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RNA-Based Therapeutics: A Future in Cancer Immunotherapy

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Research Article

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ABSTARCT

Ribonucleic acids are fundamental molecules in biology, essential for the coding, regulation and expression of genes and have recently been a source for the development of therapeutic applications in various human diseases, especially cancers, due to the advantages of high safety and efficiency along with easy synthesis. Recent trends and technologies based on microRNAs (miRNAs), small interfering RNA (siRNAs) and messenger RNA (mRNA) based vaccines highlight the various ways RNAs can increase or decrease new protein expression in cells and how this can be applied in biomedical fields as a treatment of human cancers. However, these ribonucleic-based technologies all pose their own unique set of challenges, especially regarding the safe delivery of these molecules into cells. In this review, we summarise the latest applications and progress of miRNA, siRNA and, finally, mRNA-based technologies in cancer and discuss the prospects and limitations of these fields as novel strategies for the targeted therapy of cancers with the help of nanoparticle delivery vectors. As the most recent emerging cancer therapy and ribonucleic acid technology, mRNA vaccines, in particular, have a vast potential for future applications due to mRNA vaccines providing specific, safe and tolerable treatments compared to other cancer treatments.

Keywords: Vaccines; Vectors; Ribonucleic acids; Cancer treatments

INTRODUCTION

As the demand for novel effective medical treatments rises, scientists in various fields have been working on pioneering innovative biomedical therapies for fighting against human diseases and medical conditions. Cancer remains responsible for millions of deaths around the world every year as one of the leading causes of death worldwide [1]. Despite substantial progress and improvements in conventional cancer treatments, mainly involving radiation, chemotherapy and surgery, many issues must be addressed to improve cancer therapeutics. Consequently, there is a growing interest in research for innovative and efficient therapeutics that can alleviate traditional treatments' critical side effects.

Among the biological molecules of interest for use as therapeutic agents, Ribonucleic Acids (RNAs) show prominence due to their unique properties and central role in the human body's biological processes. RNAs are a family of single-stranded complex biological molecules of nucleotide monomers that have a fundamental role in different cellular mechanisms. However, they were once thought of as simply an intermediate product in the gene expression of deoxyribonucleic acids into proteins. In contrast, discoveries in the past decade have revealed various RNA roles in almost all biochemical pathways [2]. This discovery of the vast roles of RNAs has garnered significant attention from scientists in testing RNA as therapeutic molecules, which has led to the approval of a few RNA-based drugs in recent years [3]. RNA has since gained a central role in pharmacotherapy. However, implementing RNA as an effective therapeutic agent is challenging, as the systemic delivery of naked RNA molecules to a specific targeted tissue or cell poses many unique challenges. Naked RNAs are fragile, relatively large, and negatively charged molecules [2]. Scientists have faced difficulties overcoming a cell's robust defence system to keep exogenous RNAs out of membranes while preventing the degradation of the RNA molecule during delivery [2].

For these reasons, there has been a focus on the rapid development of carriers for drug delivery applications, where the protective advantage of a carrier is particularly salient for a molecule as fragile as RNA, therefore triggering remarkable progress in RNA therapy. Ideal carriers of nucleic acids to biological systems include nanomaterials since nanoparticles are similarly sized and can be chemically synthesised to be compatible with cells [2]. Many nanostructured vehicles have facilitated the efficient and safe delivery of RNA that has been designed, manufactured and tested. These nanotechnology-based carriers make it possible for RNA to overcome the human body barriers and for scientists to exploit the biological functions of RNA in the target. The unified efforts in nanoscience and RNA therapy bring about a new era in therapeutics and pharmacological treatments of diseases, especially cancer.

MATERIALS AND METHODS

This study offers a detailed summarisation of the most significant advancements in RNA therapy. First, we introduce microRNA (miRNA), a small, non-coding, single-stranded RNA molecule that can prevent protein production by binding to target mRNA. This miRNA strategy is then compared with small interfering RNA (siRNA), a class of short non-coding double-stranded RNA of around 20-23 nucleotide base pairs in length. Although siRNA and miRNA are functionally similar in sharing a common role in gene regulation and silencing, miRNA can regulate the expression of hundreds of genes through imperfect base pairing; however, siRNA binds specifically to a single gene at a particular location. Therefore, while miRNA can have multiple targets, siRNA can only have one mRNA target, which is reflected by their different modes of action and clinical application potentials. Next, an in-depth review of mRNA vaccines' applications in cancer therapeutics is discussed.

RESULTS

Micro RNA therapeutics

Until the 1990s, the role of non-coding RNAs in our DNA was unknown [4]. The ability of an RNA molecule to inhibit gene expression was a phenomenon that was observed in plants in the 1990s by several independent groups but was not widely understood [4]. In 1993, Ambros et al. discovered the first microRNA (miRNA) gene in a nematode worm Caenorhabditis elgans, which was found to bind to RNA and prevent its translation physically. This discovery provided the first evidence that miRNA can prevent protein production by suppressing messenger RNAs [5]. Currently, miRNAs and small interfering RNAs (discussed later in the review) are widely employed RNA classes to silence genes. Both miRNAs and siRNAs have been applied in treating many diseases, from infections to cancers [6]. These molecules are highly attractive given they are highly potent and hold an advantage over traditional small therapeutic molecules, as they can be designed to alter virtually any gene of interest, and therefore have the potential to treat "non-druggable" targets, such as proteins that have a conformation inaccessible to conventional drug molecules or which lack enzymatic function [6]. Despite the similar physicochemical properties of these molecules, their distinct functions and mechanisms of action require different design requirements and serve unique therapeutic applications.

MicroRNAs (miRNAs) are double-stranded stem-loop non-coding RNA structures with dimensions of 13-15 kDNA dimensions and 21-25 nucleotides in length [2]. They are highly conserved in plants and humans and are encoded by genes. miRNAs participate in RNA interference mechanisms crucial in gene modulation and editing [7]. miRNAs are transcribed from the genome into a longer precursor molecule cleaved by the nuclear ribonuclease Drosha into a 70-100 nt long hairpin structure. This precursor molecule is further cleaved following nuclear export by the RNAse Dicer, resulting in a 17-25nt double-stranded oligonucleotide that enters the RNA-induced silencing complex (RISC) [7]. RISC is a multi-protein complex that facilitates the interaction between the mature miRNA and complementary mRNAs by separating the mature miRNA and the passenger strand.

The miRNA structure only partially complements a target mRNA sequence, enabling a single miRNA molecule to target a broad set of mRNAs [2]. Only the "seed" region of the molecule (2-6 nucleotides) can interact with the target mRNA sequence through imperfect base-pairing interactions, usually pairing with the 3'-untranslated part of the target mRNA, inducing post-transcriptional silencing. Additionally, miRNA can bind to other mRNA sites, such as the 5'-untranslated and coding regions [2].

However, this is less common. It regulates gene expression at the post-transcriptional level by selectively inhibiting an mRNA sequence by cleaving or inducing the sequence's degradation and mediating its translational repression. There are two ways of utilizing miRNAs for therapeutic applications: inhibition and replacement. miRNA inhibition involves introducing synthetic single-stranded RNAs that act as miRNA antagonists to inhibit the action of overexpressed target miRNA. In contrast, miRNA replacement is employed in cells and tissues with deactivated or repressed endogenous miRNAs (which tend to be more common in cancer) to restore miRNA levels [4].

Experimental evidence *in vitro* and *in vivo* and expression data marks miRNA molecules as molecules that frequently acquire a gain or loss of function in cancer and play a causative role in cancer development [7]. The altered expression of miRNAs has been seen in virtually all tumour types, and the introduction or repression of a single miRNA can effectively contribute to tumour progression or tumorigenesis [7]. miRNAs such as miR-15a, miR-16 and miRNAs from the miR-34 and let-7 family are tumour-suppressor miRNAs which are not limited to a particular tumour type, and the deregulation of some of these miRNAs correlates with tumour development [7]. As a unique opportunity for therapeutic intervention in cancer, miRNA replacement involves re-introducing a tumour

suppressor miRNA mimic to restore a loss of function and restrict protein-encoding genes. The synthetic double-stranded miRNA mimic is designed so that its 5'-end has a sequence partially complementary to the selected sequence in the 3' UTR unique to the target gene. Once introduced to the cell or tissue, the mimic mirrors the function of the endogenous miRNA, binding to a target gene to initiate mRNA degradation and gene silencing [4]. Traditionally, therapeutically restoring levels of tumour suppressors in tumour tissues have been achieved through gene therapy involving the delivery of relatively large viral vectors or DNA plasmids that encodes the desired protein. However, this method posed technical challenges, such as inefficient delivery to target tissues and the need for nuclear localisation [7].

In contrast, the smaller size of miRNA mimics presents an opportunity for easier delivery and simply has to enter the cytoplasm of target cells to be active and can be delivered systemically [7]. In addition, the miRNA mimic has the same sequence as the naturally occurring depleted miRNA. It is therefore expected to target the same set of mRNAs regulated by its predecessor. Hence, off-target effects are unlikely as the miRNA mimics are expected to behave analogously to their natural counterpart [7]. Nevertheless, the miRNA mimics can trigger the innate immune system and induce immunotoxicity, resulting in undesirable side effects [7].

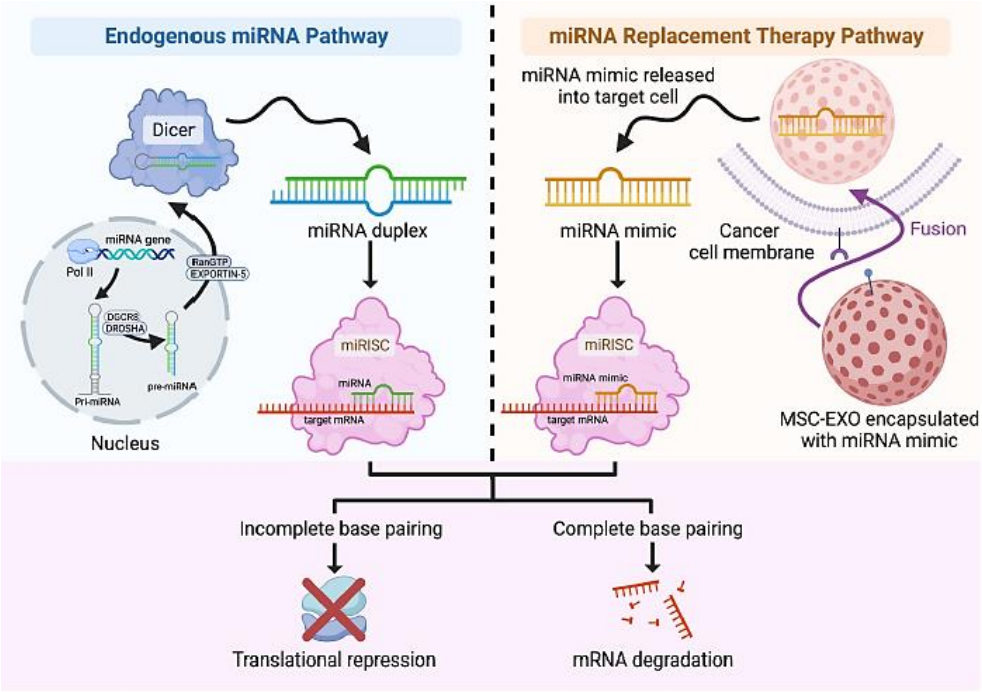
Furthermore, despite the extensive potential of miRNA in treating cancer, this technology has various limitations which must be resolved. Firstly, the negative charge of miRNAs makes them hard to permeate the cell and unstable *in vitro* due to immunotoxicity and destruction by nucleases [8]. As a result, miRNA replacement strategies have a very short systemic circulation time of unmodified miRNA mimics, as they are prone to rapid degradation and clearance by cellular mechanisms [9]. During early studies of miRNA therapeutics, naked miRNA mimics or mimics encoded in viral vectors were administered either into the systemic circulation or locally at the target tissues [10]. However, these clinical applications remained largely unsuccessful due to issues including the lack of effective delivery of miRNA to the target site, rapid clearance and poor systemic stability [10]. Several strategies have been developed and investigated to overcome the obstacles of miRNA delivery, including the chemical modifications to miRNA and the use of viral and non-viral vectors.

Despite significant advancements and achievements in the development of synthetic miRNA delivery systems, each delivery strategy possess various shortcomings: chemical modifications of miRNA lead to the introduction of accidental off-target effects, viral vectors are laden with safety and immunogenicity issues, and non-viral vectors which typically consist of synthetic Nanoparticles (NPs) face challenges due to low encapsulation efficiency [10,11]. Consequently, there has been a progressive interest in natural miRNA delivery systems that possess some of the highly favourable properties of miRNA delivery systems, including stability in different conditions, innate tropism that results in immensely effective and selective entrance into target cells, and immunologically inert [8]. Exosomes (EXOs) are a natural miRNA delivery system that has attracted significant interest in their capability as miRNA carriers due to their therapeutic safety and efficiency in transporting different cellular biological components to target cells [8]. Of the cell kinds recognised to generate EXOs, the human Mesenchymal Stem Cells (MSCs) are the most promising as they are highly proliferative and widely available to be isolated from almost all human tissues [8]. MSCs release a wide range of EXOs (MSC-EXOs), garnering attention for using MSC-EXOs as miRNA delivery systems due to their tumour-homing and immune attributes and flexible characteristics [8].

Altered MSC-EXOs have been utilised to inhibit cancer expansion and development through their use as a biological carrier for miRNA mimics. In an investigation by Shojaei, et al., an Adipose-derived-MSC-EXO (AD-MSC-EXO) was used as a carrier for a miR-381 mimic of MDA-MB-231 cells to study their effect on triple-negative breast cancer cells [12]. This study showed that AD-MSC-EXOs could suppress the proliferation, migration and malignancy

capability of MDA-MB-231 cells and improve their apoptosis *in vitro* [12]. Hence, these results provide intriguing insights into developing engineered MSC-EXOs as delivery molecules for targeted and personalized cancer therapeutics (Figure 1).

Figure 1. Comparison of the endogenous miRNA and miRNA mimic pathway.



Although both siRNA and miRNA are structurally and functionally similar, there are some key differences. miRNAs are regarded as endogenous RNAs produced from within cells, expressed as long primary miRNA transcripts from miRNA genes. In contrast, siRNAs are considered exogenous RNAs that enter the endogenous RNAi pathway [4]. As miRNA only has a short binding region coupled with the non-perfect complementarity with the target mRNA, the specificity of miRNA action is lower than that of other RNA therapeutic strategies, such as siRNA, which is perfectly complementary to its target mRNA [2].

Small-interfering RNA therapeutics

Five years after the first discovery of miRNA, Andrew and Mello, et al., discovered the process of RNA interference (RNAi) during research on gene expressions in the nematode worm C. elgans. After injecting worms with double-stranded RNA coding for specific proteins, they found that the genes carrying the same sequence were silenced [5]. These discoveries led to RNAi becoming a central tool in modern molecular biology, and soon after, in 1999, the discovery of naturally occurring small interfering RNAs (siRNAs), small antisense RNAs also involved in post-transcriptional gene silencing were discovered by David Baulcombe and Andrew Hamilton. The first synthetic siRNAs that could switch off genes in mammalian cells were produced by Tuschl, et al., an achievement that kickstarted the widespread usage of siRNAs to knock out the activity of specific genes selectively [13].

Small-interfering RNAs (siRNA) are double-stranded non-coding RNAs that are 21-23 nucleotides long, which act during RNA interference pathways in gene silencing mechanisms [2]. At the post-transcriptional stage, siRNAs can silence targeted mRNAs through interactions with an entirely complementary mRNA gene sequence, inducing mRNA degradation and translation suppressions [2]. This modulates the encoding of the specific gene into a protein, preventing gene expression.

As they can inhibit the expression of any pathological protein, siRNA-based strategies have enormous potential to become a class of pharmaceutical drugs within various fields of medicine. siRNAs have the potential to be utilised to silence any targeted gene [2]. However, they are constricted to successfully targeting only one specific gene. Therefore the therapeutic approaches for siRNA are most suited for single-gene disorders such as hemophilia and cystic fibrosis [2]. Additionally, oncology is a medical area that may benefit substantially from siRNA-based therapeutic strategies, as siRNAs allow modulation of the expression of any gene involved in tumour initiation, growth, and metastasis formation. Therapeutic siRNAs have been investigated for silencing critical cancer-associated target molecules central for tumour resistance to chemo- and radiotherapy and tumour-host interaction and have resulted in significant apoptotic and antiproliferative effects [14].

siRNAs have some significant advantages over traditional pharmaceutical drugs, such as small molecules or proteins and derivatives, as siRNAs can be designed to target and silence any gene in the body, allowing them to have broader therapeutic potential. The high specificity of siRNAs makes them less toxic to traditional drugs, and when the mRNA sequence is known, siRNA sequences targeted at the specific gene can be rapidly designed. However, siRNAs have numerous limitations that must be overcome to reach the clinical setting. siRNA-base technology has been found to induce various undesirable effects due to them interfering with the translation of other mRNAs besides the target one or potentially inducing an immune response [2].

In addition to the challenges of adverse effects of siRNA in the body, the primary barrier to the therapeutic application of siRNAs is site-specific delivery [2]. The route of administration is highly dependent on the accessibility of the target area of the body, as while local administration via intraocular, intratumor, intranasal or direct administration in the nervous system has shown favourable results, such approaches are not possible for the treatment of advanced solid tumours [15]. To most suited strategy to treat solid tumours with distant metastasis or hematologic tumours is via systemic administration, however upon intravenous administration, naked siRNAs are cleared from the bloodstream in a few minutes due to rapid renal elimination, unspecific uptake by the mononuclear phagocytic system and degradation by serum nucleases [15]. The physical-chemical properties of siRNA-negative charge, hydrophobicity and size of around 13 kDA strongly reduce their cellular internalisation.

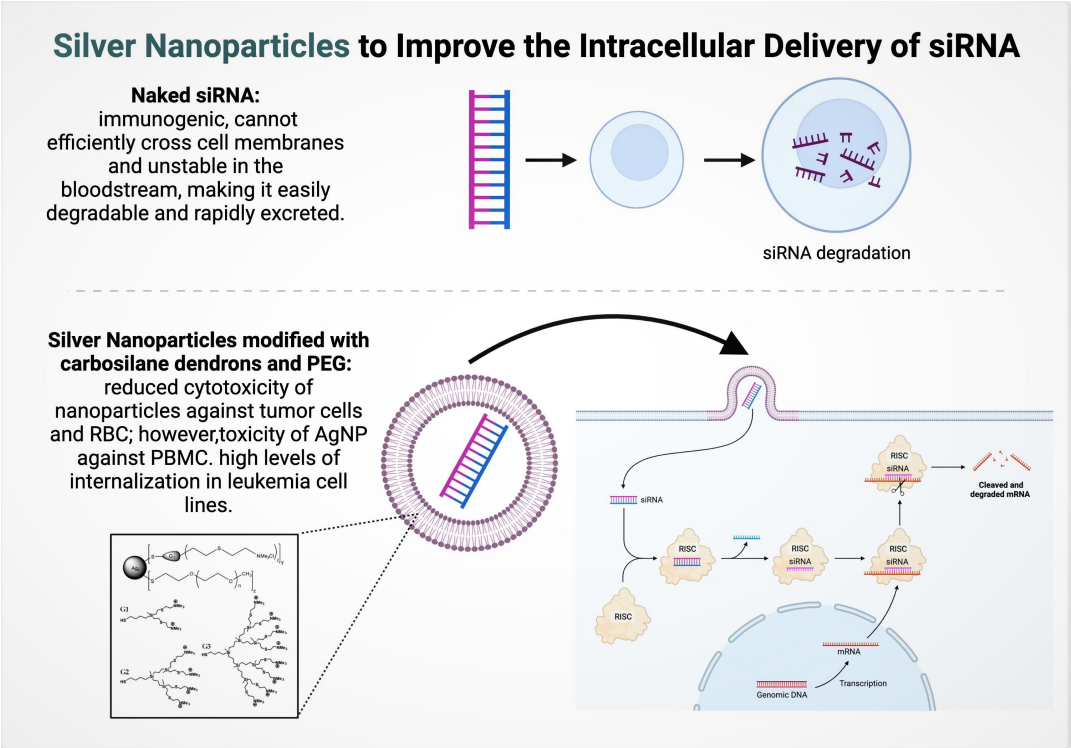
Recently, an alternative strategy for siRNA delivery is using modified silver Nanoparticles (AgNPs) as vectors for proapoptotic siRNAs, an approach that was investigated by Abashkin, et al., AgNPS have unique biofunctional and physiochemical properties, including anti-inflammatory, antiviral and antibacterial activity, which have the potential to be implemented in new biomedical strategies [16]. AgNPs can be successfully used as new nanostructured platforms for treating and diagnosing several types of cancer. Due to their broad bioactivity spectrum, they are also promising agents in critical tumour and multidrug resistance approaches.

Although AgNPs have not been studied as extensively as other nanostructures, such as gold nanoparticles, silver nanoparticles have recently shown more promise than other inorganic nanoparticles as non-viral delivery vehicles [16]. However, the challenge lies in overcoming the cytotoxic effects of the nanoparticles by modifying the nanostructures without losing the efficiency of genetic material transfection. Advantages of using AgNps include the comparative cheapness and ease of synthesis of the nanoparticles, which are also easy to modify through their ability to attach markers, ligands, and linkers to the particles. There is a promising future application of these modified nanoparticles in tumour imaging and subsequent therapy using photothermal effects. AgNPs could also be utilised as vectors in joint therapy with other drugs. However, silver nanoparticles have issues overcoming their low stability and high toxicity [16]. A study conducted by Abashkin, et al., investigated the formation of silver nanoparticle complexes modified with polyethylene glycol and carbosilane dendrons with siRNAs and the influence that the

nanoparticles have on blood cells. The potential for the delivery of siRNA through modified silver nanoparticles into malignant neoplasm cell lines and the target effect of the siRNAs of the group aimed at silencing the BCL-2 family (proteins consisting of members that either inhibit or promote apoptosis and control apoptosis through governing the mitochondrial outer membrane permeabilisation) were studied. Abashkin. et al., evaluated the possibility of using AgNPs that were modified with PEG and carbosilane dendrons to reduce the cytotoxic effects of the AgNPs. The data obtained indicated that an increase in PEGylation reduces the toxicity of AgNPs against red blood cells and tumour cells; however, it increases the cytotoxicity against peripheral blood mononuclear cells [16].

Regarding epithelial types of cancer, the cautious use of AgNPs is recommended as a noticeable proliferative activity was observed with a low level of internalisation [16]. However, the AgNPs performed well in leukemia cell lines. The results indicated high levels of internalisation and a significant decrease in viability due to cell death by apoptosis mechanisms when using proapoptotic siRNA to silence the antiapoptotic mutant gene of the BCL-2 family (Figure 2) [16].

Figure 2. The use of silver nanoparticles (AgNPs) modified by Polyethylene Glycol (PEG) and carbosaline dendrons as delivery vectors of SiRNA.



As a relatively new class of treatments and prophylactics for several chronic and rare diseases, including cancer, diabetes and tuberculosis, RNA-based biopharmaceuticals, including vaccines and therapeutics, hold great promise in the prevention and treatment of these diseases. The early development of RNA therapeutics led to RNA interference technologies that inhibit gene expression by targeting and destroying specific mRNA molecules, specifically the two central types of RNAi technology: siRNA and miRNA.

However, recent advances in RNA-based biopharmaceuticals have led to extensive research and development in the direct vaccination of mRNA molecules that can encode for a target antigen, which induces an immune response after uptake by antigen-presenting cells. In contrast to RNAi, which is a gene silencing technology, mRNA vaccines can theoretically produce any peptide via the protein synthesis process in cells and therefore has the expansive

potential for treating diseases that require protein expression and higher therapeutic effectiveness due to its continued translation into encoded peptides to trigger long-lasting expression [17].

messengerRNA vaccines

Vaccines help reduce the risk of illness, protect vulnerable people in our communities, and save innumerable lives yearly [18]. Traditional vaccine approaches introduce live attenuated and inactivated pathogens and subunit vaccines to the body, which provide long-lasting protection against numerous diseases by triggering an immune response. However, there remain several hurdles to vaccine development against non-infectious diseases such as cancer and against various infectious pathogens that can evade our adaptive immune response [18].

Messenger RNA (mRNA) is a single-stranded RNA that carries the genetic information from a DNA template necessary for protein production. mRNA is a critical component of the central dogma as the precursor translation unit for protein production and is an attractive therapeutic target as it has the potential to accomplish transgenic protein expression without the genetic manipulation of cells or organisms, as once cells finish making the protein, mRNA molecules are degraded [18]. There is a vast potential for a new type of vaccine that uses mRNA rather than a part of a bacteria or virus, which functions by introducing a piece of mRNA that codes for a viral protein usually found on the virus's outer membrane to induce the cell to produce the viral protein without exposing or infecting the individual to the actual virus. As part of the body's immune response, the immune system will recognize the foreign protein and produce antibodies that help to protect the body against infection and remain in the body long-term for immunological memory.

Hundreds of scientists have worked on mRNA vaccine-related technologies for decades before the breakthrough of mRNA-based COVID-19 vaccines, one of history's most critical and profitable vaccines [19]. A significant stepping stone towards the monumental COVID-19 vaccine occurred in late 1987 during a landmark experiment by scientist Robert Malone. Malone mixed mRNA strands with fat droplets and found that human cells immersed in this solution absorbed the mRNA, producing proteins from the messenger molecules. This was the first time a scientist realised it might be possible to "treat RNA as a drug" [19]. Despite this, for many years, mRNA was seen as too unstable and expensive to be used as a drug or vaccine, with dozens of labs and companies working on the idea but struggling to produce the right formula of fats and nucleic acids. mRNA would be taken up by the body and degraded too quickly before it could be expressed into proteins by the cells. The solution to this problem was discovered through decades of research into lipids and liposomes. The development of fatty droplets (lipid nanoparticles), wrapped around mRNA like a bubble, allows the molecule to enter the cells safely and, once inside, could be translated into proteins, priming the immune system to recognize foreign proteins for future immunity. These lipid nanoparticles consist of a mixture of four fatty molecules - three of which contribute to structure and stability, and the fourth, an ionisable lipid, which was critical to the lipid nanoparticle's success [18]. The ionisable lipid is positively charged under laboratory conditions. Still, it converts to a neutral charge under the body's physiological conditions, limiting the toxic effects of the nanoparticles on the body [20]. The first mRNA vaccines using lipid nanoparticle carriers were developed against the Ebola virus but were only used in African countries [21]. When the COVID-19 pandemic hit, decades of research and innovation in mRNA vaccine technology came to fruition all across the globe, creating a safe and effective vaccine and launching the world into a new era of vaccine technology and production.

Over the past decade, primary technological research and innovation investments have allowed mRNA to emerge as a promising alternative to conventional vaccines, a therapeutic tool for vaccine development and protein replacement therapy. The use of mRNA provides many advantages over traditional vaccines, as well as DNA-based

vaccines. Regarding safety, mRNA is a non-integrating and non-infectious platform with no risk of infection or insertional mutagenesis in a patient [18]. The production of mRNA vaccines in a cell-free manner also allows for scalable, cost-effective and rapid production. Additionally, a single mRNA vaccine can encode several antigens, allowing the targeting of multiple pathogens and strengthening the immune response against resilient pathogens in a single formulation.

There are three main categories of mRNA medicines, preventative vaccines, therapeutic vaccines and protein-encoding therapies. The recent interest in developing mRNA-based cancer immunotherapies shows promising alternative strategies to treat malignancies. Although some mRNA cancer immunotherapies aim to modify the immune-suppressive tumour microenvironment through the expression of altered or deficient tumour suppressor protein, the delivery of mRNA to every cancer cell in a patient is highly unlikely [18]. Therefore, most cancer vaccines are therapeutic, seeking to stimulate and train cell-mediated responses capable of reducing or clearing tumour burden rather than prophylactic. Most cancer immunogenic therapies aim to promote specific immune responses against tumours by utilising target mRNAs that encode for tumour antigens [2]. The ability of mRNA to encode whole antigens makes their use as cancer vaccines extremely promising.

Despite each application presenting its unique challenges, one central common challenge in mRNA medicines is preserving mRNA stability during intracellular delivery of the mRNA component to the target cell. The fundamental principle behind mRNA vaccines is the delivery of the transcript of interest, which encodes for one or more immunogen(s) into the host cell's cytoplasm for protein(s) to be translated within the membrane and are intracellularly located or secreted [22]. There are two main categories of mRNA constructs of interest: self-amplifying mRNA and non-replicating mRNA constructs, both of which have in common a cap structure, an open reading frame, a 3' poly A tail and 5' and 3' untranslated regions [22]. RNA is an intrinsically unstable and fragile molecule. Various techniques have been focused on stabilising the molecule, such as optimising the 5' cap structure and the 3' poly-A tail length and regulatory elements within the 5' and 4' untranslated regions [23].

While simultaneously needing to optimize the stability of the mRNA construct, another quintessential important factor of mRNA vaccines is the delivery of the vaccine from the bolus at the injection site into the cytoplasm of the cell. As mRNA is a transient and short-lived molecule extremely susceptible to degradation, sufficient protection is needed [23]. This has been an extensive area of research in which Lipid Nanoparticle (LNP) formulations currently produce the most successful results, making LNPs one of the most appealing and commonly used mRNA delivery tools [18]. LNPs are often comprised of four components: cholesterol which acts as a stabilising agent; naturally occurring phospholipids which support the lipid bilayer structure; lipid-linked Polyethylene Glycol (PEG), which increases the half-life of formulations; and an ionisable cationic lipid that supports the endosomal release the mRNA into the cytoplasm by promoting self-assembly into virus-sized particles [18]. LNPs help to provide sustained stability for mRNAs by protecting them from nuclease degradation. They also help to facilitate efficient cellular uptake and organ specificity and provide endosomal escape properties that increase the chance of successful cargo delivery to the cytoplasm. Recent advances in LNP formulations have focused on incorporating hydrolysable bonds to ease clearance [24]. However, these degradable bonds affect the formulation stability and continue to be a shortcoming of LNP formulations [24].

Notwithstanding the success of mRNA COVID-19 vaccines, researchers have long hoped to use mRNA vaccines as a cancer treatment method. This area has been tested in small trials for nearly a decade, showing promising early results [25]. Despite exceptional progress in the field of oncology, malignant tumors remain the second leading cause of mortality around the world, and traditional clinical treatments for tumors, such as radiotherapy,

chemotherapy, and combination therapy, struggle with limitations regarding specificity and drug resistance, sparking the need for a new type of cancer immunotherapy. mRNA Cancer vaccines are a promising means of antitumor immunotherapy by specifically attacking and destroying malignant tumor cells with high-level expression of tumor-associated and tumor-specific antigens and providing immune memory that helps achieve sustained tumor destruction. mRNA cancer immunotherapies have vast potential to provide a safer and better-tolerated treatment through their high potency, specificity, and versatility, as well as their low-cost and large-scale manufacturing potential [25].

One of the significant challenges of mRNA vaccine development is the abundance of RNases and the difficulty of mRNA molecules entering cells [17]. The development of biocompatible delivery carriers that can function to improve mRNA stability and transport mRNA into antigen-presenting cells is essential for the further development of mRNA-based vaccines. Decades of experimentation in the intracellular delivery of mRNA has started from naked mRNA into the exploration of condensation of mRNA into nanoformulations and has progressed into the focus and investigation of various viral and non-viral vectors. Despite a large spectrum of available viral vectors, their employment as delivery systems for long-term therapeutics is restricted by high production costs, potential risk of secondary carcinogenesis, unwanted genomic integration, and immunogenicity. In contrast, non-viral vectors have garnered significant attention due to their safety and biocompatibility, efficient encapsulation ability and ability to undergo endocytosis at the cell membrane. Many different non-viral vectors are being investigated and developed to protect mRNA molecules from nucleases and facilitate their uptake into cells, including Lipid Nanoparticles (LNPs), polymers, dendrimers, and others. In particular, LNPs have demonstrated success as a delivery system; however, some commercially available lipid-based vectors that display high transfection efficiency can also induce toxic responses *in vivo*. Additionally, LNPs are usually composed of various lipid components with complicated compositions that require state-of-art devices to fabricate [26]. Developing a suitable and highly efficient mRNA delivery carrier with a simple composition and a more straightforward preparation process would further the accessibility of mRNA-based biotechnology.

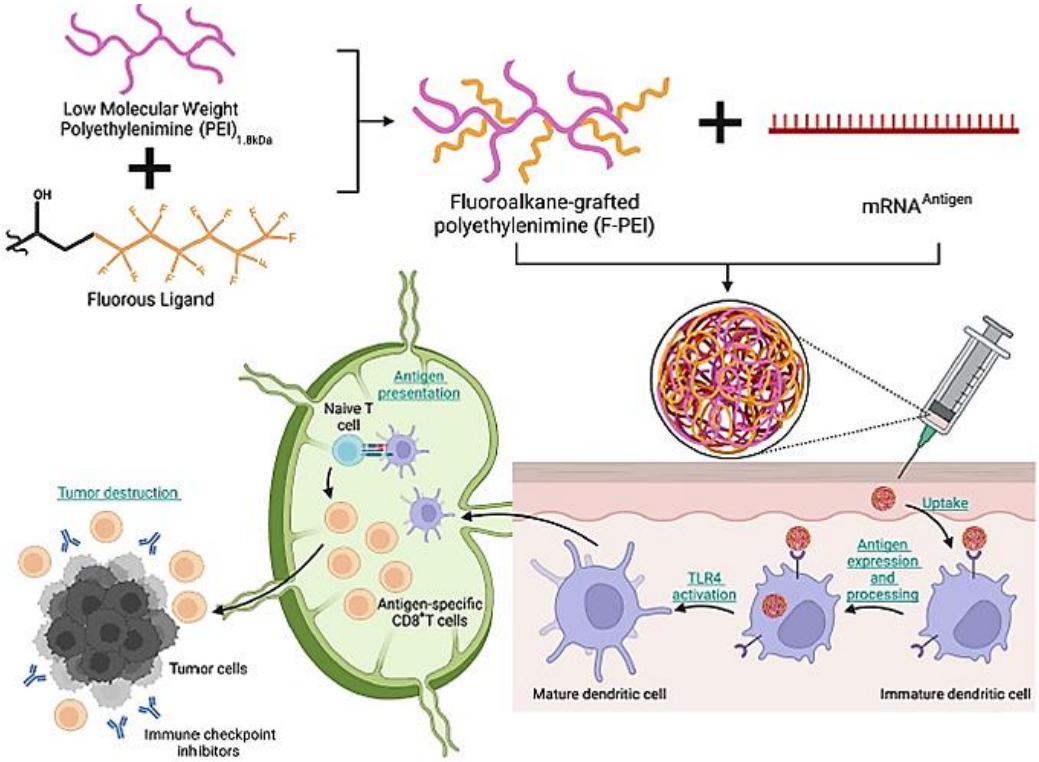
Cationic polymers have been extensively investigated as a non-viral delivery system due to their advantages over viral vectors, including their low immunogenicity and relative safety. Amongst cationic polymers, Polyethyleneimine (PEI) has emerged as the most widely studied and one of the most successful gene-delivery polymers. PEI is an organic polymer consisting of repeating units composed of an amine group and a CH2CH2 spacer. It has the highest positive charge density potential owing to the protonable amino nitrogen in every third atom of PEI. This high charge density allows PEI to form positively charged complexes with mRNA with high efficiency, providing efficient transfection and protection against degradation by nucleases in cells. Furthermore, the polymer's protonable amino nitrogens create a "proton sponge effect", buffering the pH in the endosome, causing osmotic swelling and endosomal membrane rupturing to allow the escape of the polymer-nucleic complex into the cytoplasm. PEI provides various advantages as a delivery vector, including cost efficiency, and notably, the polymer's cationic amine groups can complex with mRNA via electrostatic interactions and be packaged into ~100 nm particles to be delivered efficiently and safely into target cells while also increasing the half-life of the mRNA cargo in the cytoplasm through enhancing cellular uptake via interactions with anionic cell surface proteoglycans. Unfortunately, the high toxicity profile of PEI significantly hinders its clinical translation, as reports demonstrate that the positive charges of PEI could cause toxicity both *in vitro* and *in vivo*, inducing apoptosis and necrotic cell death. Furthermore, many studies have illustrated that PEI's molecular weight heavily impacts the delivery vector's cytotoxicity and gene transfection efficiency. Cytotoxicity increases with an increase in molecular weight; conversely,

increasing polymer size also increases gene transfer activity. For instance, a low molecular weight PEI (>2 kDa) was proven to be nontoxic but displayed poor transfection efficiency, and a PEI with a high molecular weight (25 kDa) showed high transfection activity but significant cytotoxicity. In an attempt to reduce toxicity and improve the transfection efficiency of PEI, various chemical modifications of PEI have been explored.

To date, an emerging approach for PEI modification is fluorination. In an investigation by Li, et al., Fluoroalkane-Grafted Polymers (F-PEI) with a low molecular weight of 1.8 kDa were synthesised for mRNA delivery. The nanovaccine formed through the self-assembly of F-PEI and the tumour antigen-encoding mRNA has the potential to promote intracellular delivery of mRNA and could trigger efficient antigen presentation, which elicits anti-tumour immune responses without the need for additional adjuvants [26]. Li, et al., used mRNA encoding the model antigen ovalbumin to investigate the use of this cancer vaccine to delay the growth of established B16-OVA melanoma.

The criteria for an effective mRNA delivery carrier is the ability to pack and protect the mRNA from enzymatic degradation, transport the molecule into the cytosol either directly or via escaping from the lysosome and release the cargo into the cellular translation machinery. The delivery efficiency of the mRNA molecule is affected by both the affinity of the carrier towards the mRNA and the interactions with the target cell [26]. Fluorine-containing amphiphiles have been reported to show promising protein, and gene delivery effects, as the fluorinated compounds with both lipophobic and hydrophobic features offer a high tendency of phase separation in both polar and non-polar environments, which allows their penetration across the phospholipid bilayer of the cell membrane as well as lysosomal and endosomal membranes (Figure 3) [26].

Figure 3. The synthesis and application of Fluoroalkane-Grafted Polymers (F-PEI) for the intracellular delivery of mRNA cancer vaccines



In a previous investigation, Li, et al., utilised a PEI with a high molecular weight of 25kDA as a carrier molecule. However, it was found that the high molecular weight possessed cytotoxicity, limiting its potential for biological applications despite being able to self-assemble with protein or peptide antigens to form a nanovaccine without the need for additional adjuvants. In this recent study, Li, et al., synthesised two low molecular weight PEI of 1.8kDA

with low cytotoxicity to further optimise mRNA delivery. It was found that when the F-PEI-based MC38 neoantigen mRNA cancer vaccine was combined with immune checkpoint blockade therapy could suppress established MC38 colon cancer and prevent tumour recurrence [26].

DISCUSSION

The past two decades have brought about extensive research into the potential of mRNA molecules as preventative and therapeutic vaccines for distinct infectious diseases, especially viral infections and cancer, with the COVID-19 pandemic streamlining innovation in this novel type of vaccination. The key to these developments has been developing lipid-based nanoparticles that carry and protect otherwise fragile mRNA cargoes. Hence, they are able to enter cells, escape liposomes, and generate therapeutic proteins. The ongoing and future research in mRNA vaccines holds potentially huge implications for human health. However, further modification is required to improve this technology's thermostability and limited transfection efficiency. The introduction of nanotechnology concepts holds vast potential in improving the clinical feasibility of mRNA vaccines. One promising future direction for mRNA-based vaccines is self-amplifying RNA (saRNA), a new generation of mRNA vaccines with the capacity for self-amplification. saRNA is derived from the genome of certain viruses, such as flaviviruses and alphaviruses, with the genes encoding for the viral structural proteins deleted and replaced by the target gene(s) encoding the vaccine antigen(s) [27]. Compared to conventional non-replicating mRNA, saRNA possesses several advantages. saRNA produces more sustained and higher levels of RNA amplification and transgene expression relative to conventional mRNA and hence require lower doses of RNA, making it a more appealing therapeutic considering the need for high speed and low cost for vaccine production and distribution [27]. Furthermore, compared to conventional mRNA, saRNA leads to more protein translation and generates double-stranded RNA intermediates that promote antiviral responses that cause immune stimulation, generating enhanced antigen-specific humoral and cellular responses [27]. Regarding the application as cancer therapeutic, studies conducted with various saRNA vaccines platforms found that different saRNA vaccines are capable of inducing potent, antigen-specific immune responses to a wide variety of antigens, such as tumour-associated self-antigens, viral antigens and tumour-specific neoepitopes [28]. Notwithstanding the requirement for additional studies into this emerging technology, the remarkable properties of saRNA vaccines to induce immune responses, elevated levels of antigen expression, low toxicity, and potential scalability present them as attractive targets for a new generation of cancer vaccines [28,29].

CONCLUSION

In conclusion, the development of RNA technologies, especially recent advancements in mRNA technologies, offers a plethora of potential for synthesising safe and effective rapidly and mass-produced vaccines that are versatile and can be used for various therapeutic and prophylactic applications of diseases. Furthermore, the advancement of different nanoparticle-based materials for use as nanoplatforms for biological drug delivery provides promising progress for the clinical application of RNA therapeutics. Although future research and clinical trials in this field are required to improve on limiting factors and uncover potential long-term effects of mRNA vaccines and their implications, this technology presents great promise for clinical applications in the near future.

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