step quantitative real-time PCR as previously described (112). Briefly, cDNA was synthesized from 1.2 µg of total mRNA using high-capacity cDNA reverse transcription kit according to the manufacturer's instructions. Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix and specific primers for human Bcl-2 and β-actin on a 7900HT Fast Real Time PCR instrument (Applied Biosystems) according to the manufacturer's instructions. Briefly, the reaction mixtures were incubated at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of denaturation (95 °C for 15 s) and extension/detection (60 °C for 1 min). Relative gene expression levels were calculated using the delta delta Ct (2^-ΔΔCt) method (the fluorescence threshold was set to 0.4). Results are expressed as the Bcl-2 mRNA level change between ON-treated and ON-free medium treated cells. L-ON potency (effective concentration causing 50% of target gene silencing, EC50) was calculated using a four-parameter logistic function to fit the dose-response data via SigmaPlot software.
3.2.6 Target Bcl-2 mRNA silencing study with different incubation times

HCT-116 and Caco-2 cells were seeded in a 24-multiwell plate at a density of $4 \times 10^4$ cells/well and in a 12-multiwell plate at a density of $7 \times 10^4$ cells/well, respectively. After one day, the cells were transfected with L-FANA conjugate or siRNA/LF complexes in Opti-MEM medium in the absence of serum for 5 or 15 h. Bcl-2 mRNA expression was evaluated by real time PCR 72 h after starting the transfection as described above.

3.2.7 Comparison of transfection efficiencies in upright and inverted transfection setups

The transfection medium consisted of 250 μL of Opti-MEM containing either 1 μM of carrier-free Bcl-2 targeting L-FANA conjugate or 50 nM of Bcl-2 specific siRNA complexed with LF according to the manufacturer's instructions as a particle-mediated delivery control. HCT-116 cells grown on the coverslips were washed with Opti-MEM, and the coverslips were transferred using tweezers into a new 24-multiwell plate for the transfection. For the upright transfection, the cells were placed on the bottom of the multiwell plate followed by the addition of the transfection medium. For the inverted transfection setup, the transfection medium was added to the empty well, and the cells grown on coverslips were carefully deposited upside down onto the surface of the medium. The coverslips floated on the surface of the medium due to the surface tension of the medium. After overnight incubation (15 h), the cells were transferred to a new plate and further cultured for 2.5 days in 500 μL of DMEM supplemented with 10% FBS. Subsequently, total RNA was isolated and expression levels of Bcl-2 mRNA were assessed as described above.

3.2.8 Statistical analysis

All treatment groups were compared pairwise using the one-way ANOVA test combined with Tukey’s (Holm-Sidak) post-hoc test assuming normal data distribution. The statistical analysis was performed using SigmaPlot software. The differences between treatment groups were considered statistically significant at $p$-values below 0.05.
3.3 Results

3.3.1 L-ON library screening for Bcl-2 mRNA knockdown efficacy

In a previously published study by our group, it was reported that lipophilic DSA conjugated to ONs modified with 2′-F-arabinonucleosides were more potent in transfecting prostate cancer cells than nucleic acids derivatized with cholesterol or docosahexaenoic acid (112). In a search for novel and safe amphiphilic ONs capable of modulating gene expression in intestinal cells, we synthesized and characterized a set of conjugates by linking lipophilic DSA (L) to different single-stranded antisense ONs and double-stranded siRNAs targeting the mRNA of the oncoprotein Bcl-2 (Table 3.1) as previously described (112).

Table 3.1. The sequences and modification strategies of ONs.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>Backbone</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>*5′-TCTCCAGCGTGCGCCAT-3′</td>
<td>PS</td>
<td>(27)</td>
</tr>
<tr>
<td>OMe</td>
<td>*5′-TCTCCAGCGTGCGCCAT-3′</td>
<td>PS</td>
<td>(305)</td>
</tr>
<tr>
<td>MOE</td>
<td>*5′-TCmTCmCCAGCGTGCGCmCmAT-3′</td>
<td>PS</td>
<td>(306)</td>
</tr>
<tr>
<td>FANA</td>
<td>*5′-TCTccccAGCgtgCGCcat-3′</td>
<td>PS</td>
<td>(307)</td>
</tr>
<tr>
<td>FANAnc</td>
<td>*5′-CGCagaTTAgaaACCttt-3′</td>
<td>PS</td>
<td>(35)</td>
</tr>
<tr>
<td>siRNA</td>
<td>*5′-GCAUGGCGCCUCUGUUUGAUU-3′</td>
<td>PO</td>
<td>(112)</td>
</tr>
<tr>
<td></td>
<td>3′-UUCGUACCCGGAGACAAAUACU-5′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS siRNA</td>
<td>*5′-GCAUGGCGCCUCUGUUUGAUU-3′</td>
<td>PS</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td>3′-UUCGUACCCGGAGACAAAUACU-5′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>siRNAnc</td>
<td>*5′-GUAACGACTACACGGGAGAUAUU-3′</td>
<td>PO</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td>3′-UUCAUGCGUGUGGCCUCUAU-5′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a–nc – negative control, b ATGC are DNA, AUGC are RNA, ATC are OMe- or MOE-RNA, atgc are 2′-F-ANA, Cm – 5-methylcytosine, upper sequence of siRNA – sense strand, lower sequence – antisense strand, * – site of DSA conjugation, ‘PS – all phosphorothioate linkages, PO – all phosphodiester linkages.
The antisense ONs were based on a single parent DNA sequence (oblimersen (27, 112)) and were modified by incorporating OMe-, MOE-ribonucleosides, or FANA connected via PS linkages (L-DNA, L-OMe, L-MOE, and L-FANA, respectively). These chemical modifications of nucleotides are commonly employed to enhance the binding affinity of ONs for target mRNAs as well as to increase their stability against nuclease digestion (312, 313). Typically, antisense ONs' mode of action involves recruitment of RNase H, which recognizes the thus formed DNA:RNA heteroduplex leading to a processive cleavage of the mRNA strand in a catalytic fashion (27). Since oligoribonucleotides and their 2′-O-modified analogues generally do not support RNase H binding, a “gapmer” design, in which a central DNA stretch is flanked by modified ribonucleotides (OMe and MOE) at both termini, is preferred for preserving an RNase H binding site, while providing the ON with higher nuclease resistance (28, 29). In contrast to 2′-O-modified ribonucleotides, the FANA modification can be incorporated throughout an ON sequence, since upon binding to complementary mRNA, the FANA nucleotide adopts a conformation similar to that of DNA and therefore supports RNase H recognition (30). Since an “altimer” design showed higher potency compared to “gapmer” design in our previous work (112), it was chosen for the FANA-modified ON in the present study.

In order to test whether DSA is capable of facilitating the intracellular delivery of double-stranded siRNAs, it was conjugated to the unmodified phosphodiester siRNA (L-siRNA) and its fully phosphorothioated counterpart (L-PS siRNA) (32, 112).

L-MOE, L-PS siRNA, and L-OMe caused a small but significant decrease in cell viability already 1 day after transfection (Fig. 3.1). This result indicates that the effect of the L-ON conjugate on cell viability depends on the type of ON used and on its chemical modification. Longer incubation times could potentially lead to increased cell apoptosis due to the Bcl-2 silencing (27). All L-ON conjugates exhibited significant cytotoxicity at 2.5 days post-transfection in comparison to medium treated cells, while the difference between the conjugates was not statistically significant. Interestingly, not only the conjugates targeting the Bcl-2 mRNA caused appreciable cell death, but also the conjugate with unrelated sequence (L-FANAnc) indicating the mild cytotoxicity (ca. 20%) of chemically-modified ONs, which could result from their unspecific protein binding (314, 315).
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Figure 3.1. Viability of HCT-116 cells after transfection with various ON derivatives at a dose of 1 µM in Opti-MEM medium overnight. Cell viability was assessed 1 or 2.5 days after medium exchange. Results are expressed as mean ± SD (n=3). (**p < 0.01, *p < 0.05 vs. Opti-MEM medium treated cells).

As shown in Fig. 3.2, both single- and double-stranded L-ONs decreased target gene expression levels in colon carcinoma cells in the absence of transfecting reagent. L-ON conjugates showed the following order of efficacy (based on statistically significant differences at various concentrations): L-FANA > L-OMe > L-siRNA >> L-DNA, where L-DNA did not exhibit a statistically significant reduction. There was no significant difference between the efficacies of L-FANA and L-MOE or L-PS siRNA and L-OMe at any of the concentrations tested. L-ON conjugates downregulated their target gene expression in a dose-dependent manner, and the EC$_{50}$ values of L-PS siRNA, L-OMe, L-MOE, and L-FANA were 0.86, 0.69, 0.47, and 0.28 µM, respectively (Fig. 3.2B). These results indicate that also the silencing potency of the L-ON conjugate depends on the type of ON used and on its chemical modification. Unfortunately, the potency of AONs could not be evaluated in the lipoplex-based assay due to their severe cytotoxicity on proliferating cells.
**Figure 3.2.** The silencing efficacy and cytotoxicity of L-ON conjugates. (A) Bcl-2 mRNA silencing in HCT-116 cells after transfection with various L-ON conjugates at a concentration of 1 µM. Results are expressed as mean + SD (n=3-5). *** p < 0.001, ** p < 0.01, or * p < 0.05 vs. medium treated cells. (B) Dose-dependent silencing of Bcl-2 mRNA in HCT-116 cells by transfection with L-PS siRNA, L-OMe, L-MOE, and L-FANA conjugates. Results are expressed as mean + SD (n=3-4).

DSA coupled to the unmodified siRNA was less efficient in terms of Bcl-2 mRNA silencing than its conjugate with PS siRNA in the absence of transfection reagent. Since L-PS siRNA possessed slightly lower silencing potency compared to L-siRNA upon the LF-mediated transfection (Fig. 3.3), the superior silencing properties of carrier-free L-PS siRNA could have been due to its higher resistance to extra- and intracellular nucleases (32) and/or higher uptake of PS-modified ONs (26). The conjugation of lipid to the 5’ end of the sense strands of siRNAs did not reduce their potency when transfected with the help of LF. The PS modification of L-PS siRNA
resulted in a decrease of silencing efficacy in comparison to L-siRNA when transfected with LF probably due to the impaired recognition by the RNA-induced silencing complex (RISC) machinery (32). Free PS siRNA did not decrease the bcl-2 mRNA level indicating the importance of lipid moiety for the efficient cellular delivery. All siRNA/LF lipoplexes exhibited significant cytotoxicity. Interestingly, L-PS siRNA caused significantly lower cytotoxicity in comparison to unconjugated PS siRNA when delivered as a LF-lipoplex (p < 0.01) (Figure 3.3, closed dot, right y-axis), which could result from their differing intracellular trafficking.

We selected the L-FANA conjugate as a promising candidate for further characterization based on a combination of lowest EC$_{50}$ with a favourable cytotoxicity profile.

**Figure 3.3.** Bcl-2 mRNA silencing (bar, left y-axis) and viability (closed dot, right y-axis) of HCT-116 cells after transfection with various siRNA derivatives complexed with LF in Opti-MEM medium at a dose of 0.05 µM for 5 h and free PS siRNA at 1 µM overnight. Results are expressed as mean ± SD (n=3-4). *** p < 0.001, ** p < 0.01, or * p < 0.05 vs. Opti-MEM medium treated cells, * p < 0.05 between two treatment groups.
3.3.2 Bcl-2 mRNA silencing by L-FANA conjugate in two colon carcinoma cell lines

We further investigated the target gene silencing effect of L-FANA by transfecting two human colorectal carcinoma cell lines, HCT-116 and Caco-2, with L-FANA and its controls. At a concentration of 0.7 µM, L-FANA downregulated Bcl-2 mRNA in both cell lines by 87 and 83%, respectively (Fig. 3.4). Transfection of free FANA and negative control conjugate with an irrelevant nucleic acid sequence (L-FANAnc) did not change the Bcl-2 mRNA expression levels, indicating that target gene inhibition is caused by a sequence-specific antisense mechanism and that lipid conjugation contributes to the improved intracellular delivery of FANA.

Figure 3.4. Dose-dependent silencing of Bcl-2 mRNA in HCT-116 and Caco-2 cells by L-FANA conjugate compared to the silencing efficacy of the FANA without lipid and to L-FANAnc with non-targeting sequence (nc). Results are expressed as mean ± SD (n =3-4). ***p < 0.001 or **p < 0.01 vs. medium treated cells.

3.3.3 Bcl-2 mRNA silencing in upright and inverted transfection setups

In order to assess whether sedimentation influenced the transfection efficiency, experiments with cells in inverted position were performed (Fig. 3.5). Recent studies with nanoparticles demonstrated that cellular uptake can be dramatically reduced in the inverted configuration (cells on top), as opposed to the conventional in vitro setup where cells are on the bottom of the culture
plate and sedimentation can artificially promote internalization (63, 108, 316). Indeed, we observed that transfection efficiency of siRNA/LF lipoplex was significantly reduced using the inverted setup, whereas the silencing efficiency of L-FANA conjugate was the same in both cell setups. These results indicated that L-FANA was predominantly taken up by cells via sedimentation-independent uptake routes, which was in line with the outcome of our previous work on peptide-nucleic acid conjugates (108).

3.3.4 Bcl-2 mRNA silencing with different incubation times

It could be envisaged that sedimenting particulate carriers could be rapidly taken up by cells and achieve target silencing with relatively short transfection times. Indeed, in the case of the siRNA/LF lipoplex, the target mRNA silencing efficacy did not depend on the duration of the transfection, and even a relatively short incubation time with the lipoplex (5 h) was enough to achieve potent mRNA silencing. It is likely that this is a consequence of the fast uptake of sedimented particles (Fig. 3.6) (317). This is in contrast to the effect of L-FANA, where increasing the exposure time to the ON conjugate from 5 h to 15 h resulted in significantly higher knockdown efficacy (38 vs. 65%, respectively, at 0.5 µM; Fig. 3.6), further supporting a presumed sedimentation-independent uptake.
Figure 3.6. The influence of incubation time on knockdown efficacy in HCT-116 cells transfected with 0.5 µM of L-FANA or 0.05 µM of siRNA/LF in Opti-MEM medium. The cells were transfected for 5 or 15 h, followed by incubation with complete medium for 2.5 days. Upon extending the incubation time from 5 h to 15 h, target gene knockdown efficacy by L-FANA significantly increased in contrast to siRNA/LF complex. Results are expressed as mean + SD (n=3-4). * p < 0.05.

3.3.5 Kinetics of Bcl-2 mRNA silencing by L-FANA or siRNA/LF lipoplex

Previously, it was reported that amphiphilic siRNAs could be sequestered in the endolysosomal compartment and require prolonged post-transfection times (>3 days) in order to elicit target gene silencing (117). In contrast, L-FANA conjugate exhibited rapid mRNA silencing and did not require multiple cell divisions in order to reach nucleus (Fig. 3.7). We observed strong Bcl-2 mRNA silencing already after 1 day post-transfection, which remained unaltered for at least three days. Of note, the silencing by siRNA/LF lipoplex was also achieved as early as 1 day post-transfection, and lasted for at least 3.5 days.
Figure 3.7. The Bcl-2 mRNA silencing kinetics in HCT-116 cells transfected with 1 µM of L-FANA or 0.05 µM of siRNA/LF in Opti-MEM medium overnight. Knockdown efficacy by L-FANA conjugate and siRNA/LF complex remained stable when extending the post-transfection time from 1 to 3.5 days (no statistically significant difference). Results are expressed as mean ± SD (n=3-4).

3.4 Discussion

Local delivery of nucleic acid drugs is a promising therapeutic strategy against intestinal diseases but faces numerous challenges associated with the complexity of the GI environment. In the present work, we sought to identify a potent and robust nucleic acid delivery platform based on amphiphilic ON conjugates. A set of L-ON conjugates targeting a model Bcl-2 mRNA was prepared using chemically modified AONs and siRNAs. The selected sequence of the AONs was based on oblimersen, a drug previously tested in numerous clinical trials, which has a thoroughly optimized sequence site. The sequence of siRNAs was tested in several previous studies from our laboratory showing efficient Bcl-2 mRNA and protein silencing when delivered by various particulate nanocarriers (63, 74, 112). Although all L-ON conjugates carried the same DSA lipid moiety, they induced different degrees of cytotoxicity. This could be attributed to the modification-dependent protein binding of ONs (314, 315) or Bcl-2-level-dependent cell death (27). Superior silencing efficacy of L-FANA and L-MOE conjugates compared to those of L-DNA and L-OMe may be attributed to a combination of factors including higher nuclease resistance and higher
affinity for the complementary mRNA (313, 318, 319). By introducing a few chemically modified sugar rings in the structure of AON, we could drastically increase its potency. It is worth mentioning that the order of efficacy of different ON modifications could be different for other sequences/targets. Additional efforts to increase ON's affinity towards the target mRNA could enhance L-ON silencing efficacy even further.

A fully phosphorothioated L-PS siRNA conjugate showed significant Bcl-2 silencing upon carrier-free transfection, while PS siRNA without DSA did not, suggesting that the uptake of L-PS siRNA was mediated by the lipid moiety. A phenomenon called gymnosis was recently reported in which target genes are sequence-specifically suppressed in cells by chemically modified ONs, such as locked nucleic acids and FANA, in the absence of transfection reagents or delivery moiety conjugation. For an efficient and non-toxic gymnastic transfection, prolonged exposure of proliferating cells (6-10 days) to high (µM) concentrations of ONs is required (35, 320). Considering that the average transit times in the small and large intestines of healthy humans are 3 h (321) and 27 h (322) respectively, gymnastic ON delivery is likely to be too slow for intestinal tissues, at least in the absence of frequent dosing. Moreover, the renewal cycle of intestinal epithelial cells lasts barely 3-5 days (323), therefore the intestinal ON therapy needs an efficient delivery strategy to allow the rapid knockdown of the target mRNA and protein. In the present study, DSA conjugation to ONs promoted the delivery of single-stranded antisense ONs and even double-stranded siRNAs during limited exposure times. In contrast to previous findings on amphiphilic siRNAs showing their prolonged endosomal entrapment (> 3 days) (117), DSA-FANA conjugate exhibited rapid target mRNA knockdown which lasted for more than 3 days post-transfection. The knockdown was dose-dependent in two intestinal cells lines, and longer incubation with L-FANA conjugate resulted in stronger silencing. Overall, these results present L-ON conjugates as a promising nucleic acid delivery tool capable of gene silencing in intestinal cells.
4 Effect of intestinal conditions on transfection

4.1 Introduction

Amphiphilic lipid-ON conjugates can bind to the proteins that function as lipid carriers, such as low-density lipoproteins (113), chylomicrons (115), or albumin (112, 116). Notably, a previous study from our group has demonstrated that their binding to albumin could be reversed via addition of excess of short-chain fatty acid in the medium, successfully releasing the amphiphilic ON conjugate (112). Due to their binding to serum proteins, these amphiphilic conjugates might constitute an effective delivery approach primarily for liver targeting or topical applications with reduced serum content (105, 112, 121–126). However, their applicability to the intestinal mucosa remains questionable since the harsh GI environment containing digestive enzymes coupled with the known difficulties transfecting differentiated epithelium in vitro suggests that ON delivery will be limited (Scheme 4.1) (160, 324–331). Moreover, the presence of food-derived fat in the intestine could potentially act as sink for lipid-ON conjugates, preventing their cell uptake (Scheme 4.1).

In the present work, we investigated the integrity and transfection efficiency of amphiphilic conjugates under conditions mimicking the intestinal environment (inclusion of food-derived fats (soybean oil emulsion) and pancreatin-derived digestive enzymes). To investigate the silencing of L-FANA in polarized intestinal epithelium, the non-FAE model based on differentiated Caco-2 cells was chosen. It is widely used in pharmaceutical laboratories both in academia and in industry, as it has proven to be an adequate and simple model to assess drug metabolism, toxicity, and intestinal absorption/permeation.

4.2 Materials and Methods

4.2.1 Materials

HCT-116 cell line was kindly provided by Prof. Azzalin’s group at ETH Zurich and Caco-2 cells were initially purchased from ATCC (Manassas, VA). DMEM with GlutaMAX™ medium, Opti-MEM medium, fetal bovine serum (FBS), non-essential amino acids, penicillin-streptomycin solution, trypsin, LF, phosphate-buffered saline (PBS; 1 mM KH₂PO₄, 3 mM Na₂HPO₄, 155 mM NaCl, pH 7.4), and RNase-free distilled water were obtained from Invitrogen (Carlsbad, CA). Porcine pancreatin (4×USP), ammonium persulfate, TEAA buffer 1 M, Intralipid® (20% (w/v) of soybean oil, 1.2% of egg yolk phospholipids, 2.25% of glycerol, pH 6-8.9; mimic of high-fat meal), chloroform (CHCl₃) Triton X-100, NaF, Na₃VO₄, Tris, and skim milk powder were obtained from Sigma-Aldrich (Buchs, Switzerland). Sodium decanoate was purchased from TCI (Tokyo, Japan). Duplex annealing buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5) was purchased from Integrated DNA Technologies (IDT, Coralville, IA). Potassium dihydrogen phosphate was obtained from Merck (Kenilworth, NJ). MeOH, N,N,N′,N′-tetramethylethylenediamine (TEMED), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Acros Organics (Geel, Belgium). HFIP was obtained from Fluorochem (Hadfield, UK). EDTA and polysorbate 20
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were purchased from AppliChem (Darmstadt, Germany). Gel Red nucleic acid gel stain was obtained from Biotium (Hayward, CA). MycoAlert™ PLUS Mycoplasma Detection Kit was purchased from Lonza (Basel, Switzerland). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Rockville, MD). Thermanox™ coverslips, DNA loading dye, NaCl, and micro BCA™ protein assay kit were purchased from Thermo Fisher Scientific (Waltham, MA). Transwell® inserts were obtained from Corning Inc. (Corning, NY). Low-binding microcentrifugation tubes (DNA Lobind®) were purchased from Eppendorf-Vaudaux (Schönenbuch, Switzerland). RNeasy Mini kit and specific primers for α-splicing variant of human Bcl-2 mRNA (Hs_BCL2_1_SG; QT00025011) and human β-actin (Hs_ACTB_2_SG; QT01680476) were obtained from Qiagen (Valencia, CA). High-capacity cDNA reverse transcription kit and Power SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA). Complete® EDTA-free protease inhibitors® cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Poly(vinylidene difluoride) membranes (PVDF) were obtained from Bio-Rad Laboratories (Hercules, CA). Mouse anti-human Bcl-2 monoclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG polyclonal antibody were purchased from Dako (Glostrup, Denmark). Rabbit anti-β-actin polyclonal antibody and HRP-conjugated goat anti-rabbit IgG polyclonal antibody were obtained from Abcam (Cambridge, UK). ImmunoCruz® Western blotting luminol reagent was purchased from Santa Cruz Biotechnology (Dallas, TX). Super RX X-Ray films were obtained from Fujifilm (Tokyo, Japan).

4.2.2 Cell culture

HCT-116 cells were maintained in DMEM medium supplemented with GlutaMAX™ containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a 5%-CO₂ humidified atmosphere. The cells were seeded in a 24-multiwell plate at a density of 4 × 10⁴ cells/well. Caco-2 cells were maintained in DMEM medium supplemented with GlutaMAX™ containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1% of non-essential amino acids at 37 °C in a 5%-CO₂ humidified atmosphere. For the differentiated Caco-2 cell monolayer transfection, the cells with passage number between 58 and 77 were seeded in Transwell® inserts with polyester membrane with pore size of 0.4 μm in a 12-multiwell plate at a density of 1.12 × 10⁵ cells/well as previously described (332). The medium was exchanged every other day, and cells between 13 and 17 days of differentiation were used for the experiments. The differentiation of monolayers was monitored by measuring TEER using an EVOM epithelial voltmeter with STX2 electrode (World Precision Instruments, Sarasota, FL).
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All the monolayers achieved TEER higher than 1000 Ωcm² after the two weeks of culturing, indicating the completion of differentiation process. TEER values of individual wells measured just before the transfection with ONs were set to 100% and their change was monitored for the three following days.

All experiments were performed on mycoplasma-free cell lines (regularly checked by MycoAlert™ PLUS Mycoplasma Detection Kit), and only cells in the exponential phase of growth were used for seeding.

4.2.3 Cytotoxicity assay

For the L-ON cytotoxicity experiments, Caco-2 cells were seeded in a 96-multiwell plate, and the culture medium was exchanged every other day for two weeks to obtain differentiated monolayers. The cells were treated with various concentrations of L-ON conjugates in 50 μL of serum-deficient Opti-MEM medium for 15 h. The Opti-MEM medium containing no L-ON was used as a control. The medium was exchanged for 100 μL of DMEM supplemented with 10% FBS, and the cells were further incubated for 24 h, after which the cell viability was assessed using tetrazolium-based CCK-8 reagent following the manufacturer's instructions.

4.2.4 Interaction of FANA and L-FANA with oil

For polyacrylamide gel electrophoresis (PAGE) analysis of ONs interaction with an oil-in-water emulsion, 1 μL of FANA or L-FANA (100 μM) was mixed with 9 μL of emulsion (Intralipid®) diluted with Opti-MEM to 0-2% of soybean oil, incubated for 30 min at room temperature, and mixed with 1 μL of DNA loading dye. Samples were then loaded onto 20% (w/v) polyacrylamide gel prepared in a Tris-acetate-EDTA buffer (TAE: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) by free-radical polymerization with ammonium persulfate/TEMED as an initiator. Gel was then immersed in TAE buffer and electrophoresed at constant voltage of 150 V for 60 min. ONs were revealed following manufacture's protocol for Gel Red nucleic acid gel stain and fluorescence was recorded on a ChemiDoc XRS.

4.2.5 L-FANA conjugates stability in simulated intestinal fluid (SIF) and soybean oil emulsion

To investigate the influence of digestive enzymes and food-derived fats present in intestine on the biological function of L-FANA, its stability and efficacy were tested in simulated intestinal environment containing pancreatic enzymes and lipids. For PAGE analysis 1 μL of L-FANA (100
μM) was mixed with 9 μL of USP SIF (2.5 g/L porcine pancreatin (4×USP), 50 mM KH₂PO₄, pH 6.8) or water and incubated for 2 or 15 h at 37 °C. Samples were kept at 95 °C for 15 min to heat-inactivate the enzymes and mixed with 1 μL of DNA loading dye. Alternatively, 1 μL of L-FANA was pre-mixed with 1 μL of 5% soybean oil emulsion in Opti-MEM (prepared by diluting 20% Intralipid® with Opti-MEM) for 30 min followed by incubation at 37 °C for 2 h with 8 μL of active/heat-inactivated SIF or water. Samples were then loaded onto 20% (w/v) polyacrylamide gel prepared in a TAE buffer by free-radical polymerization with ammonium persulfate/TEMED as an initiator. The gel was then immersed in TAE buffer and electrophoresed at constant voltage of 150 V for 60 min. L-FANA was revealed following manufacturer's protocol for Gel Red nucleic acid gel stain, and fluorescence was recorded on a ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA).

For the LC-MS analysis, 2 μL of L-FANA (100 μM) were mixed with 18 μL of SIF or water and incubated for 2 h at 37 °C, then at 95 °C for 15 min, and centrifuged at 14000 × g at room temperature for 10 min. The supernatants were mixed with 50 μL of CHCl₃, shaken to extract possible hydrophobic contaminants from SIF. After incubating the mixtures at room temperature for a few minutes, water layers were collected and stored at -20 °C until analysis. Analytical LC-MS was carried out on Agilent 1200/6130 system (Agilent Technologies, Santa Clara, CA) using a reverse phase column (Waters Acquity OST C18, 2.1 x 50 mm, 1.7 μm; Waters Corporation, Milford, MA) with solvent A being 400 mM HFIP and 15 mM TEAA in water, and solvent B being MeOH. The flow was set at 0.3 mL/min and a gradient was run at 65 °C from 5% to 90% B in 14 min.

### 4.2.6 Transfection of L-FANA pre-incubated with SIF and soybean oil emulsion

The influence of pre-incubation of L-FANA conjugates with SIF on the transfection efficacy was investigated. HCT-116 cells were seeded one day prior to the experiment in a 24-multiwell plate at a density of 4 × 10⁴ cells/well in DMEM containing 10% FBS. Five microliters of L-FANA were pre-incubated with 45 μL of SIF for 2 h at 37 °C followed by heat-inactivation of enzymes for 15 min at 95 °C. The mixtures were diluted ten times with Opti-MEM to a final L-FANA concentration of 1 μM, and incubated with cells overnight (15 h). ON-free Opti-MEM medium was used as a control. Subsequently, the transfection medium was changed to DMEM supplemented with 10% FBS. After 2.5 days of further incubation, total RNA was isolated and expression levels of Bcl-2 mRNA were assessed as described above. To study the silencing activity of L-FANA released from SIF-digested emulsion, 2.5 μL of L-FANA (100 μM) were pre-incubated
with 6.25 µL of 20% Intralipid® for 30 min at room temperature followed by incubation with 25 µL of SIF for 2 h at 37 °C. The released L-FANA was separated from cytotoxic oil digestion products by preparative PAGE before transfection experiments. Gels were prepared and electrophoresed as described above. After the electrophoresis, the L-FANA band was excised, weighed, and immersed in 250 µL of Opti-MEM. For L-FANA extraction, samples containing gel pieces were flash-frozen in liquid nitrogen, heated at 90 °C for 15 min, and agitated at room temperature for 3 h. The concentration of extracted L-FANA in the supernatant was 0.6 µM. The supernatants were incubated with cells overnight (15 h). The expression levels of Bcl-2 mRNA were assessed as described above.

4.2.7 Transfection of differentiated Caco-2 cell monolayers

The differentiated Caco-2 cell monolayers grown in Transwell® inserts for two weeks were washed with Opti-MEM medium from apical and basal sides. The 500 µL of Opti-MEM medium containing either naked ON derivative (0.625-5 µM) or siRNA (0.2-0.4 µM) complexed with LF according to the manufacturer’s instructions was added to the apical compartment. The basal chamber was filled with 1.5 mL of Opti-MEM medium. The Opti-MEM medium in both compartments was used as a control. Following the overnight incubation (15 h), the transfection medium was exchanged with fresh DMEM supplemented with 10% FBS in both compartments. For Bcl-2 mRNA expression analysis by qRT-PCR, cells were lysed 2.5 days after transfection as described above. For Bcl-2 protein expression analysis by Western blot, cell monolayers were lysed 4 days after transfection in 25 µL of lysis buffer (20 mM Tris–HCl pH 7.7, 150 mM NaCl, 5 mM EDTA, 1% v/v Triton X-100, 25 mM NaF, 1 mM PMSF, 1 mM Na3VO4 supplemented with Complete® protease inhibitors) in line with previously described method (63). Briefly, cell lysates were scraped from the Transwells®, centrifuged at 10,000 × g for 15 min at 4 °C to remove cell debris, and protein concentration in the supernatants was determined by the micro BCA assay according to the manufacturer's instructions. Fifty micrograms of total protein per sample were resolved on 12% SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was washed once with Tris-buffered saline buffer supplemented with polysorbate 20 (Tween-20) (TBS-T) (20 mM Tris–HCl pH 7.7, 150 mM NaCl, 0.1% v/v polysorbate 20) and blocked with TBS-T containing 5% w/v skim milk (blocking buffer) for 1 h. The membrane was cut in two at 35 kDa; the lower part containing Bcl-2 (26 kDa) was incubated with anti-Bcl-2 antibody diluted to 1:100 in blocking buffer, and the upper part containing β-actin (42 kDa, loading control) was incubated with anti-β-actin antibody diluted to 1:4000 overnight at 4 °C. Membranes were washed 3 times for 5 min with PBS-T followed by 1.5-h incubation with the corresponding HRP-conjugated secondary antibodies diluted to 1:4000 in blocking buffer. Membranes were
washed 3 times with TBS-T, and protein bands were detected with ImmunoCruz® luminol reagent and revealed on Super RX X-Ray films using an AGFA Curix 60 film processor (AGFA, Mortsel, Belgium). The relative intensities of the bands were analyzed using Image J software (National Institutes of Health, Bethesda, MD).

4.2.8 Statistical analysis

All treatment groups were compared pairwise using the one-way ANOVA test combined with Tukey's (Holm-Sidak) post-hoc test assuming normal data distribution. The statistical analysis was performed using SigmaPlot software. The differences between treatment groups were considered statistically significant at \( p \)-values below 0.05.

4.3 Results

4.3.1 Stability and silencing properties of L-FANA in SIF

The local intestinal gene silencing poses specific hurdles for nucleic acid delivery systems. Upon arrival to the desired GI site of action via enteric formulations, endoscopic tube, or enema, the L-ON conjugate will be exposed to the intestinal digestive (and/or bacterial) enzymes. Therefore, the delivery system and its nucleic acid cargo need to be stable against the attack of intestinal enzymes. To this end, we tested whether the pre-incubation of L-FANA conjugate in SIF containing pancreatic digestive enzymes would impair its transfection efficacy (Fig. 4.1). Importantly, L-FANA conjugate was stable even after 15 h of incubation with SIF, as assessed by the PAGE analysis (Fig. 4.1A), and the lipid moiety was not cleaved off upon incubation with pancreatic enzymes containing lipases (Fig. 4.1B). For the silencing experiments, L-FANA and its negative control (L-FANAnc) conjugates were incubated with pancreatic enzymes at pH of 6.8 for 2 h at 37 °C, and after the heat-inactivation of the digestive enzymes and dilution with cell culture medium the mixture was directly added to the HCT-116 cells. The difference between relative expression levels of target Bcl-2 mRNA after the treatment with L-FANA with or without pancreatin pre-incubation was not statistically significant (31% and 19%, respectively; Fig. 4.1C). This result shows that the pre-incubation of L-FANA conjugate with pancreatin did not strongly affect its silencing efficacy due to the enzymatic degradation or unspecific protein binding. Treatments with pancreatin alone or pancreatin with L-FANAnc did not change the target mRNA
expression, indicating that the silencing effect was caused by L-FANA in a sequence-specific manner.

Figure 4.1. The stability and silencing properties of L-FANA in SIF (pancreatin 1X USP). (A) PAGE images of L-FANA conjugate after incubation with SIF for 2 h and 15 h at 37 °C. Band at the top of the gel could correspond to the unspecific staining of insoluble material from pancreatin. (B) Results of LC-MS analysis of L-FANAnc conjugate in water and after 2 h incubation with SIF (1 and ii, respectively). L-FANAnc conjugate: retention time 12 min; mass 6417.9 and 6419.5 Da in water and SIF, respectively (theoretical 6419.3 Da). (C) Target Bcl-2 mRNA knockdown efficiency after the transfection of HCT-116 cells with L-FANA pre-incubated with SIF for 2 h; nc – L-FANAnc conjugate with non-specific sequence. Results are expressed as mean ± SD (n=3-4). *** p < 0.001 vs. Opti-MEM medium treated cells.
4.3.2 The effect of oil on L-FANA silencing properties

It was previously demonstrated that amphiphilic ON conjugates can bind to lipid-containing particles (e.g., low-density lipoprotein or chylomicrons (113, 115)), implying the possibility of interaction of L-ON conjugates with food-derived fats in the intestine. Using a gel-based binding assay, we observed that L-FANA indeed bound to soybean oil in a concentration-dependent manner, while the migration of unconjugated FANA was not retarded in the presence of oil (Fig. 4.2).

![Figure 4.2](image.jpg)

**Figure 4.2.** PAGE images of L-FANA conjugate and unconjugated FANA incubated with oil-in-water emulsion containing 0-2% of soybean oil in Opti-MEM for 30 min. The interaction is mediated by the docosanoyl moiety of L-FANA conjugate, as FANA without lipid group did not bind to the emulsion.

The decrease of silencing efficacy observed for L-FANA in the presence of the emulsion could be attributed to the interaction between the oil and the lipid moiety of the conjugate interfering with the cellular uptake (Fig. 4.3A, B). However, the interaction of L-FANA with the emulsion could be disrupted after the digestion of the oil droplets by the SIF (Fig. 4.3C). Importantly, the released L-FANA from SIF-digested oil fully preserved its silencing capacity (Fig. 4.3B). This finding suggests that the interaction of amphiphilic ONs with food-derived fat would probably not hamper their cellular uptake and subsequent silencing activity.
4.3.3 L-FANA silencing properties in differentiated intestinal monolayer

Intestinal epithelial cells possess characteristics that make their *in vivo* transfection by non-viral and non-bacterial carriers rather challenging; they are polarized cells that express tight
junctions and microvilli, and have reduced proliferative and endocytic capacity compared to undifferentiated cancer cells (323, 333–336). Caco-2 carcinoma cells are able to differentiate into such monolayer system, and have been used as a model to assess gene delivery efficacy to polarized intestinal epithelium (330). To compare the delivery of modified ON into proliferating cells and difficult-to-transfect epithelial cell monolayers, the L-FANA conjugate was tested on differentiated Caco-2 cells. To illustrate the resistance to transfection of fully differentiated Caco-2 cell monolayers, experiments were performed with the Bcl-2-targeting siRNA complexed with LF (Fig. 4.4A). Transfection of siRNA/LF at a concentration of 0.2 µM elicited a small decrease of Bcl-2 mRNA expression (21%, Fig. 4.4B), which was not accompanied by its downregulation at the protein level (Fig. 4.4C). We could not achieve dose-dependent Bcl-2 mRNA knockdown even with siRNA concentrations as high as 0.4 µM, at which the control siRNAnc possessing a target-unrelated sequence caused appreciable suppression of Bcl-2 mRNA, probably due to the toxicity caused by high concentrations of LF (327). In contrast, Bcl-2 silencing was successfully achieved in proliferating Caco-2 cells at a dose of 0.05 µM of siRNA (Fig. 4.5).

Strikingly, the single-molecule-based L-FANA conjugate was able to knockdown Bcl-2 mRNA in differentiated Caco-2 cell monolayers in a sequence-specific and dose-dependent manner (Fig. 4.4B). At L-FANA concentrations of 1.25, 2.5, and 5 µM the target Bcl-2 mRNA expression was reduced to 56%, 32%, and 26% of the initial level, respectively. At 5 µM the negative control L-FANAnc was inactive. Conjugation of DSA was found to be essential for successful delivery, as unconjugated FANA did not have any effect on Bcl-2 mRNA expression even at 5 µM. Consistent with mRNA silencing, western blot analysis demonstrated that L-FANA reduced efficiently the target Bcl-2 protein level (by approximately 53% at 2.5 µM; Fig. 4.4C).
**Figure 4.4.** *In vitro* evaluation of L-FANA silencing properties for differentiated intestinal monolayer delivery. (A) Schematic representation of L-FANA and siRNA/LF transfection to differentiated intestinal epithelium. (B) Target Bcl-2 mRNA knockdown efficiency by transfection of differentiated Caco-2 cell monolayers with L-FANA and siRNA/LF lipoplex in Opti-MEM medium, nc – corresponding negative controls L-FANAnc and siRNAnc with non-specific sequences. Results are expressed as mean + SD (n=3-5). ***p < 0.001, **p ≤ 0.01 vs. Opti-MEM medium treated cells, **p < 0.01 between two treatment groups. (C) Western blot assay of Bcl-2 and β-actin protein expression after transfection of differentiated Caco-2 cell monolayers with L-FANA (2.5 µM), siRNA/LF (0.2 µM), and their control groups in Opti-MEM medium.
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Figure 4.5. Transfection of proliferating Caco-2 cells with siRNA/LF and negative control siRNA (siRNAnc)/LF lipoplexes in Opti-MEM medium at a dose of 0.05 µM for 15 h. This result shows that the Bcl-2 mRNA silencing by siRNA is sequence-specific. Results are expressed as mean ± S.D. (n=3-4). ** *p < 0.01 vs. Opti-MEM medium treated cells.

Importantly, the treatment with relatively high dose of L-FANA neither caused cytotoxicity (Fig. 4.6A) nor decreased the TEER of Caco-2 monolayers (Fig. 4.6B), which is a measurable indicator of the monolayer integrity (337). In comparison, the treatment with sodium decanoate, a widely used absorbefacient disrupting the tight junctions (8), caused a pronounced drop in TEER. Based on this result, it is likely that L-FANA conjugates will be taken up primarily by epithelial cells in vivo with minimal systemic translocation via paracellular route.

Figure 4.6. In vitro evaluation of L-FANA toxicity on differentiated intestinal epithelium. (A) Viability of differentiated Caco-2 monolayer cells after treatment with 1-5 µM of L-FANAnc in Opti-MEM medium. Results are expressed as mean ± SD (n=3). (B) TEER change upon overnight treatment (15 h) with FANA derivatives (5 µM) or permeation enhancer sodium decanoate (DecNa; 4 mM) in Opti-MEM medium. Results are expressed as mean ± SD (n=3-4). *** *p < 0.001, ** *p ≤ 0.01 vs. Opti-MEM medium treated cells.
4.4 Discussion

For a successful in vivo delivery of ONs to intestinal epithelium, L-ON conjugates must remain functional in the intestinal environment, and they should be able to silence target genes in difficult-to-transfect epithelial cell monolayers. One concern regarding their biological activity in the intestine was that amphiphilic ON conjugates might bind to intestinal lipophilic content, potentially leading to reduced activity. Here, in an in vitro model system, we demonstrated that free L-FANA is released from oil phase after digestion by pancreatic enzymes and retains its silencing ability. The combination of the extreme stability of FANA-modified ONs towards nucleases (313) and hydrolysis at low pH (338), together with the high stability of DSA under similar conditions found in the GI tract (339) makes DSA-FANA conjugates particularly suitable for GI applications.

Differentiated intestinal epithelial cells featuring microvilli and expressing tight junctions represent a difficult target for nucleic acid delivery (160, 324–330). Their reduced proliferation and endocytosis rate in comparison to undifferentiated cells are largely responsible for poor transfection efficacy (335, 336). Regarding particle-based delivery systems, the resistance to transfection observed with differentiated cells may be partially attributed to the structure of their apical membrane featuring microvilli, which limits access to the absorptive membrane (330, 331). Several studies have described a variety of attempts to overcome this barrier by, for example, pre-treatment with membrane-disturbing agents (330), transfection of proliferating Caco-2 in the suspension state followed by accelerated differentiation (325), formulation with small lipid nanoparticles (331), use of β1-integrin-mediated endocytosis (55) or by electroporation (324). As previously reported for siRNA/LF, we were unable to efficiently downregulate Bcl-2 mRNA in polarized Caco-2 cell monolayers with these lipoplexes. In contrast, the single-molecule-based L-FANA conjugate was able to effectively silence the Bcl-2 gene expression at both mRNA and protein levels in differentiated monolayers. Importantly, despite lower potency of carrier-free amphiphilic conjugates in comparison to siRNA/LF lipoplexes in classical in vitro assays based on rapidly proliferating cancer cells, they by far outperform particulate delivery vehicles under more stringent conditions, such as the inverted setup or using differentiated cells. The involvement of cell surface receptors, such as fatty acid receptors, better access of L-FANA to the adsorptive cell membrane due to the smaller size, or other uptake routes different from LF-mediated endocytosis may explain the efficient target silencing by L-FANA conjugate in a non-dividing polarized epithelium. As mentioned previously, several studies have extrapolated the possible internalization routes of amphiphilic ON conjugates in proliferating cells, including adsorptive endocytosis, SID-
1 or β2-integrin-mediated endocytosis, depending on the cell line and/or the lipophilic moiety tested (112, 113, 117–121). Moreover, it is possible that the uptake mechanism and intracellular trafficking can vary among different states of cell differentiation (328); therefore, further studies are necessary to elucidate the uptake mechanism of L-FANA by the differentiated epithelium.
5 Conclusions and Outlook

In order to fully exploit the therapeutic potential of nucleic acid drugs and facilitate their clinical translation, non-toxic delivery strategies that efficiently overcome extracellular and intracellular barriers must be developed. Such delivery systems should be tailored according to the administration route and target tissue. Some success has already been achieved for the treatment of liver disorders with chemically stabilized nucleic acids or particle- or -based delivery systems, that are administered i.v. or subcutaneously. Notwithstanding, the development of oral formulations would be the most desirable way to bring these therapies forward, especially for patients with chronic disorders requiring regular dosing. However, the oral administration of nucleic acids requires their systemic translocation from the GI tract and current attempts to enable this are still quite far from reaching the clinic as they often involve complex delivery systems with questionable safety profiles.

On the other hand, the local delivery of ON therapeutics stands a better chance for clinical translation in a nearer future. The availability of the GI mucosa makes it particularly attractive for the local delivery of nucleic acid therapies. Indeed, high doses of ONs can be delivered directly to the disease site in the GI tract without the risk of complement activation or systemic target silencing. However, important barriers have to be considered, such as the thick mucus layer hampering the diffusion of delivery systems to the epithelium, the high enzymatic activity leading to nucleic acid degradation, and the tight junctions and microvilli of intestinal cells further limiting ON's uptake. Several particle-based vehicles have been investigated for the local delivery of nucleic acids to the intestinal mucosa, the majority of which relies on their uptake by the intestinal M cells and subsequently by the underlying macrophages to disseminate them to remote organs. This strategy could be applied for the treatment of inflammatory diseases, although not without the risk of systemic immunosuppression. However, other GI disorders such as familial adenomatous polyposis, cannot be treated using this approach as the therapeutic targets are located in the epithelial cells. Recently, a bacteria-mediated strategy for gene silencing in the intestinal epithelium has been proposed (55, 56), but potential issues associated with immunogenicity of the invasive
bacteria and reproducibility of dosage have to be considered. In principle, carrier-free nucleic acid drugs should have higher mucosal permeability in comparison to particulate carriers, as well as more predictable dose-effect relationships and more controllable synthesis and characterization processes. Additionally, their distribution within the intestinal lumen should be more homogeneous and unaffected by precipitation, leading to steady and evenly distributed gene silencing.

In this work, amphiphilic conjugates were tested for the first time to achieve target gene silencing in an intestinal epithelium model. The main findings have been summarized in Fig. 5.1. A series of amphiphilic conjugates consisting of DSA and nucleic acids with different architectures and mechanisms of action (single-stranded AONs and double-stranded siRNAs) was synthesized to explore the capacity of DSA as a delivery moiety. To compare their efficacy, the ONs were designed to target a model mRNA of the oncoprotein Bcl-2. While the different conjugates exhibited various degrees of cytotoxicity and target silencing, the L-FANA conjugate was selected as the most potent candidate with a favourable safety profile in an in vitro model. Presumably, although the FANA-modified conjugate was slightly better in terms of Bcl-2 mRNA silencing in comparison to the OMe- and MOE-modified counterparts, the order of efficacy could be different for other sequences/targets.

Further investigation of the silencing properties of the L-FANA conjugate in two colon carcinoma cell lines demonstrated that the delivery of amphiphilic ON conjugates was indeed mediated by the DSA group, since the unconjugated control failed to significantly downregulate the target mRNA. The rapid mRNA silencing induced by L-FANA was sequence-specific and dose-dependent, and lasted for more than 3 days. This well-defined single-molecule-based approach was compared to conventionally employed lipoplexes in an inverted transfection setup. It was found that the silencing efficacy of L-FANA was not altered by inverting the orientation of cells, whereas the knockdown induced by lipoplex particles was completely abolished, probably due to sedimentation. This suggests sedimentation-independent uptake mechanism for L-FANA conjugate, which could potentially lead to more homogeneous gene silencing within the intestinal lumen in vivo.

The oral administration of ON delivery systems implies that they retain the silencing ability in intestinal conditions. Accordingly, the L-FANA conjugate was stable in presence of digestive enzymes and its silencing activity was preserved. Indeed, the conjugate's interaction with an oil-in-water emulsion used to mimic the content of the intestinal tract was successfully disrupted upon treatment with pancreatin, which released the intact L-FANA conjugate and restored its silencing efficacy. The most encouraging finding was however that the L-FANA conjugate was able to
silence the Bcl-2 mRNA in the difficult-to-transfect fully differentiated Caco-2 cell monolayers. In this aspect our single-molecule-based approach was remarkably superior to conventionally employed lipoplexes, which were unable to transfect the intestinal epithelium. To the best of our knowledge, this is the first study showing the efficient gene silencing in polarized epithelial cells by an ON conjugate in the absence of complexation, delivery device, or membrane-disturbing agents. The TEER measurements of the differentiated intestinal monolayer revealed that L-FANA conjugates did not disturb tight junctions, which is necessary for high molecular weight drugs to achieve systemic bioavailability via paracellular transport. Therefore, there is low probability of systemic translocation during L-FANA treatment in vivo, thus limiting potential systemic off-target events and allowing these conjugates to be taken up primarily by cells lining up the intestinal mucosa. Importantly, although the L-FANA conjugate was selected as the most potent one based on mRNA silencing efficacy, the general conclusions drawn from this study regarding binding to fats and monolayer transfection are likely valid for other stabilized L-ON conjugates.

Overall, we have demonstrated successful intestinal epithelium transfection with single-molecule-based conjugates without the help of transfection or membrane-disturbing agents. This is a valuable achievement not only in view of its future pharmaceutical development but also of its application in molecular biology research, where it provides a carrier-free tool to study gene functions in differentiated intestinal epithelium. In the current work, the in vitro experiments were thus designed from a pharmaceutical technology perspective (e.g., interactions with food-derived fats, degradation by intestinal enzymes), and the characterization data gathered should serve as a basis for future in vivo experiments in suitable GI disease models.

Future work should focus on better understanding the trafficking of the L-ON in proliferating and differentiated cells, as well as elucidating its mechanism of uptake. This could be achieved by tracking the fluorescently/radioactively labelled L-ON conjugates or by using the endocytosis inhibitors for instance. Additionally, even though the bacterial load in the small intestine is relatively low, the colon hosts billions of bacterial cells and their effect on the L-ON interaction with the intestinal epithelium should thus be studied. Finally, while we have presented a proof-of-concept study in an in vitro model targeting the Bcl-2 mRNA, the L-ON silencing properties should be tested in vivo using other clinically relevant targets, such as ICAM-1 or Smad7 for the treatment of IBDs. The head-to-head comparison of efficacy of L-ON conjugates and unconjugated ONs will also be necessary to validate the efficacy of this approach. Further development of this system could include the attachment of a targeting ligand such as FANA aptamer, to enhance the cell uptake and add cell-type specificity to the system. Based on the main findings of this thesis, L-ON conjugates like L-FANA represent a suitable approach to further improve the efficacy of ON
CHAPTER 5: CONCLUSIONS AND OUTLOOK

enteral treatments currently under investigation, a road which we hope to have paved with the promising results presented herein.

Figure 5.1. Summary of the main findings of the present thesis. (A) DSA is a versatile delivery carrier for AONs and siRNAs, while the L-FANA was the most promising conjugate. (B) L-FANA preserved its silencing efficacy in the inverted transfection setup in contrast to a conventional particulate lipoplex-based carrier. (C) Given the digestion-resistant properties of L-FANA, pancreatic enzymes could disrupt its interaction with food-derived fats and restore its silencing efficacy. (D) L-FANA could efficiently silence the target Bcl-2 gene in the differentiated epithelium in contrast to the siRNA lipoplex.
6 Appendix

6.1 Synthesis of L-MOE and L-siRNA conjugates

Instruments, reagents, and conditions for standard phosphoramidite solid-phase synthesis were used for the synthesis of the L-MOE, L-PO sense strand of the siRNA, and L-PS siRNA (L-PS sense strand of the siRNA, PS antisense strand of the siRNA) as previously described (32). RNA, MOE, and DNA phosphoramidites were prepared as 0.08, 0.08, and 0.16 M solutions in dry ACN, respectively. 5-(Benzythio)-1H-tetrazole (0.24 M in ACN) was used to activate the phosphoramidites for coupling. Phosphoramidite coupling times were 2 × 120 s for DNA and 2 × 180 s for MOE. In case of PO and PS RNAs, the coupling times were 2 × 120 s and 2 × 90 s, respectively. For DSA conjugation, first a MMT-protected 6-amino-hexanol phosphoramidite linker was attached to ONs’ 5’ end using a regular coupling cycle and coupling time of 2 × 180 s, followed by three consecutive detritylation steps for MMT removal (3% DCA in DCM, 3 × 60 s). Prior to DSA coupling to MOE or PO sense strand of the siRNA, the solid support was washed three times with ACN, transferred to a screw tube and resuspended in 300 µL of anhydrous THF and DMF mixture (1:1 v/v) containing 17 mM of DSA, 34 mM of EDC HCl, and 160 mM of DMAP. The tubes were sealed with Parafilm M® and shaken overnight at 45 °C. Following the extensive washing with THF/DMF mixture, deprotection and cleavage of ONs from the solid support was accomplished with 200 µL of 25% aqueous ammonia for 16 h at 50 °C. Alternatively, in case of DSA coupling to PS sense strand of the siRNA, the solid support was successively washed with DCM (1 × 5 min), 5% DIPEA in DCM (5 × 1 min), DCM (5 × 1 min) and ACN (5 × 1 min). Subsequently, the solid support was transferred to an Eppendorf® tube and resuspended in 200 µL of anhydrous THF and DMF mixture (9:2 v/v) containing 0.25 M DSA, 0.25 M PyBOP, and 0.75 M DIPEA. The reaction mixture was shaken for 3 h at 45 °C, and washed with DMF and ACN. The deprotection and cleavage of L-PS sense strand of the siRNA from the solid support was accomplished with 500 µL of AMA (a 1:1 mixture of concentrated ammonium hydroxide and aqueous methylamine) at 65 °C for 20 min. The cleavage solution was recovered, the CPG was washed two times with 200 µL of aqueous EtOH (1:1 v/v), and all the liquid fractions were
combined and evaporated to dryness. After suspending the crude products in 200 µL of aqueous MeOH (10% v/v), the supernatants were purified by reverse phase HPLC. The purification of ONs was carried out on an Agilent HPLC using a reverse phase column (Waters XBridge OST C18, 10 × 50 mm, 2.5 µm) with solvent A being 100 mM TEAA in water, and solvent B being MeOH. HPLC flow was set at 5 mL/min and a gradient was run at 65 °C from 12.5 to 98% B in 11 min.

Analytical LC-MS was carried out on Agilent LC-MS using a reverse phase column (Waters Acquity OST C18, 2.1 × 50 mm, 1.7 µm) with solvent A being 400 mM HFIP and 15 mM TEAA in water, and solvent B being MeOH. The flow was set at 0.3 mL/min and a gradient was run at 65 °C from 8 to 95% B in 14 min for all the ONs except for the L-PS sense strand of the siRNA (7-80% B in 14 min).

6.2 Synthesis of L-DNA, L-FANA, negative control L-FANA conjugates, and FANA

Standard phosphoramidite solid-phase synthesis instruments, reagents, and conditions were used for the synthesis of the L-DNA, FANA, L-FANA, and negative control L-FANA (L-FANA n.c. (35)) as previously described (112). FANA and DNA phosphoramidites were prepared as 0.15 and 0.1 M solutions in dry acetonitrile (ACN), respectively. 5-ethylthiotetrazole (0.25 M in ACN) was used to activate the phosphoramidites for coupling. FANA phosphoramidite coupling times were 600 s, with the exception of guanosine, which was allowed to couple for 900 s, and DNA coupling times were 200 s, and 300 s for guanosine. For L-ON conjugates preparation, MMT-protected 6-amino-hexanol phosphoramidite was attached to ON 5′ end using a coupling cycle (without capping) with a coupling time of 600 s, followed by oxidation and detritylation (3% TCA in DCM, 240 s). Prior to the coupling with the DSA moiety, the solid supports were successively washed with 5% DIPEA in DCM and DCM. The solid supports were transferred to Eppendorf® tubes and resuspended in a solution of DCM containing 200 mM of docosanoic acid, 400 mM DIPEA, and 200 mM DIC. The Eppendorf® tubes were sealed with Parafilm M®, crowned with a cap lock, and shaken overnight at 40 °C. Following the excessive washing with DCM and DMF, deprotection and cleavage from the solid support was accomplished with 1 mL of concentrated aqueous ammonia and EtOH mixture (3:1 v/v) for 48 h. Cleavage solutions was removed under reduced pressure, the pellets were resuspended in 1 mL of autoclaved water, and the supernatants was purified by reverse phase HPLC. Purification of L-ON conjugates was carried out on an Agilent HPLC using a reverse phase C18 column (250 × 10 mm) with solvent A being 100 mM TEAA in water supplemented with 5% ACN (pH 7.0), and solvent B being ACN. HPLC flow was
set at 4 mL/min and a gradient was run at 50 °C from 0 to 100% B in 30 min. After collecting the target materials, volatile TEAA was removed by repeated triple co-evaporation with water and ethanol. Purification of FANA was performed using a Protein Pak DEAE 5PW analytical anion exchange HPLC column with a solvent A being water and solvent B being 1 M LiClO₄ in water. A gradient was run from 0 to 50% B in 46 min. Following purification, LiClO₄ salt was removed using illustra NAP-25 size exclusion columns (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol.

Analytical LC-MS analysis of FANA and L-ON conjugates was carried out on Agilent LC-MS using a reverse phase column (Waters Acquity OST C18, 2.1 × 50 mm, 1.7 µm) with solvent A being 400 mM HFIP and 15 mM TEAA in water, and solvent B being MeOH. The flow was set at 0.3 mL/min and a gradient was run at 65 °C from 8 to 95% B for all the ONs except for the L-FANA n.c. conjugate (10-90% B in 14 min). The data were processed and deconvoluted using the Bruker DataAnalysis software version 4.1.

6.3 Characterization of ONs

*General remarks:* Solvent A: H₂O with 400 mM HFIP and 15 mM TEAA; solvent B: MeOH.

PO sense strand of the siRNA:

3.6 min (C₁₈, gradient from 8 to 95% B in 14 min at 65 °C), 6641.3 Da (theoretical 6643.0 Da).
APPENDIX

L-PO sense strand of the siRNA:
10.8 min (C18, gradient from 8 to 95% B in 14 min at 65 °C), 7144.0 Da (theoretical 7145.4 Da).

PS sense strand of the siRNA:
3.7 min (C18, gradient from 8 to 95% B in 14 min at 65 °C), 6962.9 Da (theoretical 6964.2 Da).
**APPENDIX**

PS antisense strand of the siRNA:

3.9 min (C<sub>18</sub>, gradient from 8 to 95% B in 14 min at 65 °C), 7017.4 Da (theoretical 7016.4 Da).

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![Image of HPLC analysis for PS antisense strand](image1)

**L-PS sense strand of the siRNA:**

12.6 min (C<sub>18</sub>, gradient from 7 to 80% B in 14 min at 65 °C); 7465.0 Da (theoretical 7466.7 Da).

---

![Image of HPLC analysis for L-PS sense strand](image2)
APPENDIX

L-MOE:
11.0 min (C18, gradient from 8 to 95% B in 14 min at 65 °C), 6850.6 Da (theoretical 6848.7 Da).

L-DNA:
11.2 min (C18, gradient from 8 to 95% B in 14 min at 65 °C), 6186.0 (theoretical 6186.4).
L-FANAnc:
11.6 min (C18, gradient from 10 to 90% B in 14 min at 65 °C), 6417.7 Da (theoretical 6419.3 Da).

L-FANA:
11.1 min (C18, gradient from 8 to 95% B in 14 min at 65 °C), 6347.8 Da (theoretical 6348.3 Da).
APPENDIX

FANA:
4.4 min ($C_{18}$, gradient from 8 to 95% B in 14 min at 65 °C), 5845.3 Da (theoretical 5846.6 Da).
References


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REFERENCES

Cancer, 119, 4268–4276.


REFERENCES


List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
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<tr>
<td>AON</td>
<td>antisense oligonucleotide</td>
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<tr>
<td>ApoB</td>
<td>apolipoprotein B</td>
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<tr>
<td>ASGR</td>
<td>asialoglycoprotein receptor</td>
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<td>Bcl-2</td>
<td>human B-cell lymphoma 2 gene</td>
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<td>CD40</td>
<td>cluster of differentiation 40</td>
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<td>CPP</td>
<td>cell-penetrating peptide</td>
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<td>cRGD</td>
<td>cyclic pentapeptide Arg-Gly-Asp-d-Phe-Lys</td>
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<td>DCA</td>
<td>dichloroacetic acid</td>
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<td>sodium decanoate</td>
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<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DOTAP</td>
<td>N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride</td>
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<td>DSA; L</td>
<td>docosanoic acid</td>
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<td>DSS</td>
<td>dextran sulfate sodium</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>effective concentration causing 50% of target gene silencing</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>E. Coli</td>
<td><em>Escherichia Coli</em></td>
</tr>
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<td>EDC HCl</td>
<td><em>N</em>-(<em>3</em>-dimethylaminopropyl)-<em>N'</em>-ethylcarbodiimide hydrochloride</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>2'-deoxy-2'-fluoro-arabinonucleic acid</td>
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<td>FBS</td>
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<td>GalNAc</td>
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<td>HCO-60</td>
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<td>HFIP</td>
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<td>high performance liquid chromatography</td>
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<td>IBD</td>
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<td>IEC</td>
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<td>LC-MS</td>
<td>liquid chromatography–mass spectrometry</td>
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<td>LF</td>
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<td>LNA</td>
<td>locked nucleic acid</td>
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<td>lncRNA</td>
<td>long non-coding RNA</td>
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<td>L-ON</td>
<td>lipid-oligonucleotide conjugate</td>
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<td>LPS</td>
<td>bacterial lipopolysaccharide</td>
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<td>Map4k4</td>
<td>mitogen-activated protein kinase kinase kinase kinase 4</td>
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<td>mRNA</td>
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<td>NFκB</td>
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<tr>
<td>NiMOS</td>
<td>nanoparticles-in-microsphere oral system</td>
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<td>soybean oil-in-water emulsion</td>
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<td>ON</td>
<td>oligonucleotide</td>
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<td>PAMAM</td>
<td>poly(amidoamine)</td>
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<tr>
<td>$P_{\text{app}}$</td>
<td>apparent permeability coefficient</td>
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<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>phosphorothioate linkages</td>
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<td>PSMA</td>
<td>prostate-specific membrane antigen</td>
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<td>SELEX</td>
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<td>siRNA</td>
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<td>short hairpin RNA</td>
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<td>TEER</td>
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<td>VEGF</td>
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Curriculum Vitae

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Work experience

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Researcher, R&D projects on carbon nanotubes- and polyaniline-based composite materials, MSU – LG Chem Joint Laboratory, Moscow, Russia

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Research internship on interaction of DNA with oppositely charged carbon nanotubes, Graduate School of Environmental Studies, Nagoya University, Japan

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02/2013 – 05/2013  Teaching assistant at the galenik lab course

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English  fluent
German  advanced
Japanese  beginner
Scientific Contributions

Publications


Oral Presentations


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Lee S.H., **Moroz E.,** Castagner B., and Leroux J.-C. Cell penetrating peptide–peptide nucleic acid (CPP-PNA) conjugates for inflammatory bowel disease therapy. 2nd International Congress on Research of Rare and Orphan Diseases, Novartis Campus, Basel, Switzerland, 5 – 8 March 2014.


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