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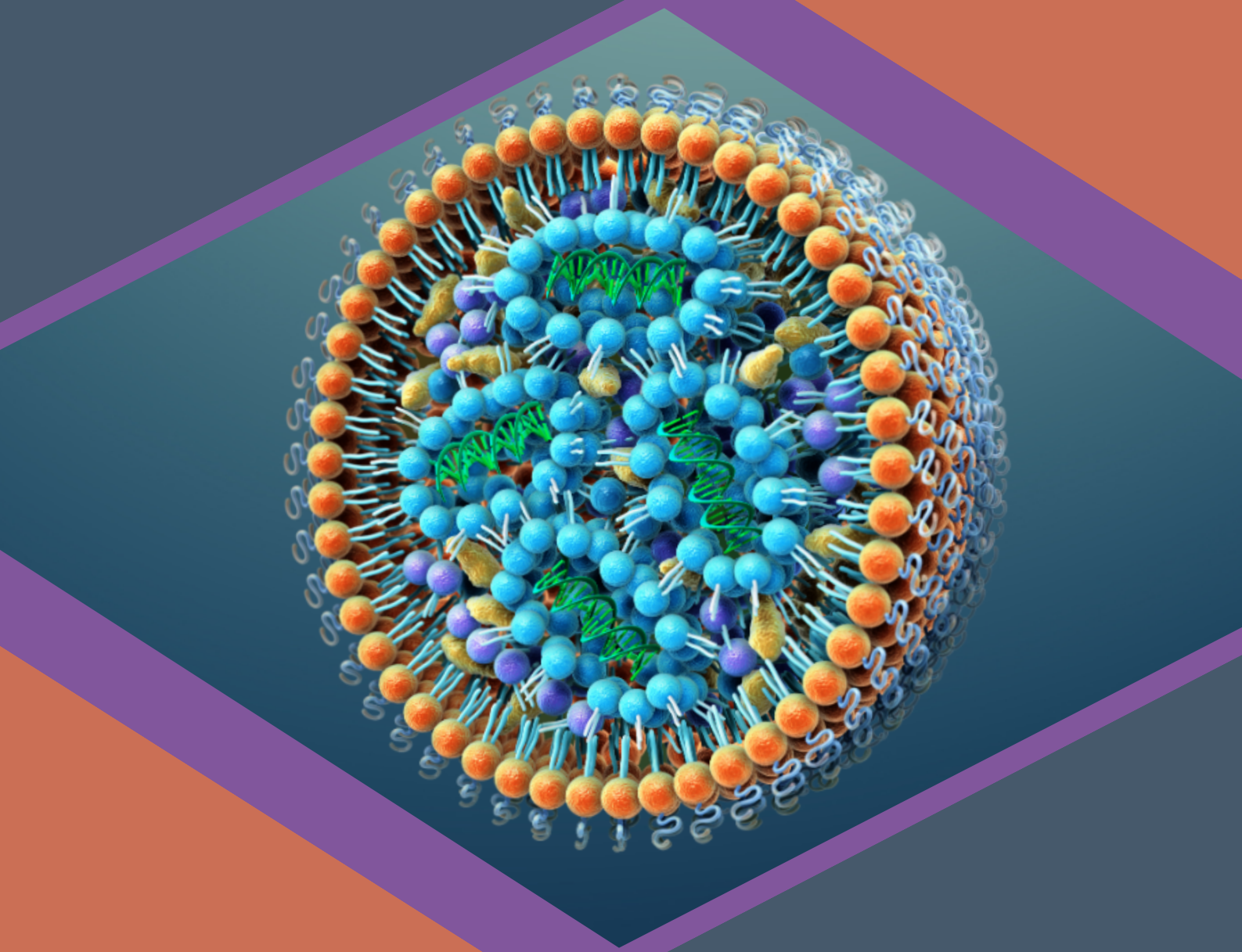

Non-viral vectors: lipid nanoparticles

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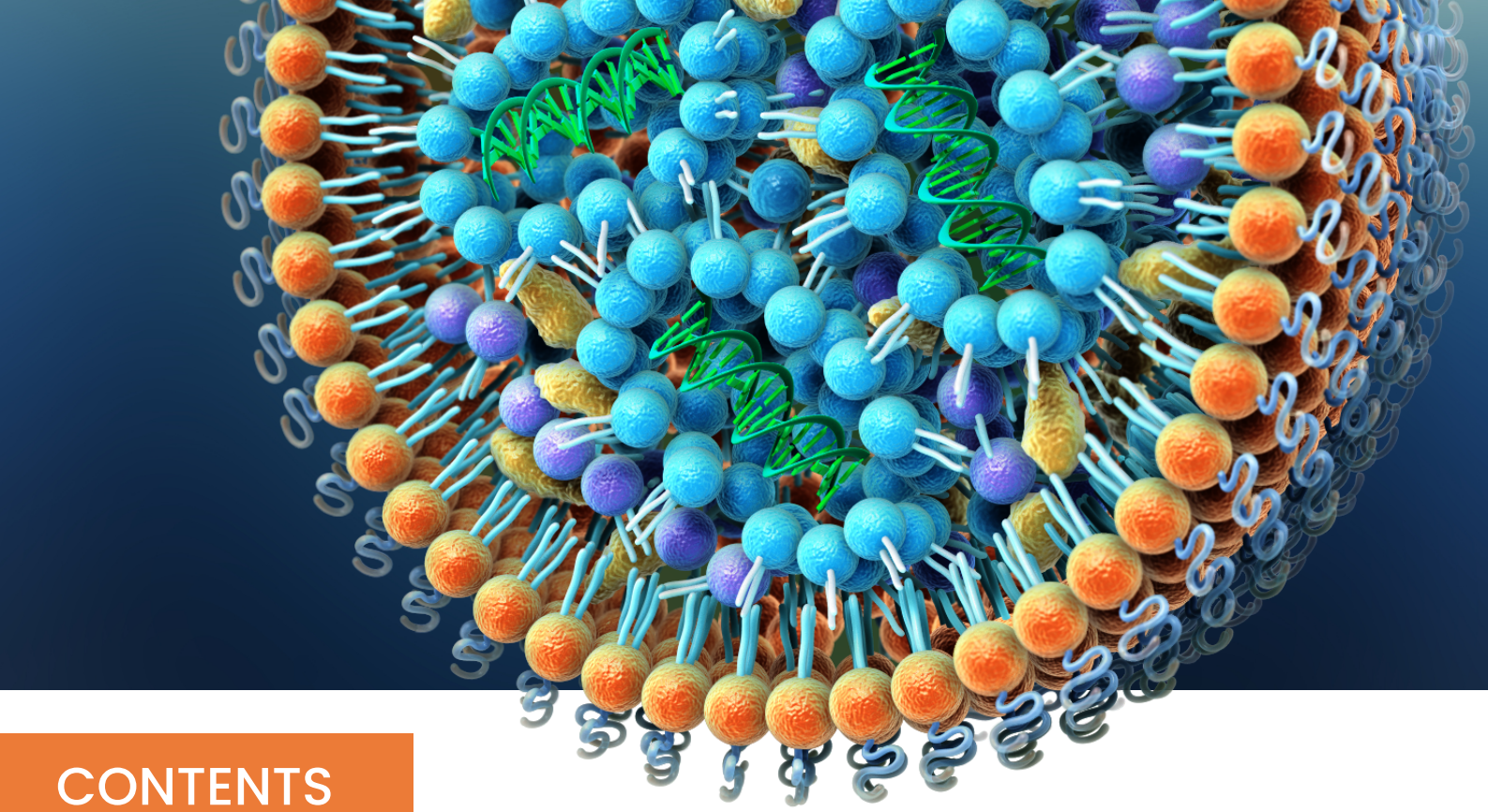

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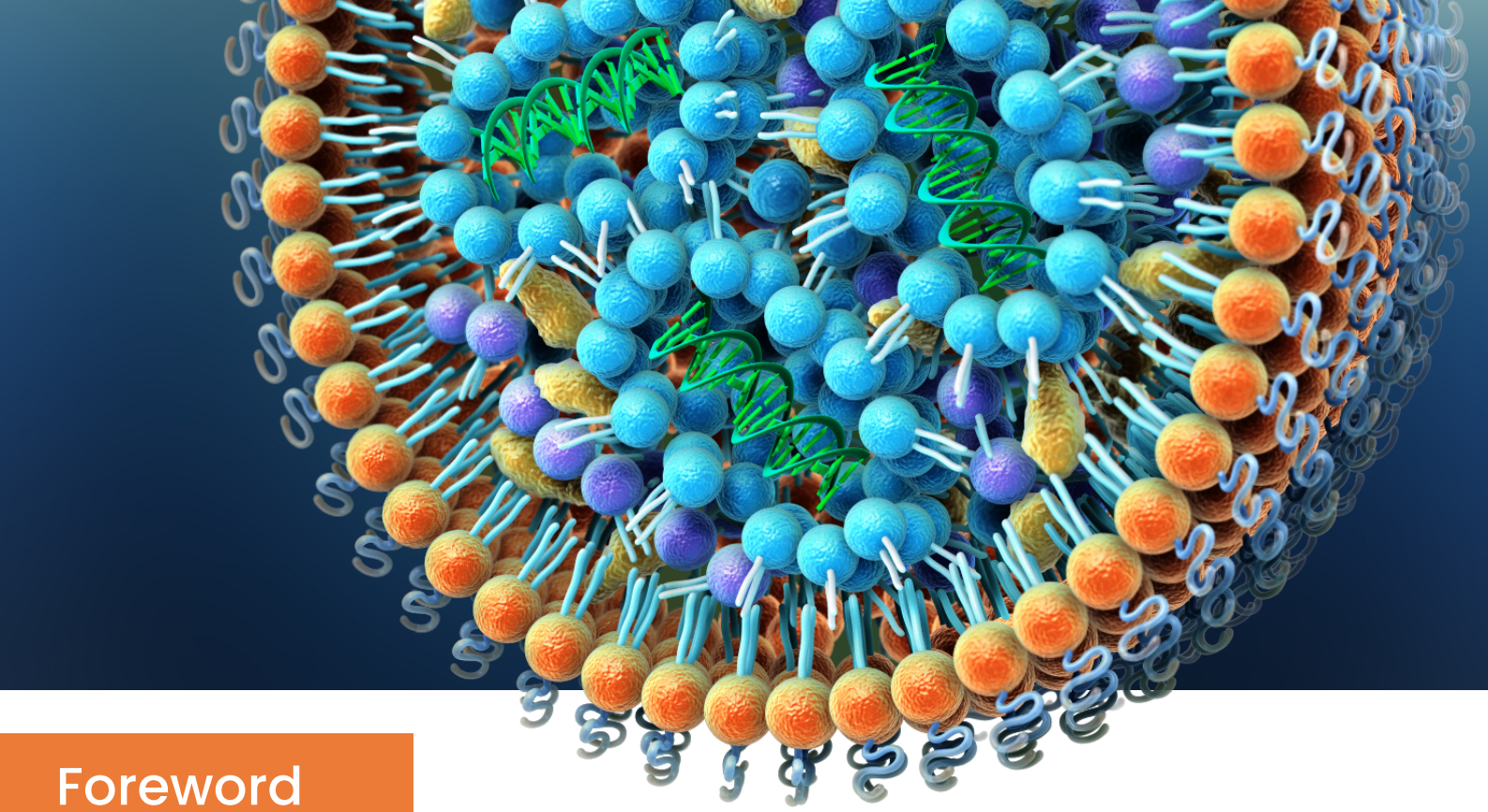

Foreword

We are pleased to present you with this eBook exploring the applications of lipid nanoparticles.

Lipid nanoparticles are the most clinically advanced non-viral gene delivery system. Their ability to encapsulate and protect fragile nucleic acids, such as DNA, mRNA, and siRNA, while facilitating their efficient delivery into cells, makes them a promising platform for gene delivery.

In this eBook, we'll explore some of the key applications of lipid nanoparticles, including in mRNA-based vaccines, like those developed for COVID-19, and for delivering siRNA molecules to target cells as a treatment strategy for conditions like cancer. We'll also explore how lipid nanoparticles are being used to advance the field of cell therapy.

In our interview with Sadik Kassim, Chief Technology Officer of Genomic Medicines at Danaher Corporation (DC, USA), we focus on the applications of how lipid nanoparticles are used in the development of cell therapies. Sadik explains some of their advantages over traditional technologies, such as viral vectors and electroporation, and gives us an insight into how they are being employed to genetically modify a variety of cell types, from T cells and natural killer cells to hematopoietic stem and progenitor cells.

We hope you enjoy reading these expert insights into lipid nanoparticles with us.

Megan Giboney
Editor, Future Science Group
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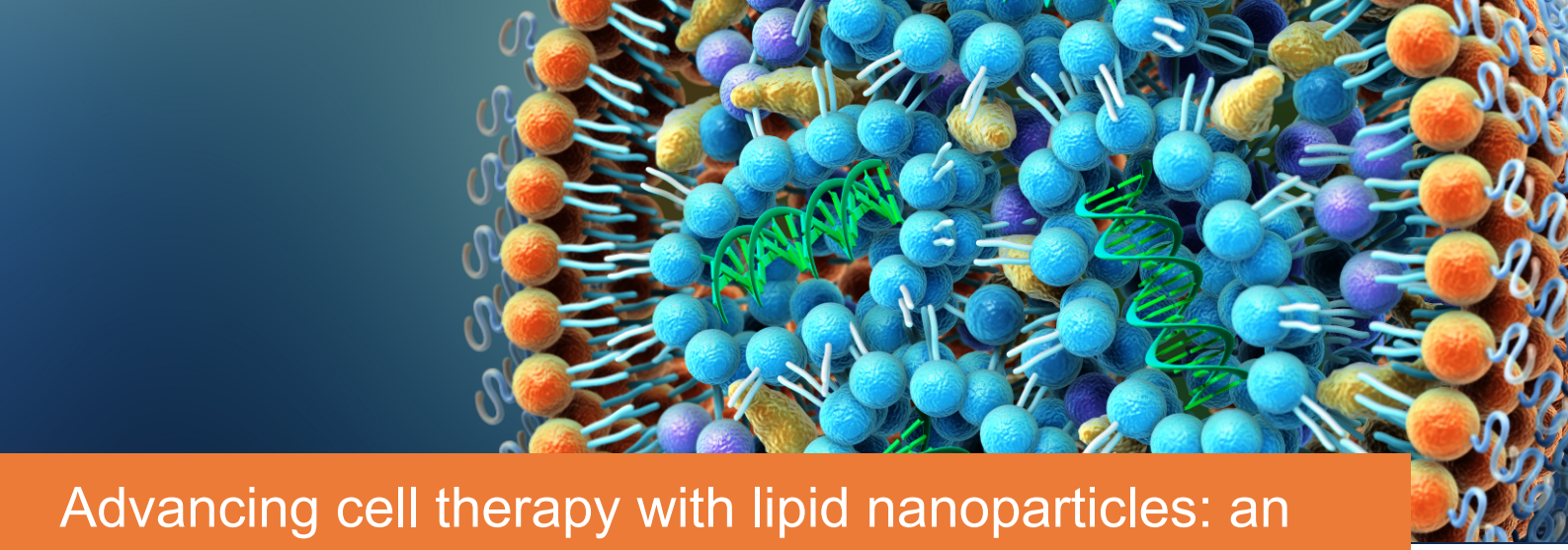


Advancing cell therapy with lipid nanoparticles: an interview with Sadik Kassim

Sadik Kassim is the Chief Technology Officer of Genomic Medicines for Danaher Corporation (DC, USA). Sadik is an immunologist by training but has been involved in the drug development side of genomic medicines for the past 16 years, having contributed to the development of 14 first-in-human clinical trials and three commercially approved CAR-T therapies (Kymriah, Yescarta, and Tecartus).

Sadik joined Danaher 1.5 years ago. Danaher is a Fortune 50 life sciences and diagnostics innovator committed to helping its customers solve complex challenges and improving quality of life around the world. Its family of world-class brands has leadership positions in the demanding and attractive healthcare, environmental and applied end-markets. With more than 20 operating companies, including Cytiva, Precision NanoSystems, Aldevron, IDT and many others, Danaher's globally diverse team of approximately 81,000 associates is united by a common culture and operating system, the Danaher Business System and its Shared Purpose, Helping Realize Life's Potential.

Sadik Kassim
Chief Technology Officer of Genomic Medicines
Danaher Corporation (DC, USA)

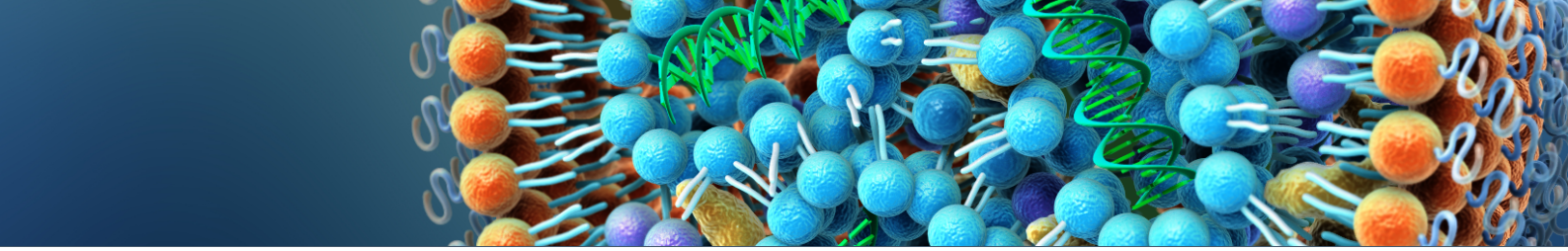
In this interview, Sadik explains the advantages of lipid nanoparticles over traditional technologies, such as viral vectors and electroporation, and gives us an insight into how they are being employed to genetically modify a variety of cell types, from T cells and natural killer cells to hematopoietic stem and progenitor cells.

1 What are some of the challenges associated with the gene editing step involved in the engineering of allogeneic cell therapies?

The fields of gene editing and allogeneic cell therapies have experienced unprecedented growth over the last decade. This year, we will likely witness the FDA approval of the first ever gene-edited cell therapy drug product and the first ever allogeneic cell therapy drug product. However, there remain significant opportunities to improve the efficacy and safety of these products. To do so, we need to overcome the following challenges:

First, we need delivery tools that enable precise, targeted, safe and reproducible gene modification of cells. Today, most of the tools used to deliver gene editing payloads are either based on electroporation, which can physiologically damage cells and reduce overall cell viability and health, or viral vectors, which modify the genome via a random or semi-random fashion.

Second, we need more precise analytical methods and assays for characterizing the impact and outcome of gene-editing events on engineered allogeneic cell therapies.



Finally, we need a better understanding of the cellular starting material. Whether the starting material is normal, healthy donor-derived cells or embryonic or iPSC-derived cell lines, there is tremendous variability inherent to the starting cellular material. Understanding and controlling this variability can potentially enable more precise and controlled gene editing of allogeneic cell therapies.

2 What role will non-viral delivery technologies play in bringing cell therapies to market faster and safer?

There are eight commercially approved gene-modified cell therapies on the market today. All of them are based on viral vector delivery technologies. Viral vector technologies have a long track record in both clinical and commercial settings and have enabled us to treat and cure thousands of patients with chronic, life-threatening diseases. Despite this significant progress enabled by viral vectors, there are still limitations to these types of approaches. Namely, viral vector approaches modify the genome in a random or semi-random fashion. Such an approach can lead to variability in manufacturing and increased manufacturing costs.

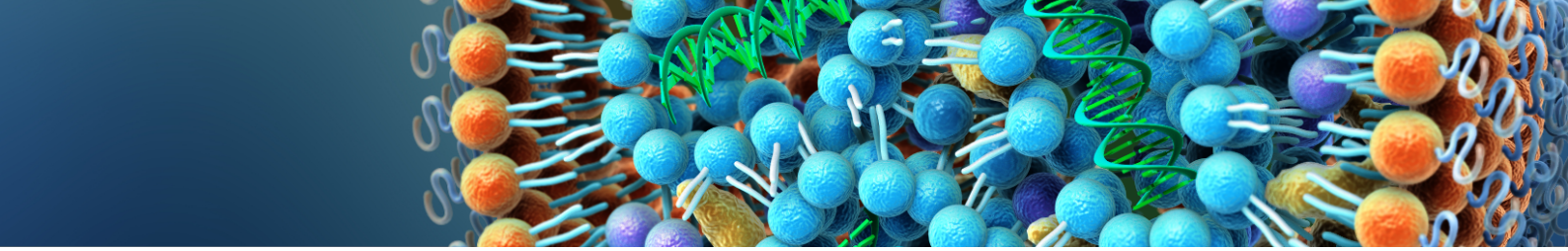
Next-generation non-viral approaches, that use CRISPR gene editing, can enable precise targeting and modification of the genome, which should enable for potentially more potent therapies that can be reproducibly manufactured at scale. Although we are only at the beginning of the use of non-viral approaches for cell therapies, there are significant potential and opportunities in this space.

3 With the success of the mRNA-lipid nanoparticle (LNP) vaccines, how can this technology be used to develop cell therapies?

Proof-of-concept data has been published from multiple labs showing that LNPs can be used to genetically modify cells by enabling the delivery of mRNA that mediate protein expression or gene editing. This has been demonstrated across a variety of cell types, from T cells to NK cells to hematopoietic stem and progenitor cells (HSPCs). One recent notable study, published by the group of Luigi Naldini, conducted a side-by-side comparison of LNPs to electroporation as a delivery vehicle for gene editing of T cells. They showed that electroporation triggers significant cytotoxicity in gene-edited T cells and HSPCs. By contrast, they found that using LNP as a delivery method for editing reagents dampens cytotoxicity, increasing the yield of *ex vivo* edited HSPCs. These types of results suggest that LNPs will play an important role as a delivery technology to enable the development of cell therapies.

4 What are some advantages and drawbacks of using lipid nanoparticles over other non-viral approaches?

A major advantage of LNPs, compared to viral and non-viral approaches, is the relatively low cost to manufacture them and the potentially rapid manufacturing turnaround time. The COVID-19 pandemic has shown us that you can manufacture a batch of mRNA-encapsulated LNPs within a month. By contrast, viral vector manufacturing takes a minimum of 6 months to one year and tends to be significantly more expensive. One disadvantage of LNPs is that few formulations have been clinically validated for cell therapy applications. Most of the LNP formulations we have today were originally developed for *in vivo* delivery of siRNA or mRNA. There remains a major need for the development and clinical validation of LNPs across a variety of applications, including gene-modified cell therapies.



5 If you could ask for one thing to improve the capability of lipid nanoparticles in the engineering of cell therapies, what would it be?

The field needs a broader library of LNP formulations that can be used to selectively target specific cell types. Additionally, we are witnessing the emergence of in vivo cell therapies. These are being enabled by the direct *in vivo* reprogramming of cells within the human body using LNPs. There will be a major need for next-generation LNPs that can be safely and effectively deployed for both ex vivo and in vivo cell therapy applications.

6 What are you most excited about in the cell therapy field in the coming years?

The cell therapy field remains at an early stage in its development and remains ripe with potential to disrupt the practice of medicine and have a major impact on human health. I am most excited about the expanded use of cell therapies in new disease areas and indications. Early clinical and commercial success in cell therapy was observed primarily within the context of cancer immunotherapy of hematologic (i.e., blood) based cancers such as leukemias, lymphomas and multiple myeloma. However, recent clinical data show that cell therapies have significant potential beyond hematologic malignancies, including genetic disorders such as sickle cell disease and beta-thalassemia, autoimmune diseases such as lupus and chronic degenerative diseases such as Type 1 diabetes. Beyond these diseases, recent data show the potential for cell therapies to make an impact in certain solid tumor cancers, which represent over 90% of the global cancer burden.

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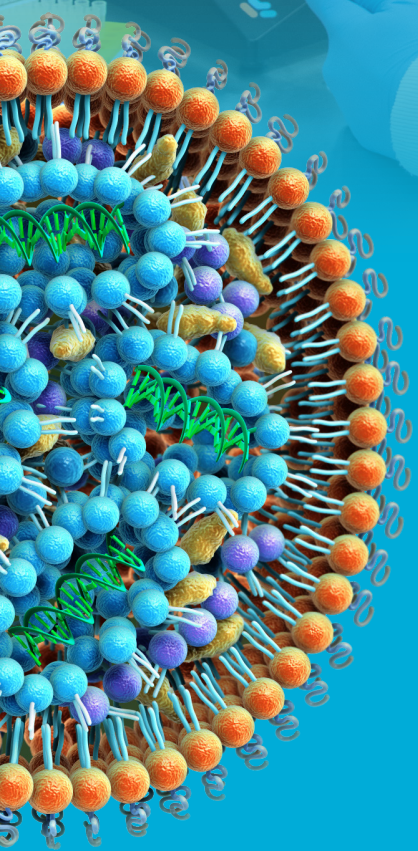

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SPECIAL FOCUS ISSUE | Applications of nanotechnology against COVID-19

Editorial

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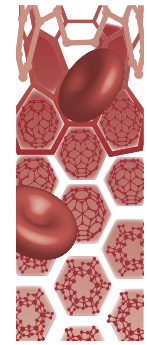

How has nanomedical innovation contributed to the COVID-19 vaccine development?

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"New technologies are intended for solving current problems. Not all of them will succeed, but even then, they can be used to solve future problems."

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Keywords: COVID-19 • lipid nanoparticles • innovation • nanomedicine • nanotechnology • vaccine

In the last 5 years, the nanomedicine community has been deeply involved in discussions on its capacity to bring real-world solutions to the market [1]. As a complex and multidisciplinary innovation process, medical nanotechnology is exposed to unique obstacles or barriers that are difficult to address individually. Nonetheless, within this perspective, all participants on this journey, including academics, industrialists and investors, are trying to improve this important discussion for the nanomedicine ecosystem [1,2].

It is not clear when it started, but it is evident that after huge investments in nanobiotechnology in the early 2000s, all of the society expected a large number of nanomedical products to reach the market, benefiting patients who were waiting for new solutions [2]. Extravagant promises presented in project proposals and preclinical results published at the time announced a forthcoming revolution in different fields of the global economy. That expectation, as we know now at the dawn of the third decade of this millennium, was not followed by industrial acceptance of the nanomedical technologies. The aftermath of the numerous R&D projects conducted so far is not exactly frustrating, but it is far from breathtaking [3].

As most of the nanomedical applications have been centered on oncology, this specific field can be taken as an example of what went on in nanomedicine. An important milestone in the evolution of oncological nanomedicine was reached after the publication of results showing that the classical enhanced permeation and retention (EPR) effect, observed in many preclinical studies, is often not observed in clinical conditions [4]. This phenomenon has been proven useful for tumor targeting in different experimental mice tumor models [5,6], so that the great majority of the preclinical studies involving oncological nanomedicines at the beginning of the 'nanotechnology era' aimed at exploring the EPR effect [5,6].

Exploring the EPR effect to increase the relative delivery of chemotherapeutic drugs to tumors by nanosystems was a very attractive model to develop a magic bullet for Ehrlich tumor therapy. Many nanomedicine researchers were co-opted by this ideal, but after decades of intense research on this strategy, we are not even close to achieving such a magic bullet. The translation of the preclinical magic bullet candidates from experimental models to patients has encountered important barriers [2,3].

Additionally, other long-promised nanomedicine tools aiming at the reduction of negative side effects, improvement of dosage, safer administration forms and the use of alternative routes of administration [7] have not hit the market as expected. Metselaar and Lammers [2,8] suggest that exploring all the possibilities of nanotechnology in pharmacotherapy, even those considered as simpler improvements, instead of only looking for revolutionary magic bullets, would bring more acceptance of nanomedicine by industry and investors.

Another point that needs to be considered in this discussion is the number of new approved nanomedicines, which has consistently risen in the last 5 years. Although it is not the number that meets former expectations, it indicates a recent rise in new nanomedicine products launched on the market. In absolute numbers, 50 new nanomedicines entered the market, most of them approved for oncological conditions. Considering clinical trials, which can potentially generate approved products, the percentage of trials for cancer was almost 70% in 2016 and

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in 2020 this number fell to close to 50%. This can suggest that some nanomedicine research is moving from oncology to other medical applications [2].

Within this context, the nononcological applications usually aim at reducing some drawbacks that are also common to several other pharmacotherapies [7,9]. Of course, all the background obtained in the oncological applications was very important to these new applications. Furthermore, it is possible that the discussion on what went wrong with oncological nanomedicines also brought other benefits from nanoscience and nanotechnology into other biomedical areas [9].

In fact, general theories about innovation always hold that innovations are not created from scratch, but are rather consequences of the constant evolution of previously available knowledge and technologies. In other words, innovation depends upon the recombination of former ideas or the combination of well-known technologies. In terms of technology evolution, the exchange of ideas and data are a fruitful strategy for flourishing innovation in a given ecosystem, such as the nanomedicine environment [10,11].

A current and striking example of this merging of ideas is lipid nanoparticle vehiculation of RNA in vaccines for SARS-CoV-2 [12,13]. Basically, the RNA molecules present in these vaccines need to be delivered to cells where they are translated into antigenic viral proteins. There is, however, a big problem with that: RNAs are extremely fragile and unstable, and readily hydrolyzed by RNAses in the extracellular media, so a free RNA molecule administered into a tissue cannot reach the cytosol of a target cell.

To overcome this intrinsic limitation, researchers encapsulated the RNA molecules in lipid nanocarriers [12,14]. This approach both protects and delivers the RNA. In this way, the half-life of the RNA is increased, and the delivery to the cytosol of target cells is optimized. In addition, it is important to highlight that the overall negative charge of polynucleotides in physiological pH impairs cellular uptake, which is also circumvented by liposomal delivery. Thus, a lipid nanoparticle has different missions in the vaccine strategy [14].

A particularly interesting point in this history is that lipid nanoparticles, especially the liposomes, were not initially designed for that task. They have been intensely investigated for decades, initially as biomimetic membrane models and later on as drug nanocarriers. More recently, these phospholipid vesicles were successfully used to encapsulate siRNA for the treatment of transthyretin amyloidosis, an autoimmune rare disease [15,16]. This was the first biomedical product to be put on the market that uses oligonucleotides as an active drug [2], proving that the protection provided by the lipid nanocarrier is useful and safe. In a retrospective analysis, however, it would be impossible to foresee that the most studied nanostructure carrier in history, the phospholipid nanoparticles, could play such an important role in the management of a future pandemic.

It is always easier to predict something after it happened. This apparently random evolution of a technology and its consequences are quite impossible to design rationally, and we need to be satisfied in understanding how the process occurred in a retrospective way. This apparent loss of control is typical of the whole scientific development process, and can be related to the frustration felt within the nanomedicine ecosystem that we discussed at the beginning of this text. How could one outline the study of biomimetic membranes aiming at the delivery of nucleic acids? New technologies are intended for solving current problems. Not all of them will succeed, but even then, they can be used to solve future problems.

This is basically how science, technology and innovation correlate to each other. Actually, they do not exist as separate entities in the real world, but are rather part of a unique, cyclic process, which has been clearly dissected. Thus, innovation is physiologically connected to basic science, as well as to technology [10]. With regard to nanomedicine specifically, the field is passing through a maturation process, with a strong scientific background that can be used as a tool to improve the effectiveness of the most varied applications in medicine, as well as in other fields. Thus, as we are experiencing a maturation stage [1], some directions cannot be clear yet, but looking at the potential applications of the field, we do believe that it made absolute sense to have invested in nanomedicine over the last few decades.

Financial & competing interests disclosure

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Genomic Medicine Drug Discovery and Screening, a digital era for RNA-LNP therapeutics

In a remarkably short period of time, genomic medicine has led to exciting revelations that are transforming the way we are able to target and treat human disease on a fundamental level. Whether the goal is to deliver genetic information to a target cell, either to replace a defective gene, or to treat or prevent disease, their effect on healthcare is already being felt. The COVID-19 vaccines are perhaps one of the biggest success stories to date, having unequivocally demonstrated their efficacy and safety in the real world.

Genomic medicine offers a faster route for drug development, clinical diagnosis, and present new opportunities for gene-editing, immuno-oncology and personalized medicine applications. Among genomic medicine modalities, Ribonucleic Acid-Lipid Nanoparticle (RNA-LNP)-based therapeutics comprise a rapidly expanding category of drugs, which are being applied to solve areas of high medical need from infectious diseases to many other areas including cancer, rare diseases, and metabolic diseases.

The ability of mRNA-LNPs to induce the expression of nearly any protein with minimal changes to their chemical characteristics makes them an ideal platform to accelerate drug discovery, development and commercialization. Once the disease gene to target is identified, whether it is a virus or a cancer, you can very rapidly find the DNA sequence—and in turn, the mRNA sequence—to use as the drug substance and have it manufactured. This unlocks a tremendous amount of innovation both in how we can treat disease and how medicine is distributed and administered as well. And fine-tuning how we deliver these genomic medicines to different tissues safely and effectively will continue to accelerate progress in the field.

OVERVIEW OF THE DRUG DISCOVERY PROCESS USING RNA-LNPS

The first step in the development of a new genomic medicine is to identify a relevant disease gene to target for modulation. A good target should be accessible to the proposed drug candidate, resulting in a drug that is safe, efficacious, and meets clinical and commercial requirements[1]. Extensive target validation needs to be conducted to confirm that the target gene has a desired therapeutic effect. This typically requires robust *in vitro* cell-based disease models and phenotypic assays. These results are further confirmed in various *in vivo* models.

Once validated, the next stage is to screen the active pharmaceutical ingredient (in this case, the mRNA), excipients, and various formulations parameters. For

mRNA-LNP formulations, parameters including the choice of cationic/ionizable lipid, lipid mix concentration, polyethylene glycol (PEG) content, N/P ratio (ratio between cationic amines in the lipid excipient and the anionic phosphates on mRNA) need to be optimized. The characteristics of an LNP for liver-specific delivery may be different than one designed for targeting cytotoxic T cells. Therefore, screening various LNP compositions is a critical step to achieving the desired payload encapsulation, stability in circulation, *in vivo* transfection efficiency and tissue-specific delivery[2]. Additionally, the molecular design of the mRNA, such as the 5' cap structure, incorporation of untranslated region (UTR), coding sequence, codon optimization and nucleosides modifications, may have an impact on the stability of mRNA molecule, protein expression, and immunogenicity of the target antigen; therefore, these parameters should also be evaluated at this stage. It is essential to assess how these variables impact nanoparticle attributes such as size and PDI since these parameters are directly linked to their *in vivo* functionality. The goal at the end of the discovery phase is to identify promising lead candidate formulations that can advance to pre-clinical development.

BOTTLENECKS IN THE DEVELOPMENT OF GENOMIC MEDICINES

Because there are, as yet no computational models that can reliably predict the *in vivo* outcomes of changing a codon, or a nucleotide modification, or a lipid species in a formulation, all of this work must be done empirically. This empirical testing of mRNA candidates and LNP formulations to identify the best candidates to advance to pre-clinical evaluation can severely bottleneck workflows.

Organizations need access to lipid libraries with which to test the numerous drug candidates, and the combination of these can generate hundreds of possible formulations that need to be created and tested. It is often a low-throughput and rate-limiting step in the drug development process that requires a significant time and financial investment. And this is especially true when dealing with raw materials like that of mRNA that are expensive, labor-intensive to produce and are limited in availability in the discovery phase. For example, the recent development of a siRNA delivery formulation for the treatment of hereditary transthyretin amyloidosis took more than 10 years of development where >300 ionizable lipids were synthesized and tested to find the optimal formulation[3].

The need to accelerate the development of genomic medicines has never been greater. To this end, access to well-characterized lipid libraries and the ability to screen LNPs at low volumes quickly is advantageous to streamline the screening process while also conserving expensive raw materials to effectively navigate the discovery space. This allows for the rapid selection of the best candidates for both RNA drug substance and lipid formulations to help researchers bring new genomic medicines to the clinic faster.

LNPS ARE EFFECTIVE AND CLINICALLY PROVEN GENE DELIVERY VEHICLES

LNPs have been widely studied as a non-viral delivery vehicle for nucleic acids, offering significant advantages over conventional methods such as electroporation, lipofection and viral vector delivery. A major drawback of electroporation is it is harsh on the cells, which can lead to poor viability and the method also does not protect the nucleic acids from nuclease degradation and is limited to *ex vivo* usage. Viral delivery modes protect the genetic payload but there are concerns of potentially dangerous immunogenic side effects and limitations with payload size and cost.

Lipid nanoparticles are composed of ionizable lipids, helper lipids, cholesterol, and and stabilizers to effectively encapsulate and protect the nucleic acid payload. The composition of the LNP allows for rapid cellular uptake via endogenous mechanisms of difficult cell types and promotes payload release into the cytoplasm. LNP technology is also gentle on the cells with minimal cytotoxicity, suitable for both *in vitro* and *in vivo* studies, easy to use and rapidly translatable and scalable as evidenced by the commercialization of LNP-based therapeutics such as Onpattro® (Patisiran) and the COVID-19 vaccines.

ACCELERATE RNA-LNP DRUG DISCOVERY

The method to create mRNA-LNP molecules involves mixing lipids dissolved in an organic solvent with RNA in an acidic buffer to induce spontaneous self-assembly. Since physical properties of the LNPs such as size and morphology are intricately linked to their biodistribution and function, highly specialized expertise and technologies are required to achieve the right combination and proportions of input materials to ensure the uniformity and quality of the particles for effective delivery of an RNA drug substance for a defined therapy[4].

NxGen™ mixing is the preferred mechanism for producing lipid nanoparticle formulations, offering non-turbulent, precisely controlled mixing environments overcoming the challenges of traditional mixing techniques that suffer from poor batch-to-batch reproducibility. Furthermore, traditional methods have limited process control and require materials far in excess of what is needed for drug discovery and screening where microliter-scale formulations are needed to conserve expensive materials.

The NxGen mixer design that is used in the full range of NanoAssemblr® instruments enables reproducible, scalable and time-invariant production of LNP formulations from µL volumes to tens of liters for drug discovery, screening, process development, clinical testing and commercialization of a genomic medicine.

The NanoAssemblr Spark™ is an instrument designed to accelerate the early stages of drug development allowing users to rapidly produce numerous formulations for *in vitro* testing with very small quantities of rare or costly raw materials. This innovative platform realizes the benefits of NxGen mixing for the controlled and reproducible ultra-low microliter volume production of LNPs allowing hundreds of formulations to be made in just hours instead of days or weeks. The resulting LNPs can be applied directly to *in vitro* cultures for functional screening

without any downstream processing, offering an efficient screening platform with considerably less hands-on time.

LNP formulation parameters can be screened systematically by varying the lipid composition, reagent concentrations, and the N/P ratio (the ratio between cationic amines in the lipid excipient and the anionic phosphates on mRNA) to evaluate their impact on nanoparticle quality and efficacy5. The platform can also be used to identify suitable formulations for different types of APIs beyond mRNA (i.e., siRNA, gRNA, plasmid DNA), or to optimize performance characteristics such as LNP size, encapsulation efficiency of target payloads, formulation stability, cell targeting.

Selecting the appropriate LNP formulation is critical to the successful development of genomic medicines and the adage of "using the right tool for the job" holds true here. Using the right technology to reproducibly formulate LNPs at small scale suitable for high throughput screening can accelerate pre-clinical programs to deliver promising lead candidate formulations faster and more efficiently. This new digital era of genomic medicine will unlock our collective ability to treat human biology in a digital way on a population basis but also on an individual level too. It will enable us to rapidly develop the life-saving therapeutics and vaccines that will define the future of medicine.

ABOUT PRECISION NANOSYSTEMS

Precision NanoSystems brings years of proven experience, scalable NanoAssemblr™ instruments, LNP deliver instruments, LNP delivery reagents, lipids and Biopharmaceutical Services to deliver unparalleled support for researchers around the globe as they traverse the path from discovery to commercialization of new genomic medicines.

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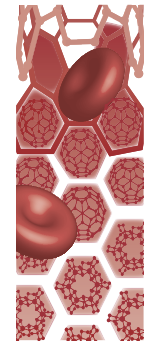

Nanoparticle-siRNA: a potential strategy for ovarian cancer therapy?

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Ovarian cancer is one of the most common causes of mortality throughout the world. Unfortunately, chemotherapy has failed to cure advanced cancers developing multidrug resistance (MDR). Moreover, it has critical side effects because of nonspecific toxicity. Thanks to specific silencing of oncogenes and MDR-associated genes, nano-siRNA drugs can be a great help address the limitations of chemotherapy. Here, we review the current advances in nanoparticle-mediated siRNA delivery strategies such as polymeric- and lipid-based systems, rigid nanoparticles and nanoparticles coupled to specific ligand systems. Nanoparticle-based codelivery of anticancer drugs and siRNA targeting various mechanisms of MDR is a cutting-edge strategy for ovarian cancer therapy, which is completely discussed in this review.

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Ovarian cancer is one of the deadliest malignancies in women and also the fifth cause of cancer death in women [1]. It is divided into several histotypes such as high-grade serous, low-grade serous, endometrioid, clear cell and mucinous [2]. This cancer often happens after the age of 40 and less than 1% of cases occur in ages less than 20 years [3–5]. Ovarian cancer often progresses to the advanced stage without any symptoms. Less than 25% of patients with advanced disease survive up to 5 years, thus it is known as the silent killer [6]. Several therapies are available for the disease, including surgery, chemotherapy, radiotherapy, monoclonal antibodies and pharmacological drugs. Unfortunately, these treatments have not been very fruitful and pose many problems, such as resistance to chemotherapy, nonspecific toxicity and resistance to apoptosis [7,8]. Several strategies have been proposed to overcome the aforementioned problems such as improving the delivery of chemotherapy. Enhancement of the delivery system includes the attachment of a powerful drug with a nanoparticle in order to encapsulate this anticancer molecule [9]. Drug-loaded nanoparticles accumulate in cancerous cells because of enhanced permeability and retention effect of nanoparticles [10], thus drug coencapsulation with a nanoparticle can improve drug delivery, even though accompanied with a weak solution [11]. While delivering a particular drug along with a nanoparticle has many benefits, chemotherapy delivery seems to be somewhat problematic. Several genes that play a role in multidrug resistance (MDR) are overexpressed in cancer cells. *MDR1* is one of the genes that account for chemoresistance in ovarian cancer cells by the efflux of anticancer drugs [2]. Therefore, high doses of chemotherapy with significant side effects must be administered in order to overcome this problem and eradicate cancer cells [12]. *Bcl-2* and other genes, which are involved in antiapoptotic responses, can also play an important role in drug resistance. Cancer cells are resistant to other cancer therapy methods like photodynamic therapy (PDT) and hyperthermia therapy,

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Table 1. Significant therapeutics siRNAs in clinical trials.

Therapeutic siRNAs	Targeted gene	Carrier system	Diseased condition	Stage	Status	Pharmaceutical factories	ClinicalTrials.gov identifiers
ALN–VSP02	*VEGF* and *KSP*	LNP	Solid tumors	I	Completed	Alnylam Pharmaceuticals	NCT01158079
ALN-RSV01	*RSV* nucleocapsid	Naked siRNA	RSV infection during lung transplantation	II	Completed	Alnylam Pharmaceuticals	NCT00658086
ARB-001467	All four HBV transcripts	LNP	Chronic HBV infection	II	Active, not recruiting	Arbutus Biopharma Corporation	NCT02631096
Patisiran (ALN–TTR02)	*TTR*	LNP	Transthyretin-mediated amyloidosis	III	Completed	Alnylam Pharmaceuticals	NCT01960348
ALN–TTRsc	*TTR*	siRNA–GalNAc conjugate	Transthyretin-mediated amyloidosis	II	Completed	Alnylam Pharmaceuticals	NCT02292186
Excellair	Spleen tyrosine kinase (*Syk*)	Naked siRNA	Asthma	II	Completed	Alnylam Pharmaceuticals	-
IONIS-HBV-RX	Conserved regions of X Orf in HBV	GalNAc-ASO	Chronic infections of HBV	II	Recruiting	Ionis Pharmaceuticals, Inc.	NCT02981602
QPI-1002	*p53*	Naked siRNA	Delayed graft function in recipients of an older donor kidney transplant (ReGIFT)	III	Recruiting	Quark Pharmaceuticals	NCT02610296
QPI-1007	Caspase-2	Naked siRNA	Optic atrophy, nonarteritic anterior ischemic optic neuropathy	II/III	Recruiting	Quark Pharmaceuticals	NCT02341560
siRNA–EphA2–DOPC	*EphA2*	LNP	Advanced cancers	I	Recruiting	M.D. Anderson Cancer Center	NCT01591356
CALAA-01	*RRM2*	Cyclodextrin NP	Solid tumors	I	Terminated	Calando Pharmaceuticals	NCT00689065
ApoB SNALP	*ApoB*	SNALP	Hypercholesterolemia	I	Completed	Tekmira Pharmaceuticals	-
SPC2996	*Bcl-2*	Naked siRNA	Chronic lymphocytic leukemia	II	Completed	Santaris Pharma A/S	NCT00285103
ARC-520	Conserved regions of *HBV*	DPC	Patients with chronic hepatitis B virus	II	Terminated	Arrowhead Pharmaceuticals	NCT02065336
Bevasiranib	*VEGF*	Naked siRNA	Macular degeneration	III	Terminated	Opko Health	NCT00499590

DPC: Dynamic polyconjugate; LNP: Lipid nanoparticle; NP: Nanoparticle; RSV: Respiratory syncytial virus.
Excellair and ApoB SNALP data modified with permission from [21,22].

as well [13,14]. In light of recent advances, molecular resistance mechanisms are more and more understood and as a result, innovative strategies are designed to tackle this problem. siRNA is a dsRNA that consists of 21–23 nucleotides. siRNA guides RNA-induced silencing complexes to binds to the specific sequence of mRNA and subsequently degrades it [15]. Given that some genes are highly expressed in many diseases including cancer, siRNAs can be used as a therapeutic agent to silence them [16]. Owing to this great potential, many siRNA-based drugs have been developed and used for clinical trials as presented in Table 1. In the early part of this decade, we have seen an avalanche of reports about shortcomings of siRNA application including enzymatic sensitivity, renal clearance and difficulty to enter cells, off-target effect, innate immune system stimulation [17]. The major of the naked-siRNA delivery defects are due to hydrophilic nature, large molecular weight and net negative. Using nanoparticle-based carriers is a powerful strategy to address these challenges [18]. In addition, a combinational therapy, which consists of siRNA-targeting MDR mechanisms and chemotherapy, has shown to be effective [19,20]. In this article, we will review the advances and setbacks in some new mechanisms of nanodrug delivery including polymeric system, lipid system and rigid nanoparticles and the development of innovative imaging methods to track and evaluate the efficacy of nanoparticle-siRNA for therapy of ovarian cancer. Nanoparticle-mediated delivery of siRNA, we will also explicate codelivery of anticancer drugs with siRNA as a prominent strategy to overcome MDR.

Lipid-based delivery of siRNA

Development of new lipid-based mechanisms for delivery of siRNA has impressively progressed in recent years [23,24]. Since unmodified siRNA is not stable in the blood and cannot cross membranes alone, lipid components are required to carry siRNA to the site of target cells [25]. Furthermore, the lipid-based system keeps siRNA safe from nuclease degradation, kidney clearance. It also makes easier cellular uptake and endosomal escape of siRNA [26].

Nanoliposomes like neutral 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) exhibit a high-loading efficacy and can control the release of the drug. DOPC efficiently encapsulates the hydrophobic molecules and protects them from renal excretion [27,28]. Guillermo *et al.* showed that hMCP1 siRNA-DOPC nanoparticles suppress tumor growth and decrease infiltration of $CD68^+$ and $F4/80^+$ macrophage cells in tumor samples obtained from mice models that are under daily restraint stress [29].

Targeting CD95 or CD95L with siRNA has proven to be toxic to some cancer cells [30]. Nevertheless, in the absence of target cells, siRNA binds to 3′UTRs of some genes that are involved in survival. This phenomenon is referred to as death induced by survival gene elimination. Template lipoprotein nanoparticles are comprised of a gold nanoparticle (AuNP) as the core. Other components include apo A-I and some phospholipids such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(3-[2-pyridyldithio] propionate) and DOPC, which are bound to AuNP/apo A-I [31]. Murmann *et al.* demonstrated that in an *in vitro* setting, siRNA along with TLP against CD95L causes death induced by survival gene elimination, hence, it is not efficacious for ovarian cancer therapy [32]. Since siRNA possesses a high anionic charge, cationic lipid-based carriers are more efficient [33]. In accordance with what has been just attested, Zhao *et al.* have shown that inhibition of Notch1 in SKOV-3 cell lines by siRNA along with a cationic cholesterol (*N*-(cholesterylhemisuccinoylamino-3-propyl)-*N,N*-dimethylamine (DMAPA-chems) can suppress tumor growth and induces apoptosis of SKOV-3 cells [34].

To overcome some limitations for the *in vivo* application of siRNA such as lack of safety and ongoing delivery, Tanaka *et al.* designed mesoporous silica particles (MSP), which were loaded with neural nanoliposome covering siRNA against the EphA2 oncoprotein that is overexpressed in ovarian cancer. In other words, they applied a multistage delivery approach, the first one was mesoporous microscale biodegradable silicon particles and the second one was DOPC nanoliposomal siRNA:siRNA-DOPC. In a mouse model of ovarian cancer, use of MSP-DOPC-siRNA suppresses this oncogene gene at least for 3 weeks, thus decreases proliferation, angiogenesis and tumor growth [35]. Moreover, Hasan *et al.* used nanoporous silicon particles instead of MSP, which contained the same nanoliposome and siRNA against EphA2. Tumor burden was reduced due to continuous silencing of this gene in mouse models [36].

Despite many advances in lipid-based siRNA carriers, there are still some significant challenges like low stability and high toxicity. To solve these problems, hydrophobic moieties, such as poly(ethylene glycol; PEG) and PLGA, can be conjugated to lipid nanoparticles and cationic polymers [37,38]. He *et al.* synthesized novel folate-modified cationic liposomes (F-PEG-CLPs) for gene delivery into SKOV-3 ovarian cancer cells. They revealed that folate-PEG-succinate-cholesterol (F-PEG-suc-Chol) have a high transfection efficiency and exhibit low toxicity [39].

Polymeric-based siRNA delivery

siRNA, with a negative charge and hydrophilic properties, cannot cross the cell membrane easily [40], so chitosan (CS) with its cationic nature seems appropriate for delivery of siRNA [41]. CS ([1,4]-2-amino-2-deoxy-D-glucan) has biocompatible and biodegradable characteristics, which make it a suitable choice for the siRNA transfer [42,43]. Steg *et al*. demonstrated that targeting of Jagged1, a Notch ligand overexpressed in malignant cells, with CS/siRNA nanoparticle leads to reduced cell viability in SKOV-3 and IGROV-AF1 and sensitization to docetaxel *in vivo*. This is partly because of overexpression of Jagged1, which is responsible for angiogenesis and chemoresistance and also a proliferation of tumor cells [44]. In another study, Kim *et al.* used siRNA against Src incorporated into CS nanoparticle *in vitro* and *in vivo*. Src belongs to membrane-associated nonreceptor tyrosine kinases, which contribute to cell division, angiogenic function and survival. Therefore, silencing of Src in SKOV-3ip1 and HeyA8 cells leads to a reduction in cell proliferation, angiogenesis and enhancement of apoptosis. The same results were observed *in vivo* [45].

Fernandes *et al.* revealed that the use of low-molecular-weight CS increases siRNA absorption and ultimately the formation of nanoparticles. Low-molecular-weight CS was conjugated with folate to improve siRNA delivery by means of folate receptor (FR) on the surface of SKOV-3 cells [46].

EZH2 is a histone–lysine *N*-methyltransferase enzyme and is functional in some cell processes. It tends to be increased in some tumor cells. EZH2 suppresses the expression of vasohibin-1 with antiangiogenic properties.

Gharpure *et al.* established that siRNA coated with CS nanoparticles, along with docetaxel against EZH2, reduce angiogenesis and tumor mass in HeyA8 and SKOV-3ip1 orthotropic mouse models [47].

Synthetic polymers such as dendrimers are used for siRNA delivery because of their well-defined and multivalent structures, proper molecular architecture and nanosized volume [48]. Ma *et al.* used siRNA targeted against $P70^{s6k}$ that is a kinase protein involved in metastasis and tumor progression in ovarian cancer. A reduction in proliferation and expansion of tumor cells *in vitro* was noted, and on the other hand, they observed diminished migration and invasion of cancer stem cell *in vivo* [49]. PI3K/Akt is another important signaling pathway in the pathophysiology of ovarian cancer and is used as a target for therapy. Triethanolamine-core poly(amidoamine) dendrimer with siRNA against Akt was used along with paclitaxel as a combination therapy. As a result, antitumor efficacy of chemotherapeutic regiment in combination with Akt siRNA *in vitro* and *in vivo* was increased [50].

Polyethyleneimine (PEI) is one of the most idealistic structures for siRNA delivery due to the fact that PEI has great buffering properties, which lead to high gene silencing. PEI-based nanoparticles have some serious challenges including nondegradable nature and more importantly, their chemical structure and high molecular weight, which result to cytotoxicity [51,52]. To address these challenges and reduce cytotoxicity and enhance delivery efficiency, PEI can be conjugated to other biodegradable polymeric systems like poly(lactide) or PEG [53,54]. Jones *et al.* used the triblock copolymer composed of PEI-graftpolycaprolactone-block-PEG (PEI-*g*-PCL-*b*-PEG-Fol) to deliver Toll-like receptor 4 siRNA into SKOV-3 cells. As a consequence, SKOV-3 cells were resensitized to paclitaxel and apoptosis was increased [55].

Rigid nanoparticles for delivery of siRNA

Rigid nanoparticles including carbon materials and inorganics are more effective siRNA carriers than soft particles because of their faster cellular internalization, and unique controllable properties (including immunologically inert surface, simple surface functionalizing to enhance uptake, stability in a physiological environment, controllable size and large surface area to volume ratios) [56,57]. However, for long-term therapeutic applications, some challenges like nonbiodegradability and slow suspension may occur. In this segment, we will explain AuNPs and carbon black nanoparticles (CBNPs).

Gold nanoparticles

AuNPs can be effective in gene delivery because of exceptional surface plasmon resonance, easy size-controlled property and facile modification with other molecules [58–60]. Arvizo *et al.* delivered MICU1 siRNA/positively charged AuNPs to human ovarian cancer cell lines (OVCAR5, OV167 and OV202). The decreased expression of *Bcl-2* simultaneous increase in the level of cytosolic $[Ca2]_{cyto}$ led to the activation of the mitochondrial pathway of apoptosis. This study introduces MICU1 as a new regulator, which prevents apoptosis in tumor cells [61].

CB nanoparticle

CBNP is mainly composed of carbon with a few other elements (including hydrogen and oxygen) [62]. Sengupta *et al.* used laser-irradiated CBNPs for the delivery of EGF receptor (EGFR) siRNA to ovarian cancer cells (Hey A8-F8). Efficient intracellular siRNA delivery and EGFR mRNA silencing have been elicited by specific qPCR assays [63].

Nanoparticle coupled to specific ligand systems

To overcome the extracellular barriers, receptor-mediated endocytosis is one of the most important strategies to increase the cellular uptake. Anisamide, hyaluronic acid (HA), antibody, luteinizing hormone-releasing hormone (LHRH), follicle-stimulating hormone (FSH), folic acid (FA), arginine and arginyl-glycyl-aspartic acid (RGD) are known as specific ligands for siRNA delivery to the tumor cells. However, among them, only RGD, antibody and FA are used for siRNA delivery in ovarian cancer. In this segment, these systems will be explained.

RGD peptides

RGD peptide motif plays an important role in cell adhesion to the extracellular matrix and many studies showed that integrin receptors can recognize this sequence [64]. Besides, RGD peptide motif can be used as a specific ligand because this sequence has a high affinity for αv integrins on tumor and tumor angiogenic endothelial cells [65]. Han *et al.* designed RGD peptide-labeled CS nanoparticle (RGD-CH-NP) to transfer POSTN siRNA, FAK siRNA and PLXDC1 siRNA in SKOV-3ip1, HeyA8 and A2780 cells *in vitro*. Afterward, they injected PLXDC1

siRNA intravenously into A2780 tumor-bearing mice *in vivo*. Silencing of multiple growth-promoting genes (FAK, PLXDC1 and POSTN) with high therapeutic efficacy have been achieved by using siRNA-coupled RGD-CH-NP *in vitro*. Selective intratumoral delivery into orthotopic animal models of ovarian cancer is considerably enhanced by RGD-CH-NP loaded with siRNA. Moreover, substantial inhibition of tumor growth was observed in the A2780 tumor-bearing mice compared with controls [66].

Arginine

Among cell-penetrating peptides, arginine as a recruiting motif has an important role and the transfection efficacy is higher in conjugated polymers than unconjugated counterparts [67,68]. Florinas *et al.* developed a nonviral siRNA delivery system composed of an arginine-grafted bioreducible polymer (ABP), microbubbles (MB) and ultrasound and used this system to transfer VEGF siRNA to A2780 human ovarian cancer cell line. ABP was used as a nonviral siRNA carrier and the focused sonication resulted in siRNA release from MB in the desired tissue. The combination of VEGF siRNA-ABP-MB with VEGF resulted in a higher silencing rate than naked siRNA [69]. In another study conducted by this team, after intratumoral injection of siRNA-ABP-MB into A2780 ovarian cancer xenograft, tumor shrinkage was reported due to improved siRNA absorption [70].

Folic acid

FA tends to bind to the overexpressed FRs in different human cancer cells with a high affinity ($K_d \sim 10^{-10}$) [71,72]. Because of the sparse distribution of the FR in normal tissues and organs, many researchers use folate-mediated delivery to target cancer cells specifically [73]. Li *et al.* developed FA-PEG-chitosan oligosaccharide lactate (FA–PEG–COL) nanoparticles to specifically deliver HIF-1α siRNA to human ovarian endometrioid carcinoma OVK18#2 cells and Alexa Fluor 647 labeled siRNA–FA–PEG–COL in BALB/c xenograft mice OVK18 #2 tumor (via the tail vein). The FA grafting considerably simplified the uptake of nanoparticles through receptor-mediated endocytosis and HIF-1α knockdown at the molecular level resulted in efficient inhibition of proliferation *in vitro*. The *in vivo* accumulation efficiency of FA–PEG–COL nanoparticles was considerably improved than that of the passive targeting COL nanoparticles [74].

Epithelial ovarian cancer (EOC) cells express high levels of PD-L1, which interact with PD-L1 on T cells and leads to immunosuppression [75–77]. Teo *et al.* delivered PEI–PEG–FA/PD-L1 siRNA (PEG was used to increase stability in a serum-containing medium) into the SKOV-3-Luc EOC cells and evaluated the sensitization of the EOC cells to T cells. In SKOV-3-Luc cells, which were treated with PEI–PEG–FA/PD-L1, siRNA sensitivity was twice as much as in controls, who were treated with scrambled siRNA [78]. In spite of noteworthy achievements in treating hematological malignancies by chimeric antigen receptor T cell (CAR-T) therapy, several challenges for solid tumors need to be addressed. Among them, the poor homing ability of CAR-T cells and immunosuppressive microenvironment of solid tumors are major issues [79,80]. It is a probability that application of nanoparticle-siRNA may help to overcome immunosuppressive microenvironment and increase the efficacy of CAR-T cell therapy for solid tumors.

Luteinizing hormone-releasing hormone

LHRH receptor is overexpressed in ovarian cancer cells. Therefore, LHRH peptide is offering a strong option for development of innovative tumor-specific carriers [81,82]. Shah *et al.* constructed a drug-delivery system (DDS), which is composed of polypropylenimine (PPI) dendrimer modified by a-maleimide-w-*N*-hydroxysuccinimide ester PEG (MAL–PEG–NHS) and LHRH peptide (as a tumor moiety) to codeliver CD44 siRNA with paclitaxel. This DDS was used to transfer the agent into cells obtained from malignant ascites in patients with advanced ovarian carcinoma. Besides, it was intraperitoneally injected into mouse xenograft model of human ovarian carcinoma. High efficacy of cellular penetration, significant siRNA secretion from the complex inside the cells *in vitro* and a reduction in tumor burden along with adverse side effects (on healthy cells) *in vivo* were observed [83].

Follicle-stimulating hormone

FSH receptor (FSHR) is a specific receptor for ovarian cancer cells. By using FSH or some FSH-derived peptides (like FSH33, amino acids 33–53 of the FSH β chain), specific drug delivery can be achieved [84]. Growth-regulated oncogene α (gro-α) plays a central role in ovarian cancer progression so suppressing this gene with siRNA might be of value. For this reason, a structure was developed by Hong *et al.*, which consisted of FSH β 33–53 peptide

and then conjugated to gro-α siRNA. Given that the FSHR is exclusively expressed on ovarian cancer cells, an FSHR-mediated delivery system is used to mediate the specific delivery of siRNA to ovarian cancer cells [85].

Antibody

Antibodies are among the most commonly investigated types of specific ligand-coupled nanoparticle systems [86], which can be used for delivery of siRNA [87]. Palanca-Wessels *et al.* created an innovative nanocarrier system that consisted of: a cationic poly(dimethylaminoethyl methacrylate) block for binding siRNA; a terminal biotin to enable linkage to a streptavidin-conjugated monoclonal antibody; and a pH-responsive ampholyte block of poly(dimethylaminoethyl methacrylate), butylmethacrylate and propylacrylic acid (PAA) groups. Thenceforth, the efficiency of linking an internalizing streptavidin-conjugated HER2 antibody to an endosome-disruptive-biotinylated polymeric nanocarrier for increasing the functional cytoplasmic siRNA delivery in ovarian cancer cells *in vitro* and in an intraperitoneal ovarian cancer xenograft model *in vivo* was examined. An 80% decline in target mRNA and corresponding proteins with constant repression was reported. After treatment with HER2 antibody-directed siRNA nanocarriers, accumulation of Cy5.5 (fluorescently labeled siRNA) in intraperitoneal human ovarian tumor mass in xenograft mice was increased and 70% of the target gene was suppressed [88].

Conquering MDR in ovarian cancer

Regularly, drug-sensitive cancer cells are killed by anticancer drug-loaded nanocarriers delivered at tumor place. However, some cancer cells survive and regenerate in the heterogeneous environment of the tumor and take on MDR properties [89]. Nowadays, MDR makes a challenging problem in the fight against various cancers such as ovarian cancer [90]. In prior studies, it has been revealed that codelivery of anticancer drugs and siRNA is an effective strategy to overcome MDR [20]. Due to the selective silence of MDR-related genes, tumor cells are exterminated more easily by drugs. In addition, because of the synergistic effects of siRNA and drugs a lower dose is required and fewer side effects are observed [91,92]. There are several mechanisms for MDR and in this part, we will explain efflux pump resistance and nonefflux pump-resistance mechanisms and we will cover codelivery methods to overcome MDR.

MDR mechanisms

Efflux pump resistance

Overexpression of ATP-binding cassette (ABC) transporters appears to be the main factor for MDR [93]. ABC transporters are members of a transport system superfamily and utilize the energy of ATP to pump substrates like sugars, ions, lipids, proteins, sterols, peptides and drugs out of the cells [94,95]. P-gp; also known as ABCB1 or MDR-1 [96] and MRP-1, also known as ABCC1 [97] are the most known efflux pumps in the cell membrane [98,99]. P-gp has excretory and protective features in the normal human body. Tissues with overexpression of P-gp under normal circumstances can progress to chemotherapy-resistant cancers [100,101]. In contrast, it has been shown that overexpression of MRPs can lead to MDR in various non-P-gp MDR cell lines. MRP-1 preferably binds to anionic hydrophobic anticancer drugs (doxorubicin [DOX], daunorubicin and etoposide) and sends them out conjugated to glutathione and glucuronate [102,103].

Nonefflux pump resistance

Nonefflux pump resistance does not involve the accumulation of drugs in tumor cells. These resistant cancer cells employ several mechanisms, which act through proteins like MCL-1, BCL-2, Toll-like receptor 4, survivin, VEGF, etc. Nonpump resistance seems to protect tumor cells from anticancer drugs by altering the checkpoints in the cell cycle, activation of detoxifying systems, the escape from drug-induced apoptosis and impaired DNA repair [89]. Among the aforementioned mechanisms, researchers have extensively studied the proteins, which are associated with the antiapoptotic defense to overcome the cancer resistance. Bcl-2 is a well-known antiapoptotic protein that belongs to the Bcl-2 family and is encoded by the *Bcl-2* gene. Anticancer drugs such as DOX lead to the overexpression of *Bcl-2* gene and subsequently Bcl-2 protein [104–106]. An increase in Bcl-2 protein concentration ensues the formation of Bcl-2/Bcl-2 homodimer on the surface of mitochondria in greater amounts. This restricts the formation of apoptosomes in the mitochondrial external membrane, which in turn leads to the circumvention of the regular apoptotic pathway in tumor cells.

Table 2. Examples of codelivery of therapeutic gene and anticancer drug for ovarian cancer therapy.

Strategy	Delivery system	Targeted gene	Drug	Used cells	Used animal models	Ref.
Codelivery of drug and the siRNA targeting nonpump resistance	CS-PLNP	*p62*, *pβ5* (for expression enhancement)	Cisplatin	2008/C13 cells	-	[107]
	FA–PEG–PEI–PCL	*Bcl-2*	Doxorubicin	SKOV-3 cells	-	[104]
	PPI-MAL–PEG–NHS-LHRH	*CD44*	Paclitaxel	Isolated from advanced ovarian cancer patients	Mouse xenograft model of human ovarian carcinoma	[83]
	Cationic Au-Fe_3O_4	*Notch3*	Cisplatin	SKOV-3/DDP cells	-	[108]
	YSA-conjugated nanogel	*EGFR*	Docetaxel	Hey cells and SKOV-3	-	[109]
	PEI-coated MSN and YTZ3-15	*TWIST*	Cisplatin	A2780R cells	-	[110]
Codelivery of drug and the siRNA-targeting pump resistance	HA-PEI/HA-PEG	*MDR1*	Paclitaxel	-	Mouse xenograft model of human MDR ovarian cancer	[111]
Codelivery of drug and the siRNA-targeting both pump and nonpump resistance	HA-PEI/PEG	*PKM-2* and *MDR-1*	Paclitaxel	SKOV-3TR cells	Mouse xenograft models of paclitaxel-resistant tumor	[112]
	NCP-1	Survivin, Bcl-2 and P-glycoprotein	Cisplatin	ES-2, OVCAR-3 and SKOV-3	Cisplatin-resistant SKOV-3 subcutaneous xenograft mice	[113]

LHRH: Luteinizing hormone-releasing hormone; MDR: Multidrug resistance; MSN: Mesoporous silica nanoparticle.

Codelivery of siRNA & anticancer drugs to overcome MDR in ovarian cancer

In this section, we will describe prominent strategies regarding the codelivery of anticancer drugs and siRNAs. The classification is based upon different targeted MDRs concerning ovarian cancer therapy: codelivery of nonpump resistance siRNA and the drug, codelivery of pump resistance siRNA and the drug. Some studies benefiting from the codelivery of siRNAs and anticancer drugs for ovarian cancer therapy are summarized in Table 2.

Targeting pump resistance through codelivery of siRNA & anticancer drugs

As already have been described, MDR1 could be the Achilles heel of the pump resistance MDR mechanism and thus many studies have been conducted to silence this gene. Yang *et al.* created HA-based self-assembling nanoparticles (HA-PEI/HA-PEG), which target CD44 receptors overexpressed on MDR ovarian cancer cells. They delivered HA-PEI/HA-PEG/MDR1 siRNA with paclitaxel into a human xenograft MDR ovarian cancer model. As a consequence, the expression of MDR1 is downregulated. Therefore, the sensitivity of tumor cells to paclitaxel is enhanced [111,114].

In another study, Lingegowda *et al.* used a siRNA targeting the platinum resistance genes *ATP7A* and *ATP7B* in ovarian carcinoma. For *in vivo* delivery, they utilized neutral nanoliposome DOPC with incorporated siRNA to decrease the expression of ATP7B in 48 h. Tumor shrinkage, cancer cell apoptosis and proliferation reduction have been reported [115]. Therefore, targeting MDR1, ATP7aA and ATP7B have great potential to overcome pump resistance mechanisms and as a consequence, for development of new combinatorial approaches.

Targeting nonpump resistance through codelivery of siRNA & anticancer drugs

As previously mentioned, Bcl-2 plays a pivotal role in MDR. Many studies designed to overcome MDR are based upon silencing this gene. Zou *et al.* codelivered DOX and Bcl-2 siRNA with a folate-conjugated ternary copolymer consisted of PEG, PEI and poly(ϵ-caprolactone) (PCL) to SKOV-3 cells *in vitro*. Simultaneous downregulation in Bcl-2, as an antiapoptotic protein and upregulation of Bax, a proapoptotic protein, led to an increase in apoptosis rate [104].

The amplification of the Notch3 locus in ovarian high-grade serous carcinoma has been demonstrated by many types of research [116]. Notch3 signaling has a critical role in nonpump resistance ovarian cancer propagation via antiapoptotic regulations [117]. Thus, downregulation of Notch3 as a potential target for ovarian cancer therapy has been extensively studied [118,119].

Recently, to overcome the MDR caused by Notch3, codelivery of Notch3 siRNA and anticancer drugs has been studied as an innovative and efficient approach. Chen *et al.* applied VEGF RNA aptamer and Notch3 siRNA to make an innovative chimera delivery system mediated by cationic Au-Fe_3O_4 nanoparticles. They codelivered this

structure with cisplatin to SKOV-3 cell line and cisplatin-resistant SKOV-3/DDP cells. A significant suppression of Notch3 expression was observed. As a result, apoptosis and necrosis rates increased in cisplatin-resistant SKOV-3/DDP cells [108].

EGFR is mostly known for its prototypical angiogenic and metastatic properties. It is another protein related to nonpump resistance MDR, which prevents apoptosis by increasing the expression of Bcl-2 [120–122]. Codelivery of EGFR siRNA and anticancer drugs via its synergistic inhibitory effects on tumor growth and angiogenesis appears to be a promising strategy for the treatment of ovarian cancer [123–125]. Dickerson *et al.* used core/shell hydrogel nanoparticles (nanogels) functionalized with peptides (YSA), which particularly target the EphA2 receptor to deliver EGFR siRNAs (EGFR siRNA-loaded/YSA-conjugated nanogels) to SKOV-3 cells. A significant reduction in EGFR expression was noted. To estimate the effectiveness of EGFR siRNA-loaded/YSA-conjugated nanogels in intensifying cell line sensitivity to taxanes, HEY cells were incubated with EGFR siRNA-loaded nanogels. In order to allow for a maximum decline in EGFR expression, cells were treated with increasing concentrations of docetaxel. As an outcome, the docetaxel sensitivity was roughly eightfold greater in treated Hey cells than untreated controls [109].

TWIST and other genes involved in EMT tend to take part in cancer metastasis [126,127]. They also regulate chemoresistance and cancer cell stemness [128–130]. *TWIST* is responsible for the upregulation of *EMT* effectors including vimentin and *N*-cadherin and downregulation of E-cadherin [131]. Roberts *et al.* tested TWIST siRNA and two nanoparticle delivery platforms, in other words, YTZ3-15 third-generation dendrimer, which is polyamidoamine dendrimer and mesoporous silica nanoparticles (MSN) that electrostatically attached to PEI to create a positive charge, which intrinsically attracts negatively charged siRNA, in A2780R cells. They observed sustained TWIST knockdown with both nanoparticle delivery platforms. Nevertheless, PEI-coated MSN needed more incubation with cells to ensue knockdown as compared with YTZ3-15 but lasted longer. PEI-coated MSN-siTWIST plus cisplatin was intraperitoneally injected in mouse xenograft models of cisplatin-resistant tumor. Lower tumor burden was seen in mice treated with PEI-coated MSN-siTWIST plus cisplatin than mice treated with cisplatin alone [110].

As previously mentioned HA has a significant role in the specific delivery due to recognizing CD44 [132]. In the study which conducted recently has been revealed that codelivery of siTWIST-MSN-HA with cisplatin results in shrinking the tumor burden in both *in vitro* and *in vivo* and also higher delivery efficacy than other delivery methods [133].

Recent studies have been shown that a reduction in the expression of the β5 subunit of the proteasome and/or increased autophagy regulatory protein P62/SQSTM1 (P62) expression is associated with MDR [134–136]. Babu *et al.* designed an innovative multifunctional nanoparticle structure to codeliver p62siRNA, β5 plasmid DNA and the cancer drug cisplatin to 2008/C13 ovarian cancer cells that are resistant to cisplatin. Multifunctional nanoparticle contains two layers including the inner and outer layers, which are respectively comprised of cisplatin-loaded polylactic acid nanoparticle and cationic CS. The outer layer is ionically connected to β5-expressing plasmid DNA (pβ5) and/or P62 siRNA (siP62). Considerable reduction in the resistance to cisplatin was observed following codelivery of siP62, pβ5 and cisplatin compared with the delivery of either siP62 or pβ5 alone [107].

Targeting both pump & nonpump resistance through codelivery of siRNA & anticancer drugs

Codelivery of siRNA, targeting mechanisms of both pump and nonpump resistance, with anticancer drug impedes the antiapoptotic defense of cells and bypasses drug efflux pump and as a result, increases drug sensitivity outlined in Figure 1 [137–140]. Most of the time, the strategies targeting merely a single factor contributing to drug resistance are not sufficient to circumvent MDR [141,142] due to simultaneous activation of both pump and nonpump resistances in the heterogeneous tumors.

To surmount the aforementioned challenge, Talekar *et al.* encapsulated siRNA duplexes against PKM2 (siPKM-2, glycolysis protein-silencing siRNA) and siMDR-1 (drug efflux pump-silencing siRNA) in HA-based self-assembling nanoparticles (HA-PEI/PEG). They transfected SKOV-3TR cells with siRNA-loaded NPs and reported downregulation in MDR1 and PKM2. Afterward, paclitaxel with siRNA-loaded NPs was intravenously injected in a mouse xenograft model of the paclitaxel-resistant tumor. A considerable tumor shrinkage by nanoparticles and hindrance in tumor volume doubling time were noted ($p < 0.05$) with combination therapy in both the nonresistant type (twofold) and resistant (eightfold) xenograft models [112].

He *et al.* developed a nanoscale coordination polymers composed of cisplatin prodrug, *cis,cis,trans*-[$Pt(NH_3)2Cl_2(OCONHP(O)\ (OH)_2)_2$] coated with DOTAP, cholesterol and DSPEP-EG2k to transfer pooled

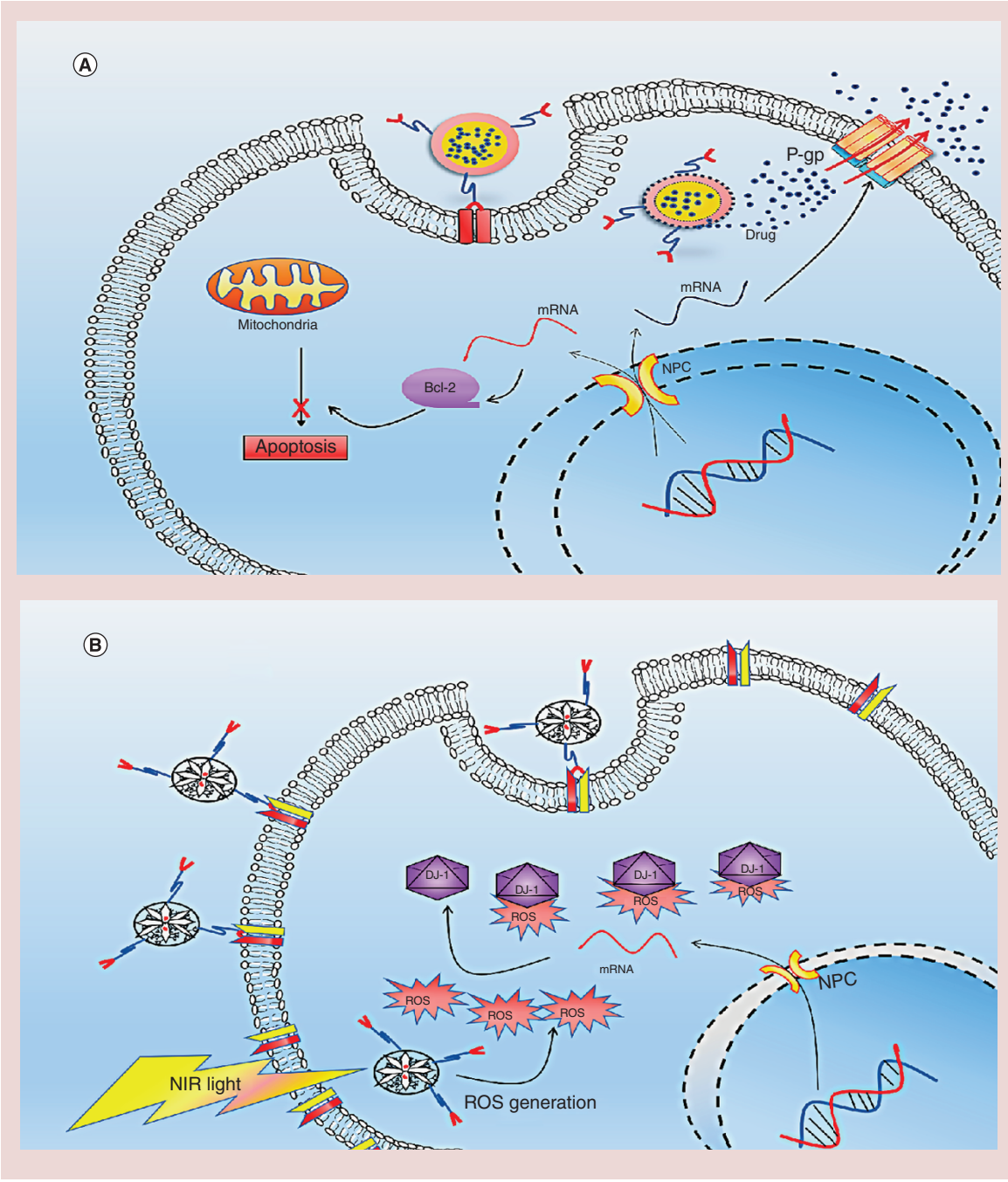

Figure 1. Codelivery of nanocarrier-mediated siRNA targeting both pump and nonpump resistance with an anticancer drug. (A) Simultaneous activation of both pump and nonpump resistance mechanisms in cancer cells. **(B)** Codelivery of siRNA targeting both pump and nonpump resistance with drug leads to silencing *MDR* genes (like *MDR1* and *Bcl-2*) and as a consequence, the drug can induce cell apoptosis.

siRNAs (siP-gp, sisurvivin and siBcl-2) in SKOV-3, OVCAR-3 and ES-2 cells *in vitro*. The agent was intratumorally injected into mouse xenograft model of human ovarian carcinoma. Cellular uptake of siRNA and cisplatin and overall efficiency of chemotherapy increased. Meanwhile, endosomal escape ability was provided *in vitro*. Also, reduction of tumor burden in cisplatin-resistant SKOV-3 subcutaneous xenografts was reported [113].

Advantages of nano-siRNA in other therapeutic methods

In recent years, we have seen an increase in the number of studies that use nanoparticle-loaded siRNA modalities for cancer treatment such as PDT and hyperthermia therapy. Nanoparticle-loaded siRNA is used for various purposes like silencing-resistant genes and increasing the efficacy of treatment methods. In this part, the role of nanoparticle-mediated siRNA delivery in PDT and hyperthermia therapy will be discussed.

Nano-siRNA in photodynamic therapy

PDT, alternatively called photochemotherapy, is composed of a photosensitizer and a light emitter [143]. The photosensitizer embedded in tumor cells generates reactive oxygen species in response to light exposure at a specific wavelength [144,145]. Owing to its ability to focus the light on tumor cells, PDT has a minimum side effect profile. Dj-1 protein is an antiapoptotic agent in cancer cells, which has a critical role in the antiapoptotic pathway [146]. Use of nanoparticle-mediated siRNA delivery can be an effective approach to address this issue as shown in Figure 2. Schumann *et al.* developed dendrimer nanoplatform functionalized with PEG and LHRH peptide (PPI) to deliver near-infrared photosensitizer (phthalocyanine) and Dj-1 siRNA into A2780/AD cells *in vitro* and then PPI-Pc, PPI-Dj-1siRNA were intravenously injected to mouse xenograft model of A2780/AD cells. Combinational therapy proved to be more efficient than PDT alone *in vitro*. Furthermore, complete tumor eradication was observed in the mice with ovarian cancer, which were treated with a single dose of combinational therapy [147].

Nano-siRNA in hyperthermia therapy

Hyperthermia therapy (thermal therapy or thermotherapy) is an important way in the treatment of cancer [148]. This type of treatment utilizes heat to eliminate cancer cells and is capable of inducing antitumor immunity [149]. However, there are still some challenges like thermo-resistance (thermotolerance) [150]. To wind up, Hatakeyama *et al.* delivered CTGF (a key factor in hypothermia resistance) siRNA-DOPC nanoliposome to xenograft HTR SKOV-3 and HeyA8, mice. And then PEG-CuS NPs were intravenously injected. Due to CTGF underexpression and hyperthermia, tumor burden was decreased in the HeyA8 model. In addition, local hyperthermia and CTGF silencing led to decreased metastasis rate and tumor burden in HTR SKOV-3 tumors [151]. Moreover, it is evident that hyperthermia can improve drug delivery [152,153]. Thus, the simultaneous application of hyperthermia and nano-siRNA delivery can be a promising option for ovarian cancer therapy.

Imaging & evaluation of siRNA

The incapacity to trace and identify biodistribution and expression of the nanotherapeutics in the target tissue is one of the challenging obstacles in clinical trials of ovarian cancer [154]. Even though biopsy or autopsy can provide data on delivery and effects of RNAi-mediated therapy, these methods are invasive and are not commonly used for evaluation of nanoparticle-based delivery systems. Accordingly, recent modalities are evermore focused on noninvasive methods such as imaging in gene therapy. In consequence, different imaging technologies have been developed and applied in RNAi-mediated therapy. In light of what has been just mentioned, both nanoparticles or siRNAs can be tracked and evaluated regarding *in vitro* and *in vivo* effect by optical (fluorescence, nonfluorescence) imaging, nuclear imaging (positron emission tomography/single photon emission-computed tomography) and MRI [155,156].

Lin *et al.* used facial layer-by-layer engineered upconversion nanoparticles (UCNPs) for delivery and tracking of siRNA. They assembled the PAA layer negatively charged, PEI layer positively charged and siRNAs-targeting MDR1, respectively, on the surface of UCNPs. UCNPs with the feature of intrinsic photon upconversion are unique particles for following the attachment and detachment of the siRNA. Emission of color lights is induced by near-infrared-initiated fluorescence resonance energy transfer (FRET), which is shaped by the UCNPs as the donor and fluorescence dye-labeled siRNA as the acceptor. In case of attachment of siRNA to UCNP, red light is emitted through FRET and if the siRNA detach the UCNP, green light is emitted through non-FRET. They delivered UCNP/PAA/PEI/MDR1-siRNA to the OVCAR8 cells and observed the increased efficacy of the following chemotherapy with paclitaxel [157].

Conclusion

The application of RNAi-based therapies especially for cancer has been sharply accelerated because of recent advances made in DDSs. In spite of the longtime availability of conventional chemotherapy, nonspecific toxicity and drug-resistance are challenges yet to be addressed. A promising strategy to tackle drug-resistance is the use

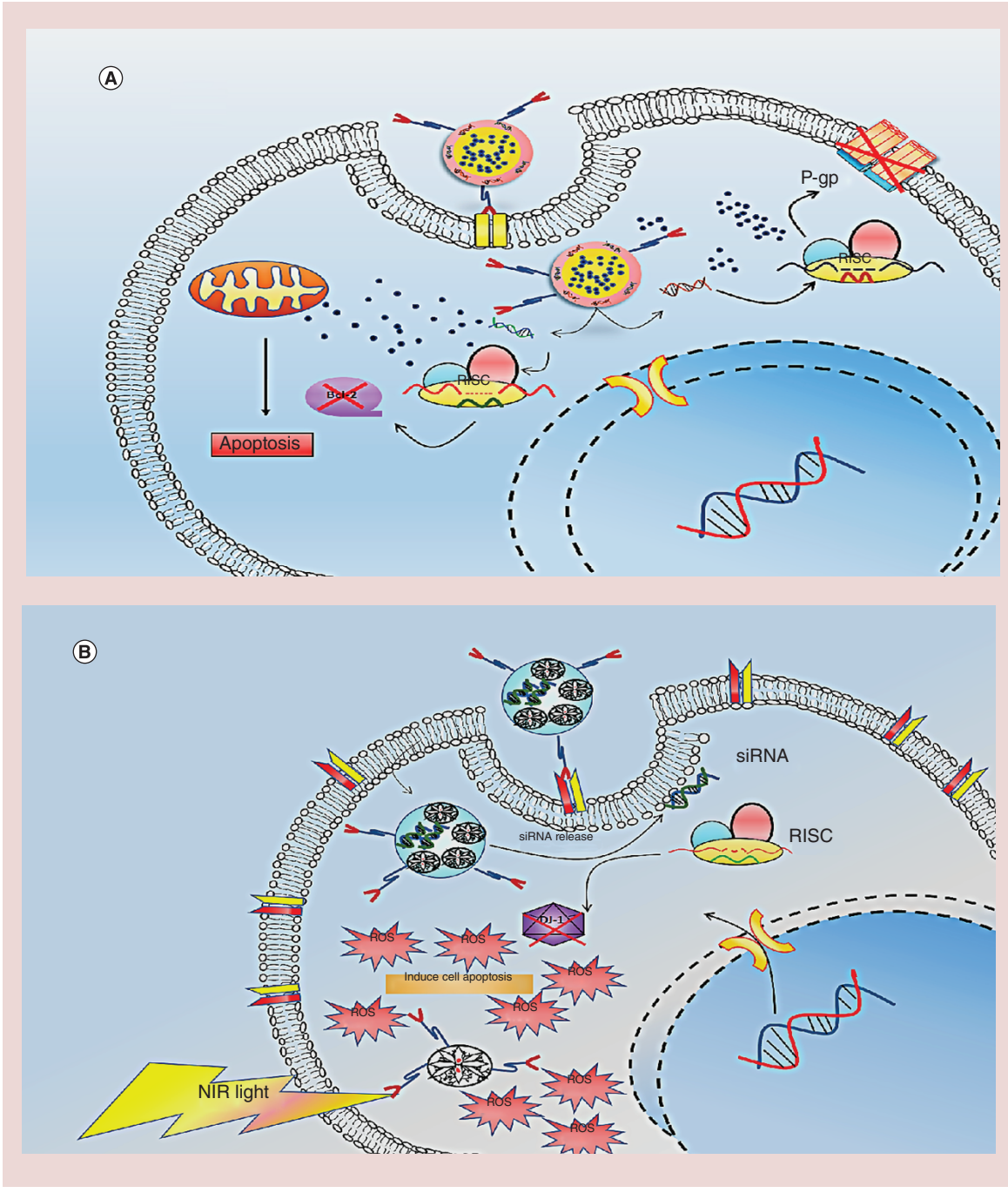

Figure 2. Nanoparticle-mediated delivery of siRNA to overcome the photodynamic therapy resistance. (A) Dj-1 protein inactivates ROS, which is generated by an exposed photosensitizer and has antiapoptotic features. **(B)** siRNA that is delivered by nanoparticle causes degradation of Dj-1 mRNA by RISC complex and subsequently, ROS induces cell apoptosis.
RISC: RNA-induced silencing complex; ROS: Reactive oxygen species.

of siRNA to directly silence MDR-relevant genes or oncogenes. Recently, a combination of anticancer drugs and siRNA has appeared as a powerful strategy to address MDR. The combination therapy has strong synergistic effects on ovarian tumor cells. The application of siRNA to knockdown the genes related to MDR leads to the evasion of the efflux pump by anticancer drugs, as well as efficiently initiates apoptosis. This can increase the tumor-killing ability of anticancer drugs. In addition, a decreased nonspecific toxicity can be achieved by means of encapsulating

multiple payloads of drugs with nanocarriers. The emergence of new experimental techniques including noninvasive imaging methods to monitor siRNA delivery has been a major contributor.

Altogether, nanoparticle-mediated siRNA delivery as a revolutionary strategy can be used for MDR overcoming as a result, cancer treatment.

Future perspective

Despite the mentioned improvements, overstimulation of immune system and toxicity are limiting factors for application of this method. Moreover, signaling pathways of RNAi and target molecules are not totally identified. We believe that future works should concentrate on designing and optimizing of novel carrier systems to address these challenges. Recently, black phosphorus has been designed and used for efficient gene delivery and it can be expected that further works will follow in the footsteps of this pioneering study in the foreseeable future. It is noteworthy that no clinical trial has hitherto been conducted regarding this type of treatment for ovarian cancer. This warrants the critical need for clinical trials to be carried out to evaluate the efficacy of this method.

Executive summary

Background

- Ovarian cancer is one of the most aggressive forms of cancer now without any effective treatment.
- Multidrug resistance and efficient delivery of chemotherapy drugs are the most challenging problems in the treatment of ovarian cancer.

Lipid-based delivery of siRNA

- Lipid components have great potential to carry siRNA to the site of target cells because siRNA is not stable in blood and cannot cross the membranes alone.
- Some polymers like poly(ethylene glycol) and poly(lactic-co-glycolic acid) can be conjugated to lipid nanoparticles to increase stability and decrease the toxicity.

Polymer-based siRNA delivery

- Synthetic polymers are among the best carriers for siRNA delivery due to their well-defined and multivalent structures, proper molecular architecture and nanosized volume.

Nanoparticle coupled to specific ligand systems

- To overcome the extracellular barriers, receptor-mediated endocytosis is one of the most important strategies to enhance the cellular uptake.
- Anisamide, hyaluronic acid, antibody, luteinizing hormone-releasing hormone, follicle-stimulating hormone, folic acid, arginine and arginyl-glycyl-aspartic acid are known as specific ligands for siRNA delivery to the tumor cells.

Targeting both pump & nonpump resistance through codelivery of siRNA & anticancer drugs

- Targeting both pump and nonpump resistance via codelivery of siRNA and chemotherapy drugs is a strong strategy for sensitizing tumor cells and as a result cancer treatment.

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Genome Editing Using mRNA-Lipid Nanoparticles for CAR-T Cell Therapy

The development of genome editing technologies has provided scientists with ways to directly target and modify the genomic sequences of a living organism. It has extended our understanding of the genetics behind human disease by enabling the creation of more accurate cellular and animal models. It has extraordinary potential across a variety of fields from basic research to applied biotechnology and biomedical research[1]. However, delivery of gene editing tools to target cells has been a challenge. New technologies for safe and efficient gene delivery that can overcome payload limitations will enable researchers to realize the full potential of gene editing strategies to explore new avenues for cancer treatment and beyond.

The basis of gene editing relies on initiating a double-strand break (DSB) at a chromosomal site of interest to trigger one of two endogenous cellular DNA repair pathways: nonhomologous end joining (NHEJ) or homology directed repair (HDR) resulting in gene disruptions or targeted integration, respectively. Up until 2013, engineered nucleases, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) were the dominant technologies used for gene editing. However, long development times coupled with their relatively low editing efficiency limited the speed of progress in the field.

In 2013, the discovery of clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-associated nucleases derived from a bacterial adaptive immune defense system transformed the field of genome editing. The CRISPR/Cas9 system uses short guide RNA (sgRNA) to direct Cas9-mediated cleavage and insertion of a donor HDR template. The simplicity, versatility, and highly tunable nature of RNA design to retarget Cas9 provides significant advantages over ZFNs and TALENS and has ushered in a new era of genomic engineering. The gene editing field continues to innovate and evolve rapidly, with alternative or engineered CRISPR nucleases (i.e., Cas12 and dead Cas9) and other modalities, such as base and prime editing, being investigated to increase efficiencies and reduce off-target effects.

Gene engineering of T cells to produce new cancer immunotherapies like chimeric antigen receptor (CAR)-T cell therapy, have revolutionized cancer treatment. Current autologous CAR-T immunotherapies have demonstrated high clinical success, but there is a need to improve safety and efficacy profiles. Next generation CAR-T designs focus on enhancing CAR-T cell potency, limiting off-target effects, broadening the therapeutic targets beyond liquid cancers, and manufacturing universal CAR-T cells from allogeneic donors[1]. These new strategies require more complex CRISPR/Cas9-enabled genetic engineering strategies where the chosen gene delivery method plays a central role in determining the gene editing efficiency, safety, and scalability.

DELIVERY MECHANISMS FOR GENE EDITING TOOLS

Therapies based on genome editing of T cells can be divided into *in vivo* and *ex vivo* methods (Figure 1). In *in vivo* therapies, gene editing machinery is directly inserted into the body. On the other hand, in *ex vivo* therapies the target cells are isolated, genetically engineered and reinfused back into the patient. While viral vectors have been used clinically for T cell engineering with high efficiency, the method suffers from several drawbacks, such as high cost, potential for insertional mutagenesis, and off-target effects that pose safety concerns for patients[2]. Non-viral modes of delivery have emerged as a alternative to viral vectors in terms of their safety, simplicity, and flexibility.

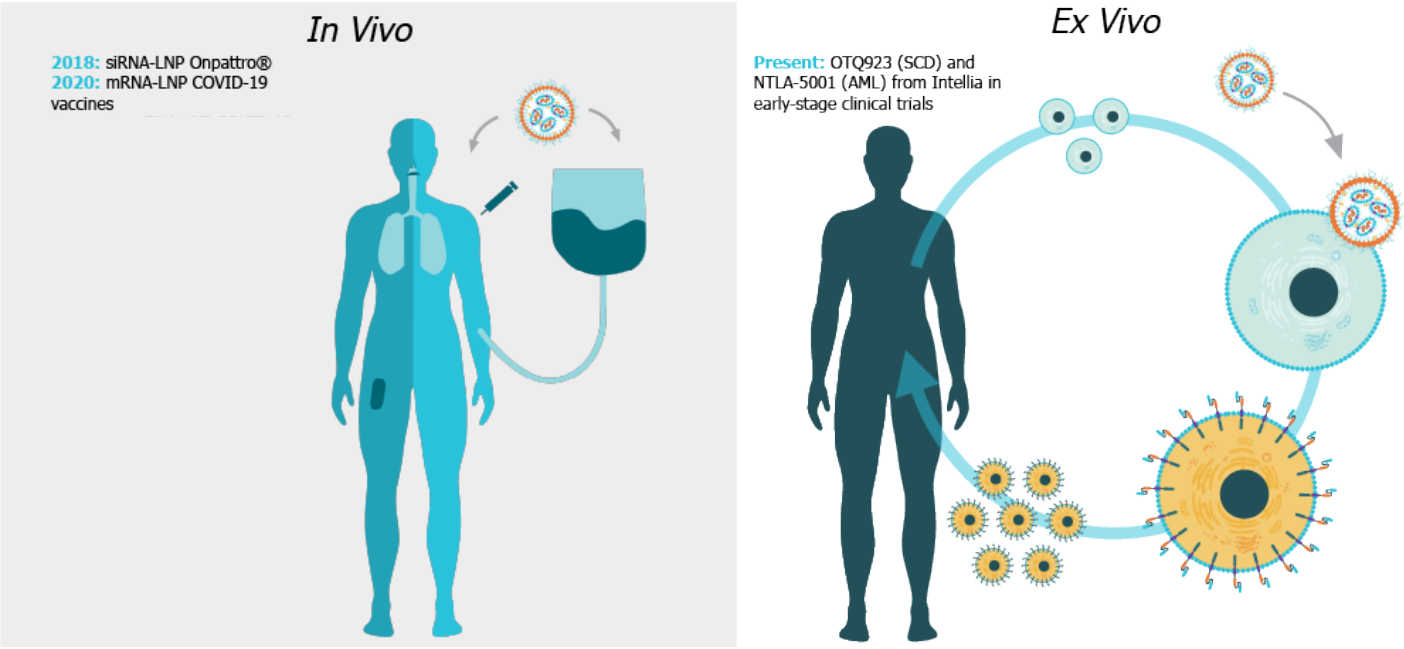

Figure 1. *A schematic depiction of in vivo and ex vivo gene editing is shown. For in vivo methods, gene editing machinery is directly injected into the body. For ex vivo gene editing, the target cells are isolated prior to genetic manipulation and subsequent reinfusion into the patient.*

One of the long-standing non-viral methods for the *ex vivo* delivery is electroporation. This technique utilizes pulsed high-voltage electrical currents to transiently open nanometer-sized channels in the cell membrane to deliver nucleic acids into the cell. For CRISPR/Cas9, sequential electrical pulses are required to separately deliver the Cas9 mRNA and the sgRNA. This results in a dramatic trade-off between efficiency and cell viability, which poses barriers to using electroporation for sequential or multiplex genetic manipulations.

This trade-off is not observed with lipid nanoparticles (LNPs), making it an attractive alternative for effective RNA delivery over electroporation. LNPs are entirely synthetic lipid formulations designed to encapsulate and protect RNA from nuclease degradation before delivering it into

target cells. The RNA-LNP complex structurally resembles low density lipoproteins (LDL) and can use the endogenous uptake pathway of LDL to enter the target cells via receptor-mediated endocytosis. This gentle uptake mechanism enables successful and highly efficient genome engineering of T cells compared to electroporation with more uniform gene expression, higher transfection efficiency, and viable cell yield. In fact, Intellia Therapeutics, a leading clinical-stage genome editing company, released data showing that LNPs effectively replaces electroporation for delivery of CRISPR/Cas9 gene edits to T cells in their allogeneic TCR-T and CAR-T program. LNPs have been shown to lower the risk of chromosomal translocations observed with multiplexed editing, as well as the negative effect of electroporation on T cell health.

Precision NanoSystems has leveraged their deep knowledge of LNP chemistry and cell biology to design a T cell-specific LNP composition for gene editing. Because the LNP characteristics are sensitive to manufacturing conditions, precise and reproducible control of the manufacturing process is required to ensure consistent particle production. The NanoAssemblr instruments paired with specialized reagent kits like the GenVoy-ILM™ T Cell Kit for mRNA (available for the NanoAssemblr Spark™ and Ignite™ instruments) provides researchers with the tools needed to establish a robust and scalable method for *ex vivo* gene delivery within their own labs. mRNA-LNPs can be easily and seamlessly integrated into a standard primary T cell culture workflow to facilitate the production of next generation CAR-T cell therapies from concept to clinical practice.

LNPS SUPPORT NEXT-GENERATION GENE-EDITED CAR-T CELLS THERAPIES

The effectiveness and versatility of LNPs using the GenVoy-ILM T Cell Kit for mRNA for both autologous and allogeneic CAR-T cell therapy development is highlighted in several proof-of-concept experiments (Figure 2)[3].

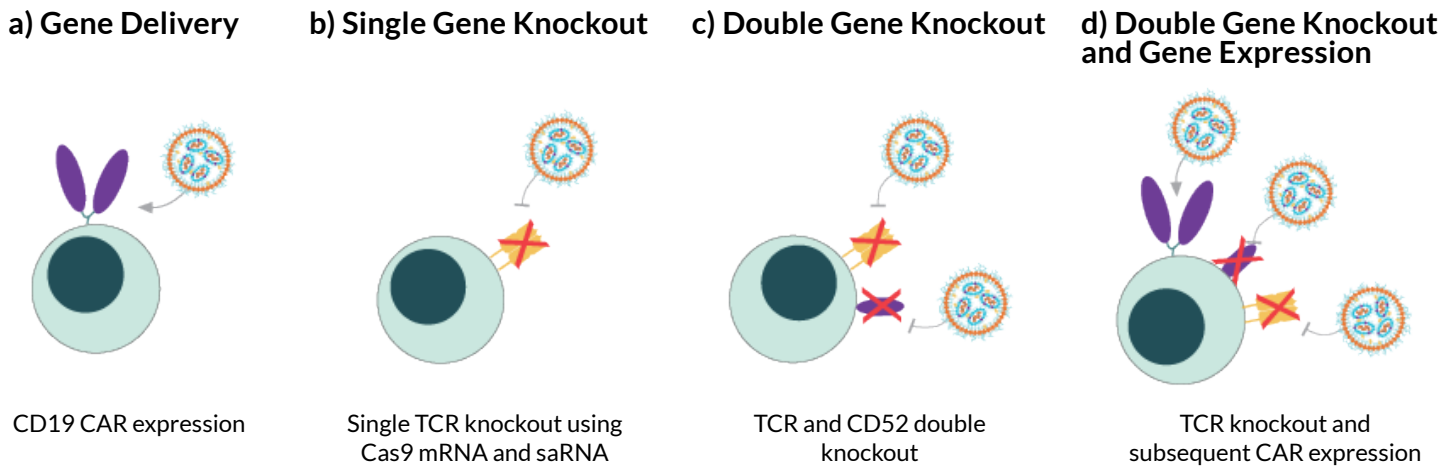

Figure 2. *LNPs can be easily integrated into ex vivo CAR-T cell gene engineering strategies, including gene expression (a), single gene knockout (b), double gene knockout (c), and multi-step gene knockout and expression (d).*

Autologous Cell Therapies

In experiments comparing the delivery of anti-CD19 CAR mRNA into T cells via LNPs and electroporation, the transfection efficiency (TE) was found to be significantly higher than electroporation. Increased cell viability and more homogenous CAR expression was also observed—important parameters that positively influence the final cell yield of the CAR-T product[3]. Studies have also shown that LNP-mediated generation of CD19 CAR-T cells demonstrated equivalent tumor killing potency compared to electroporation and/or lentiviral transfection[4,5].

Allogeneic Cell Therapies

Allogeneic CAR-T cell products generated from healthy donor cells have the potential to overcome the well-documented shortcomings associated with autologous therapies, but challenges including the risk of host allogeneic rejection and graft-versus-host-disease (GVHD) must be addressed. CRISPR/Cas9 gene editing using LNPs as the delivery vehicle supports single gene knock out (KO) of native αβ TCR and double KO of both the TCR and the T cell marker CD52, to allow antibody-mediated (anti-CD52) lymphodepletion. KO of CD52, a T cell marker, allows for antibody treatment of the patient (anti-CD52) to enhance lymphodepletion without affecting infused allogeneic CAR T cells to reduce both alloreactivity and GVHD. As well, multi-step engineering for gene KO followed by CAR transgene expression executed using LNP-mediate CRISPR/Cas9 successfully produces highly functional TCR–CD19 CAR-T cells that demonstrate effective *in vitro* tumor cell killing[3].

The demonstrated ease of LNP-mediated gene delivery across a variety of genetic modification scenarios offers researchers to accelerate the development of next-generation CAR-T cell therapies.

FINAL REMARKS

Gene delivery platforms need to support a diversity of genetic strategies for the development of new genomic medicines. Workflows that require multiplex or sequential genetic manipulations are becoming more commonplace as the field turns its focus on tackling new disease targets and work on allogeneic approaches to create “off-the-shelf” products to serve a greater patient population. LNPs are being utilized in clinical evaluation in several gene editing programs. Intellia Therapeutics is rapidly advancing both *in vivo* and *ex vivo* products in their clinical pipeline based on their LNP CRISPR system. Both OTQ923/HIX763 focused on *ex vivo* gene editing of hematopoietic stem cells to treat sickle cell disease and their lead *in vivo* genome editing candidate to treat transthyretin (ATTR) amyloidosis NTLA-2001 have reported promising preliminary clinical results.

LNPs are a clinically validated and scalable technology used in the formulation of the FDA- approved COVID-19 mRNA vaccines and ONPATTRO® (patisiran), the first small interfering RNA-based drug. This delivery technology has been shown to be effective, gently, and scalable for gene delivery and editing applications to help accelerate T cell therapy research and drug development in a rapidly evolving clinical landscape.

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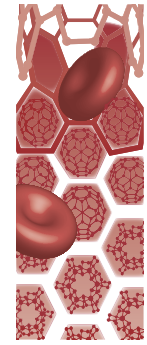

Nanotherapeutic systems for delivering cancer vaccines: recent advances

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With an increase in the global burden of cancer-related deaths, the quest for developing new therapeutic solutions has taken momentum. In this regard, the idea of using cancer vaccines came to existence approximately 30 years ago, where gene therapy interventions have shown significant improvement in the therapeutic outcomes against several types of cancers. Cancer vaccines usually encounter a number of challenges with limited targeting ability to the tumors. Nanocarriers have been studied as a technological innovation for tumor targeting of gene therapeutics. This article provides a critical insight into the recent progress made in nanotherapeutic strategies for genetic vaccine delivery for treatment against various types of cancers. Moreover, the article intends to provide a summary of the research work being done on this topic.

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Cancer has been a significant cause of mortality for the past few decades. However, developing an effective therapeutic strategy for cancer treatment is highly challenging [1]. It primarily depends on understanding the etiopathology of cancers and associated mechanisms for the metastasis of cancer cells. After cardiovascular diseases, it is considered as the second most common cause of death in the USA and across the globe. According to the 2015 WHO report, 7.6 million deaths out of 58 million worldwide were caused by cancer alone [1]. The cancer-related death toll is projected to rise by 10 million cases per year, reaching 15.4 million cases in 2030. The growing number of deaths due to cancer creates extreme pressure on healthcare systems, as well as on biopharmaceutical industries to develop new and effective therapeutic solutions against cancer [2].

Surgery, radiation therapy and chemotherapy have been used for many years but have shown minimal effectiveness for reducing cancer mortality. Such therapies are only effective against tumors of different organs and organ systems of the body [2]. If tumor cells have spread by metastasis from one part of the body to another, these therapies are less effective. Chemotherapy treatments for advanced cancers like breast, lung, colorectal, prostate and pancreatic cancers can sometimes be considered as palliative therapies. The rapidly increasing number of deaths, however, has considerably triggered the development of cancer vaccines, with the aim to prevent repetitive treatment regimens and instead deliver an ultimate solution to patient [3]. Research has demonstrated that cancer vaccines can be more effective than conventional chemotherapy-based treatment strategies, as these acts on the immune system of the patient in order to avoid metastasis of the cancer cells [3].

Future Medicine

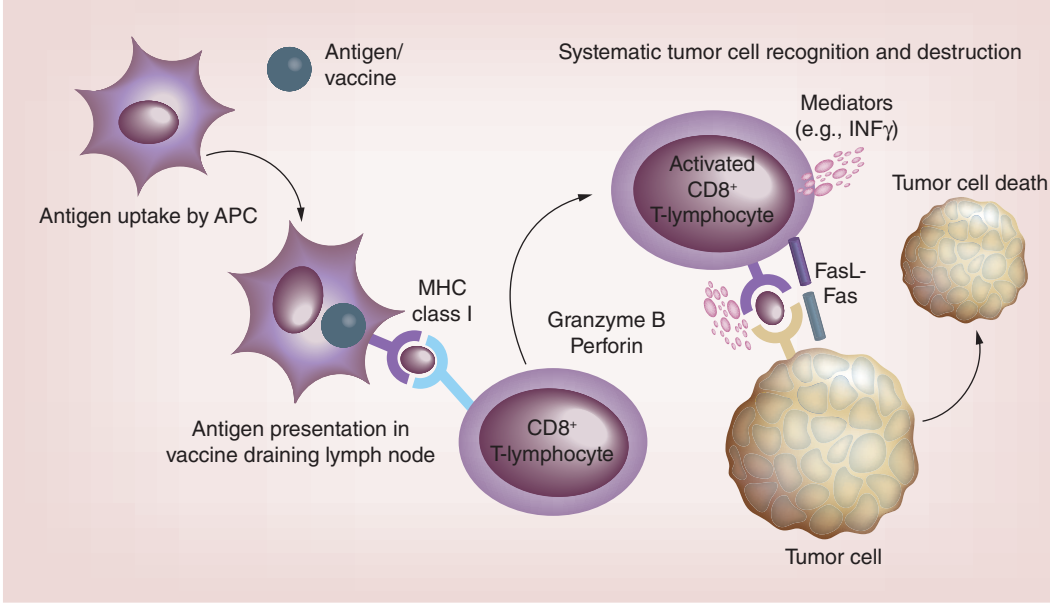

Figure 1. Proposed mechanism of action for cancer vaccines. Tumor antigens (e.g., administered as proteins, peptides or whole tumor cells) are taken up and processed by specialized APCs such as dendritic cells. Dendritic cells migrate to the vaccine-draining lymph nodes and present relevant antigens to $CD8^+$ T lymphocytes, which, in turn, are able to recognize tumor cells throughout the body and destroy them by several effector mechanisms such as the perforin/granzyme pathway, direct cell–cell interaction (e.g., Fas/Fas ligand) or certain mediators (e.g., INFγ). Not shown but also of importance are B lymphocytes, $CD4^+$ T helper cells and cells of the innate immune system such as natural killer cells and macrophages.
APC: Antigen-presenting cell.
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Cancer vaccines & their applications

Cancer vaccines are used to stimulate the tumor-specific immune response, providing defensive protection by activating the adaptive immune system to slowly destroy the cancer cells. This technique ensures that cancer cells of various organs or organ systems in the body have limited chances for forming recurrent tumors [4–6]. This is attributed to the body's innate ability to identify the tumor cells in the body directly through an immune recognition mechanism. The antigens act as immune system modulators or rejuvenators to prepare the body for the fight against the cancer cells. Figure 1 depicts the mechanistic pathways involved in inducing the immunization by the cancer vaccines in the human body for fighting against cancer and its recurrence.

Smart carriers in cancer vaccine delivery

Among the different domains of drug delivery and biomedical research, the use of nanotechnology has been extensively studied. Nanosystems are made of miniaturized devices with a diameter of less than 1 μm [7]. Often known to be 100–10,000-times smaller than the size of a mammalian cell, these nanosized particles are considered to be highly useful for versatile drug-delivery applications [8]. Due to their tunable material properties, high aspect ratio and surface functionalization properties, nanostructured systems have shown their worth in treating several human ailments [9]. In this context, nanostructured devices have been rigorously tested for their function in the treatment of diseases such as cancer [10]. Nanocarriers can be utilized to deliver antigenic components for the induction of immunization in order to protect the human body against cancers.

Nanocarriers are helpful in overcoming the challenges associated with the delivery of cancer vaccines [11]. Such nanocarriers are primarily constituted of a blend of lipidic and polymeric excipients [12]. Examples of some of the very widely investigated nanocarriers include liposomes, nanoparticles and nanoplexes that have demonstrated excellence in delivering cancer vaccines. Some reports have indicated that nanocarriers can be used for localized delivery of cancer vaccines, with the help of targeting ligands. In addition, multifunctional nanosystems are also reported to be very helpful for site-specific delivery of the gene cargos [12]. Figure 2 shows select instances of the nanotherapeutic carriers investigated for cancer vaccine delivery.

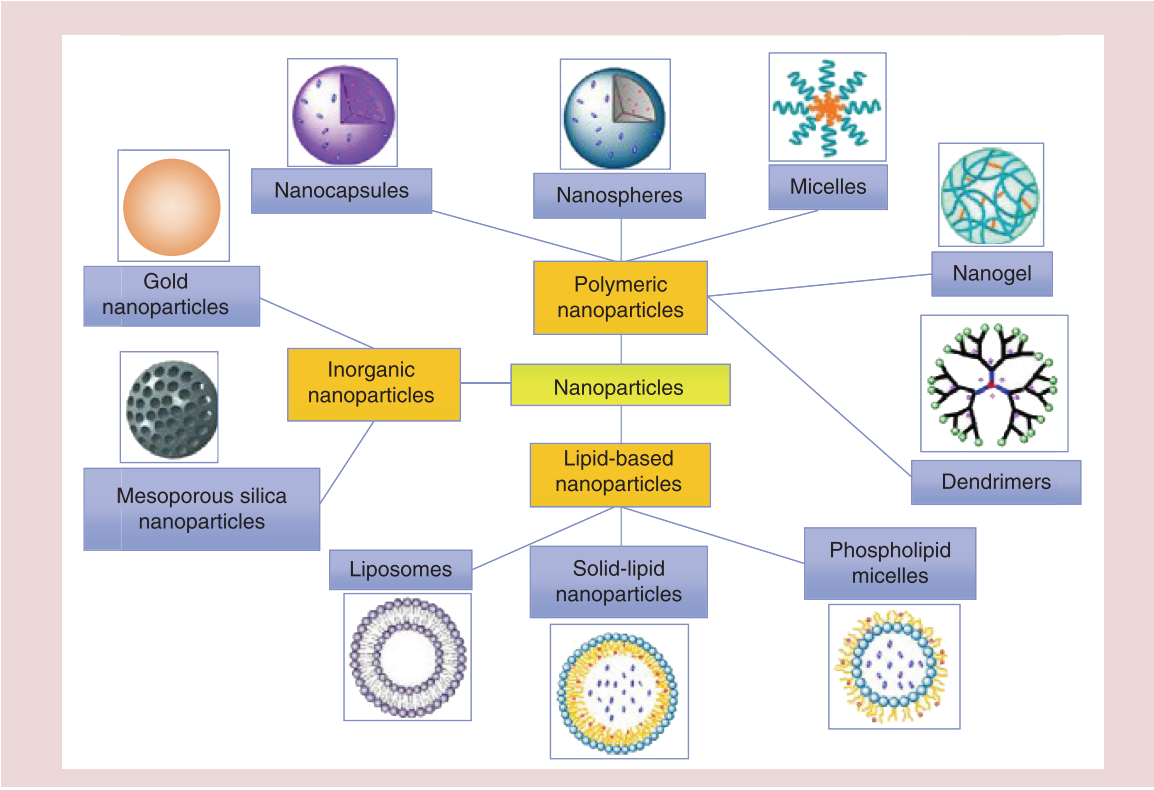

Figure 2. Pictorial depiction of various nanostructured vehicles used for cancer vaccine delivery.
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Nanoparticles

Polymeric nanoparticles have been used for many decades for drug delivery and biomedical applications. These are spherical particles (10–500 nm in size) with hollow or solid cores that can hold genetic materials. Usually, they are composed of natural, semisynthetic or synthetic polymers with biocompatible and biodegradable properties. They can accommodate a wide range of therapeutic products and deliver them to any site of action in the human body [13]. Previous studies have demonstrated that cancer vaccines in nanoparticulate systems can induce strong antitumor activities by potentiating immunotherapeutic proteins such as modified cytokines and monoclonal antibodies [14].

Poly(D,L-lactic-*co*-glycolic) acid (PLGA) and poly(lactic acid) (PLA) are the most widely used polymers approved by the US FDA for delivering therapeutic biomolecules [15]. Nanoparticles prepared using these polymers require critical control of the formulation and process parameters for obtaining desired particle size, ζ potential, entrapment efficiency and drug release characteristics [15]. PLGA/PLA-based nanoparticles are nontoxic and nonimmunogenic in nature for delivery of vaccine adjuvants and possess good transfection efficiency for internalization into the immune triggering cells of the body [15,16]. It has been reported that nanoparticles with size 1–100 nm can induce the cellular response employing MHC-I antigen expression [15]. However, the exact mechanism behind the cellular immune response by which nanoparticles internalized into the target cells for transfection is unknown [16].

Besides the aforementioned polymers, literature reports have demonstrated the applications of antigenic proteins and peptides derived from the natural sources for preparing the polymeric nanocarriers for immunization against specific types of cancers [17,18]. In a recent study, Hamdy *et al.* observed the effective delivery of TAA peptide and TLR-4 ligand using PLGA nanoparticles. These peptide-functionalized nanocarriers showed activity against the cytotoxic T lymphocytes to inhibit tumor growth in mice [19]. Table 1 summarizes the select instances of polymeric nanoparticles use in cancer vaccine delivery.

Poly(butylcyanoacrylate)-based nanocarriers have shown high acceptability for applications in tumor immunotherapy [20]. Chesson and Zloza tested the use of such nanoparticles for active immunization against human glioblastoma cells by improving the treatment efficacy for dual delivery of doxorubicin and anti-TGF-β genes. The study demonstrated a high death rate for cancer cells due to the activation of $CD25^+$ T cells [21].

Table 1. Some important examples of nanomedicine-loaded cancer vaccine for biomedical application.

Nanocarrier-loaded vaccines	Outcomes	Ref.
TAA peptide (TRP-2180-188) and TLR-4 ligand (7-acyl lipid A)-loaded PLGA nanoparticles	These peptide functional nanocarriers influence cytotoxic T lymphocytes to inhibit the growth of tumors in mouse	[19]
Encapsulating oval albumin liposomes	This found a safe antitumor response to B16 melanoma cell expression	[26]
CpG codelivery vaccine and gastric cancer antigens MG7 by nanomedicine	The payload by high-vacuum shear ultrasound emulsifier led to 70 and 93% while the mouse resistant to MG7 and CpG nanoemulsion resulted in a strong cell growth inhibition	[30]
Diphtheria-loaded nanobilosomes	It achieved systemic immune reaction by releasing of hepatitis surface antigen to lymphoid tissues	[36]
Tetanus-loaded PLGA microsphere	It achieved greater antigen loading and enhanced immune response	[41]
Hepatitis B surface antigen-loaded chitosan microspheres	It found mucosal antibody titres and subsequently increased after peroral immunization	[42]
Peptide-based malaria vaccine (SPf66)-loaded IRIVs	The intramuscular injection provokes the immune response in BALB/c mice, which revealed higher efficacy of IRIV-based vaccine over conventional formulation	[52]
Plasmodium falciparum GLURP-MSP3 chimeric protein-loaded virosomal formulation	The said formulation augmented the immune response against malaria and improves stability and efficacy	[53]

DC: Dendritic cell; IRIV: Immunopotentiating reconstituted influenza virosomal; PLGA: Poly(D,L-lactic-*co*-glycolic) acid.

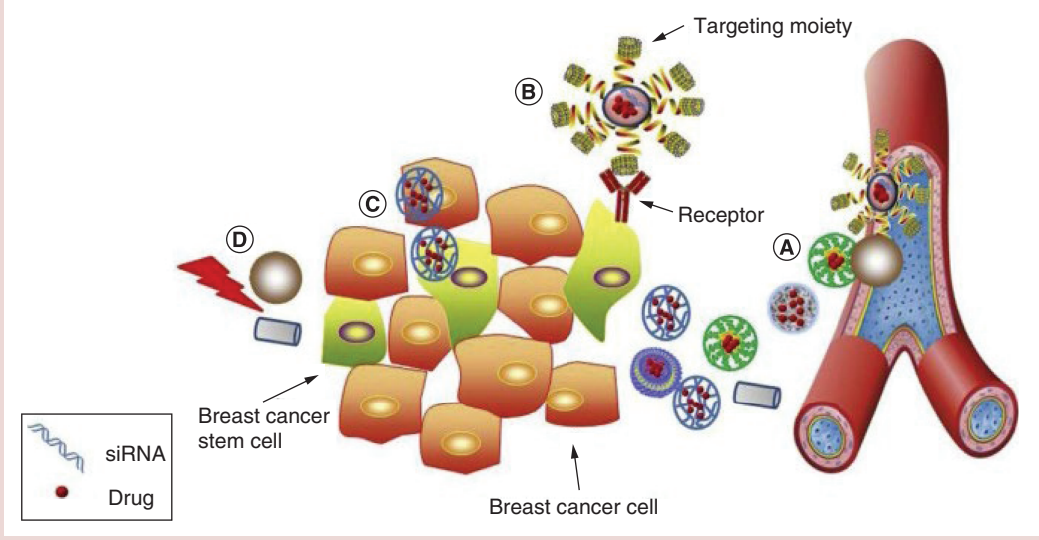

Figure 3. Biological fate of nanotherapeutic systems in cancer vaccine delivery.
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Liposomes

Liposomes are the most widely studied vesicular carriers for drug-delivery applications. Such carriers usually exist in uni-/multi-lamellar spherical lipid vesicles containing phospholipid bilayers and a hydrophilic core. Several liposomal systems have been reported in the literature for delivery of DNA vaccine [22]. These can be used for encapsulation, injection and/or adsorption of the antigens for vaccine delivery to the immune cells. Furthermore, adjuvants may be used to enhance antigenic immune response and to strengthen the humoral immunity, as normal liposomes have little or no intrinsic adjuvant properties, as shown in Figure 3.

Several liposomal systems with differences in their immunization capacities have been investigated for vaccine-delivery applications. In majority, liposomal vaccines primarily help in delivering the loaded antigens with their ability to activate and/or supply antigens to the target cells [22]. Immunogenic peptide liposomes can be fused with the cell membranes by the pinocytosis process. Traditional liposomes containing phosphatidylcholine are nontoxic but undergo rapid systemic clearance by the reticular endothelial system [23]. Zhao *et al.* worked on the protein

Table 2. Summary of various nanomedicine-loaded vaccines and their clinical stages.

Disease (s)	Type of vaccination	Manufacturer	Clinical development	Ref.
Liposomal-based vaccines				
Diphtheria, tetanus and hepatitis A	Diphtheria, tetanus/hepatitis A combined vaccine (im.)	Swiss serum, Switzerland	Developed	[26]
Hepatitis A	Epaxal Berna vaccine (im.)	Swiss serum, Switzerland	Developed	[25]
Hepatitis A & B, diphtheria, tetanus and influenza	Hepatitis A7 B/diphtheria/tetanus/influenza super combined vaccine (im.)	Swiss serum, Switzerland	Under development	[27]
S. flexneri 2A infection	*Shigella flexneri* 2A vaccine (oral)	Novavax	Under development	[28]
IRIV-based liposome vaccines				
Influenza	IRIV liposomes (trivalent influenza vaccine)	Swiss serum, Switzerland	Phase III	[50]
Hepatitis A & B, diphtheria, tetanus and influenza	IRIV liposomes (hepatitis A & B, diphtheria, tetanus)	Swiss serum, Switzerland	Phase I	[50]
Hepatitis A	IRIV liposomes (Epaxal-Berna)	Berna Biologics	Approved in Switzerland	[51]
Diphtheria, tetanus and hepatitis A	IRIV liposomes (diphtheria, tetanus, hepatitis A combined vaccine)	Swiss serum, Switzerland	Phase I	[50,52]
Hepatitis A & B	IRIV liposomes (combined HAV/HBV)	Swiss serum, Switzerland	Phase I	[54]
Virosomes-based vaccines				
Hepatitis B	Recombivax Engerix-B Recombinant HBV	Merck & Co. GlaxoSmithkline	Developed & marketed	[48]
HPV cervical cancer	Gardasil® Self-assembled particles of HPV	Merck & Co.	Developed & marketed	[49]

HAV: Hepatitis A virus; HBV: Hepatitis B virus; HPV: Human papilloma virus; im.: Intramuscular; IRIV: Immunopotentiating reconstituted influenza virosomal.

antigen carriers using mouse model tumors in liposome-polycation-DNA complex thus achieved faster immune recognition. Owing to its high effectiveness, it has been lately studied as a vaccine adjuvant in clinical trials for delivery of antibiotic protein, HPV16 E7 responsible for causing cervical cancers [24].

Epaxel® is a vaccine used for immunization against hepatitis A, a primary cause of liver carcinoma that contains formalin-inactivated antigens adsorbed onto the surface of liposomes. Such vaccines have shown superior tolerability and therapeutic efficacy over the conventional vaccines [25,26]. Another example includes amalgamation of the replicative protein antigens with regular Sendai virus protein (UV inactive) liposomes. These liposomes can convert the Sendai fusion protein into several forms of mammalian cells and facilitate uptake of them directly into the cytoplasm [26]. Clinical evaluation of such vaccines by intradermal injection revealed reduced risk of cancer as compared with the conventional liposomes. Apart from this, a literature report on the administration of hepatitis A7B/diphtheria/tetanus/Influenza antigens in a super combined vaccine system for intramuscular injection has also gained popularity due to high safety, less immunogenicity and enhanced efficacy as compared with the individual components [27]. Similar instances on the application of liposomal vaccines are summarized in Table 1.

Shigella is a bacterium with high prevalence can trigger acute diarrhea and dysentery in the developing countries. Intranasal immunization using *S. flexneri* 2A vaccine (oral) in mouse pneumonia model have shown enhanced protection against *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6 and *S. sonnei* viruses [28]. Some other instances of liposomal vaccines for cancer treatment are under development and their clinical status is summarized in Table 2.

Nanoemulsions

Nanoemulsions are colloidal dispersions with globule size 20–200 nm [29]. Significant efforts have been undertaken to explore the utility of nanoemulsions as vectors for cancer vaccine delivery [29]. Nanoemulsions have versatility for local or systemic delivery of vaccine antigens to boost the cellular immune response. Shi *et al.* developed an immunostimulatory vaccine for codelivery of CpG and MG7 antigens for protestation against gastric cancer [30]. The vaccine showed 70–93% loading capability for the antigens in the nanoemulsion system and showed high immunization ability for significant inhibition of growth of cancer cells [30]. Such vaccine improved tumor protection ability owing to the presence of unique MG7 antibodies, while copresence of CpG antigen in nanoemulsion increases the response of MG7 antigen for effective immunization alone [30].

Magnetic nanoparticles

Magnetic nanoparticles are useful in cancer therapy owing to their ability for producing hyperthermia [31]. These nanoparticles showed significant reduction in the progression of the tumor by improving the formation of MHC-I and enhanced immune response through T-lymphocyte-mediated antitumor activity [31]. Ito *et al.* investigated the combinational effects of immunotherapy and intracellular hyperthermia on melanoma, where magnetic nanoparticles administered to C57BL/6 mice melanoma nodule showed signification reduction in the tumor volume [32]. This provides inkling for further research opportunities on these carriers for exploring their applicability in cancer vaccine delivery.

Niosomes

Niosomes are bilayer vesicles containing nonionic surfactants rather than phospholipids that offer greater stabilization as compared with the conventional liposomes. These are formed by hydration of nonionic surfactants using nonsolvent evaporation, detergent removal, reverse evaporation, extrusion, ultrasonication and microfluidization techniques [33]. These are highly useful for the delivery of vaccine antigens to the antigen-presenting cells, as they are easily uptaken by oral route through M cells of the intestinal payer's patches and gut-associated lymphoid tissues [33]. New biopharmaceutical applications of niosomal vaccines include antigens-specific pathogenic organisms, such as diphtheria, coarse tetanus and pertusis. Recent instances have shown modification of the lamellar structure of the niosomes to obtain the nanovesicles for vaccine-delivery applications to treat cancers [33].

Bilosomes

Bilosomes have been formed recently with the technological developments around nonionic surfactant vesicles. Bile salts together with nonionic surfactants tend to produce bilamellar vesicles [34]. These are explored in research for mucosal immunization achieved after peroral administration of the antigens, toxoids and subunit vaccine. Conacher *et al.* observed higher antibody titer and immune adjuvant properties of the bilosomes through the oral route of immunization using bovine serum albumin-based influenza vaccine [35]. Shukla *et al.* used nanobilosomes loaded with diphtheria toxoid for enhanced mucosal immune response by the transmission of recombinant hepatitis B surface antigen to the M cells [36] and lymphoid tissues in the gut for systemic immunization.

Archaeosomes

These are typical vesicular carriers made from polar phospholipids obtained from the archebacteriae *Sulfolobus acidcaldarius*. Such carries are produced by aggregation of the said lipids below their critical micelle concentration to form a self-assembled structure. Archaeosomes are effective for the delivery of antigens through oral route of delivery to elicit their systemic immune response [37]. Archaeosomes possess unique structure and better stability at higher temperature, alkaline pH and serum proteins. Literature reports have revealed that archaeosomes offer better self-adjuvant property for effective immunization as compared with the conventional liposomes [37]. Archaeosomes encapsulated antigens can be delivered orally to provide effective mucosal vaccination. Upon oral administration, the macrophages are activated to release the charged antigens for immune activation by triggering the MHC class I and II antibody-forming pathways [38].

However, archaeosomes also provide strong, long-term immunity regulated by antigen-specific cells with $CD8^+$ CTL responses, thus allowing the body to interpret the immune response in memory cells T. In short, archaeosomes are effective in triggering both humoral and cell-mediated immunization pathways. Patel *et al.* found higher antibody titer for oval albumin-based archaeosomes for inducing systemic and oral mucosal immunization [38]. Although much research has not been done in this area for designing vaccines against the cancers, yet there is significant potential exist with the use of these carriers *vis-à-vis* the liposomal systems.

Microspheres

Microspheres are spherical particles with size less than 125 nm in diameter. Such carriers contain a monolithic structure consisting of polymers derived from the natural, semisynthetic and synthetic origin [39]. It is commonly used in the delivery of vaccines to promote oral immunization against infectious diseases. It is also used as a carrier for the delivery of antigens and subunit vaccines via inhalational and parenteral routes. The effective delivery of antigens using microspheres depends on characteristics like particle size, antigen loading capacity, swelling and erosion tendency, and biological stability at physiological pH [39]. Two key mechanisms for antigen release from the microspheres are deaggregation and/or dissolution. Microspheres prepared from natural polymers

like chitosan, alginate, dextran, among others, primarily exhibit swelling and surface erosion phenomena, while synthetic polymers like PLA and PLGA exhibit bulk corrosion as the principal mechanism for releasing antigen [39]. Kirby *et al.* designed PLGA microspheres for delivery of subunit tuberculosis vaccine prepared using modified dual-emulsion solvent evaporation process, which further opened the possibility of use of such carriers in cancer vaccine delivery [40]. Furthermore, the instances of the application of microspheres in delivering the cancer vaccine have been summarized in Table 1.

Gupta *et al.* observed higher antigen loading and enhanced immune response for the tetanus toxoid delivered through PLGA microspheres [41]. The results indicated improved stability of tetanus toxoids PLGA microspheres. Such a system was appropriate for delivering the rHsp65 protein and KLK antigens for immunization against tuberculosis [41]. Premaletha *et al.* observed that mucosal antibody titers increased significantly after peroral immunization with hepatitis B surface antigen-loaded chitosan microspheres [42]. Recent advances have also shown that chitosan microparticles are effective in delivering vaccines through the inhalational route. Due to the highly vascular nature of the nasal mucosal, microspheres prepared using chitosan-alginate polymeric composites exhibit enhanced mucoadhesion properties and provides efficient delivery of antigenic materials over a long period of time [42]. Surface functionalization by mannosylation of chitosan microspheres ensures smooth delivery of antigens to the antigen-presenting cells [42]. FluMist®, the first Medimmune Inc. nasal vaccine, includes influenza antigen-loaded in microspheres demonstrated successful immunization against type A and type B influenza viruses [43].

Viral nanovectors

These include noninfective viral particles that are composed of the viral envelope protein (50 nm in diameter) produced by self-assembling of protein sheaths without containing the genetic material. Such innovations in vaccine delivery have opened new doors for cancer treatment, which can be utilized for delivering highly complicated macromolecular structures for specific delivery to a particular site by overcoming the biological barriers for effective immunization [44]. The main benefits include ease method of preparation and improved antigen-loading potential for vaccine-delivery applications. The viral nanovectors exhibit unique potential to undergo fusion process with the endosomal antigenic membrane that enables easy access to Class I MHC, thus allowing cytotoxic T-lymphocyte activation for inducing both cellular and humoral immune responses [45].

Inflexal® V is the first virosomal flu vaccine developed by Berna Biologics (Bern, Switzerland) for immunization against seasonal pneumonia and influenza [46]. Epaxel™ is the first virosomal vaccine by Berna Biologics for immunization against hepatitis A, which has been marketed in a number of European, Asian and South American countries [46]. Bio Hep B is the first vaccine focused on virosomes developed by Berna Biologic (Bern, Switzerland) which is marketed with pre-S1, pre-S2 and HBV surface antigens [47]. Engerix-B (Hep-B [Eng]) is a hepatitis B surface antigen vaccine given through intramuscular (im.) injection shows superior immunogenicity in healthy neonates and infants, children, adolescents and adults. It is well tolerable, highly nonimmunogenic and exhibits excellent protective efficacy against the hepatitis B virus [48]. Various other virosomal vaccines developed include Cervarix®, Gardasil®, Gardasil9® and hepatitis B, including Sci-B-VacTM (third generation) are commercially available [49]. Apart from this, the applications of virosomes based cancer vaccine are given in Table 2.

Immunopotentiating virosomal carriers

These are typical virosomal vectors composed of spherical hexagonal unilamellar vesicles up to the size of 150 nm in diameter [50]. Such vectors are primarily used in oral immunization against influenza viruses, retrovirus, hepatitis A & B virus and polyvalent hepatitis A, diphtheria and tetanus. Owing to their versatile applications, such viral nanocarriers have extended their utility for cancer vaccine delivery [51]. For delivery of peptide-based malaria vaccine (SPf66) by immunostimulating complexes, the said formulation was administered through intramuscular injection for inducing the immune response in BALB/c mice that revealed high efficacy over the conventional vaccine formulations [52,53]. Similarly, the virosomal formulation of *Plasmodium falciparum* GLURP-MSP3 chimerical protein developed by Tamborrini *et al.* showed significant improvement in the immune response against malaria [53]. Hepatitis A virus (HAV) and hepatitis B virus (HBV) are also considered as one of the leading causes of deaths worldwide due to the increased risk of individuals for developing cancers. Such viruses are a major public health issue since both HAV and HBV cause significant morbidity and both can be lethal. Furthermore, a combination of HAV/HBV vaccine is well tolerated and equally effective [54].

Lipid-based drug-delivery systems

Lipid-based nanocarriers are useful in delivering the cancer vaccines by loading the antigenic materials in it [55]. The following are recent alternatives for the vaccine delivery such as triglyceride emulsions, solid lipid nanoparticles and self-emulsifying drug-delivery systems. The antigens encapsulated within the lipid matrix can easily enter into the lymphatic system via intestinal lymphoid tissues to trigger the immune response through the oral route. This method is particularly helpful in stimulating the immune response in case of Hodgkin's and non-Hodgkin's lymphoma, and other types of cancers [56]. In addition, the intranasal delivery of influenza and cholera vaccines using the immunostimulating complexes has shown superior antibody titer against the respiratory syncytial virus [57].

Key challenges: formulation challenges & regulatory hurdles

The delivery of genetic vaccines using viral vectors has now been substituted with nanocarriers as chemical vectors, which are highly stable, long-lasting and biodegradable ones [58]. Due to the high safety and strong biocompatibility of these carriers, vaccines containing genes and antigens have gained popularity for cancer treatment [58]. However, the successful clinical translation of nanomedicines for cancer vaccination requires thorough evaluation of their safety and efficacy for human use. Moreover, the commercial production of such cancer vaccines also require critical monitoring of the key challenges associated with optimization of the product and process parameters for attaining desired therapeutic performance. Some of the critical quality attributes highly important for the cancer vaccines include antigen loading efficiency, particle size, ζ potential and controlled release delivery profile of the nanocarriers. In this regard, use of predictive *in vitro–in vivo* performance evaluation tools and combinatorial approaches can be helpful in accelerating the faster clinical translation of the genetic vaccine candidates. Besides, additional studies for evaluating the *in vivo* effectiveness, biodistribution, clearance and toxicity profiling of the nanovaccines are required for the cancer treatment.

Other technical challenges highly important include large-scale development of the nanocarriers for antigenic vaccine delivery [58]. At present, most nanocarriers are manufactured in laboratory on small-scale batches and then scale-up to larger scale industrial capacity. As the scale-up process involve high cost, thus require critical monitoring of the scale-dependent parameters that are directly linked with the therapeutic performance. Besides, evaluation of economic feasibility of scale-up process is also critically monitored in order to avoid quality crisis [59,60]. Apart from the formulation challenges, the regulatory challenges for approval of genetic vaccines for cancer treatment are highly crucial. In this regard, the key formulation challenges with respect to the excipients used for preparing nanocarriers for delivering the genetic vaccines and their safety status is essential for the regulatory approval [60].

Conclusion & future perspective

The current scenario of development of cancer vaccines has been significantly improving with the escalation of number of products into the market. The applications of nanocarriers have shown supremacy of the effectiveness of the cancer vaccines to strengthen the body's immune response over the conventional vaccines. Many of the nanocarriers containing cancer vaccines have shown excellent outcome with respect to their optimum formulation characteristics and ability to induce a strong immune response. The US FDA has recently approved a number of nanomedicines-based cancer vaccines based on the liposomes, transferosomes and microspheres technology, while many others are under the clinical evaluation and translation stage.

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Executive summary

Current challenges

- The use of chemotherapeutic treatment strategy against various types of cancer possess efficacy but produces high toxicity in patients.
- Application of genetic vaccines for cancer treatment, especially are highly capable of reducing the current cancer mortality rate.
- The first cancer cell-based dendritic vaccine was approved by the US FDA in 2010, which has been proven to be very effective in controlling the immune response.
- Despite efficacy and enormous capabilities for tumor targeting to produce effective immune response, cancer vaccines encounter a number of biopharmaceutical issues.

Therapeutic opportunities

- Nanocarriers have emerged as a substitute for the delivery of genetic vaccines over the viral vectors, as they are stable, long-lived and biodegradable in nature.
- Due to the high stability and high biocompatibility, the nanocarriers have been highly effective for delivering the vaccines.
- Nanoengineered therapeutic systems have attracted great attention for their enormous capabilities in delivering the gene cargos to the cancer cells through active targeting approach.
- Several genetic vaccines have been developed and evaluated through preclinical and clinical studies have demonstrated significant therapeutic potential.

Formulation challenges & regulatory hurdles

- The successful and complete clinical evaluation of nanomedicine requires addressing major technical challenges, such as improving the loading efficacy of therapeutics, ensuring controlled drug release, evading immune cells and maximizing the nanocarrier accumulation at the target sites.
- The design of optimal formulations with all the characteristics desirable for specific applications is a challenge that could hinder clinical translation.
- Currently, nanocarriers are prepared on a small scale in a laboratory setting, and it is difficult to produce nanocarrier with identical properties on a large scale in an industrial setup.
- In financial point of view, multiple fabrication steps are not preferred because they usually increase the manufacturing cost.
- Another important aspect is of evaluation of nanocarriers for their potential adverse effects on the biological system, which shall be carefully studied through the clinical trials.
- The development of scalable methods with highly predictable *in vivo* characteristics can enable faster clinical translation.

Review focus

- This special report provides an overview of the prospects and precepts in the design, development, evaluation and application of nanotherapeutic carriers in cancer vaccine delivery.

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•• **Highly useful piece of the literature on liposomal vaccines for cancer treatment.**

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PRECISION NANOSYSTEMS
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Accelerating the Development and Scale-Up of mRNA Vaccines

The need to accelerate vaccine development has never been more important as we continue to navigate life amidst a global pandemic. The approval of the first COVID-19 vaccines was an enormous milestone for messenger RNA (mRNA) therapeutics that altered the course of the pandemic and highlights the rapid response potential of the technology. While it may seem as though the mRNA technology underlying the vaccines was an overnight success, it was based on decades of scientific research and innovation, making synthetic RNA safe for injection. However, getting these highly sensitive RNA molecules into cells without degradation while maintaining safety, potency and efficacy was a major challenge. Lipid nanoparticles (LNPs) have solved many of these problems and are now the key to making mRNA vaccines a reality. The demonstrated clinical efficacy of the COVID-19 mRNA vaccines has driven explosive growth in the development of RNA-based vaccines and concomitantly propelled LNPs into the mainstream as an effective drug carrier for complex polynucleotide- and peptide-based therapeutics.

The LNPs used in the COVID-19 vaccines are composed of positively charged ionizable lipids which undergo an electrostatic interaction with negatively charged mRNA molecules. The LNP shell effectively encapsulates the mRNA, forming a protective barrier against metabolic enzymes. Mimicking endogenous low-density lipoproteins (LDLs), LNPs are taken into the target cells by endocytosis. Within the endosome, the pH-sensitive ionizable lipids facilitate endosomal escape and release of the mRNA payload into the cytoplasm. While LNPs are complex delivery systems, their low toxicity, ability to efficiently encapsulate a variety of genomic payloads (or multiple payloads) and be engineered to specifically target a type of cell present new opportunities for emerging nanomedicines.

With growing global interest, demand for LNPs is at an all-time high. The move from a niche application to mainstream has increased investment into bioprocessing development efforts to establish reliable and robust manufacturing with clear scalability and compliance goals in mind. As the Quality by Design (QbD) concepts and Design of Experiment (DoE) approaches gain momentum in process development, opportunities to leverage vertically (up/down) scalable platform production technologies, predictive process models and automation are providing deep process knowledge. Importantly, evaluation of both upstream and downstream steps at scale is needed to gain end-to-end process insight across the entire manufacturing workflow, which is critical to identify any gaps or unanticipated effects resulting from process or analytical changes on product critical quality attributes (CQAs). With nanoparticles, scale-up of downstream formulation and fill-finish operations can have huge impacts on functionality and stability. Therefore, paying attention to downstream considerations can make the difference between success and failure on the path towards commercialization.

THE IMPACT OF DOWNSTREAM PROCESS DEVELOPMENT ON BIOACTIVITY

The goal of process development is to define and optimize critical process parameters while ensuring process scalability for long-term success. The production of LNP-based nanomedicines can be challenging because of their size and complexity, since nanoparticle morphology can be impacted by downstream filtration processes that can impact the bioactivity of the resulting drug product. Therefore, a thorough understanding of how to mix the lipids and RNA to form the nanoparticles in a robust and reproducible manner is key to successful LNP formulation and delivery. Critical process parameters (CPPs) such as flow rates, temperatures and mixing ratios can affect the physicochemical characteristics of the resulting nanoparticles. Appropriate analytical and biological assays to assess how changes in processing variables affect nanoparticle properties, which include particle size, polydispersity (PDI) and drug encapsulation efficiency (EE%), are needed to confirm that product identity, potency and safety are maintained across all developmental stages to guide formulation and process development.

Traditional methods for nanoparticle manufacture have previously involved turbulent mixing processes where organic solvents containing LNPs meet the aqueous solutions of RNA in an uncontrolled manner. However, heterogeneous particle size, inconsistent encapsulation and poor batch-to-batch reproducibility pose barriers to scale-up. Non-turbulent mixing devices were developed to overcome the shortcomings of these production techniques to improve the consistency and reproducibility of nanomedicine production. Precision NanoSystems' (PNI) NxGen™ technology has enabled flow rates thousands of times higher than conventional microfluidic designs while maintain controlled mixing conditions. Non-turbulent flow brings together the fluid streams containing the lipids dissolved in an organic solvent and the nucleic acids dissolved in an aqueous buffer in a controlled manner, creating a solvent polarity change and triggering the formation of LNPs loaded with RNA. Precise control of the chemical and physical environment enables highly predictable, time-invariant mixing for reliable and repeatable nanoparticle self-assembly.

PNI has implemented NxGen™ technology across a range of NanoAssemblr® systems to support LNP formulation through all drug development stages with increasing throughput, from preclinical, clinical to commercial production that also meet phase-appropriate regulatory requirements. The conserved mixing element across production volumes offers developers a risk-based approach to chemistry, manufacturing, and controls (CMC) studies since operations modeled on small-scale preclinical instruments can be more easily translated to large-scale platforms. This helps to minimize variability during tech transfer and reduce the number of engineering runs prior to cGMP manufacturing. Of course, process development is often not linear and may require movement between scales to revisit and reoptimize process parameters. Scalable technology that supports this flexibility enables rapid and streamlined process development and thus offers significant advantages over static technologies.

The next stage in the downstream workflow after LNP-RNA assembly is tangential flow filtration (TFF), which encompasses both ultrafiltration and diafiltration. For nanoparticles, diafiltration is used to exchange the organic solvent used during formulation for a buffer that is suitable for storage stability and administration. Ultrafiltration is used to concentrate the therapeutic to its final formulation concentration.

Process development is a challenge for any drug developer, and mRNA vaccines are no different. Process optimization is needed to achieve a sustainable, cost-effective and

robust manufacturing process that ensures the safety and efficacy of the end product. Platform technologies such as the NanoAssemblr® enable small-scale modeling of unit operations that is predictive of performance at scale and accelerates process optimization. The ability to accelerate the development and commercialization timelines for new mRNA vaccines and other LNP-based therapeutics holds tremendous potential to ensure global readiness against future pandemics and bring life-saving treatments to patients faster.

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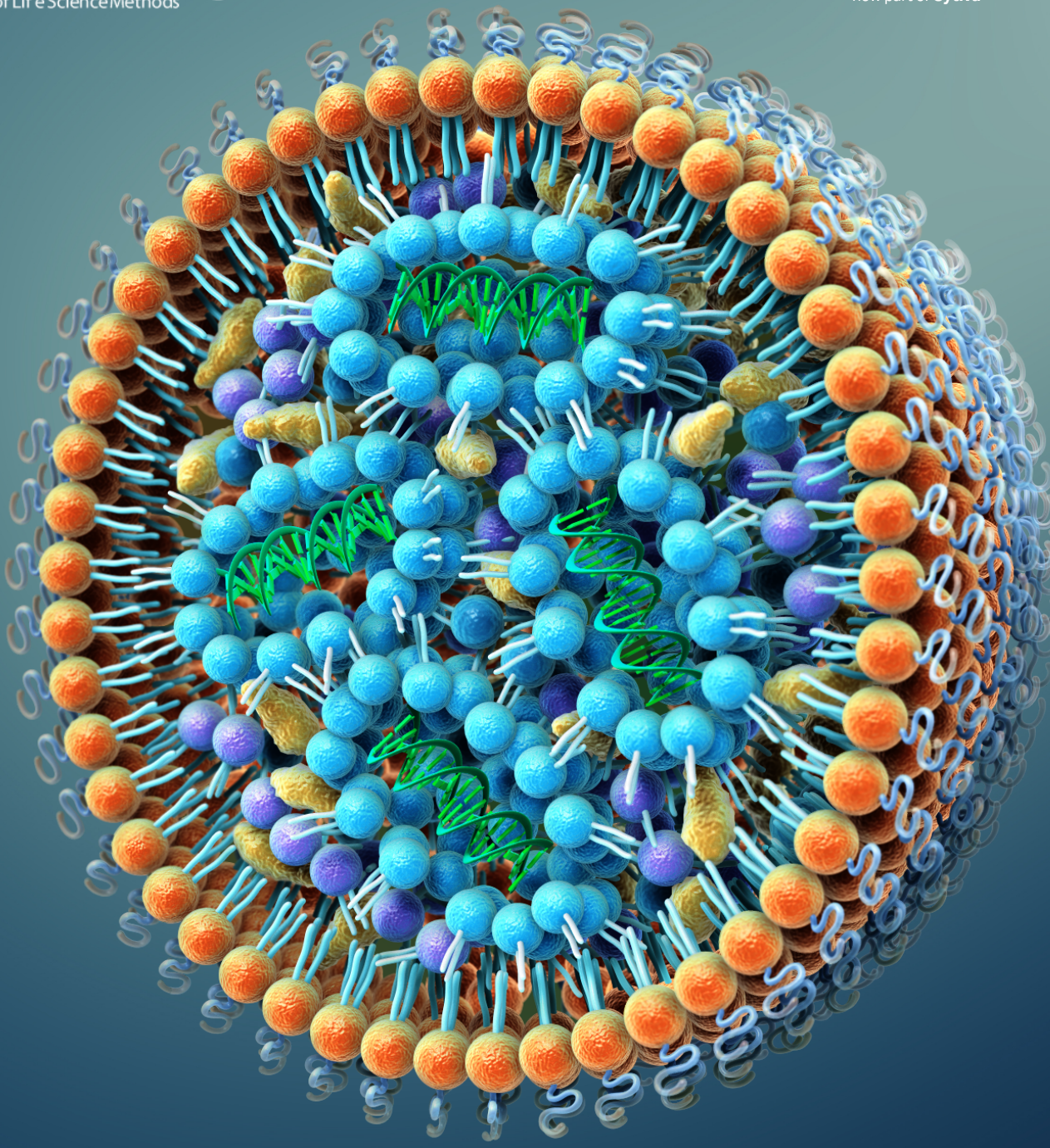

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