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# Nanomedicine-based delivery strategies for nucleic acid gene inhibitors in inflammatory diseases

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# NANOMEDICINE-BASED DELIVERY STRATEGIES FOR NUCLEIC ACIDS GENE INHIBITORS IN INFLAMMATORY DISEASES

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## Nucleic acids



ASO



siRNA

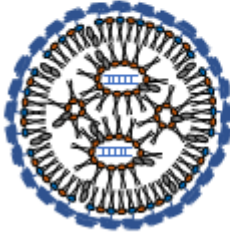


shRNA

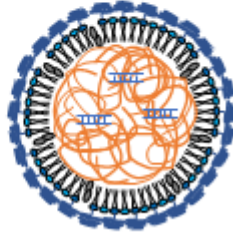


Aptamer

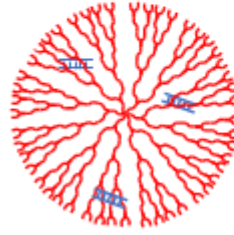
## Nanocarriers



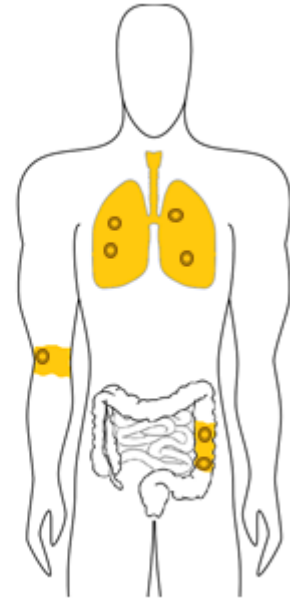
SNALPs



Polymer-lipid NP



Dendrimers



## **Abstract**

Thanks to their abilities to modulate the expression of virtually any genes, RNA therapeutics have attracted massive research efforts. Among the strategies focusing on nucleic acid gene inhibitors, antisense oligonucleotides and small interfering RNAs have reached advanced clinical trial phases with several of them having recently been marketed. These successes were obtained by overcoming stability and cellular delivery issues using either chemically modified nucleic acids or nanoparticles. As nucleic acid gene inhibitors are promising strategies to treat inflammatory diseases, this review focuses on the barriers, from manufacturing issues to cellular/subcellular delivery, that still need to be overcome to deliver them to sites of inflammation other than the liver. Furthermore, key examples of applications in rheumatoid arthritis, inflammatory bowel, and lung diseases are presented as case-studies of systemic, oral, and lung nucleic acid delivery are presented.

## **Keywords**

Gene inhibition; Nucleic acid; Nanoparticle; Inflammation

## 1 INTRODUCTION

In the last decades, nucleic acid-based gene inhibitors have attracted growing attention as therapeutic strategies due to their key abilities to modulate the expression of virtually any genes involved in human diseases [1]. Nowadays, the pipeline of almost all major pharmaceutical companies comprises an array of nucleic acid-based drugs controlling gene expression such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), microRNAs mimics (miRNAs), anti-microRNAs (Antagomirs), and aptamers (Apts) [1]. While only the latest block directly protein function, all the other classes can modulate the translation steps before the proteins are expressed. Whereas ASOs, Antagomirs or Apts can origin from RNAs or DNAs, all others are only RNAs. The most advanced nucleic acids in clinical trials are the ASOs, Apts, and siRNAs with most of the nucleic acids on the market having been identified for ocular delivery (*i.e.* Formivirsen<sup>®</sup> or Macugen<sup>®</sup>) or liver diseases (*i.e.* Onpattro<sup>®</sup>)[1].

Despite these pivotal clinical successes, the effective delivery of nucleic acids remains a major challenge, especially for extra-hepatic sites of disease. While local delivery may be considered for lung or oral delivery, reaching tissular and cellular sites other than the liver, after systemic administration, remain a key issue. Numerous studies have shown that extravasation and subsequent cell uptake of nanoparticles can preferentially occur in inflammatory sites due to the specific physiopathology and vascular enhanced permeability of most inflamed tissues. Nanomedicines can therefore be used to take advantage of these characteristics to address encapsulated molecules to the tissue of interest by passive diffusion [2,3].

Since nucleic acid-based gene inhibitors present great potentials in treating inflammatory disorders [4] such as rheumatoid arthritis (RA) [5], inflammatory bowel diseases (IBD) [6], sepsis [7], atherosclerosis [8], or respiratory inflammation [9], nanomedicines have to be tailored in agreement with the extravasation process at the inflammatory site and immune cells specificity. Among all the studies describing the use of nanomedicines to treat the aforementioned inflammatory diseases, most of them have focused on the inhibition of classical signaling pathways involved in inflammation and the secretion of proinflammatory cytokines among which, TNF- $\alpha$  has been particularly targeted due to its central role in the inflammatory processes [10].

In the present review, we propose to first discuss the development of different nanomedicine-based delivery strategies and the recent progress achieved in terms of i) stability of the nucleic

acids, ii) formulation processes and iii) intracellular trafficking while keeping in mind the biological specificities of inflammation-related diseases. Nucleic acids delivery applications in three inflammatory diseases *i.e.*, rheumatoid arthritis, inflammatory bowel, and lung diseases will then be discussed in light of the above characteristics. Those three examples were chosen as they provide strong case studies of respectively systemic, oral, and lung delivery and clearly demonstrated that inflammatory diseases can be one of the major fields for extrahepatic delivery of nucleic acid-based gene inhibitors.

## 2 NUCLEIC ACID-BASED GENE INHIBITORS

Nucleic acid-based gene inhibitors are a class of therapeutic molecules based on deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Table 1 provides the key differences between the different classes of nucleic acid-based gene inhibitors. In most cases, they are based on RNA or DNA molecules such in the case of ASOs and Antagomirs. Again, apart from Apts, most of them, single or double-stranded, have short sequences. Their targets are mainly cytoplasmic except for a few cases such as triple helix ASOs, that interact with genomic DNA. Finally, while some ASOs act as translation modulator/blocker and most Apts are developed to bind to proteins, all other aforementioned strategies lead to messenger RNA molecules (mRNA) degradation.

Table 1: Comparison of several properties of the different nucleic acid-based inhibitors

	<b>Antisense Oligonucleotides</b>	<b>Small interfering RNAs</b>	<b>Antagomirs or AntimicroRNAs</b>	<b>microRNA mimics</b>	<b>Aptamers</b>
Abbreviation in the text	ASOs	siRNAs	AntimiR	miRNAs mimics	Apts
Structures	Single strand RNA/DNA	Double strand RNA	Single strand DNA/RNA	Double strand RNA	Single strand DNA/RNA
Length (mer)	12-23	20-25	12-16	20-25	20-100
Site	Intracellular (nucleus, cytoplasm)	Intracellular (cytoplasm)	Intracellular (cytoplasm)	Intracellular (cytoplasm)	Intracellular (cytoplasm) Extracellular
Target	mRNA pre-mRNA	mRNA	miRNA	mRNA	Protein
Function	mRNA degradation Translational inhibition Splicing inhibition	mRNA degradation	miRNA degradation	mRNA degradation Translational inhibition	Inhibition of protein function

### 2.1 Antisense Oligonucleotides

ASOs are single-stranded RNA or DNA sequences of 12–23 bases, which are complementary to specific mRNA. ASOs were first synthesized in Novosibirsk (Russia) by the Grineva's group

in 1967 [11]. In 1978, Zamecnik and Stephenson used a synthetic ASO, which was complementary to a 13 nucleotides sequence in the RNA genome of the Rous sarcoma virus, to inhibit the translation of the viral RNA and subsequently block the virus replication in chicken embryo fibroblasts [12]. ASOs have been shown to bind to mRNA by Watson-Crick base-pairing, and therefore modifying the translation and the production of the associated protein [13]. The first observations were explained by a steric blockage translation process, in which the presence of the bound ASO prevents the binding of translation factors and modulators, and impedes the movement of the ribosome along the mRNA strand [14,15] (Figure 1A). A second related mechanism is the catalytic action of ribonuclease H [16] (RNase H). This enzyme specifically cleaves the RNA strand within the RNA-DNA duplex (Figure 1A). The ASO is then released can bind to a new mRNA strand. Both mechanisms involve mRNA and occur in the cytoplasm. However, ASOs can also exert an activity in the nucleus, where they can form triple helixes with DNA, inserting themselves into the major groove of the double helix. This occurs preferentially when a sequence composed entirely of purines is base-paired with one composed of pyrimidines [17]. The formation of this triple helix structure leads to the cleavage of the DNA, inhibiting gene expression at the transcription stage. The treatment of inflammatory bowel disease (IBD) using an ASO targeting the p65 subunit of NF- $\kappa$ B and inhibiting the production of the inflammatory cytokine was first explored in the '80s [18,19]. This early proof of concept was later confirmed as downregulation of NF- $\kappa$ Bp65 with an ASO was shown to induce symptoms attenuation in dextran sodium sulfate (DSS)-induced colitis [20] and intestinal fibrogenic processes murine models [21,22]. Additionally, Alicaforsen, a 20-base ASO inhibiting ICAM-1 production, which is an important adhesion molecule involved in leukocyte migration and trafficking to the site of inflammation, was tested in the treatment of IBD up to a phase II clinical study [23]. Similarly, Mongersen, an ASO against SMAD7 mRNA, demonstrated a preclinical and early clinical efficacy in Crohn's disease, but unfortunately, the phase III clinical trial was stopped due to a lack of efficacy [24]. However, other ASOs have also been developed to modulate additional cytokines such as IL-4 in pulmonary inflammation [25], TNF- $\alpha$  in colitis [26], IL-10 on skin burned [27], and IL-5 in asthma [28].

## **2.2 Small interfering RNA**

SiRNAs have been discovered by studying how the introduction of long double-stranded RNA (dsRNA) into a variety of hosts induces post-transcriptional silencing of homologous host



genes and/or transgenes. The first observation published in 1990 was made in petunias as the plants were transfected with a chalcone synthase gene to get them to be more purple, but turned white instead [29]. The siRNA mechanism was later elucidated by Fire and Melo [30] who in 2006, were awarded the Nobel Prize for their work. Once in the intracellular compartment, dsRNAs are metabolized into siRNA by the action of DICER, an endogenous ribonuclease. SiRNAs consist of double-stranded RNAs of 21–23 base pairs that bind in the cytoplasm to a multiprotein complex, known as RNA-Induced Silencing Complex (RISC) (Figure 1B) [31]. During the assembly of RISC, one of the two siRNA strands known as the passenger strand is released, whereas the second strand (the guide strand) binds to the Argonaute protein Ago2 [32]. This guide strand then directs RISC to bind to its complementary target mRNA, which is then cleaved by the RNase activity of Ago2, preventing its translation to a protein sequence and triggering its destruction (Figure 1B) [33]. Other nucleic acids known as short hairpin RNAs (shRNAs), are recognized by the cellular RNA interfering machinery, and are processed to form active siRNAs. Those shRNAs, as opposed to siRNAs, are synthesized within the nucleus of cells, and once transported to the cytoplasm they are further processed and incorporated into RISC [34].

In inflammatory diseases, many of the siRNA/shRNA applications have focused on rheumatoid arthritis. Targets for siRNAs in RA are primarily cytokines such as interleukin-1 (IL-1), IL-6, IL-18 [35], and TNF- $\alpha$  [36–39], or signaling pathway modulators including NF- $\kappa$ B [40–42], Notch1 [43,44], c-Rel [45], TAK1 [46], CCR5 [47], STAT1 [48], and PLK-1 [49]. Noticeably, siRNA-based strategies targeting TNF- $\alpha$  expression have been extensively tested to treat other inflammatory diseases such as IBD [50] or acute lung injury [51–53].

### **2.3 MicroRNA mimics and Antagomirs**

MicroRNAs (miRNAs) represent a class of small endogenous non-coding RNA molecules that play important roles in the native regulation of gene expression impacting cell differentiation, proliferation, and survival [54]. MiRNAs typically bind to complementary target mRNA sequences, resulting in mRNA translational inhibition or degradation (Figure 1C). MiRNAs dysregulation can lead to numerous diseases, including cancer, hepatitis, cardiovascular and inflammatory diseases [55,56]. Most miRNA sequences are encoded within introns of protein-coding genes with some miRNAs having their promoter region. The biogenesis of miRNAs begins with the transcription of a long primary-microRNA transcript (pri-miRNA) by an RNA polymerase. The pri-miRNA is then cleaved by the Drosha/DGCR8 protein complex generating

a pre-miRNA that is transferred to the cytosol. There, the terminal loop of the pre-miRNA is cleaved by DICER, and the resulting two miRNA strands can bind to AGO proteins to form RISCs. Within the complex, the two miRNA strands are unwound and one of the strands is selected as a guide strand. The selected mature single miRNA strand is then able to bind to mRNAs with complementary nucleotide sequences resulting in translational repression or mRNA degradation (Figure 1C) [57]. Unlike siRNA that are highly specific, miRNAs can regulate multiple mRNA targets leading to a potentially enhanced efficacy in controlling gene expression.

MiRNA profiling analyses of patients with autoimmune disease have revealed several alterations in the expression of particular miRNAs such as miRNA-155, -146a, -326, -21, 125b, and -181 [58,59]. For example, overexpression of miRNA-146a and miRNA-155 has been shown to stimulate macrophages NF- $\kappa$ B pathway and the secretion of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-17 [60,61]. Consequently, new strategies have been developed focusing on miRNA as therapeutic candidates (in the form of miRNA mimics) or as molecular targets using antimicro-RNAs (Antagomirs) as therapeutic moieties [4]. Recent studies have revealed that various miRNAs also play essential roles in the endogenous modulatory mechanisms of several inflammatory pathways [62]. As suggested by Tahamtan *and coworkers*, this opens new exciting opportunities in using miRNA mimics in inflammatory diseases [62]. In the last few years, proinflammatory miRNA-155 has particularly attracted the attention of several teams, which have focused on delivering Antagomir-155 to treat RA [63], DSS-induced colitis [64] as well as neuroinflammation [65,66] atherosclerosis [67], and sepsis [68]. Similarly, the delivery of miRNA-181 and miRNA-34a have been shown to regulate inflammation respectively in osteoarthritis [69] and murine collagen-induced arthritis [70].

## **2.4 Aptamers**

Aptamers (Apts) are single-stranded DNA or RNA molecules (20-100 bases) that can fold into specific 3D structures with a high affinity toward specific targets (Figure 1D). Apts are typically selected *in vitro* from combinatorial oligonucleotide libraries by a high-throughput process known as systematic evolution of ligands by exponential enrichment (SELEX). Since the discovery of the SELEX method [71] and the progresses made in nucleic acid amplification, numerous Apts have been successfully selected to bind to a wide array of targets such as ions, small chemical molecules, proteins, as well as animal cells or unicellular organisms. Apts are capable to bind to their target with a high-affinity range (from picomolar to nanomolar) and a

high selectivity being able to distinguish between very closely related molecules. Although Apts share common properties with antibodies in terms of binding and specificity, they differ in structure, chemistry, and production methods. Indeed, while antibodies display several advantageous pharmacokinetic properties such as low renal filtration, and nucleases resistance, Apts also hold key advantages. First, they can be chemically custom synthesized with ease and good reproducibility, while each antibody production required a long optimization and a heavy biological setup. Furthermore, as Apts are significantly smaller than antibodies they are generally not immunogenic and do not induce unwanted toxicity. As such they represent a compelling alternative for several biomedical applications such as receptor agonists/antagonists, gene expression modulator, and targeting ligand for small drug molecules, contrast agents, and drug delivery systems [72,73]. Several clinical trials have been carried out focusing on the use of Apts as therapeutics. The most advanced results have been obtained for ocular delivery with molecules such as Macugen<sup>®</sup>, Zimura, and Fovista with the first being marketed, and the two others in phase III clinical trials. Concerning inflammation, past research works have enabled the selection of a dozen of Apts with an affinity toward cytokines or their related receptors including IL-2, IL-6, IL-10, IL-11, IL-17, IL-32, TGF- $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , CCL2, and IP-10 that have shown promising results in the diagnosis, prevention, and treatment of various inflammatory diseases [74].

### **3 OVERCOMING POOR STABILITY IN BIOLOGICAL FLUIDS**

#### **3.1 Stability of nucleic acids-based gene inhibitors in biological fluids**

It is generally admitted that nucleic acids have poor stability in biological fluids. However, only a few papers have studied how the structures of the different types of nucleic acid-base gene inhibitors influence this stability. The few comparative studies published have revealed that double-stranded RNAs tend to be more stable against nucleases than single-stranded RNAs [75,76]. These results are consistent with the relative stiffness of the RNA duplex, which stabilizes the torsion angles, between the phosphodiester and the ribose, in conformations that are less sensitive to serum ribonucleases. When comparing double-stranded RNAs of different sizes, it has been shown that shorter (18-mer) strands have a faster degradation rate than longer 21-mer and 24-mer. This may be linked to the existence of protective RNA:protein complexes that favor longer double-stranded RNAs [77]. Nevertheless, siRNAs have typically a very short half-life of less than 20 minutes in serum [78,79]. Natural phosphodiester-based

ASOs display an even shorter half-life of about 5 minutes [80]. For the last 40 years, several groups have investigated potential chemical modification strategies to improve nucleic acid stability and cell penetration while in parallel others have been worked on new formulation concepts to carry and protect nucleic acids.

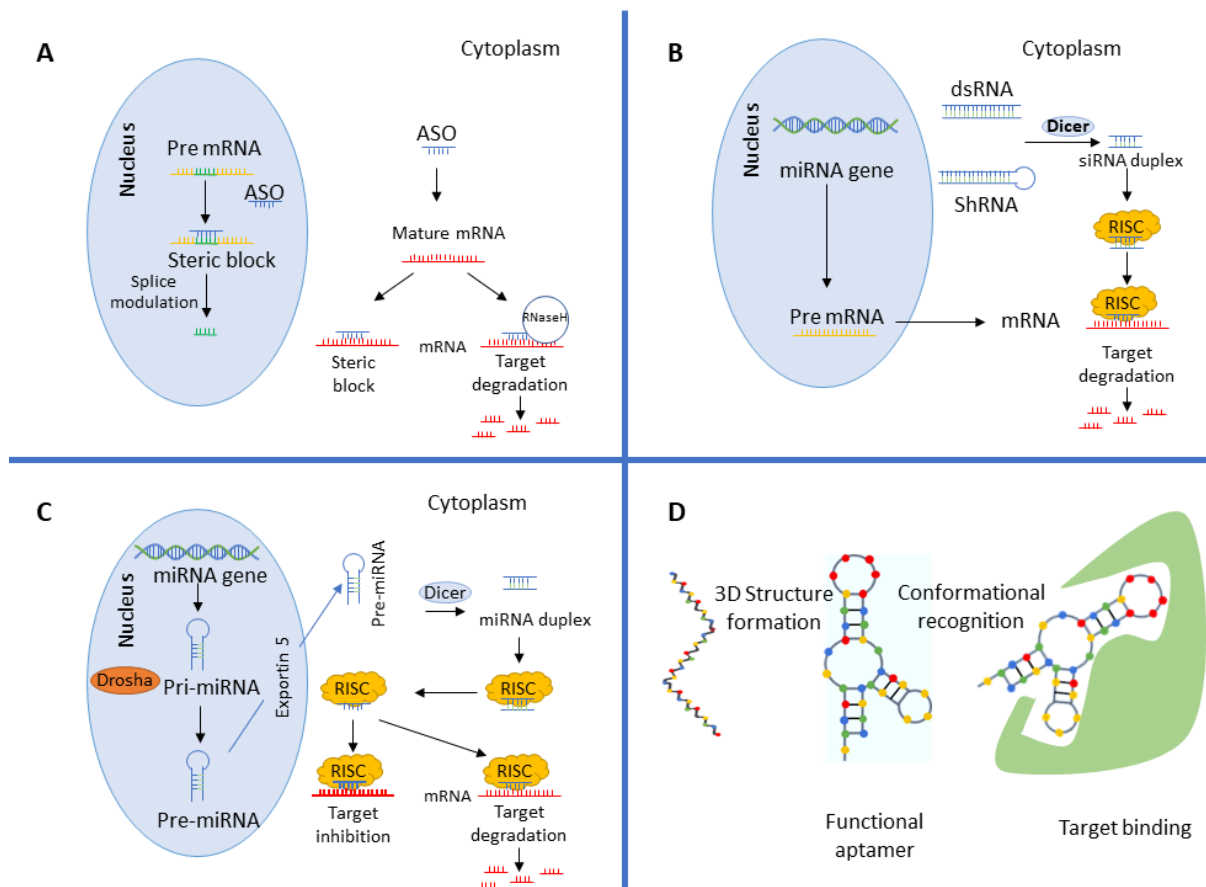


Figure 1: Mechanisms of Action of A) antisense oligonucleotides, B) siRNA, C) miRNA, D) Aptamers

## 3.2 Chemical modifications

### 3.2.1 Conventional modifications

The first chemical modification strategy developed to improve the stability of nucleic acids has focused on the conversion of the phosphodiester bond (Figure 2) into phosphorothioate (PS) [81]. The substitution of the non-bridging oxygen by a sulfur atom results in the enhanced resistance of the nucleic acids to enzymatic degradation. Although this modification induces a decrease in the stability of the duplex formed between the nucleic acid and the target RNA, the duplex is still a substrate for the RNase H. In the '90s, this PS-based strategy became very popular for the stabilization of antisense therapy and led to the development and approval of the first intraocular administered ASO (Fomivirsen®) targeting the viral mRNA of

cytomegalovirus (CMV) for the treatment of CMV-related retinitis. However, the main disadvantage of PS-modified nucleic acids is their tendency to bind to plasma and cellular proteins and subsequent off-target and toxic effects [82]. As phosphorothiate-modified siRNAs (PS-siRNA) have been shown to display similar off-target problems [83], PS chemistry has only been marginally used for siRNA therapeutics. Additionally, early studies suggested that PS-siRNA did not induce a superior silencing activity compared to their phosphodiester counterparts [84]. As an alternative, boronophosphate based-modification has been tested and was demonstrated to improve nuclease resistance, with boronophosphates-modified siRNAs acting similarly to unmodified siRNAs and therefore being significantly more efficient than PS-siRNAs [85].

Other common modification strategies have focused on the ribose 2' position and the replacement of 2'-hydroxyl by different chemical groups such as 2'-O-methyl, 2'-O-methoxyethyl, and 2'-fluoro (Figure 2). Similarly, these chemical modifications have induced an increase in the overall stability of the nucleic acids without reducing their inhibitory properties *in vivo* [86]. In siRNAs, synthetically, 2' modifications can be introduced throughout the duplex [87]. However, to preserve the inhibitory activity while enhancing stability, it is preferable to alternate modified and unmodified nucleotides [78]. 2'-Fluoro modifications have also been successfully carried out in Apts sequences [88] where they are selectively introduced on the pyrimidine bases and are often associated with 2'-O-methyl modifications on the purine bases to combine stability and high binding efficiency [88].

Locked nucleic acid (LNA), also referred to as "*inaccessible RNA*", is a family of conformationally locked nucleotide analogs that displays unprecedented hybridization affinity toward complementary DNA and RNA [89,90]. Commonly used LNAs contain a methylene bridge connecting the 2' oxygen with the 4' carbon of the ribose ring (Figure 2) [91,92]. LNA ASOs are highly stable in blood serum and cell extracts while keeping a potent efficacy toward RNase H activation [89]. However, *in vivo*, LNA ASOs have been linked to acute hepatotoxicity as measured by serum transaminases, organ weights, and body weights [93]. Despite these side effects, several siRNAs [88,92,94–96] and Antagomirs [97–99] have been developed using an LNA-modified backbone and displayed a dramatically improved plasma half-life with no alteration of their biological activity [88,92,94–96]. Importantly these modifications should not be introduced on the nucleotides at positions 10, 12, and 14 of the

guide strands since modifying these particular nucleotides has shown to significantly reduce the inhibitory effect of the siRNA [94].

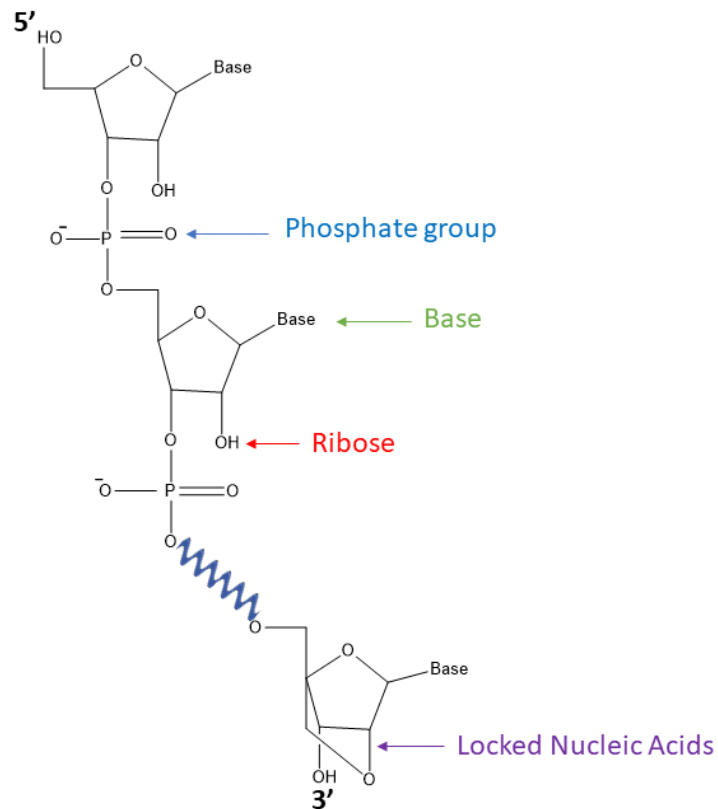


Figure 2: Main chemical modification on nucleic acids

Another major strategy to improve the delivery of nucleic acid has been the development of prodrugs from nucleic acids and more specifically lipid prodrugs. Numerous groups have tested the association of ASOs and siRNA to cholesterol [100,101] or squalene [102], a cholesterol precursor which conjugates can self-assemble into nanoparticles [102]. Fatty acids have also been conjugated to ASOs. For instance, palmitic acid conjugated ASOs have demonstrated increased plasmatic concentration and improved tissue delivery [103]. The same strategy has been applied to siRNA [104]. Another common modification consists of attaching a short fatty acid, coupled to an N-acetyl-galactosamine (GalNAc) molecule, to a phosphate group at the 3' terminus of one of the two strands of siRNA. The addition of GalNAc enables active targeting of hepatocytes via recognition of the asialoglycoprotein receptor and improved cell accumulation as discussed in a later paragraph [105]. Finally, similarly to

peptides and proteins, numerous nucleic acids have been conjugated to polyethylene glycol (PEG) to induce longer blood circulation time. This strategy has been successfully applied to ASOs [106] and aptamers such as with pegaptanib (Macugen®), the only approved therapeutic aptamer [107].

While chemical modifications have been widely applied to different nucleic acids, the most recent approach consists of building chimeric structures to introduce several chemical modifications within the same sequence. Gapmers ASOs for example are oligonucleotides designed around a gap sequence of six to eight unmodified or PS backbone that binds to the target and induces RNase-H associated mRNA degradation (Figure 3) [108]. Several chemically modified nucleic acid analogs are then inserted at each end of the gap sequence to increase its binding affinity and to protect it from exonucleases (Figure 3) [109].

Steric-blocking ASOs are synthetic, fully modified, single-stranded oligonucleotides that typically range from 15 to 30 mer in length and contain chemical modifications in the sugars as well as in the backbone. Many of the chemical modifications are similar to gapmers ASOs and are intended to increase nuclease resistance, reduce the immune response, and increase binding affinity. However, unlike gapmers, steric-blocking ASOs do not elicit RNase H-mediated degradation of the target RNA, but rather block the target RNA via sequence-specific Watson and Crick base-pairing [1].

The initiation of alternative splicing is another major clinical application of steric blocking ASOs. ASOs can bind to targeted pre-mRNA and disrupt its interaction with the splicing machinery leading to an altered splicing result and a different protein sequence being produced (Figure 1A). This strategy is particularly compelling to treat diseases caused by mutations that lead to abnormal splicing such as Duchenne muscular dystrophy and spinal muscular atrophy for which splice-switching oligonucleotides are currently being tested clinically [110].

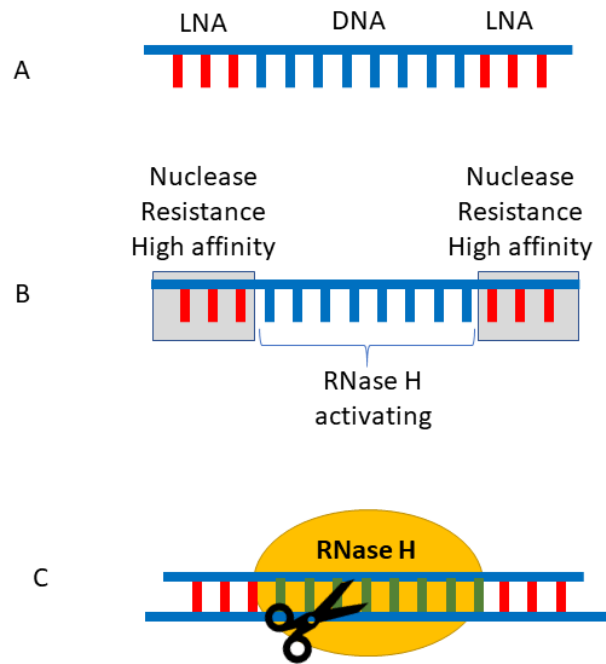


Figure 3: Example of one configuration of gapmers

### 3.3 Nanoparticle design for nucleic acid delivery

In the last decades, numerous studies have focused on the development of nanocarriers to protect and deliver nucleic acids. The vast majority of those strategies have focused on protecting nucleic acids against degradation through an association with colloidal carriers. Several reviews have well described the array of nanocarrier systems used to deliver RNA and DNA [111]. Thus, in the next paragraphs, we will focus on lipid and polymer-based colloidal nanoparticles with an emphasis on manufacturing issues.

#### 3.3.1 Lipid-based delivery systems

Since the first observations by Bangham *and coworkers* in the 60's liposomal formulations have been developed as drug delivery carriers using a variety of synthetic lipids and have led to several clinically approved products [112]. While they were first employed to formulate small anticancer or anti-infectious molecules [113,114], the tremendous progress achieved during the last decades in terms of formulation, characterization, *in vitro/in vivo* evaluations and regulatory process have greatly supported the development of lipid-based systems for nucleic acids. One of the key characteristics of the liposomal formulations used for nucleic acid delivery is the presence of positively charged lipids that have been introduced to interact with the negatively charged nucleic acids. The resulting lipoplexes have been shown to not only protect ASOs, siRNA, miRNA, and Antagomirs from nucleases degradation, but also to enhance



cellular internalization, and lower the renal clearance of the different nucleic acids [115–117]. Commonly used positively charged lipids are either the commercial 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), N-(1-[2,3-dioleoyl]-propyl)-N,N,N-trimethylammonium chloride (DOTMA) or various proprietary ionizable lipids. Liposome formulation may also need the presence of a helper lipid such as dioleoyl-phosphatidylethanolamine (DOPE) as in Lipofectamine® 2000 [118]. Regarding siRNA, it was initially considered that the electrostatic interactions between siRNA and a cationic liposome/lipid system could create two potential problems: (i) a relatively uncontrolled interaction process leading to a siRNA–lipoplex particle of excessive size and poor stability and (ii) incomplete shielding of siRNA molecules exposing siRNA to enzymatic or physical degradation [119]. Nevertheless, several reports have described a high association rate and a subsequent complete change in the morphology of the liposomes. Nascimento *and coworkers* [120] for instance, demonstrated that the association of siRNA to unilamellar vesicles modifies the morphology turning the unilamellar vesicular structure into a multilamellar one, with siRNAs located between the different lamellae [120]. However, lipoplexes have usually a high surface charge density, which can induce undesirable non-specific interactions with serum proteins leading to aggregation and further adverse effects [121]. To achieve a better systemic delivery of siRNA, various research groups have considered the use of only neutral lipids and/or the addition of PEGylated lipids to reduce protein interactions. However, with no positive charges to interact with the nucleic acids, neutral and negatively charged liposomes have required the introduction in their aqueous core of auxiliary cationic polymers, such as protamine to interact with the nucleic acids [122]. This polymer-core-encapsulation of nucleic acid allows high loading efficiency without significantly increasing the size [73,123] leading to improved stability [122]. In addition to cationic, neutral, and negative liposomes, another widely studied strategy consists of stable nucleic acid-lipid particles (SNALPs). SNALPs are based on a bilayer composed of a mixture of low cationic lipid content, Polyethylene Glycol attached to phospholipids (PEGylated phospholipids), which provide a long circulation time and ionizable/fusogenic lipids to enable cellular uptake and endosomal release of the siRNA cargo. These ionizable lipids, whose mechanisms of action are described later, have initially consisted of the ionizable aminolipid (1,2-dioleoyl-3-dimethylammonium propane) DODAP (also referred to as DLin-DMA) [124]. In the last few years, several groups have worked on the optimization of the ionizable the components such as the DLin-KC2-DMA 2,2-dilinoleyl-4-

dimethylaminoethyl-[1,3]-dioxolane [125]. In the marketed Onpattro<sup>®</sup>, the ionizable lipid is DLin-MC3-DMA ((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate) [126].

### 3.3.2 Polymer-based delivery systems

Initial studies to deliver ASOs and siRNA were carried out with biodegradable polymers such as poly(alkylcyanoacrylate) (PACA) or poly(lactide-co-glycolide) (PLGA) with positive charges introduced by adsorbing cationic surfactant or polymer onto the surface of the particles [127–129]. Polymer microparticles were also used for the controlled delivery of ASOs or siRNAs mostly for local delivery [130–132]. True encapsulation of nucleic acids in polymeric systems encounters the problem that nucleic acids are hydrophilic while the polymers used to form nanoparticles are hydrophobic. As one solution, Lambert *and coworkers* [133,134] developed a method to produce nanocapsules with an aqueous core and a PACA shell that was shown to be efficient for the encapsulation of both ASOs [133,134] and siRNA [135].

Alternative approaches have used complexation by cationic polymer entities. The first studies were based on polyethyleneimine (PEI) to deliver ASOs [136] or siRNA [137]. PEI has been the most commonly used cationic polymer for nucleic acid delivery as it is available in a wide range of molecular weights and bear many protonated amino groups, resulting in high cationic charge densities under physiological pH values and an excellent ability to bind with nucleic acids [138]. However, as previously mentioned for lipid-based formulations, this constant positive surface charge has been responsible for off-target effects and severe toxicities [139,140]. In-depth, studies showed that the toxicity of PEI tends to deteriorate as both the molecular weight [141] and the number of branching increase [142]. Nevertheless, it had been shown that the cytotoxicity of PEI can be reduced by removing terminal groups that are not complexed with nucleic acid [139].

Cationic dendrimers have also attracted the attention of researchers [143–145]. Similarly to other cationic formulations, cationic dendrimers can complex nucleic acids and protect them from nuclease degradation, improving their biopharmaceutical properties [146–149]. One of the advantages of the dendrimers over other cationic polymers is their step-by-step synthesis process that enables the production of precise spatial structure of a specific molecular weight. The choice over many functional terminal groups is another advantage of dendrimers which provides numerous options for customized functionalization [150]. Furthermore *In vivo* studies

have shown that dendrimers can have a prolonged circulating time [151] making their application for nucleic acids delivery very appealing [52,152,153].

### **3.3.3 Overcoming manufacturing issues**

As seen in the previous paragraphs, nucleic acids are fragile molecules. Therefore, they may be denatured or destroyed during the manufacturing process. As a result, preparation methods involving nucleic acids deriving from standard nano-formulation processes need to be adapted. Indeed, the majority of early preparation protocols involved the use of organic solvents (chloroform, dichloromethane) that can induce aggregation of the nucleic acids. Size reduction processes (sonication, extrusion microfluidization) may also damage the nucleic acids through shear stress and/or heat production [154]. Fortunately, nucleic acids tend to be relatively stable to mild and high temperatures and their complexation with a carrier seems to have protective effects. However, it must be noted that the sensitivities of a specific nucleic acid depend on its structure and mechanism of action as the 3D shape of Apts is typically more sensitive to heat than siRNA duplexes or miRNA stem-loops structures. While those considerations may not be critical for processes during which the nucleic acids are only mixed with the preformed nanoparticles (PEI polymer, dendrimers, lipofectamine®), they are central and need to be carefully monitored when nucleic acids are encapsulated within the carrier [155,156].

Another critical manufacturing issue has been the difficulty of scale-up formulation methods based on lipid film hydration followed by size reduction [118]. Interestingly a detergent/dialysis method was developed in the late '90s, in which the different components (nucleic acids, structural lipid, and cationic lipid) were mixed in the presence of a detergent, which was later removed by dialysis [157]. While this method did not involve any mechanical forces, it led to the formation of 65nm lipid-based particles with a 70% encapsulation efficiency. However, this method was also not optimized for large-scale production. Thankfully, this early work paved the way for new scalable formulation methods. A very effective one consists of flow mixing the lipids solubilized in an ethanol solution with an aqueous solution containing the nucleic acids to induce instantaneous ethanol dilution and formation of nucleic acids-loaded lipid nanoparticles. This method was first carried out with T-connector [158] but was quickly adapted with the use of microfluidic T shape mixing channels or Herringbone structures that enable parallel continuous production [159].

Once produced, the shelf-stability of the nucleic acid formulations may also be a barrier. This issue is particularly delicate as it involved not only the nucleic acids but also the structure of the nanoparticles, which is central to the overall efficacy of the product. Therefore, as RNAs hydrolysis and nanoparticle structure instability have multifactorial causes, both need to be carefully preserved and assessed [160]. Thankfully the Onpattro®'s package insert reveals that the product needs only to be between 2 and 8°C, and can even be stored for up to 14 days at a temperature below 25°C. This is very encouraging for future applications. Furthermore, several groups are investigating RNAs stability during lyophilization and spray-drying process to produce inhalable formulations to treat lung inflammation [161].

Finally, one extra barrier worth mentioning in the time of writing is the supply chain. In an interesting study, Jarzebska *and coworkers* pointed out the significant quality heterogeneity of the different protamine commercially available [162], and the necessity to strictly control it for potential human use. Furthermore, in the early months of 2021, the mass production of mRNA lipid nanoparticles for the COVID-19 vaccine was slow down by several bottlenecks with news reports indicating that the manufacturing of ionizable custom lipids being one of them.

## **4 OVERCOMING THE PLASMA MEMBRANE**

### **4.1 Internalization**

Once the nucleic acids have reached the targeted cells, they still need to access the cytosol where they can interact with mRNA and mitigate gene expression. As nucleic acids are large and negatively charged molecules, crossing the plasma membrane is considered the most difficult part of the process [163]. In non-immune cells, clathrin and caveolae-mediated endocytosis are the prevalent internalization mechanisms. However, when working in the context of inflammation and cells such as macrophages, neutrophils, and dendritic cells, phagocytosis becomes the predominant mechanism [164].

Clathrin-mediated endocytosis is the main process used by non-phagocytic eukaryotic cells to internalize nutrients, growth factors, or antigens with clathrin-coated pits typically occupying 2% of the cell surface. Clathrin-mediated endocytosis is a relatively rapid process that may only last less than 60 seconds and has been associated with membrane receptor-mediated internalization such as transferrin, low-density lipoprotein, and epidermal growth factor receptors [165–167]. Complementary to clathrin-mediated endocytosis [168–170], nanoparticle internalization has also been shown to occur through caveolae-mediated

endocytosis [171,172]. Caveolae pits are small (50-80nm) flask-shaped membrane invaginations present in various cell lines that can represent up to 10-20 % of endothelial cell surfaces. Caveolae pits have been linked with membrane lipid raft recycling [173,174] and cell internalization. Caveolae-mediated endocytosis is involved in the uptake of nutrients such as calcium and iron [175] as well as diverse toxins (cholera, tetanus) and viruses (simian virus, human immunodeficiency virus) [176]. Interestingly, several groups have worked on the modulation of the carriers' sizes, charges, shapes as well as ligand natures and densities to specifically favor one endocytosis mechanism over the other [177–179].

Conversely, the main mechanism of nanomaterials' uptake by immune cells is phagocytosis [164]. To our knowledge, no specific studies have been carried out to thoroughly study the influence of external factors on phagocytosis of nanomaterials other than the opsonization of their surfaces. However, researches focusing on the phagocytoses of bacteria and cancer cells by immune cells have pointed out the impact of key receptors such as scavenger, pattern recognition, and complement recognition receptors [180]. Additionally, several groups have been studying the influence of cell polarization state (M1/M2) on the phagocytic mechanisms and rate, with most of the studies showing higher phagocytic activity in M2 monocytes/macrophages [180,181] including toward nanoparticles [182].

#### **4.2 Intracellular trafficking**

Following nanoparticle clathrin-mediated internalization, endocytosis vesicles undergo active and rapid coating disassembly leading to clathrin recycling to the cell surface. The uncoated vesicles and their contents then migrate within the cell lumina and fuse with early endosomes [183]. At this stage, various internalized lipids and receptor proteins are recycled back to the membrane. This redistribution can be done directly or via the trans-Golgi network [184]. The remaining molecules are collected in multivesicular endosomes known as multivesicular bodies, transported into the cytosol. There, they fuse with lysosomes forming secondary lysosomes where entrapped proteins and nucleic acids are fully degraded. Using confocal microscopy to study the localization of fluorescent particles, numerous reports have demonstrated the accumulation of internalized nanoparticles within the endo/lysosomal pathway [185–188]. Along this processing pathway, the luminal pH of the organelles decreases from pH 6.3 in early endosomes to pH 5 in late lysosomes [189]. This acidification controls the activity of the diverse enzymes present in the lamina such as lipases, carbohydrases, proteases, phosphatases, and nucleases [190]. The severe acidic and

enzymatic conditions reduce most of the molecules internalized into their simplest constituents. The lysosomal breakdown products are finally released either into the cytoplasm or outside the cell [190,191]. In a very interesting study, Gilleron *and coworkers* compared the intracellular fate of 2 different siRNA formulation strategies [192]. Using an array of biomolecular and imaging tools, the authors demonstrated that the superior efficacy of their lipidic formulations over gold-based nanoparticles was not due to level of cellular uptake but primarily by the rate of siRNA escaping out of the endosomal pathway [192].

The fate of NP internalized through caveolae-mediated endocytosis remains unclear. Studies have shown some distribution of internalized molecules within the endoplasmic reticulum or the Golgi complex supporting the concept of the fusion of the caveosomes with the Golgi complex [175]. However, other reports have instead presumed the existence of a specific pathway between caveosomes and early endosomes that alternatively drives internalized biologics to the endosomal pathway [193].

Similarly, nanoparticles engulfed by phagocytosis have been shown to accumulate in large vesicles called phagosomes, that fuse with lysosomes to form phagolysosomes. Those phagolysosomes are characterized by acidic pH and the presence of enzymes such as nucleases [194]. Very interestingly within the immune cells, those intracellular mechanisms seem to vary based on the cell types and polarization states. In an interesting study, Foote *and coworkers* isolated monocytes, neutrophils, and dendritic cells from human plasma and analyzed how the different cells phagocytosed *Candida albicans* yeasts [195]. Furthermore, polarization states appear to also have a significant influence. For example monocytes “classically” activated by LPS/IFN- $\gamma$  (M1) showed an alkalization process to pH 8.5 of their phagosomes followed by neutralization, while non-classical M2 monocytes underwent phagosomal acidification to pH 5. Similar striking differences in phagosomal acidification kinetic and intensity were also demonstrated between M1 and M2 macrophages [196]. As discussed by the authors [195], further variations may also be observed when comparing different immune cell subpopulations such as lung alveolar and interstitial macrophages. Therefore, as acidification of the intracellular vesicles is key in the endosomal escape mechanisms of some of the delivery systems presented in this review, those variations may constitute either a barrier or an opportunity, for the design of general nanocarriers or the targeting of a particular immune cell population.

### 4.3 Cells penetrating moieties

Cells penetrating peptides (CPPs) are small peptides that have been characterized to be able to cross/disturb cellular membranes and can be used to improve the delivery of nucleic acid within the cytosol. Those peptides can be of natural origins such as penetratin (isolated from *Drosophila*) and TAT protein (isolated from HIV), synthetically developed *i.e.* oligo-arginine, or selected from libraries by phage display screening processes [197]. As those peptides are of different natures and structures, their mechanisms of action also differ [198]. Pep1, for example, can directly translocate within the cell plasma membrane through its amphipathicity [199]. Other, typically cationic peptides, such as TAT, penetratin, or oligo-arginine have been shown to interact with the anionic phospholipids present in the endosomal membrane. These interactions led to the formation of pores within the endosomal membrane or help the peptide's partition across the membrane. An alternative strategy consists of using an Apt-siRNA chimera, in which the Apt targeted the prostate-specific membrane antigen. This approach was demonstrated to induce both cell specificity targeting and potent internalization [200].

It is also important to acknowledge the excellent results obtained by conjugating SiRNA and ASO to *N*-acetyl galactosamine (GalNAc) for hepatocyte delivery [201]. While using this strategy only a very small fraction (<1%) of the SiRNAs reaches the cytoplasm, it is still sufficient to induce a significant physiological response [105,202]. As described above, most CPPs are directly conjugated to the therapeutic RNAs [203,204], however, another popular method consists of conjugating them on the surface of a nanocarrier [205,206]. For example, Yang *and coworkers* tested the efficiency of PEGylated liposomes bearing both a photoresponsive capped CPP (CGRRMKWKK) and asparagine-glycine-arginine targeting peptide. In their study, the authors showed that the conjugation of both CPP and targeting peptides on the same carrier improved the silencing efficacy compare to liposomes bearing only one of either peptides [207]. In the context of inflammation, the use of CPP needs to be carefully assessed as several of those peptides may have immunomodulatory effects themselves. For example, TAT peptide has been isolated from a protein that was shown to activate monocytes [208]. Similarly, intratracheal administration of penetratin-siRNA conjugates in mice led to the induction of an innate immune response in the lung observed by an increased level of interferon  $\alpha$  and TNF- $\alpha$  [209].

#### **4.4 Polycations.**

Historically, several polycations of polymeric and protein origins such as PEI, chitosan, or protamine have been shown to induce endosomal escape through different mechanisms collectively referred to as the “Proton Sponge Effect”. This effect has been very well described elsewhere [210] and includes polymer swelling, the interaction of the polymer with the endosomal membrane, and the increase of the osmotic pressure within the endosome. This latest mechanism has been linked to the buffering action of the polycations, which promotes the accumulation of a large number of protons and in parallel with chloride ions and water. This buildup induces the swelling of the vesicles and membrane destabilization and/or burst [211]. As those mechanisms are complex and intertwined, the choice of the polycations should not be only based on pKa and buffering capacity but also on swelling capacity and rigidity [212]. In parallel, in a very interesting paper, Vermeulen *and coworkers* studied the efficiency of PEI/pDNA polyplexes with different cell lines. Using microscopy, they revealed that the difference in endosomal size and endosomal membrane leakiness between cell lines clearly impact transfection efficiency, as the cells with large and leaky endosomes showing the least endosomal escape [213]. This could be of crucial importance for designing strategies that specifically target immune cells as phagosomes are quite large ( $\geq 1 \mu\text{m}$ ) compared to endosomes (few hundreds of nm) [213,214].

#### **4.5 Cationic/ ionizable lipids**

One common way to improve intracellular delivery is to use cationic lipids such as DOTAP and DOSAP which can be already employed to complex nucleic acids. Once in the endosome, those cationic lipids are believed to interact with anionic lipids from the endosomal membrane inducing the release of the genetic material in the cytosol. While the exact processes behind this endosomal release have not been fully characterized yet, two mechanisms have been identified. The first mechanism is based on the fusion of the membrane of the lipid nanoparticles and the membrane of the endosome. The second model is explained by lipid exchange between the lipid nanoparticles and the lysosomal/endosomal membrane leading to the formation of inverted nonbilayer structures and the leakage of the content of the nanoparticles inside the cytosol [215–217]. Since the development of cationic lipid carriers in the early '80s [218], numerous studies have focused on improving those formulations. Early consensus was that lipid-based carriers had to include a cationic lipid, typically DOTAP, a structural lipid such as cholesterol and phosphatidylcholine [219], as well as a pegylated



phospholipid. However, the permanent positive charges present on DOTAP-based liposomes have been described to cause poor tissue distribution and cell toxicities. Therefore, several academic groups and companies have spent a tremendous effort and develop new ionizable lipids. The idea is to obtain lipids that are neutral in the extracellular environment but become positively charged once in the endosomes [220]. Based on this idea Prof. Cullis's team and others developed a new kind of lipid-based platforms containing ionizable lipids with pKa values below 7 and therefore exhibiting little to no positive charges at physiological pH but once in the endosomes, the ionizable lipid becomes cationic, and through its interaction with the ionic lipid from endosome as explained before. This induces the rapid destabilization of the endosomal membrane and the release of the nucleic acid in the cytosol [221–223] (Figure 4). The development of these ionizable lipids such as DLin-MC3-DMA has been demonstrated to spectacularly improve the nucleic acid therapeutic efficacy [126]. Indeed, key examples of those types of formulations are now clinically used to deliver siRNA to the hepatocytes (Onpattro®) [126,224] and mRNA to muscle cells and antigen-presenting cells (BioNTech and Moderna's COVID-19 vaccines) [225].

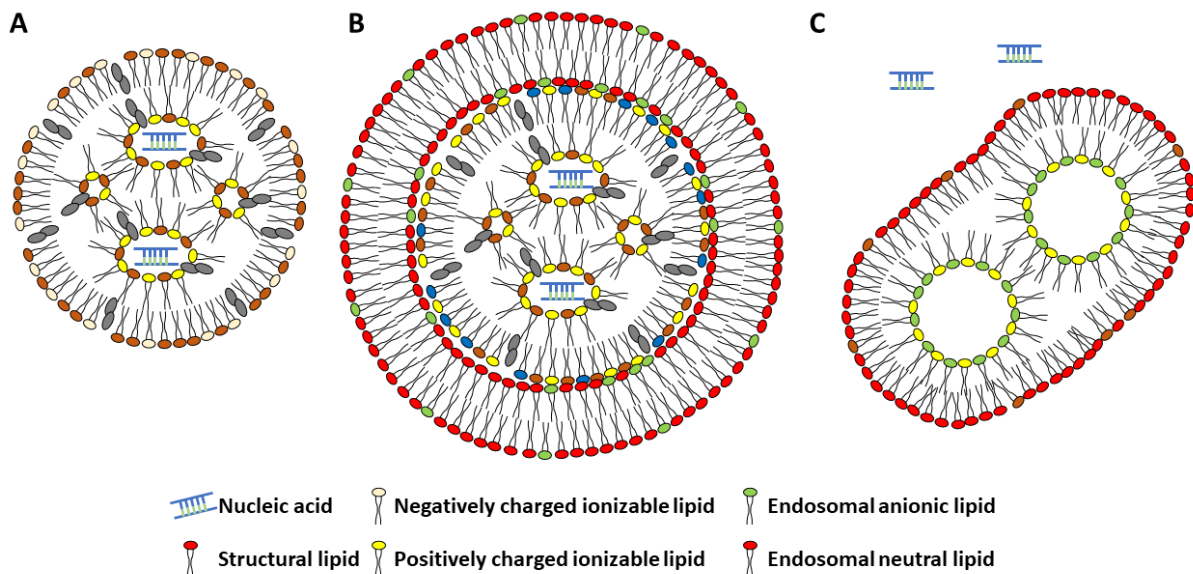


Figure 4: Potential mechanism behind ionizable lipid nanoparticles' endosomal escape.

A) In the extracellular environment the lipid nanoparticles exhibit an overall neutral charge. B) Within the endosomes, as the pH decreases below their PKa, ionizable lipids are positively charged and interact with the anionic lipids present in the endosomal membrane. C) The interactions lead to the formation of inverted nonbilayer structures and the release of nucleic acids in the cytosol. Adapted from [217].

#### 4.6 Unwanted activation of the immune cells by delivering nucleic acids.

In the context of delivering RNA to treat inflammation, one specific barrier is the fact that immune cells are specialized in recognizing extracellular nucleic acids through their toll-like receptors (TLR) [226]. TLR3, 7, 8, and 9 which are present in endosomal/phagosomal membranes can detect specific features present on nucleic acids such as dsRNA (TLR3), ssRNA (TLR7/TLR8), and un-methylated CpG motifs (TLR9) [227]. The activation of those TLRs induces the activation of the immune cells. This feature is so prevalent that CpG containing oligonucleotides are clinically used as an adjuvant in vaccines such as the recombinant vaccine HEPLISAV-B® against hepatitis B [228].

Activation by RNA/nanoparticle complexes is quite challenging to study. In a very interesting experiment, Yasuda *and coworkers* first demonstrated that plasmid DNA did not induce macrophage activation on its own, and cationic liposomes only a faint TLR independent one. However, when they incubated macrophages with DNA/liposomes, this led to a strong proinflammation reaction from both TLR9-dependent and -independent activation pathways [229]. In a follow-up study, the same group showed a slightly different pattern for dendritic cells as those cells were not activated by the cationic liposomes, a little by DNA, but strongly by DNA/liposome complexes [230]. Subsequent studies have been carried out with mRNA and protamine and demonstrated similar results. Incubated separately both components did not induce activation of peripheral blood mononuclear cell however, mRNA/protamine complexes led to a strong immune stimulation and interferon- $\alpha$  production [162]. This is not surprising as previous works have used protamine/mRNA complexes as vaccine strategy and demonstrated potent *in vivo* immune activation through TLR7 activation [231]. Additional studies have shown that the carrier may have a direct immune effect. Cationic liposomes have for example been demonstrated to induce reactive oxygen species (ROS) production [232] that can lead to cell death and immune activation. Indeed, several studies have observed activation of the NLRP3 inflammasome by cationic liposomes [233–236] as well as polystyrene nanoparticles [237]. While the exact biological pathways are not fully understood, several studies have pointed out the contribution of ROS production in pro-inflammatory mechanisms [233]. A recent study has also investigated the effect of lipofectamine® on murine RAW 264.7 macrophage cell line and primary bone marrow-derived macrophages. Using bone marrow from wild-type and TRIF knock-up animals, Guo *and coworkers* demonstrated that incubation of these cells with lipofectamine® alone induced the production of type I IFN through the TRIF/

IRF3 and IRF7 pathways but not through extracellular signal-regulated kinase (ERK) and I $\kappa$ B $\alpha$  [238]. Additionally, Wijagkanalan *and coworkers* demonstrated that intrathecal injection of cationic liposome/DNA complexes led to an increase of TNF- $\alpha$  in Bronchoalveolar lavage fluid (BAL) [239]. Finally, 20 years ago, Kisich *and coworkers* administered by intraperitoneal injection a solution cationic liposome-siRNA targeting against TNF- $\alpha$  to rats. While they did observe a significant reduction (70%) of TNF- $\alpha$  cellular production, they also detected a 400% increase in the number of inflammatory cells present in the peritoneal cavity [240].

All together those studies demonstrated that nanocarriers may catalyze cellular immune responses toward nucleic acids by favoring their endosomal accumulations and subsequent interactions with TLRs. Furthermore, some of these cationic nanocarriers also appear to trigger ROS production and proinflammatory activation by themselves. Those important points may have been overlooked by a large ratio of studies that used *de facto* nanoparticles to induce an inflammatory response toward cancer cells or promote antigen presentation. Therefore, we believe that extensive works remain to be carried out to understand the immune activation effect of the different nucleic acids/carriers complexes as it could undermine the success of anti-inflammatory strategies [241].

## **5 NANOMEDICINES FOR NUCLEIC ACID BASED GENE INHIBITORS DELIVERY IN MAJOR INFLAMMATORY DISEASES**

### **5.1 Systemic delivery in rheumatoid arthritis**

In most inflammatory diseases, monocytes accumulate within inflamed lesions and differentiate into tissue-resident macrophages. This accumulation can generate a cascade of mechanisms such as local macrophage proliferation, the release of proinflammatory cytokines, proteases, and cellular vesicles, which can exacerbate the inflammation leading to the recruitment of further inflammatory cells. Interestingly, the worsening of the inflammation is also characterized by an increased level of vascular endothelial growth factors and other cytokines that can trigger angiogenesis and leading to enhanced microvascular permeability to macromolecules. This is a key characteristic as this permeability can be exploited to help to deliver drug-associated-nanomedicines systematically. Therefore, a large array of nanomedicines has been developed to tackle a variety of inflammatory mechanisms

such as monocyte infiltration and differentiation, cytokine and chemokine release, local cell proliferation, and pathological angiogenesis [242].

Concerning nucleic acid delivery, one of the most investigated inflammatory diseases is rheumatoid arthritis as summarized in table 2 and discussed in two recent review articles [5,243]. Among the first experiments carried out in collagen-induced arthritis (CIA) murine model, complexes made between cationic liposomes and a siRNA silencing TNF- $\alpha$  were produced and systemically delivered in animals [35]. Results obtained after weekly injections demonstrated that systemic and local joint secretions of TNF- $\alpha$  as well as IL-6 and MCP-1 were significantly inhibited [35,244]. Similar results were found with cationic liposomes covered by PEG [245]. In another study, Wang *and coworkers* developed a CPP-conjugated liposome-polycation-siRNAs complex. This platform was loaded with a siRNA targeting the ribonucleotide reductase M2 (RRM2) to increase the levels of apoptosis and inhibit the proliferation of fibroblast-like synoviocytes in RA (RA-FLS). Results confirmed that the siRNA treatment led to significant inhibition of RRM2 gene expression and protein level by about 80% *in vivo*. It is worth mentioning that the liposomes effectively targeted RA-FLS, resulting in significantly reducing their proliferation and increasing their apoptotic level [246]. Targeted-TNF- $\alpha$  siRNA was also complexed with biocompatible cationic lipids and then incorporated into an acid-sensitive PEG-hydrazone-stearic acid derivative to form lipid nanoparticles [36]. These lipid nanoparticles were then used in a mouse model of collagen antibody-induced arthritis and induced a reduction of paw thickness and bone loss, as well as an improvement of the histopathological scores [36]. The same group also developed acid-sensitive sheddable PEGylated nanoparticles conjugated with mannose to target mannose receptors and further increased the delivery of the nanoparticles within chronic inflammation sites [247]. In a study, focusing on SNALP to deliver an anti-TNF- $\alpha$  siRNA, it was shown that two doses of those particles administered in an experimental arthritis model by intra-articular injection efficiently suppressed inflammation at a relatively low anti-TNF- $\alpha$  siRNA dose due to the direct administration into the joint [248]. Interestingly, this treatment further led to a reduced Th1 response [248]. A hybrid-nanoparticulate system made of calcium phosphate and liposomes covered with folic acid was designed to deliver NF- $\kappa$ B-targeted siRNA and methotrexate in collagen-induced arthritis (CIA) murine model [249]. The authors observed a strong inhibition of NF- $\kappa$ B signaling pathways, a decrease in pro-inflammatory cytokines expression, and the reduction of the arthritis progression [249]. Finally, in a recent paper, a lipidoid-polymer

hybrid nanoparticle made of poloxamer 407 and spermidine-based lipidoid (C14) loaded with a siRNA targeting IL-1 $\beta$ /siRNA were injected intravenously in a CIA murine model. The authors observed a rapid accumulation of the carrier in arthritis joints and a reduction of expression of proinflammatory cytokines in those joints [250]. Polymeric nanoparticles have also been frequently used as siRNA delivery systems [251] [252]. Shi *and coworkers* synthesized a folate-PEG-Chitosan-diethylethylamine conjugate loaded with anti-TNF- $\alpha$  siRNA [253]. These nanoparticles, not only significantly lowered TNF- $\alpha$  concentration in tissue but also reduced articular cartilage destruction and bone loss in the murine CIA model. Alternatively, Song *and coworkers* cross-linked low molecular weight PEI through a degradable linker to deliver anti-TNF- $\alpha$  shRNA. This complex was demonstrated to have good biocompatibility, a relatively high transfection efficiency, and to be very effective in reducing the severity of arthritis in the CIA model [254]. PLGA nanoparticles covered with cationic lipids have also been used to deliver a siRNA in RA. The intravenous administration of nanoparticles carrying an anti-BTK siRNA to the CIA murine model led to dramatic reductions in joint inflammation and other RA symptoms [255]. Another study associated a siRNA targeting the C5 fraction of the complement with protamine conjugated to a C5aR1 blocking antibody to treat a collagen antibody-induced arthritis murine model [256]. This anti-C5aR1ab-protamine-C5/siRNA conjugate was shown to induce significant reductions in arthritis burden (by 83%) as well as in histopathology score, C3 deposition, and neutrophil/macrophage infiltrations [256].

Dendriplexes have not been widely employed in the field of RA. However, Jensen *and coworkers* demonstrated that polyamidoamine dendrimers associated with anti-TNF- $\alpha$  siRNA were able to significantly induce gene-silencing in LPS-activated macrophages [257]. This observation was further confirmed *in vivo*, but in a psoriasis model [258]. We believe that the main reason behind the infrequent use of polyamidoamine dendrimers for systemic delivery of siRNA is probably due to their toxicity and strong affinity to the monocyte phagocytic system, in particular in the liver. Another report has described the inhibition of a transmembrane enzyme involved in cleaving and releasing bioactive soluble TNF- $\alpha$ , when using an shRNA complexed to a bone resorption site-specific peptide carrier composed of aspartate repeating as well as arginine repeating sequences [259]. The strategy strongly reduced arthritic symptoms in the murine CIA model together with enhanced anti-inflammatory and anti-osteoclastogenic effects [259]. One recent paper has described methotrexate-conjugated polymer hybrid micelles/miR-124 complexes that accumulate in

inflamed joints of adjuvant-induced arthritis in rats. These complexes displayed a superior therapeutic efficacy through both anti-inflammatory and direct bone protective effects. Interestingly the combination of miRNA-124 and methotrexate in these micelles was shown to induce disease remission demonstrating the potentialities of using miRNA to treat RA [260].

## **5.2 Oral and rectal delivery in inflammatory bowel disease**

Due to the physicochemical properties of nucleic acids and their poor stability in biological fluids, oral administration has not been widely considered. However, several groups have focused on improving the bioavailability of ASOs during oral delivery. In most published studies, the ASOs have been associated with a matrix to protect them against degradation. For instance, an enhanced bioavailability of ASOs targeting NF- $\kappa$ B mRNA was obtained by entrapping the nucleic acids in an inert, biodegradable albumin matrix. While control ASOs in solution had a low oral bioavailability (9%), the microencapsulation of the nucleic acids led to a significant increase (up to 70%) of their bioavailability [261]. In another study, the nucleic acid bioavailability was improved by the addition of large amounts of sodium caprate, a penetration enhancer [262]. Oral absorption in humans has been characterized by a strong interindividual variability. Indeed, when studying different formulations to deliver 2'-O-(2-methoxyethyl) modified ASOs, Tillman *and coworkers* revealed that while one of their formulations showed a plasma bioavailability average of 12.0%, the actual results varied from patient to patient from 1.96 to 27.5% [263]. Due to these bioavailability issues, oral administration has been primarily employed for local applications such as in IBD (Table 2). One example is the development of Mongersen<sup>®</sup> an ASO targeting SMAD7 (an inhibitor of TGF- $\beta$  signaling) that has been shown in phase II clinical trial to induce remission in patients with active Crohn's disease [264,265]. Unfortunately, the phase III clinical trial was recently discontinued due to the apparent inefficacy of the drug, although all the factors contributing to the failure of this study remain unknown [266]. In recent years, researchers have identified new targets, and several therapeutic approaches have been tested, with many of them targeting TNF- $\alpha$  [267]. One of these studies focused on the encapsulation of a triple-helical  $\beta$ -glucan poly(dA)-PS-modified anti-TNF- $\alpha$  ASO inside a colon-specific chitosan-alginate hydrogel and achieved a loading efficiency of 83.5 % [268]. The ASOs carried by this chitosan hydrogel were orally administered in a murine model of DSS-induced inflammation and were shown to be specifically released to the inflamed intestine and internalized by intestinal cells and

particularly macrophages. Such cell-specific targeting led to a significant reduction in local production of TNF- $\alpha$ , myeloperoxidase, and malondialdehyde [268].

Similar strategies centered on siRNAs have been carried out using polyplexes or cationic lipids inserted in a matrix. Among the first studies, siRNAs targeting Map4k4 gene, a germinal center protein kinase involved in TNF- $\alpha$  signaling, were complexed with PEI and encapsulated in  $\beta$ 1,3-D-glucan shells. Oral administration of these shells was shown to significantly improve mice survival after LPS intraperitoneal injection through an 80 % inhibition of TNF- $\alpha$  and IL-1 $\beta$  production [37]. SiRNAs have also been encapsulated in thioketal nanoparticles (TKNs), which degrade in response to reactive oxygen species (ROS). Oral administration of those TKNs loaded with an anti-TNF- $\alpha$  siRNA in a murine model of ulcerative colitis induced a decrease in TNF- $\alpha$  mRNA level in the colon and protection from IBD inflammation [269].

As intestinal CD98 expression has been shown to play a crucial role in controlling homeostatic and innate immune responses in the gut, modulation of CD98 expression in intestinal cells represents another promising strategy for the treatment and prevention of IBD [270]. Hence, Xue *and coworkers* as part of their extensive work on this topic, have developed poly(lactic acid)/t nanoparticles coated with poly(vinyl alcohol) and used those formulations to deliver anti-CD98 siRNA/PEI complexes orally to the DSS-induced colitis mouse model [271]. This treatment was shown to effectively downregulate CD98 expression in intestinal epithelial cells and intestinal macrophages leading to the reduction of inflammatory features [271]. In a follow-up study, the same group produced particles composed of the same anti-CD98-siRNA but associated with urocanic acid-modified chitosan and conjugated with an antibody also against CD98 [272]. These nanoparticles were administered orally in a murine colitis model and led to a significant reduction in CD98 colon expression as well as a decreased level of pro-inflammatory cytokines (TNF- $\alpha$ , IL-6, and IL-12) [272]. Furthermore, the accumulation of nanoparticles within the colon and the efficacy of the strategy were also observed through significant reduction of several colitis markers such as body weight loss, myeloperoxidase activity, and histological analysis [272]. The same anti-CD98-siRNA was also co-delivered with curcumin, a known anti-inflammatory agent, using HA-functionalized PLGA nanoparticles encapsulated within a chitosan/alginate hydrogel. Results observed *in vivo* in ulcerative colitis models revealed that this co-delivery of CD98-targeted siRNA and curcumin was able to modulate the inflammation process and protect the mucosal layer [273]. In parallel, this group has also worked on mannosylated nanoparticles to target mannose receptors expressed on

macrophages [274] as well as combining administration of anti-TNF- $\alpha$  siRNA with pro-healing IL-22 [275]. Both siRNA and IL-22 were loaded into galactosylated polymeric nanoparticles for further macrophage targeting, and the nanoparticles were given orally to DSS-induced colitis mice using an alginate/chitosan hydrogel. Mice that received the combination therapy demonstrated quick recoveries with no significant differences compared to healthy controls in key colitis markers such as TNF- $\alpha$  protein expression, colon length, myeloperoxidase levels, or histological score [275]. As an alternative to hydrogels, Kriegel *and coworkers* developed an administration strategy based on poly( $\epsilon$ -caprolactone) microspheres to carry siRNA-loaded gelatin nanoparticles. They used this multicompartamental platform to deliver a combination of siRNA targeting TNF- $\alpha$  and cyclin D1 (CyD1) in the DSS-induced acute colitis murine model. Results obtained demonstrated that this approach induced significant decreases in TNF- $\alpha$  and CyD1 expression in colon cells as well as associated pro-inflammatory IL-1 $\alpha$ , IL- $\beta$ , and interferon- $\gamma$ . Furthermore, the modulation of both TNF- $\alpha$  and CyD1 expression led to an increase in animal body weight, and a reduction of tissue myeloperoxidase activity [276].

While most of the works centered on IBD have been focusing on oral delivery, a few published studies have been exploring rectal administration as an alternative. Using an amphiphilic cationic cyclodextrin (CD) as a carrier, McCarthy *and coworkers* successfully administered rectally TNF- $\alpha$ -targeted siRNA to DSS-induced acute colitis model. Clinical signs of colitis such as total colon weight and colonic mRNA expression of TNF- $\alpha$  and IL-6 were significantly improved for the mice treated with CD TNF- $\alpha$ -targeted siRNA [277]. Alternatively, a strategy based on multi-shell nanoparticles made of siRNA:calcium phosphate complexes encapsulated into PLGA nanoparticles with a PEI outer layer, has been investigated for the local treatment of colonic inflammation. *In vitro*, those nanoparticles loaded with siRNAs directed against TNF- $\alpha$ , KC, or IP-10 exhibited a rapid cellular uptake, little toxicity, and excellent *in vitro* gene silencing efficiencies. Subsequent intrarectal application of these nanoparticles to the DSS murine model led to the significant decrease of the gene expression within colonic biopsies and mesenteric lymph nodes, and an improvement of the overall intestinal inflammation [278].

### **5.3 Lung delivery in inflammatory respiratory diseases**

Local administration of nucleic acid gene inhibitors through inhalation has been naturally investigated to treat lung inflammatory disorders (Table 2). The first studies were carried out



with free nucleic acids and led to encouraging results. In one of them, Danahay *and coworkers* investigated the pulmonary distribution of a PS-ASO targeting human C-raf kinase (ISIS 5132, also known as CGP69846A) after intratracheal (i.t.) instillation [279]. Results obtained in an inflamed airways rat model demonstrated that following i.t. administration, CGP69846A displayed dose-dependent lung retention which was unaffected by local inflammation. Further autoradiography and immunohistochemistry analyses demonstrated the cellular accumulation of the oligonucleotides within alveolar macrophages, eosinophils, and bronchial/tracheal epithelium [279].

Following a study in transgenic mice showing the implication of transcription factor GATA-3 in T helper cell type 2-mediated allergic airway inflammation [280], Finotto *and coworkers* developed a therapeutic strategy based on anti-GATA-3 ASO. Intranasal administration of the ASO to a murine model of model allergic pulmonary inflammation and hyperresponsiveness led to nucleic acid uptake in lung cells, associated with a reduction of intracellular GATA-3 expression [281]. Furthermore, local administration of the anti-GATA-3 ASO resulted in the abrogation of lung inflammation features including eosinophils infiltration and Th2 cytokine production [281]. Similarly, a multi-target approach using a mixture of ASOs targeting phosphodiesterases type 4 (PDE4) and 7 (PDE7) led to positive results in smoke-induced lung inflammation in mice. In this study, the tracheal administration, through a microsyringe, of a solution of free ASOs induced a significant decrease of targeted mRNA in BAL as well as reductions in chemoattractants and metalloproteases levels leading to a decrease in recruitment of neutrophils in the lungs [282].

PEI-based vectors have also been commonly considered for the delivery of siRNA. To treat lung inflammation. For example, intranasal administration of IL-4 ASO/PEI complexes in an ovalbumin-sensitized murine model of airway inflammation led to a 30% reduction of IL-4 concentration in BAL and a subsequent reduction of the number of infiltrated inflammatory cells obstructing the airways [283]. Another strategy using PEI has focused on the delivery of siRNA targeting macrophage migration inhibitory factor (MIF) gene to modulate particle matter-induced airway inflammation in mice. Choi *and coworkers* used a dexamethasone-conjugated PEI polymer to complex the Anti-MIF siRNA and administered the complexes by intratracheal inhalation to SiO<sub>2</sub> particles-exposed mice [284]. This Anti-MIF siRNA treatment

led to the decrease of pulmonary inflammation as observed by the reduction of cellular infiltration and histologically [284]. Similarly, nasal administration of anti-CXCL-1 siRNA complexed with PEI was used in LPS challenged rats and demonstrated to induce a significant decrease in macrophages number in BAL [285]. Finally, Merkel *and coworkers* developed a novel carrier made of transferrin-polyethylenimine (Tf-PEI), to selectively deliver siRNA to activated T cells in the lung [286,287].

Several other cationic polymers have also been used to deliver siRNA. Our group has for instance successfully used polyamidoamine [51] and phosphate-based dendrimers [52] to deliver anti-TNF- $\alpha$  siRNA in a murine lung injury model. Among the two dendrimers made of morpholinium or pyrrolidinium that were tested, pyrrolidinium dendrimers were the most effective and induced a strong reduction in immune cell lung infiltration and cytokine production [52]. An interesting alternative approach has been focusing on new vectors based on penetrating peptides to deliver siRNA and pDNA encoding for a shRNA that both targeted TNF $\alpha$ . Intranasal delivery of the nucleic acids in both an acute lung inflammation model and asthma model in mic led to efficient anti-inflammatory effects [288]. Finally, recent studies have shown that the endogenous production of anti-inflammatory miR-146a following injurious ventilation is not sufficient to prevent lung injury [289]. Consequently, Bobba *and coworkers* used cationic lipid-based nanoparticles to deliver miR-146a by intratracheal instillation and were able to induce significant reductions in lung inflammation and alveolar-capillary permeability [289].

## **6 CONCLUSION**

As presented in this review, a wide array of delivery systems has been developed to deliver nucleic acids to inflamed tissues using diverse routes of administration. The most advanced molecules in preclinical experiments are ASOs and siRNA whereas exciting studies on molecules exploiting the miRNA mechanism are emerging. The use of aptamer is more marginal although these molecules are also being applied with much success as targeting moieties (and not discussed here). Due to the large variety of the systems examined, it is very difficult to determine which are the most adequate ones for each route of administration. However, analyses of the different studies have revealed common required properties such as good entrapment rate, long stability, and endosomal escape capacity. Interestingly, as most of the strategies described in this review have been focused on targeting phagocytic immune

cells, the presence of a cell-specific ligand on the surface of the particles does not appear to be as critical as for other diseases. However, we noticed that most studies do not mention or study immune cell's intracellular specificities, as well as the potential of the pro-inflammatory effect of the delivery systems themselves. We believe that those critical aspects will need to be carefully characterized in the future as nucleic acid-based nanomedicines are promising strategies to treat inflammatory diseases.

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**Table 2: In vivo delivery of nucleic acid-based gene inhibitors in rheumatoid arthritis, inflammatory bowel diseases, and lung inflammation**

BAL: Bronchoalveolar lavage, CIA: Collagene-induced arthritis, DDA: Dimethyl Dioctadecyl Ammonium Bromide, DSS: Dextran sodium sulfate, HA Hyaluronic acid, i.a.: intraarticular, i.v.: Intravenous, LPS: Lipopolysaccharide, PEG: Poly(ethylene glycol), PEI: Polyethyleneimine, PLA: Poly(lactic acid), PLGA: Poly(lactide-co-glycolide), ROS: Reactive Oxygen Species, SNALP: Stable Nucleic Acid Lipid Particles

Formulation	Route of administration	Target/inhibitor	Animal model	Results	Reference
<b>Rheumatoid arthritis</b>					
Cationic liposomes 2-(3-[Bis-(3-amino-propyl)-amino]-propylamino)-N-ditetradecylcarbamoylethyl-acetamide)/DOPE	i.v	TNF- $\alpha$ /siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Inhibition (50-70%) of articular and systemic TNF-<math>\alpha</math> secretion</li> <li>▪ Reduction of IL-6 and MCP-1</li> </ul>	[244]
Cationic liposomes DOTAP/DSPE-PEG	i.v.	TNF- $\alpha$ /siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Efficient delivery of siRNAs to synovial cells</li> <li>▪ Decrease of the severity of arthritis and reduction of TNF-<math>\alpha</math> mRNA in joints</li> </ul>	[245]
Calcium phosphate/liposome-based hybrid nanoparticles covered with folic acid	i.v.	NF- $\kappa$ B/siRNA (Combined with methotrexate)	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Inhibition of NF-<math>\kappa</math>B signaling pathways and reduction of the expression of pro-inflammatory cytokine</li> <li>▪ Folate receptor-targeted nanoparticles suppress the arthritis progression in the mice model</li> </ul>	[249]
SNALP	i.a.	TNF- $\alpha$ /siRNA	DDA-Induced Arthritis/Mice	<ul style="list-style-type: none"> <li>▪ Suppresses inflammation at a low dose</li> <li>▪ Reduces Th1 response</li> </ul>	[248]
Solid-lipid nanoparticles	i.v.	TNF- $\alpha$ /siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Targeting inflamed tissues and reduction of paw thickness, bone loss, and histopathological scores.</li> </ul>	[36]

Nanoparticles of thiolated glycol chitosan	i.v.	TNF- $\alpha$ /siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ High accumulation in arthritic joints.</li> <li>▪ Inhibition of inflammation and bone erosion</li> </ul>	[39]
Nanoparticles thiolated glycol chitosan	i.v.	Notch1/siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Higher targeting efficiency in the arthritic joints</li> <li>▪ Retarded progression of inflammation, bone erosion, and cartilage damage</li> </ul>	[43]
Nanoparticles of chitosan diethylethylamine coupled to polyethylene glycol-folic acid.	i.v.	TNF- $\alpha$ /siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ siRNA uptake facilitated via the folate receptor</li> <li>▪ Reduction of inflammation demonstrated by improved clinical scores and lower TNF-<math>\alpha</math> protein concentrations in target tissues</li> </ul>	[253]
Nanoparticles made of 2,6-pyridinedicarboxaldehyde and low-molecular-weight polyethyleneimine	i.v.	TNF- $\alpha$ /shRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ In vivo inhibition of TNF-<math>\alpha</math></li> <li>▪ Reduction of clinical arthritis score. and paw swelling</li> </ul>	[254]
anti-C5aR1ab - protamine - C5 siRNA conjugate	i.v.	C5/siRNA	Anticollagen antibody induced arthritis /Mice	<ul style="list-style-type: none"> <li>▪ Reduction of arthritis score</li> </ul>	[256]
Micelles of arginine-histidine-cysteine based oligopeptide-modified polymer	i.v.	NF- $\kappa$ B/siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Effective suppression of mRNA expression and inflammatory cytokine levels in the arthritic paws of CIA mice</li> <li>▪ Reduction of clinical scores and paw thickness.</li> </ul>	[40]

Micelles of polyethylene glycol–poly $\epsilon$ -caprolactone–polyethylenimine	i.v.	NF- $\kappa$ B/siRNA +Dexamethasone	CIA/Mice	<ul style="list-style-type: none"> <li>▪ The combination of siRNA and Dexamethasone significantly reduces NF-<math>\kappa</math>B signaling in murine macrophages</li> <li>▪ The co-delivery system was able to switch macrophages from M1 to M2 phenotype.</li> </ul>	[41]
Lipidoid-polymer hybrid nanoparticle Poloxamer 407 and spermidine-based lipidoid (C14)	i.v.	IL-1 $\beta$ /siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Rapid accumulation in arthritis joints</li> <li>▪ Reduction of expression of proinflammatory cytokines in joints</li> </ul>	[250]
Cationic lipid coating PEG- <i>b</i> -PLGA nanoparticles	i.v.	Bruton's tyrosine kinase/siRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Reduction of joint inflammation and other RA symptoms</li> </ul>	[255]
Hybrid Micelles of MTX-PEI-Linoleic acid and mPEG-Linoleic acid	i.v.	miRNA-124 +Methotrexate	CIA /Rat	<ul style="list-style-type: none"> <li>▪ Reduction of arthritis score</li> <li>▪ Reduction of pro-inflammatory cytokine</li> </ul>	[260]
Complex with bone resorption site-specific peptide carrier composed of aspartate and arginine repeating sequences	i.v.	TNF- $\alpha$ converting enzyme/shRNA	CIA/Mice	<ul style="list-style-type: none"> <li>▪ Reduction of arthritic symptoms</li> <li>▪ Enhanced anti-inflammatory effect</li> <li>▪ Enhanced osteoclastogenic effects</li> </ul>	[259]
<b>Inflammatory bowel diseases</b>					
Polysaccharide-based nanocomposites encapsulated in a colon-specific hydrogel made of chitosan-alginate	Oral	TNF- $\alpha$ /PS-ASOs	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>▪ Reduction of TNF-<math>\alpha</math> production by 36%</li> <li>▪ Reduction of myeloperoxidase and malondialdehyde (markers of inflammation)</li> </ul>	[268]

PEI polyplexes in $\beta$ 1,3-D-glucan shells	Oral	Map4k4/siRNA	LPS-induced inflammation in mice	<ul style="list-style-type: none"> <li>Depletion of TNF-<math>\alpha</math> mRNA in macrophages recovered from the peritoneum, spleen, liver, and lung</li> <li>80% decrease in TNF-<math>\alpha</math> and IL-1<math>\beta</math> in peritoneal exudate cell macrophages</li> </ul>	[37]
ROS-sensitive thioketal nanoparticles	Oral	TNF- $\alpha$ /siRNA	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>Reduction of TNF-<math>\alpha</math> messenger RNA levels in the colon and protection from ulcerative colitis</li> </ul>	[269]
PLA nanoparticles loaded with CD98 siRNA/ PEI	Oral	CD98/siRNA	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>Reduction of CD98 expression in mouse colonic tissues</li> </ul>	[271]
CD98-PEG-urocanic acid-modified chitosan nanoparticles carrying CD98 siRNA	Oral	CD98/siRNA	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>Reduction of colon expression of CD98.</li> <li>Reduction of the severity of colitis based on loss of body weight, myeloperoxidase activity, inflammatory cytokine production, and histological analysis</li> </ul>	[272]
HA-functionalized siCD98/CUR-loaded PLGA nanoparticles embedded in a chitosan/alginate hydrogel	Oral	CD98/siRNA Curcumin	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>Accumulation in inflamed colonic sites</li> <li>Suppression of TNF-<math>\alpha</math> and reduction of myeloperoxidase</li> <li>Reduction of the histological score</li> </ul>	[273]
Galactose-functionalized TNF $\alpha$ siRNA-loaded PLGA nanoparticles (Gal-siTNF-nanoparticles embedded in a chitosan/alginate hydrogel	Oral	TNF- $\alpha$ /siRNA IL-22	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>Stronger capacities to down-regulate the expression of pro-inflammatory factors and promote mucosal healing</li> </ul>	[275]

Gelatin nanoparticles encapsulating a combination of siRNA duplexes targeting TNF- $\alpha$ and cyclin D1 entrapped in a poly( $\epsilon$ -caprolactone) microsphere	Oral	TNF- $\alpha$ /siRNA CyD1/siRNA	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>▪ Successful gene silencing with the aid of dual siRNA treatment</li> <li>▪ Decrease colonic levels of TNF-<math>\alpha</math> or CyD1,</li> <li>▪ Suppression of inflammatory cytokines (interleukin-1<math>\alpha</math> and -<math>\beta</math>, interferon-<math>\gamma</math>)</li> <li>▪ Reduction of tissue myeloperoxidase activity</li> </ul>	[276]
Amphiphilic cationic cyclodextrin	Rectal	TNF- $\alpha$ /siRNA	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>▪ Mild amelioration in clinical signs of colitis</li> <li>▪ Significant reductions in mRNA expression of TNF-<math>\alpha</math> and IL-6</li> </ul>	[277]
Calcium phosphate (CaP) core coated with siRNA, encapsulated into PLGA nanoparticles, and coated with a final outer layer of polyethyleneimine	Rectal	TNF- $\alpha$ /siRNA KC/siRNA IP-10/siRNA	DSS-induced inflammation in mice	<ul style="list-style-type: none"> <li>▪ Significant decrease of the target genes in colonic biopsies and mesenteric lymph nodes</li> <li>▪ Distinct amelioration of intestinal inflammation</li> </ul>	[278]
<b>Lung inflammatory diseases</b>					
Aerosol	Intranasal	GATA-3/ASO	ovalbumin-sensitized mice	<ul style="list-style-type: none"> <li>▪ Abrogation of signs of lung inflammation including infiltration of eosinophils and Th2 cytokine production</li> </ul>	[267]
Aerosol	Intratracheal	PDE4/ASO PDE7/ASO	Mouse models of cigarette smoke-induced lung inflammation	<ul style="list-style-type: none"> <li>▪ Significant protection against the smoke-induced recruitment of neutrophils and production of KC and pro-MMP-9</li> <li>▪ Inhibition of target mRNA in cells from lung lavages</li> </ul>	[282]



Polyplexes with PEI	Intranasal	IL-4/ASO	ovalbumin-sensitized murine model of airway inflammation	<ul style="list-style-type: none"> <li>▪ Decrease of circulating IgE levels</li> <li>▪ Alleviation of airway inflammation</li> </ul>	[283]
Polyplexes with PEI and dexamethasone	Intratracheal	MIF/siRNA	SiO <sub>2</sub> particles-induced inflammation in mice	<ul style="list-style-type: none"> <li>▪ Reduction of MIF mRNA</li> <li>▪ Reparative lung response</li> <li>▪ Reduction of cellular infiltration</li> </ul>	[285]
Phosphated dendrimers containing pyrrolidinium surface groups	Intranasal	TNF- $\alpha$ /siRNA	Acute lung inflammation in mice	<ul style="list-style-type: none"> <li>▪ Reduction of TNF-<math>\alpha</math> in BAL</li> </ul>	[52]
Polyamidoamine (PAMAM) dendrimer	Intranasal	TNF- $\alpha$ /siRNA	Acute lung inflammation in mice	<ul style="list-style-type: none"> <li>▪ Reduction of TNF-<math>\alpha</math> and Interferon <math>\gamma</math> in BAL</li> </ul>	[51]
CPP: Stearyl- AGYLLGKLLLOOLAAAALLOOL L-NH <sub>2</sub> (Stearyl- AGYLLG) $\delta$ - OINLKALAALAKAIL-NH <sub>2</sub>	Intranasal	TNF $\alpha$ /siRNA and shRNA	Acute lung inflammation and asthma in mice	<p>In ALL:</p> <ul style="list-style-type: none"> <li>▪ Both peptides are efficient in reducing lung inflammation</li> <li>▪ Both nucleic acid types are efficient</li> </ul> <p>In Asthma:</p> <ul style="list-style-type: none"> <li>▪ Both carriers and nucleic acid types induce reduction of PVI score</li> <li>▪ Mild reduction of inflammation</li> </ul>	[288]
Cationic lipid-based nanoparticles	Intratracheal	miRNA-146a	Injurious mechanical ventilation in mice	<ul style="list-style-type: none"> <li>▪ Reduction in lung inflammation</li> <li>▪ Reduction of alveolar-capillary permeability</li> </ul>	[289]

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