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Non-coding RNA-directed therapeutics in lung cancer: Delivery technologies and clinical applications

Non-coding RNA-Directed Therapeutics in Lung Cancer: Delivery Technologies and Clinical Applications

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Abstract

Lung cancer is one of the most aggressive and deadliest health threats that encounter humans. There has been an increasing interest in non-coding RNA (ncRNA) recently, especially in the areas of carcinogenesis and tumour progression. However, ncRNA-directed therapies are still encountering obstacles on their way to the clinics. In the present article, we provide an overview on the potential of targeting ncRNA in the treatment of lung cancer. Then, we discuss the delivery challenges and the recent approaches enabling the delivery of ncRNA-directed therapies to the lung cancer cells, where we illuminate some advanced technologies including chemically-modified oligonucleotides, nuclear targeting, and three-dimensional *in vitro* models. Furthermore, advanced non-viral delivery systems recruiting nanoparticles, biomimetic delivery systems, and extracellular vesicles are also highlighted. Lastly, the challenges limiting the clinical trials on the therapeutic targeting of ncRNAs in lung cancer and future directions to tackle them are explored.

Keywords: Lung cancer; non-coding RNA; oligonucleotides; nanocarriers.

1. Introduction

Lung cancer, either primary or metastatic, is a leading cause of mortality that accounts for almost 2 million deaths across the globe every year. The World Health Organization (WHO) estimated that among all cancer types, there were 2.2 million new cases of lung cancer and 1.80 million lung cancer-related deaths in 2020, representing 18% of all cancer-related deaths.[1] The one-year and five-year survival rate of lung cancer patients are 26% and 5% respectively, making lung cancer the highest cause of cancer deaths worldwide.[2] Although surgical intervention is effective for several early-diagnosed tumors, its therapeutic capacity is compromised in case of disseminated and metastatic tumors. The lack of tools for early diagnosis, non-feasibility of surgical intervention for late stage patients, as well as the development of drug resistance necessitate the development of new therapeutic approaches to tackle this disease.[3] The progression of diseases can be manipulated on the genetic level via the augmentation of missing genes or knocking down upregulated faulty ones.[4, 5] Furthermore, nucleic acids can serve as platforms for the expression of antigens for vaccination purpose, which is epitomised by the success of messenger RNA (mRNA)-based vaccines against COVID-19. [6, 7]

Despite the availability of diverse modalities for gene therapy, including zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs), Clustered regularly interspaced short palindromic repeats (e.g., CRISPR-Cas9), or small molecules, oligonucleotides (ONTs) are the most widely used and preferred therapeutic tool to accomplish gene therapy owing to their ease of synthesis, modification, and scale-up.[4] ONTs are short nucleic acids that present an amazing tool for the manipulation of cellular RNAs thanks to their ease of synthesis, economic prices, high specificity, and relative high stability compared to their longer counterparts.[8] There are diverse forms of synthetic ONTs that are recruited in gene therapy and its associated research including antisense oligonucleotides (ASO),

short/small interfering RNA (siRNA), short hairpin RNA (shRNA), and miRNA.[4, 9] Moreover, other forms of ONTs are invested in additional applications beyond gene therapy, such as oligodeoxynucleotides (ODNs) that are widely used as adjuvants in immunotherapy and vaccines.[6, 10] Over the past 30 years, ONT therapeutics have emerged as promising candidates to address several diseases such as ophthalmic conditions,[11] hypercholesterolemia,[12] neurodegenerative diseases,[13] and cancer malignancies.[14] Fomivirsen (Vitravene) was the first ONT-based drug approved by the US-FDA in 1998 for the treatment of Cytomegalovirus retinitis in Acquired immunodeficiency syndrome (AIDS) patients.[15] Since then, 15 ONT-based therapeutics have reached the market in addition to a considerable number that are currently under phase I/II clinical trial investigations.[16, 17] **Table 1** lists the FDA-approved ONTs as of 2022.[18] However, the benefits of ONTs are limited by their tricky delivery obstacles, including their susceptibility to the attack of nucleases, instability in plasma, short shelf-life, immunogenicity, and low specificity towards the intended tissue targets. Therefore, efficient delivery vectors are essential to accomplish successful gene therapy using ONTs.[19]

Please, insert Table 1 here

Nanocarriers allow efficient delivery of ONTs compared to the viral delivery vectors, owing to their attractive features including: versatile synthesis and applications depending on the material chosen; tunable physico-chemical properties offering tailored characteristics in relation to the target organ and the delivery route; lower immunogenicity; higher stability; and higher scalability.[4, 7, 20] Unsurprisingly, ONTs complexed within nanocarriers have been approved for the treatment of various diseases. Among the several types of nanocarriers developed during the past decades, lipids nanoparticles (LNPs) and polymeric nanoparticles (polymeric NPs) are the most used. For example, Patisiran (Onpattro®), an FDA-approved therapy for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR), is an

LNPs-based formulation.[21] The characteristics and applications of LNPs and polymeric NPs in nucleic acids delivery have been extensively reviewed in literature.[10, 19, 22]

Most existing ONTs focus on protein-encoding genes, which represent a small portion of the transcribed human genome. A previous study showed that DNA coding for proteins presents only a small part of an organism's genome that make less than 2%.[23] This is in line with another study by Hangauer *et al.*, which showed that the exons encoding for protein compose less than 3% of the human genome.[24] Even though most of the human genome is transcribed at some point, with new ways to identify non-coding transcripts being developed.[25] Hence, non-coding RNAs (ncRNAs) have gained increasing attention as diagnostic and prognostic tools, or as therapeutic targets.[26]

The involvement of ncRNAs, such as microRNA (miRNA) and long non-coding RNA (lncRNA), in the initiation, development and progression of various cancer malignancies has been widely studied and reviewed elsewhere and it has become clear that their upregulation or downregulation can be a key driver of tumour phenotype and progression.[27, 28]

Herein, we shed light on the novel ONT therapeutics that target ncRNAs and their applications in the treatment of lung cancer. We focus on the role of miRNA and lncRNA at the cellular level, and their potential as drug-targets. The principles, challenges and delivery technologies are discussed. Moreover, the clinical potential and future directions of this area of endeavour are expounded.

2. ncRNAs in molecular medicine: theory and applications

2.1. ncRNAs as emerging therapeutic targets

Most of the genome is transcribed into RNA, while only approximately 3% of the transcript encodes for proteins. The remaining 97% comprises ncRNAs that play a crucial role in regulating gene expression.[28] According to their function, ncRNAs can be divided into structural ncRNAs (ribosomal RNA, rRNA, and transfer RNA, tRNA) and regulatory ncRNAs (including miRNA and lncRNA). The latter group influences diverse cellular pathways, hence their involvement in developing several pathologies and diseases.[29]

miRNAs are the most studied class of short ncRNAs. Since the discovery of a small RNA transcribed, lin-4, in *Caenorhabditis elegans* in 1993 [30] and the first mammalian miRNA, *let-7*, in 2000,[31] numerous studies have clarified their mechanism of action and their involvement in cellular pathways, including tumorigenesis, hematopoietic cell differentiation, development timing control, cell proliferation, apoptosis, organ development,[32, 33] inflammation,[34, 35] cardiovascular diseases,[36] and neurodegenerative diseases.[37, 38] The biogenesis of miRNAs, from a precursor stem-loop pre-miRNA, is a multi-step process, the result of which is one strand (the guide strand) being retained within an RNA-induced silencing complex (RISC) to recognize the target mRNA.[39] The other strand (the passenger strand) was previously thought to undergo degradation, although many recent studies demonstrates that it can also regulate gene expression.[40-42] Once the miRNA-RISC complex is formed, it binds the complementary 3′ untranslated region (UTR) of target mRNAs, leading to translational repression and/or mediating cleavage and degradation of the target mRNAs.[43] Recently additional modes of action for miRNA have been discovered, e.g. miRNAs do not only bind to the 3’ UTR of mRNA, but can also recognize other regions along the mRNA transcript; miRNAs are capable of upregulating protein expression under certain conditions.[44]

lncRNAs, which have a limited coding potential, constitute a large class of RNA transcripts longer than 200 nucleotides. The term lncRNA is somewhat misleading, as this RNA species can give rise to small peptides through certain sequences called small open reading frames (smORFs).[45-47] However, primarily lncRNA function as regulatory RNA molecules, regulating gene expression at the epigenetic level.[48-50] Although all functions of lncRNAs have not yet been elucidated in detail they are also known to participate in cellular functions through transcriptional, and post-transcriptional regulation. Mutations in lncRNAs or dysregulation of lncRNA expression have been increasingly associated with many human diseases, such as neurodegeneration, cancer, heart diseases, and Type II diabetes.[51-53] lncRNAs can be grouped based on the types of interactions they make with their targets: RNA-RNA pairings, RNA-DNA hybrids, RNA structure-mediated interactions and protein linkers.[45] More usefully, lncRNAs may be classified based on their targeting mechanisms:[54-56]

- Signal: show cell type-specific expression and respond to diverse stimuli, such as *Xist, COLDAIR;*
- Decoy: bind and titrate away a protein target, but does not exert any additional functions, such as *DHFR upstream transcripts, PANDA;*
- Guide: bind proteins and then direct the localization of ribonucleoprotein complex to specific targets, such as *Xist, HOTAIR;*
- Scaffold: serve as central platforms to bring together multiple proteins to form ribonucleoprotein complexes, such as *HOTAIR, 7SL.*

Lately, targeting ncRNAs have emerged as a very promising tool for the treatment of various diseases including several types of cancer, infectious pathologies, cardiovascular, and respiratory diseases.[57, 58] There is great potential of using therapeutic ONTs as a heavily

studied method to successfully block small and lncRNAs. This is because ONTs can interact with their targets via Watson-Crick base pairing.[59] Additionally, ONTs have biomimetic properties, enabling them to mimic the naturally occurring ncRNAs.[60] Furthermore, lncRNAs can serve as a sponge for the endogenous miRNAs, and subsequently, their targeting can alter the gene expression profile of certain genes which can be valuable from a therapeutic point of view.[61]

The ability of miRNA to influence specific mRNA translation and therefore expression of proteins that are involved in various diseases makes them interesting candidates to develop new therapeutic platforms. Strategies to target miRNAs are based on the fact that miRNAs are either downregulated or upregulated in diseases compared to normal tissues, and consequently miRNA functions can be addressed via two approaches:[62]

(i) Up-regulated miRNA can be targeted with the use of miRNA inhibitors, which are novel derivatives of ASOs.

(ii) Down-regulated miRNA in lacking cells can be replaced by delivering miRNA mimics, which are generally double-stranded RNAs (dsRNAs).

The main strategy to target lncRNAs is represented by the blockade of their function via ASO or siRNAs. Therefore, the expression of specific protein involved in diseases in modulated.[63] However, because lncRNAs are largely resident in the nucleus, their inhibition poses additional challenges compared to the inhibition of mature miRNAs, which reside predominantly in the cytoplasm. Further challenges of targeting ncRNAs and some approaches to tackle them are discussed in subsequent sections of this article.

2.2. Approaches to target ncRNAs in lung cancer

Several studies reported the involvement of ncRNAs in the genesis, development and growth of lung cancer. Among the most studied ncRNAs involved in lung cancer are LINC00313,[64]

MALAT1,[65] *XIST,[66] HIF1A-AS1*,[67] *HOTAIR* p21WAF1/CIP1,[68] *MEG3*,[69] miR-21,[70] and miR-184,[71] which have been broadly reviewed elsewhere. In this section, we focus on the most recent findings about miRNA and lncRNA which have been discovered to play a crucial role in lung cancer and their potential as therapeutic targets. **Fig. 1** outlines some of these novel pathways.

Please, insert Fig. 1 here

Tai *et al.* reported on the involvement of GLIDR in lung adenocarcinoma via the miR-1270/TCF12 axis. Particularly, GLIDR was upregulated in lung cancer cells, and its downregulation via silencing plasmids increased apoptosis and decreased cell proliferation and colony formation. Moreover, the upregulation of GLIDR was concomitant with the downregulation of miR-1270, which was confirmed to be sponged by GLIDR in lung adenocarcinoma. Subsequently, the investigation of potential miR-1270 targets revealed that transcription factor 12 (TCF-12) is directly targeted by miR-1270, thus confirming the influence of the GLIDR/miR-1270/TCF-12 axis in the development of lung adenocarcinoma. Such functional studies establish a firm basis for the targeted mode of action of novel therapeutics.[72]

LINC00520 is an oncogene in several tumour malignancies. However, its role in lung cancer was not investigated until the study carried out by Huang *et al.*, who reported on LINC00520 upregulation in lung adenocarcinoma and its association with poor prognosis in patients. To prove LINC00520 involvement in lung cancer, lung adenocarcinoma tissues were transfected with silencing plasmids. The lowered expression of LINC0520 was related to repression of cells viability and colony formation, hence confirming the association of the lncRNA with cell proliferation.[73] Sheng *et al.* focused on the role of Linc00284 in lung cancer: their studies on human tissues samples from patients and on A549 and NCI-H1975 cell lines revealed

Linc00284 upregulation in lung cancer. Furthermore, it was found to be related to cancer progression via miR-205-3p/c-Met axis. Specifically, Linc00284 silencing was responsible for attenuated cell proliferation, migration and invasion compared to controls.[74]

Regarding miRNAs, Liu *et al.* studies suggested that miR-199a-3p acts as a tumor-suppressor in lung cancer.[75] Calu3 cells and PC9 lung cancer cell lines express lower levels of miR-199a-3p compared to controls and *in vivo* studies demonstrated that miR-199a-3p mimic resulted in the inhibition of Calu3 tumour xenograft growth. Guo *et al*. found that miR-374a-5p correlated with tumor proliferation and migration.[76] miR-374a-5p was found to be downregulated in non-small-cell lung cancer, and the overexpression of miR-374a-5p in A549 cells led to a decrease in cell proliferation and in the ability of cells to migrate. Those findings suggest that novel therapeutics might be viable based on miR-374a-5p mimic to treat lung cancer.

miR-1260b and miR-23a have been reported to be associated with increased exosome release activity from lung cancer cells, with a subsequent promotion of distal organ metastasis and angiogenesis.[77, 78] In addition, Choe and co-workers reported on the tumor-suppressing activity of miR-550a-3-5p through its inhibitory effect on Yes-associated protein (YAP) in various cancer cell lines.[79] On the contrary, Wei *et al.* reported on an opposite tumour-promoting effect of the same miRNA on the metastasis of lung cancer to the brain.[80] Similar alternative and opposite roles for miRNAs in different cellular (or cancer) contexts have been reported elsewhere, so it is important to take this into account when proposing them as ONTs.

3. Delivery challenges of ncRNA-targeted therapeutics

Most ncRNA-targeted therapeutics rely on ONTs that can inhibit or modulate the activity of their targets. Nevertheless, one of the major limitations of ONT therapeutics clinical translation is the numerous issues related to their delivery. In this section, we shade the light on the obstacles that hamper the journey of these novel therapeutics from the bench to the bedside.

First, the poor *in vitro-in vivo* correlation of most experimental research on ONT therapeutics is troubling.[81] In most cases, the promising results shown on cell cultures cannot be reproduced *in vivo*. One possible reason is the dramatic differences between the relatively static two-dimensional status 2D cell cultures and the dynamic three-dimensional status of the *in vivo* environment.[9] Another factor is difference in behaviour between the normal tissue cells and those which were immortalized and cultured ex vivo for a massive number of generations.[82] Moreover, the differences in the origin and methods of preparing various cell lines result in individual variations between them, even though they are used to represent the same disease model.[83]

Second, upon shifting to the *in vivo* evaluation, the Systemically-delivered ONTs face multiple challenges immediately after injection. Particularly, the major obstacle is nuclease degradation that can drastically reduce their bioavailability.[7] In addition, the binding of ONTs to plasma protein may alter their pharmacokinetics or hinder their delivery to their target tissues.[84] On the other hand, some recent reports revealed that plasma protein binding can increase the bioavailability of ONTs under certain circumstances.[85] For example, binding of ONTs to plasma protein prevents them from being rapidly excreted by glomerular filtration.[86] Therefore, proper control of pharmacokinetics of ONTs is a crucial step that must be considered in their *in vivo* delivery. Moreover, the presence of exogenous nucleic acids in the blood stream triggers the innate immune response due to the interaction with pattern recognition receptors, including membrane-bound Toll-like receptors (TLRs) or cytosolic RIG-I family receptors.

The latter fact may result in serious immune reactions such as hypersensitivity or anaphylaxis.[87] Furthermore, ONTs are subject to rapid renal clearance following intravenous administration, where the average half-life of most ONTs is as low as few minutes.[14, 88] One additional factor to consider is the poor specificity of ONTs to the target tissues leading to off-target adverse side effects.[9] One successful approach to target hepatocyte while limiting systemic effect is represented by the conjugation of siRNA with N-acetylgalactosamine (GalNAc), that is recognised by the asialoglycoprotein receptors located on the surface of hepatocytes and hepatocellular carcinoma cells.[89] Such an approach has been successfully applied in Givosiran, a hepatocytes-targeting siRNA that has been approved by FDA in 2019 for the treatment of acute hepatic porphyria (AHP).[90] Some lung-specific targeting approaches are discussed in a subsequent section of this article. However, it should be noted that the attachment of targeting ligands to ONTs or their carriers increases the complexity of the delivery system with a negative impact on its scale-up and clinical translatability.[7] The development of alternative ligand-free targeting methods is currently a hot area of interest.[5]

Third, upon reaching their target cells, the ONTs face additional barriers to access their intracellular targets. The therapeutic cargo needs to be internalized into the desired cells, the process that is limited by the hydrophilic and anionic nature of ONTs which makes them infeasible for passive diffusion. Following endocytosis, the ONT payload needs an efficient tool to escape from lysosomal degradation.[4, 19] Eventually, a substantial proportion of lncRNAs prefers to stay in the nucleus rather than the cytosol, representing an additional obstacle that hampers their targeting.[91, 92]

Fourth, the successful *in vivo* delivery of ONTs does not guarantee a successful clinical translation owing to the differences between the small experimental animal models (e.g., mice) and humans. A famous example is the great success of the enhanced permeability and retention

(EPR) effect in delivering various therapeutics to murine tumor models versus the disappointing results obtained in clinical trials.[7, 14]

4. Advanced delivery technologies

Despite the challenges that encounter the delivery of ONTs targeting ncRNAs, researchers have developed several innovative approaches to tackle them. **Table 2** summarizes the approaches that have been developed in response to the above challenges.

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4.1. Selection of route of administration

Given the additional limitations in systemic delivery of ONTs compared to local administration, topical delivery has been preferred in the development of ONT therapeutics. Not surprisingly, most FDA-approved ONT therapeutics are delivered locally (e.g., intrathecally, intravitreally). Only two of the 15 FDA-approved products (Patisiran and Vutrisiran) are delivered intravenously, which reveals the challenging systemic delivery of ONT therapeutics.[18] Patisiran is complexed within lipid nanoparticles (LNPs), ensuring its successful delivery to the liver. Vutrisiran uses enhanced stabilisation chemistry, which Alnylam Pharmaceuticals has embraced fully, in combination with GalNAc for liver targeting.

The selection of the route of administration of ONT-based drugs depends mainly on the intended application and on the organ that needs to be targeted. Parenteral administration (intravenous and subcutaneous) is chosen when a systemic effect is aimed, while a local route is preferred to deliver drugs in specific compartments. To date, the most exploited routes of administration of ONTs are intravenous (IV) and subcutaneous (SC), followed by intrathecal administration which has gained much interest for the delivery of ONTs to the central nervous system. Patisiran,[93] Mipomersen,[94] and Spinraza[95] are examples of FDA-approved drugs which are delivered intravenously, subcutaneously and intrathecally respectively. Lately, local routes of administration such as oral, pulmonary, intravitreal and rectal are being widely investigated as potential alternatives for ONTs delivery. However, there are still no FDA-

approved locally-delivered ONTs for the use in human diseases, although many Phase I to III clinical trials are showing promising results. **Table 3** highlights some of the registered clinical trials on locally-delivered ONTs therapeutics as of 2022.[96]

Please, insert Table 3 here

Currently, there are no ONT-based drugs approved for the treatment of lung cancer and/or for respiratory diseases. Nevertheless, delivery of ONTs via inhalation is an emerging tool to tackle common respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD) and Cystic Fibrosis (CF). Drug delivery via the lungs offers many advantages to treat pulmonary pathologies over other routes of administration, including large surface available for absorption, direct targeting of the organ bringing about the possibility of lower the dose administered, and non-invasiveness.[97] This may be practical for potential preventative therapies (i.e. targeting early lung cancer development). Still, the same concept may not be applicable to tumours once they are established or disseminated. Nevertheless, the large air/blood interface offers opportunities for less invasive delivery.[98] A study carried out by Templin *et al.* on ISIS-2105, an antisense Phosphorothioate ONT targeting the E2 gene of human papilloma virus (HPV), reported more than 80% of intact ONT in the lungs after its inhalation via saline solution nebulisation. ISIS-2105 lung concentration was significantly higher after inhalation compared to intravenous injection. Moreover, ISIS-2105 systemic concentration after inhalation was negligible for the lowest doses tested and only significant for the higher dose employed in the study.[99] A study by Gottlieb *et al.* on ALN-RSV01, an siRNA targeting the nucleoprotein gene encoded in the RNA of respiratory syncytial virus (RSV), revealed that minimized systemic exposure is achieved when administrating ONTs through inhalation. The drug was not included in any carrier, though it was dissolved in saline solution and administered via inhalation. The results showed minimum systemic availability of the drug suggesting a high local concentration of the inhaled ONT. However, the subsequent

studies did not allow the detection of any antiviral effect of ALN-RSV01.[100] Another study by Drevinek *et al.* on Eluforsen, an inhaled ASO targeting the cystic fibrosis transmembrane conductance regulator (CFTR) mRNA, reported on undetectable serum concentrations of the ASO for 25 mg doses and very low concentration for 50 mg dose in F508del-CFTR homozygous CF patients. Moreover, Eluforsen serum concentration remained low following multiple doses administration. Hence, the study showed that the treatment was safe and well tolerated, outlining as an promising therapeutic tool to treat cystic fibrosis.[101] In addition to the above, improvements in the pharmacokinetic properties of inhaled ONT might be brought about by their encapsulation within nanoparticles, as suggested by the study carried out by De Santi and co-workers, in which an ASO targeting CFTR mRNA was loaded into poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) and nebulized, showing promising *in vitro* results to restore CTFR activity in patient-derived CF cells.[102] An in-depth discussion of the value of nanocarriers in the delivery of ONT therapeutics is provided in the subsequent sections of this article.

Despite the numerous advantages of local delivery of ONTs, intravenous (IV) administration is still dominant in a substantial proportion of the published *in vivo* studies on animal models, probably due to its technical feasibility, economic methodology, rapid results, and uniform distribution of the administered dose to the cells of the target organ.[4] This is even though IV administration is known to confer several adverse effect in off-target organs and to cause more systemic effects such as weight loss. Although the comparative studies between the different routes of administration in the treatment of lung cancer are limited, there are increasing evidence favouring the adoption of local administration in comparison with the systemic routes. The first findings in this area were reported by Garbuzenko and co-workers, who performed an *in vivo* comparative study between intravenous and intratracheal delivery of ASO, siRNA, and a small molecular drug, Doxorubicin, via PEGylated liposomes. Interestingly, the local

intratracheal administration of ASO and siRNA via nanoparticles resulted in a decreased tumor growth compared to the systemic delivery of drug or the intratracheal delivery of the free drug, which demonstrated a great potential for nanoparticles as carriers for ONTs via a local route of administration.[103] More recently, Perry *et al.* investigated the *in vivo* delivery of CpG oligodeoxynucleotide (ODN), an ONT which has been widely explored as anticancer immunotherapy due to its capability of activating B cells and plasmacytoid dendritic cells. The complexation of CpG within polymeric nanoparticles delivered via orotracheal instillation in mice resulted in a prolonged release of cytokines compared to untreated mice and to soluble CpG-treated mice. Further studies on tumor-bearing mice showed that the local delivery of CpG NPs was responsible for 100% survival whereas soluble CpG-treated mice had only a reduction of tumor growth in comparison with untreated mice that reported a steady increase of tumor growth. However, following administration of the first dose of CpG NPs, mice experienced a significant yet transient weight loss that did not worsen after receiving the second dose.[104]

4.2. Chemically-modified ONTs

Synthetic ONTs that are mainly employed in gene therapy including ASO, siRNA, miRNA, and aptamers have been originally composed of naturally-occurring nucleotides that are assembled together. However, chemically-modified nucleotides have emerged later to improve the performance of nucleic acid therapeutics and overcome the pitfalls of their naturally-occurring counterparts.[21] Chemical modification of nucleotides can be performed either at the sugar backbone or at the nitrogenous bases in order to improve their pharmacokinetics (particularly, their bioavailability once administered) and pharmacodynamics properties (affinity to the target). **Table 4** summarizes the most common chemical modifications to ONTs and their advantages.

Please, insert Table 4 here

Chemical modification of ONTs can be beneficial to prolong their shelf-life and to preserve the nucleic acid molecules from excessive plasma protein binding, nucleases, and lysosomal degradation. Moreover, conjugation of ONTs with specific molecules such as peptides, lipids, or other nucleic acids (such as aptamers) can drive the ONTs to the tissue of interest with increased selectivity or facilitate a better complexation within nanocarriers such as LNPs, polymeric nanoparticles, or novel biomimetic vectors.[105, 106] An interesting example of chemical modification at the nitrogenous base level is the incorporation of neutral phopsphoester ONT within siRNA to generate a prodrug that is called "short interfering ribonucleic neutral molecules (siRNNs)", which was introduced by Hamil and Dowdy.[107] While siRNNs are stable and neutral *ex vivo*, once they internalize into the cells, they are enzymatically-cleaved by cytoplasmic thioesterases into native wild-type anionic siRNA molecules that can exert their biological function efficiently. siRNA can be protected from serum nucleases and delivered in vivo without needing carriers or transfection vectors.[107]

Another example of base modifications is the Phosphorothioate (PS), where the non-bridging oxygen atom in the phosphate backbone is replaced with sulphur. Such a modification improves the stability of ONTs against nucleases and promotes their pharmacokinetics via the enhanced binding to plasma proteins to avoid rapid renal clearance. In addition, phosphorothioated ONTs are more hydrophobic than their wild-type counterparts, which improves their cellular uptake.[108] On the other hand, phosphorothioation has been associated with a reported reduced binding affinity to the mRNA targets, which may represent a technical shortcoming that limits their wide applicability.[105] Meanwhile, 2'-O replacements represent a typical model of sugar backbone modifications, where the 2'-OH group of the ribose is replaced with methoxy group (2'-OMe), fluoro moiety (2'-F), methoxyethoxy group (2'-MOE), or 2',4'-o-methylene bridge (Locked Nucleic Acid, LNA). The rationale of such modifications

usually aims at imparting nuclease stability and increasing binding affinity to the target RNA.[105, 109] A combination of two or more of the above-mentioned strategies is also an applicable approach to take the integrative benefits of all of them.[105]

A study by Segal *et al.* provides an example of how chemical modification and conjugation can improve pharmacodynamic properties of ONTs. This involved the synthesis of a 2'-OMe or 2'-Fluorinated versions of *miR-let7* with a Phosphorothioate backbone and its *in vitro*/*in vivo* testing against non-small cell lung cancer (NSCLC). In addition, miR-let7 was conjugated with eicosapentaenoic acid (EPA) or docosanoic acid (DCA) to further improve the ONT stability. Unmodified *miR-let7* and the chemically-modified versions were exposed to serum to mimic bloodstream conditions. The results showed that the ONT modification significantly improved stability, suggesting a beneficial role of chemical modifications to protect *miR-let7* from nuclease degradation. Moreover, the adopted modifications enhanced the biodistribution of *miR-let7* to NSCLC and downregulated the target High-mobility group AT-hook 2 (HMGA2) *in vivo*.[110] Moreover, a study carried out by Russo *et al.* reported the successful conjugation of *miR-34c* with an aptamer to generate a molecular aptamer-miRNA chimera called GL21.T-miR-34c. *In vitro* studies on Calu-1 (NSCLC cell line) and MCF-7 (breast cancer cell line) showed that the chosen aptamer was responsible for the targeted delivery of *miR-34c* into Calu-1 cells, as evidenced by comparing the gene expression profiles obtained after the treatment of both cell lines in question with GL2.T-miR34c which revealed a significant increase in *miR-34c* expression on Calu-1 cells only, thus confirming the selectivity of the aptamer-miRNA chimera for NSCLC cells. Hence, this study gives evidence on how conjugation represents a potential way to improve ONT delivery in lung cancer while avoiding side effects in non-targeted tissues.[111]

4.3. Nuclear targeting

As alluded to above, the fact that a substantial proportion of ncRNAs resides in the nucleus represents a formidable obstacle that hampers the efficient delivery of ncRNA-directed therapies. In addition to the general challenges that encounter ONT therapeutics in the cytosol, the eukaryotic nucleus is surrounded by a double-layered lipid membrane that is called nuclear envelop, with a restricted permeability that is mediated through embedded junctions between the inner and outer membranes, which are referred to as "nuclear pore complexes, NPCs".[112] NPCs consist of cytoplasmic filaments, cytoplasmic outer ring, central channel, transmembrane ring, inner ring, nuclear outer ring, and nuclear basket. A typical structure of NPCs is presented in **Fig. 2**.[113]

Please, insert Fig. 2 here

The transport of cargos through NPCs is regulated by nuclear transporters, which are a wide class of proteins with importins representing their most important member. NPCs are a natural mechanism through which important endogenous proteins (including cell cycle regulators, histones, and transcription factors) gain access to the nucleus following their synthesis in the cytosol; however, they might be exploited by ONTs that target nuclear lncRNA. NPC-trafficked proteins possess certain sequences of amino acids, called nuclear localization signals (NLS), which are recognized by importins to facilitate the transport of the cargo in question from cytosol into the nucleus through NPCs. The mechanism of such a transport is illustrated in **Fig. 3**.[114]

Please, insert Fig. 3 here

NLS were first reported by Adam and co-workers in 1989, who analysed the mutants of simian virus 40 (SV40) and identified a 7-aminoacid NLS (PKKKRKV) that mediates the viral import into the nucleus.[115] Subsequently, several NLS were recognized which are mostly of viral

origin and have been reviewed elsewhere.[114, 116] From a delivery point of view, the identification of NLS has resulted in a revolution in the nuclear targeting, not only to target ncRNAs, but also for the delivery of other gene therapies that require nuclear import such as plasmid DNAs (pDNA). For example, the HIV-derived TAT peptide (YGRKKRRQRRR) has been recognized as both a cell-penetrating peptide to improve the cellular uptake of cargos as well as a NLS to promote nuclear delivery.[117] In spite of its high potential, TAT peptide was limited by its low stability against endogenous proteolytic enzymes.[118] Therefore, octaarginine (R8) peptide, a synthetic biomimetic version of TAT peptide, has emerged to cope with this challenge. R8 has been reported to retain the cell-penetrating and NLS activities of TAT peptide, while demonstrating a higher stability, ease of synthesis, and ease of modifications to suit the various delivery vectors.[119] A stearyl-modified version of R8 peptide (STR-R8) has been developed to facilitate easy incorporation into LNPs.[120] Protamine is another example of NLS, which has been recruited as a complexing agent of pDNA thanks to its highly cationic nature. Following cellular uptake, protamine acts as NLS to mediate the nuclear delivery of its genetic cargo owing to its mimicry to nuclear proteins.[121] Additional examples include the Human cytomegalovirus UL79 protein-derived PY-nuclear localization signal (PY-NLS),[122] BP NLS,[123] and MP NLS.[124]

4.4. Advanced experimental models

The process of drug discovery with a perspective of clinical translation from the bench to the bedside requires intensive preclinical screening and evaluation to confirm the efficacy, selectivity, and biosafety of the drug in question. The same rule applies to ONT therapeutics. However, the classic *in vitro* testing based on two-dimensional (2D) cell cultures has repeatedly reported misleading results with a poor *in vitro-in vivo* correlation, especially in the area of anticancer therapeutics where the wonderful *in vitro* data cannot be reproduced *in vivo*. This mainly can be attributed to the differences between the static 2D cultures versus the dynamic

in vivo environment, lack of cell-cell interactions, lack of blood supply, lack of immune responses, irrelevant pharmacokinetics and distribution, big differences in doses, and variable responses among different cell lines representing a single disease model.[4, 7, 125]

Advanced three-dimensional (3D) *in vitro* models have emerged to tackle the above drawbacks. A graphical summary of some of these models that are used in the evaluation of lung cancer therapeutics is presented in **Fig. 4**.[126]

Please, insert Fig. 4 here

Spheroids have been the first generation of 3D culture models in which cellular aggregates of usually a single cell type are generated to mimic the 3D tumor internal structure and signalling. In addition, the growth of the cellular aggregates leads to the formation of a hypoxic core area in the central part of spheroids, which mimics the hypoxic microenvironment of most lung cancers.[127] Moreover, the growth of spheroids is associated with the deposition of extracellular matrix which mimics the stroma-rich microenvironment of some tumors including lung cancers.[128] Li and co-workers compared the results of gene delivery efficiency of siRNA-loaded nanoparticles to A549-derived lung cancer spheroids versus an *in vivo* murine orthotopic model. The data obtained in the spheroid model were in line with the *in vivo* findings, suggesting a high degree of correlation.[129] However, spheroids that are based on a single cell type cannot reflect the real cellular interactions in the *in vivo* tumor microenvironment, which includes multiple cell types. Furthermore, spheroids derived from laboratory-cultured cell lines do not necessarily reflect the features of clinical tumors.[130] The first challenge has been resolved via co-culturing spheroids with endothelial cells (for angiogenesis studies), immune cells (for immunotherapy studies), or tumor-associated fibroblasts.[128] Meanwhile, the latter challenge can be addressed through the generation of

patient-derived spheroids, which facilitate a better resemblance to clinical tumors and allows the development of personalized therapies.[131]

Organoids represent a more advanced form of 3D *in vitro* models , which are made of stem cells or organ-specific progenitor cells that undergo self-assembly in the presence of a scaffolding extracellular environment like Matrigel® or collagen to give a complex 3D structures with a better resemblance of the organ in question compared to simple spheroids.[132] Studies recruiting lung cancer organoids have started to appear since 2017, when Pauli *et al.* reported a patient-derived organoid of lung adenocarcinoma with a success rate of 50%,[133] followed by Finnberg and co-workers who succeeded in the same year in the development of a patient-derived organoid of NSCLC with a 100% success rate.[134] Since then, multiple lung cancer organoids have been developed which are mostly derived from patient biopsies, which are reviewed and listed by Rossi *et al.*[135] Nevertheless, recent reports have raised concerns regarding the purity of lung cancer organoids as revealed by the possibility of dominance of normal lung cells over their malignant counterparts despite being derived from primary lung tumors. Therefore, more sophisticated protocols are necessary to confirm the purity and identity of the generated organoids, which subsequently complicate the process and raise the costs.[136, 137]

To facilitate a higher mimicry to the dynamic *in vivo* environment, microfluidics technology has been introduced to enrich the performance of spheroids and organoids, which has led to the evolution of "organ-on-chip" systems. Microfluidic channels can maintain a dynamic flow of the culture media supplemented with nutrients to simulate the blood supply.[7] This also enabled the study of cell migration and tumor metastasis. Moreover, the extravasation process from the blood vessels to the tumor cells can be simulated as well. For example, Kühlbach *et al.* developed a microfluidic system composed of two channels separated by a porous membrane, where one channel represented the vascular compartment and contained primary

endothelial cells derived from the pulmonary artery and the other channel represented the reservoir compartment that received the extravasated payloads. Under flow conditions, the endothelial cells behaved like the *in vivo* conditions and the extravasation of fluorescently-labelled cancer cells into the recipient chamber could be tracked.[138] Furthermore, microfluidics technology can be integrated with 3D bioprinting and hydrogel biomaterials to simulate the tumor microvasculature. Meng and co-workers designed 3D bioprinted capsule for the controlled release of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) into a microfluidic organoid system. The programmed release of the above growth factors allowed the accurate study of the metastasis, angiogenesis, and cell migration processes with a further potential in evaluating anticancer treatments.[139]

The epithelial cells lining the airways of the lung are thought to play a pivotal role in the development of respiratory tract malignancies. However, 2D cultures of these cells demonstrate a significantly different morphology and biological performance compared to the real microenvironment. Moreover, animal models based on rodents demonstrate different anatomical features from humans. For example, rodents possess 6-8 levels of branching airways, while humans owe 20 or more levels of branching. In addition, mice are lacking the complicated structure of human bronchioles.[140] Therefore, air-liquid interface (ALI) culture systems have emerged to address these shortcomings.[141] In ALI models, the cells are cultured so that their apical parts are exposed to air while their basal parts are immersed into the cell culture media (liquid phase). Such unique architecture allows cells to differentiate into the mucociliary phenotype that mimics the *in vivo* situation.[142] Meenach and co-workers reported a significant difference in the half-maximal inhibitory concentration (IC_{50}) of the cytotoxic drug, paclitaxel, upon being tested on A549 lung cancer spheroids that were submerged or cultured on ALI system, which revealed the importance of ALI systems in providing accurate data compared to submerged culture systems.[143] Furthermore, ALI

systems have been utilized in studying other respiratory diseases including viral infections, cystic fibrosis, and asthma.[144-146]

There have been an increasing number of reports in favour of the recruitment of non-human primates to replace rodents and *in vitro* models in preclinical studies. Yet, the wide application of this approach is limited by the high cost, complex handling tools, and the necessity for special facilities, high therapeutic doses, and highly-trained personnel.[7]

4.5. Nanocarriers

To reach their target transcript and carry out their functions, ONTs must be delivered into the desired tissues and cells. This has prompted massive efforts to search for new delivery systems that deliver nucleic acids into target cells. The delivery system is expected to protect ONTs in transit and improve cell targeting, but needs to do so without limiting cell entry and intracellular bioavailability at the desired biological target. After entering the cells, the chosen delivery vector should ensure that ONTs can escape from the endosomes and enter the cytoplasm.[10, 19] Although viral-based vectors such as Adenoviruses, Lentiviruses, and Adeno-associated viruses (AAVs) have shown high gene delivery efficiency, they are limited by the challenges of immunogenicity, low flexibility for surface modifications, and immunogenicity.[4, 19] Researchers have recently begun to focus on nanotechnology as potential non-viral delivery systems such as lipid nanoparticles (LNPs), polymeric nanoparticles, biomimetic nanoparticles, and extracellular vesicles. With regards to lung cancer treatment, some novel studies reported promising *in vivo* results after ONTs delivery via nanoparticles, both systemically (intravenous and subcutaneous injections) and locally (intratracheal delivery) that are summarized in **Table 5**. The following section will focus on the advances of the employment of nanoparticles for the development of miRNA and lncRNA therapeutics for lung cancer treatment.

Please, insert Table 5 here

4.5.1. Lipid nanoparticles (LNPs)

LNPs have been considered the gold standard non-viral gene delivery vectors, especially following the enormous success of LNPs-based vaccines targeting COVID-19. This may be attributed to their rapid fabrication, natural abundance of their precursor lipids, and mimicry to biological and cellular membranes.[5, 6] LNPs have witnessed a wide variety of developments starting from the simple cationic or PEGylated liposomes, reaching stimuli-responsive and self-targeted LNPs.[7] In addition, the preparation methods of LNPs have also been developed. Traditional preparation methods of LNPs were based on tedious, non-scalable, and hard-to-reproduce methodologies such as thin lipid film hydration method,[9] alcohol dilution method,[147] extrusion,[148] and reverse-phase evaporation method.[149] Over the past decade, microfluidic mixing technology has emerged to substitute the classic preparation methods, enabling scalable preparation of LNPs with a high degree of uniformity, particle size control, high loading capacity, and high encapsulation efficiency of nucleic acid cargos.[14] Furthermore, a novel concept of self-homing LNPs that can reach their target without the need of attaching targeting moieties has been introduced. In such an approach, the composition and physico-chemical properties of LNPs are tweaked to manipulate the endogenous protein corona that encapsulate them following their *in vivo* administration, which subsequently allows the recruitment of endogenous transport mechanisms for the highly-efficient and selective delivery of LNPs to their target cells. Thus, simple, economic, and scalable delivery systems can be developed to substitute their sophisticated counterparts that rely on ligand-based targeting.[5]

In the area of lung cancer ONT therapeutics, several LNPs systems have been reported. Yung *et al.* developed LNPs based on a cationic lipid combination of tertiary amine and quaternary amine, which were called “QTsome”. The developed LNPs were recruited for the delivery of

anti-miR-21 to A549 xenograft mouse model following intravenous administration. The results revealed that knocking down miR-21 resulted in a significant tumor suppression and prolongation of survival rate of mice.[150] Abd Elwakil *et al.* prepared multifunctional envelope-type nanodevice (MEND) based on a pH-sensitive cationic lipid, YSK05, and modified with GALA peptide to target the lung endothelium. Upon loading MEND with siRNA targeting cluster of differentiation 31 (CD31), a significant tumor eradication was obtained in an aggressive murine model of metastatic lung cancer.[151] Other studies are summarized in **Table 5**.

4.5.2. Polymeric nanoparticles

Polymers are some of the most common used materials for nanoparticles preparation. This is due to their numerous innate properties, such as the versatility of structural conformations, biodegradability, and ease of synthesis. Polymeric nanoparticles could play a crucial role in advancing ONT therapeutics. Cationic polymers have emerged as promising candidates for non-viral gene delivery systems, given they can be easily conjugated with genetic material via electrostatic attraction at physiological pH (the negative charges of nucleic acids will easily bond with the positive charges of polymeric chains). Several factors can influence the ability of cationic polymers to carry nucleic acids, including their structure, molecular weight, and surface charge. Thus, flexible properties together with their facile synthesis, allow researchers to have a broad range of materials available to develop the best delivery system for the specific purpose.[7] Moreover, few *in vivo* studies revealed the potential of using polymeric nanoparticles instead of LNPs. Yang *et al.* reported the successful complexation of miR-155 inhibitor within nanoparticles composed of a biodegradable poly(ester amine) (PEA) and hyaluronic acid, and their delivery in tumor-bearing mice. A considerable decrease in tumor size was reported in mice treated with miR-155-loaded nanoparticles compared to controls.[152] In addition, Zhang *et al.* investigated the effect of functionalization of

poly(lactic-co-glycolic acid) (PLGA) nanoparticles with epidermal growth factor receptor (EGFR)-targeting aptamer as a tool to deliver the anticancer agent, homoharringtonine (HHT), selectively to lung cancer. Results showed a diminished tumor growth in mice treated with the functionalized nanoparticles compared to those received free drug or non-functionalised nanoparticles, suggesting an interesting application for ONTs in tumor-targeting drug delivery.[153]

Chitosan is a naturally derived polymer that has been widely investigated as a starting material for the preparation of nanocarriers, due to its beneficial properties, notably mucoadhesiveness and antibacterial activity. In addition, the presence on its backbone of hydroxyl and amino groups are beneficial in manufacturing water-soluble or target-oriented chitosan derivatives.[154] Chitin is the naturally-occurring precursor of chitosan, which is abundantly found in nature (in crustacean shells or fungi). Chitosan, obtained via the deacetylation of chitin, is a polysaccharide composed of repeated glucosamine and N-acetyl-glucosamine units. It has been widely used in biomedical application for the preparation of micelles, hydrogels and nano-complexes given its properties, including good biocompatibility, favourable biodegradability, low cytotoxicity, absorption enhancer, anticholesterolemic and antibacterial properties.[155, 156] Several studies revealed its potential as a starting material to produce nano-structures able to act as drug carriers. The presence on its backbone of positively charges carried by the amino groups, held great promise for chitosan to create complexes with negative charged molecules including nucleic acids.[157] Numerous studies have confirmed the safety and efficacy of nucleic acid delivery via chitosan *in vivo* as well.[158-160]

However, the protonation of amino groups on chitosan backbone at physiological pH render the polymer insoluble in water, which represents the major limitation in chitosan utilisation. To expand the application of chitosan in biomedicine, improving chitosan structure was necessary to impart new properties to the polymer. This was possible through exploiting the

two reactive groups on chitosan backbone being the amino group (-NH) and the hydroxyl group (-OH). Moreover, the production of chitosan derivatives not only improved its chemical and physical properties, but also broadened its applications: -OH and -NH groups render chitosan a highly customizable candidate to have a polymer with improved targeted delivery, drug release ability or mucoadhesive properties.[161] **Fig. 5** shows the chemical structure of chitosan, its synthesis, and some common derivatization strategies.

Please, insert Fig. 5 here

A favourable example of the application of chitosan derivatives is the chitosan-based delivery system developed by Ragelle *et al.*[162, 163] The study revealed that modifying chitosan with a small peptide (Arginine-Glycine-Asparagine) would not only improve targeted delivery of miR-34a but also improve its anti-cancer activity *in vivo*. Further developments of chitosan-based delivery systems were exploited by several researchers over the past years, revealing the potential of chitosan as a versatile polymer. **Table 6** summarizes the most recent chitosan modifications which were beneficial in delivering ONT therapeutics for cancer-related applications.

Please, insert Table 6 here

Regarding the utilization of chitosan to deliver ONTs in lung cancer, studies showed its promising application in enhancing drug delivery and uptake. Nafee *et al.*[164] as well as Taetz *et al.*[165] demonstrated the ability of the water-soluble chitosan hydrochloride to enhance cellular uptake of an ASO directed against human telomerase when the polymer was used to coat PLGA NPs. Confocal microscopy with a fluorescent-labelled ASO revealed that the chitosan hydrochloride coating of PLGA NPs was responsible for the enhanced uptake of the nanostructures in A549 and Calu3 cells. Furthermore, cytotoxicity studies showed a decrease of A549 cell survival when treated with the ASO-complexed chitosan-coated PLGA NPs,

confirming the successful internalisation of the drug into the desired cells. Later on, the same research group tested the nanocarrier on lung cancer cells derived from human patients and the results revealed similar outcomes in terms of uptake and cell-survival.[166] More recently, studies carried out by Zhu *et al*.[167] and Huang *et al*.[168] provide examples of the benefits of using chitosan derivatives-based NPs for the detection and treatment of lung cancer, respectively. Zhu *et al*. investigated the manufacture of chitosan-molecular beacon (MB) NPs and their application in the detection of lung cancer. Hairpin-ONT molecular beacons are gaining an increasing interest over the past years given their ability to produce fluorescence after binding with miRNA (or RNA and DNA) targets, thus being perfect candidate for genetic material detection and diseases diagnosis. However, given their structure and charge, they cannot penetrate cell membrane. The results obtained by the authors revealed the successful complexation of chitosan with the designed anti-miR-155 molecular beacon and secondly that chitosan represented a valuable tool to deliver the molecular beacon to the tissues of interest in lung cancer-baring mice. Whilst not a therapeutic study, this does suggest the ability of chitosan to deliver ONTs to lung cancer and to enhance its uptake into cells. The latter study by Huang *et al*. investigated the formation of α-linoleic acid-modified chitosan nanocarriers, complexed with phenylboronic acid and loaded with gefitinib and an antimiR-21 ONT. Uptake studies by flow cytometry and confocal microscopy on lung cancer cell lines demonstrated efficient internalization of the nanocarrier by the cells. In addition, the treatment of cells with the nanomedicine considerably increased the expression of Phosphatase and tensin homolog (PTEN), usually downregulated by miR-21, compared to the treatment with naked anti-miR-21, thus suggesting the promising ability of the employed chitosan-derivative in the delivery and uptake of ONTs.

4.5.3. Biomimetic NPs

Recently, the paradigm of drug delivery has shifted from overcoming the natural barriers to "learning from nature" and imitating the natural pathways, which has led to the flourishment of the concept of biomimetic drug delivery systems. Biomimetic nanoparticles have emerged as a novel tool for the delivery of biologically-active molecules to tumor malignancies, recapitulating the properties of naturally-occurring particles. Their ability of mimicking endogenous molecules significantly reduces innate-immune system activation and can provide an enhanced and accurate targeted delivery of active pharmaceutical ingredients.[10, 169, 170] However, their application regarding delivering ONTs for the treatment of lung cancer is still limited. One of the studies that provided a proof-of-concept of biomimetic NPs for cancer therapy is the study by Vázquez-Ríos *et al.,* in which exosome-mimetic nanosystems (EMNs) were designed to resemble A549-derived exosomes. In addition, EMNs were functionalized with specific proteins to increase the tropism of EMNs towards lung cancer cells. Results showed that functionalized-EMNs (F-EMNs) have similar characteristics compared to natural exosomes in terms of size, composition, drug loading capacity and *in vivo*-targeting ability, while providing the advantages of faster preparation and higher batch-to-batch reproducibility. Moreover, *in vivo* data on A549-bearing mice models demonstrated that F-EMNs have similar pharmacokinetics properties in comparison with natural exosomes.[171]

Hyaluronic Acid (HA) has been widely investigated as a material for biomimicry purposes given its unique features, among which is its ability to bind and activate cluster of differentiation 44 (CD44) receptor, that is upregulated in several cancer malignancies.[172] In lung cancer, upregulation of CD44 receptor has been found to be related to tumor progression and metastasis formation. The study carried out by Kim *et al*. highlighted the role of HA in NP coating when aiming for targeted drug delivery. Particularly, the generation of ASO-containing NPs, hybridized with MU peptide and coated with HA (named HMA nanoballs) seemed to be

a promising approach for the successful delivery of ASOs to CD44-overexpressing cancer cells. The study revealed a preferential tumor accumulation of HMA nanoballs compared to uncoated nanoballs in mice that were previously injected with skin cancer cells (KR). Hence, given the role of CD44 in lung cancer. These findings suggested that HMA nanoballs might be useful instruments to deliver ASO to lung cancer cells.[173]

4.5.4. Extracellular vesicles

Much natural cell-to-cell communication occurs via cellular secretion of biomolecules (proteins, nucleic acids, lipids) loaded into extracellular vesicles. Unlike biomimetic NPs, these are naturally-occurring biological carriers that are less-likely to be recognised as xenobiotics, thus minimising side effects. Extracellular vesicles come in a variety of particle sizes with different surface properties, including intrinsic targeting properties. Among the various kinds of extracellular vesicles, circulating exosomes are nanovesicles of endocytic origin measuring 40-120 nm in diameter. As vehicles of intercellular communications between healthy and cancer cells, they are involved in several steps of the carcinogenesis process, from tumour growth to angiogenesis and metastasis.[174] Since their first discovery and isolation in the 1980s,[175, 176] exosomes have gained particular attention amongst researchers for their pronounced potential as diagnostic and theranostic tools for various pathologies.[177]

One main application of exosomes is their use as carriers for biomolecules such as proteins or ONTs that are challenging to deliver via lipid or polymeric carriers. The use of exosomes offers several advantages over traditional carriers such as low immunogenicity and toxicity, wide distribution throughout the human fluids, ability to carry a variety of molecules across impervious membranes, such as the blood brain barrier, capability of targeted delivery to specific cells due to the presence of protein on their surface.[178] Although there are several studies regarding ONT delivery via exosomes for the treatment of malignancies such as breast,

gastric and colon cancers,[178-180] the application of such a technology in lung cancer is still minimal. A recent study by Jeong *et al*., utilizing two different *in vitro* approaches (canonical 2D cell culture study and a novel microfluidic 3D lung cancer model), demonstrated that HEK293T-derived exosomes loaded with miR-497 mimic were able to reduce tumor growth and migration in A549 cells and angiogenesis formation in human umbilical vein endothelial cells (HUVECs).[181] Moreover, another study by Nie *et al*. proved that miR-216 loaded into breast cancer cells-derived exosomes (miRNA-231-Exo) represent not only a promising tool for delivery of miR-126 mimic, which has previously been reported to inhibit tumor growth in A549 cells via PTEN/PI3K/AKT signalling pathway,[182] but they also have a unique tropism towards A549 lung cancer cells compared to normal human embryonic lung fibroblasts. Thus, highlighting the importance of exosome-surface protein expression in target recognition and metastasis formation process. In addition, miRNA-231-Exo were able to lessen tumor proliferation and migration in mice bearing A549 tumors.[183]

Despite the promising features of exosomes, they are still limited by multiple technical obstacles in terms of isolation, purification, scale-up, and drug loading techniques, which collectively hamper their wide clinical applications. Such an area of endeavour is still in need of tremendous research efforts to bring them into the clinics.[184]

5. Clinical potential and future perspectives

ncRNAs were first reported in *E. coli* followed by eukaryotic cells in 1970s, however, their important role as regulators of gene expression has not been elucidated until 2000s. Since that time, the contribution of a huge number of ncRNAs, especially miRNAs, in the carcinogenesis and progression of various tumors has been extensively investigated.[185] Moreover, there have been 45 clinical trials dealing with ncRNAs in the area of lung cancer as of February 2023.[186] Nevertheless, the vast majority of these trials involved the use of ncRNAs as diagnostic or prognostic tools. A limited proportion of clinical trials attempted the therapeutic delivery of ncRNA-directed therapies to lung cancer. For example, MRX34 is a lipid nanoparticle formulation of miR-34a that reached clinical trials stage for treatment of several malignancies including lung cancer. Although the study was withdrawn due to adverse effects in most of the patients recruited in the study, the encapsulation of miR-34 in the lipid formulation was a key to promote miR-34a therapeutic value.[187, 188] The present situation shades the light on the limitations that hamper the clinical translation of ncRNA technology in the field of anticancer therapeutics. The delivery challenge has been considered one of the major obstacles associated with gene therapy in principle, taking into consideration the delicate and complicated nature of nucleic acids compared to other drugs. However, the dramatic advances in delivery technologies have enabled several gene therapies to reach the pharmaceutical market within the past few years.[7] Thus, a specific focus on the shortcomings of ncRNA-directed therapies is needed.

First, most reported gene therapies target the coding RNA fragment (mRNA) to modulate the production of proteins in question. This can be understood considering the well-characterized nature of this area of interest, where most of the cellular proteins have been identified, characterized, and their relevant expression pathways have been elucidated. On the contrary, ncRNA is still a virgin area with much less clear background. Upon searching the literature, a

substantial proportion of the published research on ncRNA fits the area of bioinformatics, where the relationship between the differential expression of certain ncRNAs and tumor prognosis is studied.[189] Although these studies are important to give us idea on the potential of such ncRNAs as therapeutic targets, there are still a lot of efforts needed for their experimental characterization, especially in terms of their chemistry and production pathways, in order to make them qualify as therapeutic targets. Second, the lack of integration between the finding of researchers in the overlapping areas of bioinformatics, nucleic acid chemistry, molecular biology, and drug delivery makes it difficult for any of them to complete the journey alone from the bench to the clinic. While the current economic crisis post COVID-19 and Russia-Ukraine war led to a significant shrinkage in academic funds that can gather the multidisciplinary researchers together into collaborative projects, artificial intelligence (AI) may offer a promising solution that can help researchers to match the reported findings on a certain target so that a comprehensive overview is generated to stimulate the research to go on without the need of starting from scratch.[190] Third, chemically-modified ONTs are increasingly substituting their wild-type counterparts, which is a promising direction to maximize the efficiency, selectivity, and biosafety. An integration between such a technology and the drug delivery nanotechnology will enable highly-potent and selective therapies to be developed.[6] Fourth, the compositions of the delivery nanosystems need to be simplified with a more focus on the implementation of biomaterials and ligand-free targeting strategies that will improve their scalability and subsequently, their clinical translatability. In addition, the recruitment of novel large-scale production techniques such as microfluidic mixers will speed up the manufacture and encourage the investment on these therapies.[5, 7, 191] Fifth, shifting the dependency of laboratory research from 2D cell cultures and rodents models to organ-on-chip and non-human primates will provide more reliable and clinically-relevant results to be built on during the clinical trials stage. In conclusion, targeting ncRNAs is a novel and

promising non-classic endeavour in the humanity's battle against cancer. We expect to see enormous achievements in such an area in the upcoming years.

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The authors report no conflict of interests associated with this work.

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Figure legends

Fig. 1. Some novel pathways for the involvement of ncRNAs in the tumorigenesis of lung cancer, which can offer promising therapeutic targets. Abbreviations: TCF-12, Transcription factor 12; YAP, Yes-associated protein; c-Met, Mesenchymal-epithelial transition factor. The figure was created using BioRender.com software with a publication license.

Fig. 2. A typical structure of NPC. The NPC consists of an inner ring and two outer rings; cytoplasmic and nuclear, and is embedded within the nuclear envelope through transmembrane Nups. FG-repeat NUPs form a central channel that controls the transport through NPC. The nuclear basket faces the nuclear side, while eight filaments face the cytoplasmic side. The right panel of the figure shows the names of the involved NUPs in each substructure. Abbreviations: NPC, nuclear pore complex; NUPs, nucleoporins. The figure is reproduced from Mitic *et al.*[113]

Fig. 3. A schematic illustration showing the mechanism of nuclear import through NPCs, which is triggered by NLS, and the subsequent regulation of the process. A) The mechanism of nuclear import via importins. The NLS-containing cargo is recognized and bound to importin α, which subsequently binds to importin β1 to form a trimer that is directed to NPCs for the nuclear import. The process is regulated by the cytoplasmic protein, RanGDP, that is transformed into RanGTP post nuclear import. B) Recycling of importins following the nuclear import process. RanGTP binds to importin β1 leading to the dissociation of the imported trimer, which is catalyzed by NUPs such as Nup50 to prevent their re-association. Subsequently, importin α and importin β1 are exported back to the cytosol to be used in further transport cycles. The figure is reproduced from Lu *et al.*[114]

Fig. 4. A graphical summary for the advanced *in vitro* models that are used in the study of lung cancer therapeutics. The figure is reproduced from Boucherit *et al.*[126]

Fig. 5. Chemical structure, synthesis, and chemical derivatization of chitosan. The functionalisation of –OH groups (red) and/or $–NH_2$ groups (Blue) with various molecules can lead to the formation of chitosan derivatives with customisable properties.

Tables

Table 1. List of FDA-approved oligonucleotide therapeutics as of 2022, their approval year, indication and administration route.[18] [(a)]

Oligonucleotide type	Brand name, Drug name	Manufacturer	Indication	Administration route	Approval year
ASO	Vitravene (Fomivirsen)	Ionis	Cytomegalovirus infection	Intravitreal	1998 [(b)]
Aptamer	Macugen (Pegaptanib)	Eyetech/Pfizer	Neovascular, elderly macular degeneration	Intravitreal	2004
ASO	Kynamro (Mipomersen)	Ionis	Hypercholesterolemia	Subcutaneous	2013
ASO	Exondys 51 (Eteplirsen)	Sarepta	DMD	Intravenous	2016
ASO	Spinraza (Nusinirsen)	Ionis	Spinal muscular atrophy	Intrathecal	2016
ssON	Defitelio (Defibrotide)	Jazz	Hepatic veno-occlusive disease	Intravenous	2016
ASO	Tegsedi (Inotirsen)	Ionis	hATTR	Subcutaneous	2018
siRNA	Onpattro (Patisiran)	Alnylam	hATTR	Intravenous	2018
ASO	Waylivra (Volanorsen)	Ionis /Akcea	Familiar chylomicronaemia syndrome	Subcutaneous	2019
ASO	Vyondys 53 (Golodirsen)	Sarepta	DMD	Subcutaneous	2019
siRNA	Givlaari (Givosiran)	Alnylam	Acute hepatic porphyriasis	Subcutaneous	2019
ASO	Viltolarsen (Viltepso)	NS pharma	DMD	Intravenous	2020
siRNA	Oxlumo (Lumasiran)	Alnylam	Primary hyperoxaluria type 1	Subcutaneous	2020
ASO	Amondys 45 (Casimirsen)	Sarepta	DMD	Subcutaneous	2021
siRNA	Leqvio (Inclisiran)	Alnylam /Novartis	Hypercholesterolemia	Subcutaneous	2021

[(a)] The data were collected from FDA database and classified by the authors.

[(b)] The drug was discontinued in 2002.

Abbreviations: ASO, Anti-sense oligonucleotide; ssON, Single-stranded oligonucleotide; siRNA, Small interfering RNA; hATTR, Hereditary transthyretin-mediated amyloidosis; DMD, Duchenne muscular dystrophy.

Table 2. A summary of the major challenges that encounter ncRNAs-targeting oligonucleotide therapeutics and some recent approaches to tackle them.

Nature of the challenge	Challenge	Approach to overcome	References
Experimental	Poor *in vitro-in vivo* correlation	3D cell culture models	[7]
		Dynamic culture models	[192]
In vivo	Poor stability	Chemically-modified oligonucleotides	[193]
	Poor pharmacokinetics	Nanocarriers	[7, 19, 20]
	Immunogenicity		
	Poor selectivity	Targeted delivery systems	[4, 9]
Cellular	Poor cellular uptake	Targeted delivery systems	[9, 191]
		Cell-penetrating peptides	[194]
Intracellular	Lysosomal degradation	Endosomal escape devices	[6]
		Fusogenic lipids	[151]
	Poor nuclear penetration	Nuclear-localization signals	[114]
Translational	Poor scale-up	Ligand-free targeting	[5]
		Microfluidic devices	[7, 14]
	Poor animal-human correlation	Representative animal models	[4, 195]
		Patient-derived xenografts	[196, 197]

Table 1. oligonucleotide therapeutics delivered by local routes of administration that reached advanced clinical trials.[96] [(a)]

Oligonucleotide type	Oligonucleotide name	Administration route	clinical Phase	Indication	ClinicalTrials.gov Identifier
ASO	Mongersen	Orally	Phase II [(b)]	Chron	NCT02685683
ASO	Exc 001	Intradermally	Phase IIb	Hypertrophic scar	NCT01038297
ASO	Alicaforsen	Rectally	Phase III	Chronic antibiotic refractory pouchitis	NCT02525523
miRNA mimic	Remlarsen	Intradermally	Phase II	Skin Fibroplasia	NCT03601052
siRNA	Cotsiranib	Intradermally	Phase II	Hypertrophic scar	NCT02956317

(a) The data were collected from National Institute of Health (NIH) database and classified by the authors.

(b) Withdrawn later due to lack of effectiveness.

Table 4. A summary of the most common chemical modifications of oligonucleotides, their description, and the advantages and disadvantages they brought about in the oligonucleotide therapeutics.

Site of modification	Mode of modification	Description	Advantages	Disadvantages	Application
Nitrogenous base	Phosphorothioate (PS)	Non-bridging oxygen atom is replaced with sulphur group	Improved stability against nucleases; Improved pharmacokinetics; Increased binding to plasma proteins; Increased hydrophobicity	Reduced binding affinity to the mRNA targets	ASO
	Phosphoester	Neutral phopsphoester oligonucleotides are incorporated within siRNA to generate a prodrug (short interfering ribonucleic neutral molecules)	Massive RNAi response (due to the cleavage of Phosphoester group once the molecule is internalized)	-	siRNA
	Peptide nucleic acids (PNA)	Sugar backbone is replaced with synthetic poly ethyleneimine scaffold with nucleobase acetic acid connected to every second backbone nitrogen atom via amine bond	Improved stability against nucleases and proteases; Stronger affinity to RNA targets	-	
Sugar	2'-OMe	2'-OH group of the ribose is replaced with 2'-methoxy group	Increased affinity to RNA; Increased stability against nucleases	Sensitivity to serum nucleases	siRNA, ASO
	2'-F	2'-OH group of the ribose is replaced with 2'-fluoro	Increased binding affinity to RNA	-	

2'-MOE	2'-OH group of the ribose is replaced with 2'-methoxyethoxy group	Increased affinity to RNA; Increased serum stability; Increased miRNA inhibitory activity		
Locked Nucleic Acid (LNA)	2'-OH group of the ribose is replaced with 2',4'-o-methylene bridge	Increased RNA affinity (reached by reducing the conformational flexibility of nucleotides)	Anti-miRNA activity is only slightly higher	
Combination of chemical modifications	More than one of the abovementioned modifications co-exist in the same oligonucleotide molecule	Combined benefits of multiple modifications in a single molecule	Complexity of synthesis	All types

Abbreviations: RNAi, RNA interference.

Table 5. Preclinical studies regarding oligonucleotide therapeutics delivered via nanoparticles as novel treatments for lung cancer.

Study	Context	oligonucleotide cargo	Nanocarrier type	Delivery route/animal model
(Loira-Pastoriza *et al.*, 2021)[198]	Immunotherapy of metastatic lung cancer	Phosphorothioate-linked CpG ODNs (C274 and B1826)	Cationic liposomes	Intraperitoneally or intratracheally in murine model of metastatic lung cancer
(Perry *et al.*, 2020)[104]	Immunotherapy of metastatic lung cancer	CpG ODNs	Polymeric nanoparticles	Orotracheal instillation in mice instilled with KAL-LN2E1 cells
(Zhang *et al.*, 2020)[153]	Targeted delivery of anticancer, homoharringtonine (HHT), to lung cancer cells	EGFR aptamer (as a targeting ligand)	Stimuli-responsive PLGA nanoparticles	Intraperitoneal injection in A549 athymic mouse xenograft
(Cheng *et al.*, 2018)[199]	Dual Modulation of Bcl-2 and Akt-1 in Lung and Cervical Carcinomas	ASO	T7 Peptide-Conjugated LNPs	Tail vein injection (IV) in A549 athymic mouse xenograft
(Yang *et al.*, 2018)[152]	Anti-MicroRNA-155 delivery for lung cancer therapy	miR-155 inhibitor	PEA NPs	IV in A549 athymic mouse xenograft
(Cheng *et al.*, 2017)[200]	Knocking down Bcl-2 for treatment of lung cancer	Chemically-modified ASO (G3139-GAP)	LNPs	IV in A549 athymic mouse xenograft
(Yung *et al.*, 2016)[150]	Therapeutic delivery of AntimiR-21 for lung cancer	Phosphorothioate-modified antimiR-21	LNPs	IV in A549 athymic mouse xenograft
(Garbuzenko *et al.*, 2010)[201]	Targeting MRP1 and BCL2 to supress chemoresistance in lung cancer	ASO	Liposomes	Inhalation in an orthotopic murine model of human lung carcinoma

Abbreviations: PEA, Poly(ester amine); EGFR, Epidermal growth factor receptor; MRP1, Multidrug resistance-associated protein 1; Bcl-2, B-cell lymphoma 2 protein.

Table 6. Some recent studies on chitosan modifications which are beneficial for oligonucleotides delivery.

Study	Cargo	Application	Chitosan modification	Experimental model	Advantages of the modification
(Motiei *et al.*, 2021)[202]	miRNA (miR-34a)	Breast cancer	Chitosan grafted with PGA	MDA-MB-231 cells	Increased core stability against pH variation and improved the nanoparticle loading capacity
(Khatami *et al*., 2021)[158]	Anti-miR-21	Colorectal cancer	Chitosan functionalised with antimiR-21	MCF-7, C26 cells, and mice	Increased uptake of nanoparticles into target cells, reduced tumor growth *in vivo*
(G. Huang *et al*., 2021)[168]	Anti-miR-21	Tyrosine kinase inhibitors-resistant NSCLC	α-Linolenic acid-modified chitosan	H1975 cells and mice	Increased nucleic acid complexation, cellular uptake, and tumor accumulation
(Zhu *et al.*, 2020)[167]	MB ONT	Detection of miR-155-5p and imaging of lung cancer	Chitosan is self- assembled with MB ONT	A549, SPC-A1, H446 cells, and mice	High detection and imaging efficiency
(Dowaidar *et al.*, 2017)[203]	siRNA	Gene delivery to cancer cells	Chitosan conjugated with a cell-penetrating peptide and used to coat Fe3O4 NPs	HeLa cells	Improved colloidal stability and transfection efficiency
(Dong *et al*., 2011)[166]	ASO	Inhibition of telomerase activity in lung cancer	Chitosan-coated PLGA NPs	A549, primary NSCLC, and patient-derived tissues	High complexation of ASO and high delivery efficiency
(Nafee *et al*., 2007)[164]	ASO	Gene delivery to cancer cells	Chitosan-coated PLGA NPs	A549 cells	Improved colloidal characteristics of NPs, high loading capacity, increased cellular uptake

Abbreviations: PGA, Polyglutamate; NSCLC, Non-small cell lung cancer; MB ONT, Molecular Beacon Oligonucleotide.