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Review article

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Targeting cancer with mRNA–lipid nanoparticles: key considerations and future prospects

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Abstract

Harnessing mRNA–lipid nanoparticles (LNPs) to treat patients with cancer has been an ongoing research area that started before these versatile nanoparticles were successfully used as COVID-19 vaccines. Currently, efforts are underway to harness this platform for oncology therapeutics, mainly focusing on cancer vaccines targeting multiple neoantigens or direct intratumoural injections of mRNA–LNPs encoding pro-inflammatory cytokines. In this Review, we describe the opportunities of using mRNA–LNPs in oncology applications and discuss the challenges for successfully translating the findings of preclinical studies of these nanoparticles into the clinic. We critically appraise the potential of various mRNA–LNP targeting and delivery strategies, considering physiological, technological and manufacturing challenges. We explore these approaches in the context of the potential clinical applications best suited to each approach and highlight the obstacles that currently need to be addressed to achieve these applications. Finally, we provide insights from preclinical and clinical studies that are leading to this powerful platform being considered the next frontier in oncology treatment.

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Key points

- mRNA–lipid nanoparticles (LNPs) are a powerful, versatile platform that hold great potential as anticancer therapies; LNPs have been tested in clinical applications and can successfully and safely deliver mRNA payloads designed to target various tissues and cell types.
- mRNA–LNPs can be administered to specific tissues through various routes and using different approaches for targeting specific tissues and cell types.
- Passive targeting approaches do not involve modifications of mRNA–LNPs for delivery to specific tissues and cells; these approaches typically rely on inherent tendencies of different particles to accumulate in different tissues or tumours.
- Active targeting approaches involve modification of the surface of mRNA–LNPs for their delivery to a specific cell type; improvements in the ability to target nanoparticles to specific cell types is key for expanding their applications in clinical oncology.

Introduction

Lipid nanoparticles (LNPs) are vehicles for delivering nucleic acids to cells. Three RNA–LNPs are currently approved by the US Food and Drug Administration (FDA) and European Medicines Agency: patisiran, a small interfering RNA (siRNA)–LNP for the treatment of hereditary transthyretin-mediated (hATTR) amyloidosis, and BNT162b2 and mRNA-1273, two mRNA–LNP-based COVID-19 vaccines[1,2]. LNPs are lipid-based delivery vehicles that: (1) protect nucleic acid payloads from being degraded or from activating RNA-sensing mechanisms and, subsequently, innate immune responses; (2) enable the introduction of nucleic acid payloads into the cell cytoplasm; and (3) function by themselves as adjuvants when used for vaccination[3,4]. LNPs are a versatile platform that can encapsulate various types of RNA as payloads and be administered by different routes. mRNA–LNPs have been successfully implemented as prophylactic vaccines against pathogens, and they have also been tested in various oncology clinical trials either to achieve intratumoural expression of immune-stimulating cytokine combinations or as cancer vaccines (Table 1). Both applications have demonstrated that mRNA–LNPs are efficient drug-delivery vehicles. New potential avenues involve the development of targeting strategies to deliver payloads selectively into cell types previously considered unreachable, which would enable the use of payloads that might be ineffective or toxic when expressed in a broad range of tissues. In this Review, we provide an overview of various approaches for targeting cancer cells using mRNA–LNPs, highlighting the advantages and challenges of each approach in terms of selective cellular expression. We also discuss how improvements in mRNA–LNP targeting strategies from the past few years are creating therapeutic opportunities in oncology.

mRNA–LNPs: an overview

LNPs commonly comprise four lipid components that are mixed to form uniform spheres with a diameter of 50–150 nm that encapsulate RNA payloads[5]. The four common components are: a phospholipid, cholesterol, a 'stealth' lipid, and an ionizable lipid[2,6,7] (Box 1; Table 2). The ionizable lipid has a head group that is charged at lower than physiological pH levels and yet is neutral at physiological pH levels, thus preventing immediate inflammatory toxicities driven by cationic lipids[8]. The ionizable lipid both encapsulates the negatively charged nucleic acid payloads and enables the release of payloads to the cytoplasm at the acidic pH levels present in endosomal compartments (Box 1; Fig. 1). Although the ionizable lipid has been the most extensively researched, all components of the formulation and how their ratios affect biodistribution, mRNA expression, and biological and/or physiological effect are areas of ongoing research interest[9].

In their natural forms, mRNA molecules are unstable and challenging to work with; however, owing to progress in mRNA design (with improvements such as use of modified nucleotides, sequence modifications and mRNA capping modalities for increased stability and reduced immunogenicity), mRNA-based therapies have become a highly attractive and financially lucrative therapeutic platform. At present, mRNA molecules are synthesized using in vitro transcription systems (Box 1). Non-modified mRNA molecules are recognized by cellular RNA sensors, resulting in the activation of an innate immune response[10] and thus, nucleoside modifications are considered one of the most important breakthroughs in the field of mRNA-based therapies (Box 1).

Targeting strategies

Passive targeting

Passive targeting approaches are those used to deliver mRNA–LNPs to tissues and cells without modifying their surface with targeting moieties. In oncology, this approach is used to target accessible tumours as well as non-malignant tissues, such as the spleen and lymph nodes, for antitumour immune modulation.

Passive targeting via intratumoural administration. Intratumoural administration of mRNA–LNPs is the simplest method for delivering nanoparticles, and is used in scenarios in which most of the mRNA translation can be physically constrained to the tumour area. The poor vasculature organization, lack of lymphatic drainage and high extracellular matrix (ECM) density of most tumours can limit the diffusion of LNPs from the injection site to such tumours[11]. These features can also restrict LNPs from getting into deep areas of the tumour, even when administered intratumourally, but can also limit the off-target expression of payloads owing to leakage[11]. Accordingly, several preclinical studies of mRNA–LNP-based cancer therapies have used intratumoural injections to express cytokines and toxins, and for CRISPR–Cas9-mediated targeting of oncogenes[12–19]. In clinical settings, to decide whether intratumoural administration is relevant, physicians must consider the number of doses required, and tumour physiology in terms of tumour stage, tumour type and accessibility for injection; this approach, for example, would be appropriate in patients with melanoma (Table 2). Despite the propensity of tumours to retain particles[20], LNPs injected intratumourally are not necessarily restricted to the tumour area, and imaging studies have demonstrated LNP accumulation in the liver and lymphatic organs after intratumoural injections[11,14]. The cellular distribution of injected particles also varies within the tumour cellular populations: injection into the tumour mass does not guarantee expression primarily in cancer cells, which might be problematic with therapies with direct cytotoxic activity, such as cell cycle modulators and toxins. Preclinical studies have shown that large proportions of mRNA payloads are expressed by immune cell populations in the tumour area, notably macrophages[14,15]. Consequently, cancer-targeted mRNA–LNP-based therapies solely intended for intratumoural injection are mostly reserved for applications in which expression of the

Table 1 | Selected oncology clinical trials testing mRNA–LNPs

LNP name	Indication	Payload	Administration	Phase	NCT number	Outcomes	Ref.
Cancer vaccines							
BNT112	Prostate cancer	Fixed combination of five antigens commonly expressed in prostate cancer	i.v.	I/II	NCT04382898	Administered as monotherapy or with i.v. cemiplimab; acceptable safety profile; vaccine-induced immune responses detected using ELISpot	168
BNT113	Head and neck cancer	Human papillomavirus 16 oncoproteins E6 and E7	i.v.	II	NCT04534205	Administered in four cycles as monotherapy or with i.v. pembrolizumab; acceptable safety profile for both strategies	169
mRNA-4157	Solid tumours	Individualized cancer vaccine against up to 34 neoantigens	i.m.	I	NCT03313778	Administered in up to nine cycles as monotherapy or in combination with i.v. pembrolizumab; acceptable safety profile was reported; three CR and eight PRs in 63 patients in the combination group were reported	170
	Melanoma	Individualized cancer vaccine against up to 34 neoantigens	i.m.	II	NCT03897881	Administered in up to 18 cycles in combination with i.v. pembrolizumab; 18-month DMFS 91.8% and 76.8% with combination and pembrolizumab monotherapy, respectively; granted Breakthrough Therapy designation by the FDA in combination with pembrolizumab for high-risk melanoma following resection	171
Other immunotherapies							
BNT211	CLDN6$^+$ R/R solid tumours	CLDN6 CAR antigen vaccine (CARVac) as adjuvant for CAR T cell therapy	i.v.	I/II	NCT04503278	Patients received CLDN6-targeted CAR T cells ± adjuvant CARVac; manageable safety profile; 7 of 21 patients had a PR	172
mRNA-2752	R/R solid tumours or lymphomas	IL-23, IL-36γ and OX40L	i.t.	I/II	NCT03739931	Administered in up to seven cycles as monotherapy or in combination with i.v. durvalumab; reported to be safe and tolerable when combined with durvalumab; treatment resulted in sustained increase in IFNγ and TNF levels, and PD-L1 expression	173
MEDI1191	Solid tumours	IL-12	i.t.	I	NCT03946800	Administered with i.v. durvalumab; combination reported to be safe and tolerable; treatment resulted in increased serum IL-12 and IFNγ levels, and increased infiltration of CD8$^+$ T cells; 3 of 31 patients had a PR	26

CAR, chimeric antigen receptor; CLDN6, claudin-6; CR, complete response; DMFS, distant metastasis-free survival; ELISpot, enzyme-linked immunosorbent spot assay; i.m., intramuscular; i.t., intratumoural; i.v., intravenous; LNP, lipid nanoparticle; OX40L, OX40 ligand; PR, partial response; R/R, relapsed and/or refractory.

translated protein in non-malignant tissues is tolerable or desired. This effect is most commonly achieved with mRNA–LNPs encoding various cytokine mixtures combined with immune-checkpoint inhibitors (ICIs)[21]. The local expression of certain cytokines, such as IL-12, could ameliorate the dose-restricting adverse events resulting from their systemic administration[12].

Regardless of the mRNA payload, LNPs have an innate adjuvant effect that promotes a CD4$^+$ T helper 1 (T_H1)-mediated cytokine response[4]. T_H1 responses are generally tumour-suppressive whereas T_H2 responses are associated with reduced cell-mediated immunity and the activation of immune populations with a cancer-promoting phenotype[12,22]. Based on these observations, investigators administered mRNA–LNPs encoding cytokines (including, but not limited to, IL-12, IL-15 and IL-36γ) intratumourally to mice, resulting in a tumour microenvironment (TME) favouring antitumour activity[12,23,24]. This phenotype supported the activation of CD8$^+$ and T_H1 cells, recruitment of natural killer and natural killer T (NKT) cells, and activation of CD103$^+$ and CD8$^+$ dendritic cells (DCs), which are needed for antigen cross-presentation and activation of CD8$^+$ T cell-driven responses[12,15,23–25].

In a mouse model of cancer, non-encapsulated mRNA-mediated expression of a combination of IL-15 and IL-12 in the tumour bed increased the infiltration and proliferation of T cells in the tumour and inhibited its growth[16]. An ongoing clinical trial is testing the injection of mRNA–LNPs encoding IL-12 in combination with the anti-PD-L1 antibody durvalumab[26] (Table 1). mRNA–LNPs encoding cytokines often also include OX40 ligand (OX40L) to protect T cells from anergic cell death during activation and promote a memory T cell phenotype[25]. Of note, high levels of OX40L in tumour tissues are associated with improved 5-year overall survival in patients with non-small-cell lung cancer[25]. In a mouse model of cancer, intratumoural administration of mRNA–LNPs encoding OX40L, IL-23 and IL-36γ elicited an antitumour immune response and protected against tumour rechallenge[14]. This response was mediated by CD8$^+$ T cells, DCs and NKT cells recruited and activated in the tumour area. A phase I clinical trial is testing intratumoural injection of an mRNA–LNP encoding the same three cytokines combined with durvalumab[27] (Table 1). An important feature observed with local administration of mRNA–LNP-based immunotherapies in mice is that intratumoural injections generate an immune response

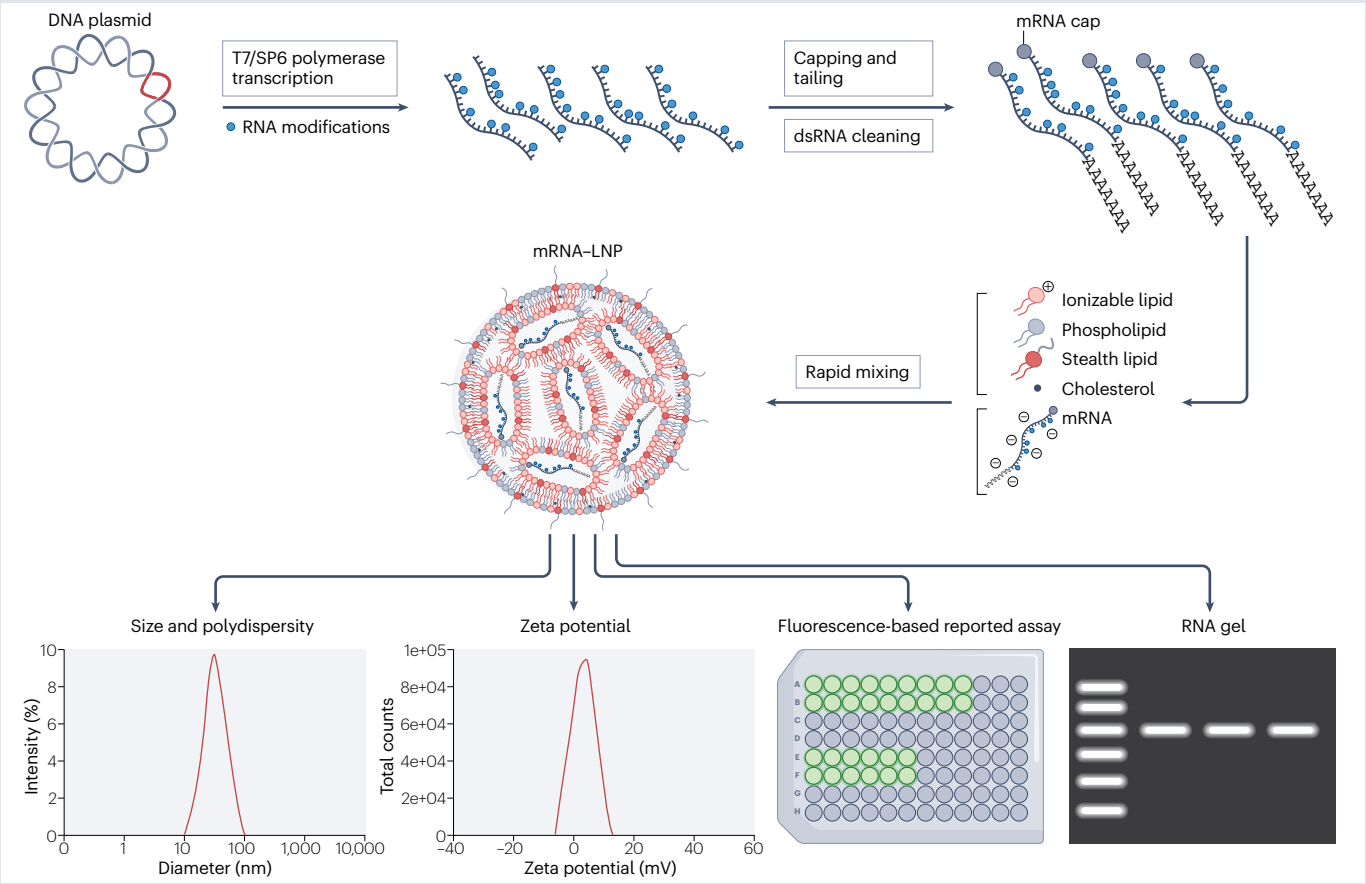
Box 1

Generation of mRNA–LNPs

mRNA payloads are synthesized via in vitro transcription reactions[130], in which mRNAs are synthesized based on a DNA template encoding the desired payload downstream of a bacteriophage promoter site, most commonly T7 or SP6. The 5′ and 3′ untranslated regions (UTRs) of mRNA are encoded on the transcript, and an mRNA cap and a poly(A) tail that can be added co-transcriptionally or post-transcriptionally via enzymatic processes (see top part of the figure)[2]. These components contribute to mRNA stability and enhanced expression. Furthermore, the mRNA payloads can incorporate modified nucleosides, such as N1-methylpseudouridine, to increase expression by inhibiting recognition by intracellular innate immune sensors[183–185]. N1-methylpseudouridine has been used in both approved mRNA–lipid nanoparticle (mRNA–LNP) COVID-19 vaccines[183].

Next, the mRNA payloads are purified from double-stranded RNA (dsRNA) impurities[130] to limit dsRNA-sensing pathways, which drive innate immune responses leading to a decreased amount of proteins expressed[186–188]. mRNA–LNPs are formulated by mixing these payloads with various lipid components (Table 2). Structurally, the ionizable lipids hydrostatically interact with the nucleic acid payloads to form inverted micelles around the nucleic acid. This nucleates a spontaneous self-assembly of the helper lipids and sterols to create solid-core LNPs. Hydrophilic components of the stealth lipids (for example, polyethylene glycol (PEG)) can be found facing the hydrophilic exterior of the LNPs (see central part of the figure)[189,190].

Generally, LNP size and uniformity are validated by dynamic light scattering, surface charge is determined by ζ-potential measurements, payload encapsulation is determined by fluorescence-based reporter assays, and payload purity and stability can be validated in RNA gels (see bottom part of the figure). The resulting mRNA–LNPs commonly have a diameter of 50–150 nm, are uniformly distributed and encapsulate close to 100% of nucleic acid payloads. The ionizable lipid is a synthetic lipid comprising lipid tails with a cationic ionizable head group. At acidic pH levels, the positive charge on the head group at acidic pH levels interacts with the negative charge on RNA molecules and facilitates the encapsulation of nucleic acid payloads within LNPs and their release into the cytoplasm after internalization. The ionizable lipid is considered the pivotal component in terms of activity and is the focus of many research attempts to optimize LNP formulations. The ionizable lipids in the mRNA–LNPs clinically approved thus far are ALC-0315, SM-102 and DLin-MC3-DMA, used in BNT162b2, mRNA-1273 and patisiran, respectively[2].



sufficient to inhibit dissemination of metastases outside the injected area. This effect was observed in mouse models in which intratumoural injections of mRNA–LNPs encoding IL-12 or IL-15 caused regression of non-injected distal lesions[12,13].

In summary, intratumoural injection is an interesting administration route for immune-stimulating mRNA–LNP therapies. This approach presents an opportunity to manipulate the TME to promote antitumour immune responses and enables local expression of cytokines that might be overly toxic when administered systemically. Nonetheless, the use of this approach is limited to tumours that are accessible and to scenarios in which payload expression outside cancer cells is tolerated.

Endogenous targeting

Passive targeting of inaccessible solid tumours beyond the liver with mRNA–LNPs entails an arduous journey. To reach the tumour bed, intravenously administered mRNA–LNPs must safely pass through the vascular system, avoid systemic clearance, extravasate to the tumour site, penetrate within the tumour and be delivered intracellularly to cancer cells. Each one of these stages encompasses multiple barriers that also depend on disease status and tumour architecture.

Once LNPs are injected intravenously, they rapidly adsorb serum proteins that can alter the pathways involved in their trafficking and internalization, such as those involved in the clathrin-mediated and macropinocytosis pathways[28–30]. Generally, LNPs accumulate in the liver upon systemic administration owing to the adsorption of serum proteins in the circulation. Most notably, apolipoprotein E coats LNPs and facilitates their uptake into hepatic cells via binding to LDL receptors[31–33]. To enhance circulation time, PEG-linked lipids are frequently incorporated into LNP formulations[2]. PEG groups are hydrophilic and provide the LNPs with stealth properties by enabling them to evade uptake by the reticuloendothelial system and confer colloidal stability[6]; however, the PEG molecular ratio of the formulation must be adjusted because a high proportion of these groups can prevent cellular LNP uptake and therefore impede activity[34]. Furthermore, PEG molecules can promote LNP-mediated reactogenicity via complement activation-related pseudoallergies[35–38]. Although PEG has been traditionally considered non-immunogenic[39], anti-PEG antibodies have been detected that facilitate accelerated blood clearance of LNPs and activate the classic complement pathway[40]. These two properties can hamper the clinical translation of systemically delivered mRNA–LNPs, and thus treatment schedules will most probably involve multiple injections. To this end, investigators are attempting to identify potential replacements for PEG[40,41]; yet, to date, PEG remains the stealth lipid most commonly used to increase the circulation time of LNPs. To control inflammatory reactions, in the APOLLO trial, patients received dexamethasone before patisiran, which was administered by slow bolus infusion[42]. Of note, patisiran is indicated in patients with a type of neuropathy. However, dexamethasone administration could be counterproductive in patients with cancer receiving immunotherapies, one of the main applications of mRNA–LNPs in oncology. For example, in a phase I study of an mRNA–LNP vaccine targeting the virus in patients infected with chikungunya virus, those receiving prior steroids had a ~1.7-fold reduction in IgG levels[43].

The accurate prediction of which serum proteins adsorb on LNPs and how this process can affect LNP biodistribution holds great potential; this targeting approach is referred to as endogenous targeting. Indeed, the past few years have seen substantial advances in the understanding of how LNP formulations affect protein adsorption and alter in vivo biodistribution[33,44,45]. However, some researchers have suggested that LNP alterations need to be restricted within acceptable physicochemical characteristics pertaining to size, uniform distribution and surface charge[8,46]. For example, a negative surface charge on LNPs can prevent their uptake and a positive charge carries the risk of systemic toxicity via activation of TLR4-mediated signalling in immune cells[8,46]. Nevertheless, the role of distinct formulations on differential biodistribution, and the general relationships between lipid formulation, structure and activity remain to be fully understood. Efforts to optimize formulations for endogenous targeting of different tissues focus on both in vitro and in vivo screens that commonly use reporters for LNP biodistribution, the most common of which are DNA barcodes[29,47–49]. The tissue distribution of LNP reporters is then reversely engineered to identify the mechanisms underlying endogenous passive uptake of mRNA–LNPs in certain tissues (Fig. 2). This approach remains challenging, however, as evidenced by the fact that in vitro and in vivo adsorption has been measured and their correlation can vary[50]. Regarding in vivo screens, two important considerations are that barcodes generally rely on readouts that include distribution but exclude the extent of protein translation; and that DNA–LNPs, commonly used in barcodes, do not necessarily have the same distribution as mRNA–LNPs[51]. Regarding formulation and endogenous targeting, lipid formulations that can alter biodistribution without incorporating exogenous targeting moieties are easier to manufacture than those incorporating modifications. Currently, targeting strategies designed using formulation optimization generally enable preferential uptake by certain organs but rarely achieve high levels of target cell specificity. Furthermore, certain cell lineages, such as myeloid cells, are expected to have greater uptake of mRNA–LNPs than others, such as lymphocytes, which are notoriously hard to transfect. Indeed, delivery to T and B cells has been achieved after combinatorial screening of lipid formulations, yet the percentage of cells transfected is low (1.5–2% and up to 10% of T and B cells, respectively)[52,53], although one group achieved up to 30% silencing in splenic T cells using an optimized ionizable lipid in the LNP formulation, encapsulating siRNA without the addition of targeting moieties[54].

Next, LNPs must extravasate from the bloodstream into the tumours. The hypothesis that drugs can diffuse from the bloodstream into tumours primarily relies on the assumption of enhanced blood

Table 2 | Lipid components in LNPs and their function

Lipid component	Examples	Function	Refs.
Phospholipid	DOPE, DSPC, DOPG	Helper lipid considered to improve the structural stability of formulations	174
Sterol	Cholesterol and cholesterol analogues	Structural stability	175–177
PEGylated lipid	PEG–DMG, PEG–DSPE	Stealthiness: provides colloidal stability and enables evasion of the mononuclear phagocytic system	178,179
Ionizable lipid	DLin-MC3-DMA, ALC-0315, SM-102	Encapsulates mRNA payload, improves transfection efficacy and enables differential delivery	54,152,180

DMG, dimyristoylglyceride; DOPE, dioleoylphosphatidylethanolamine; DOPG, dioleoylphosphatidylglycerol; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DSPE, distearoyl-*sn*-glyceride; LNP, lipid nanoparticle; PEG, polyethylene glycol.

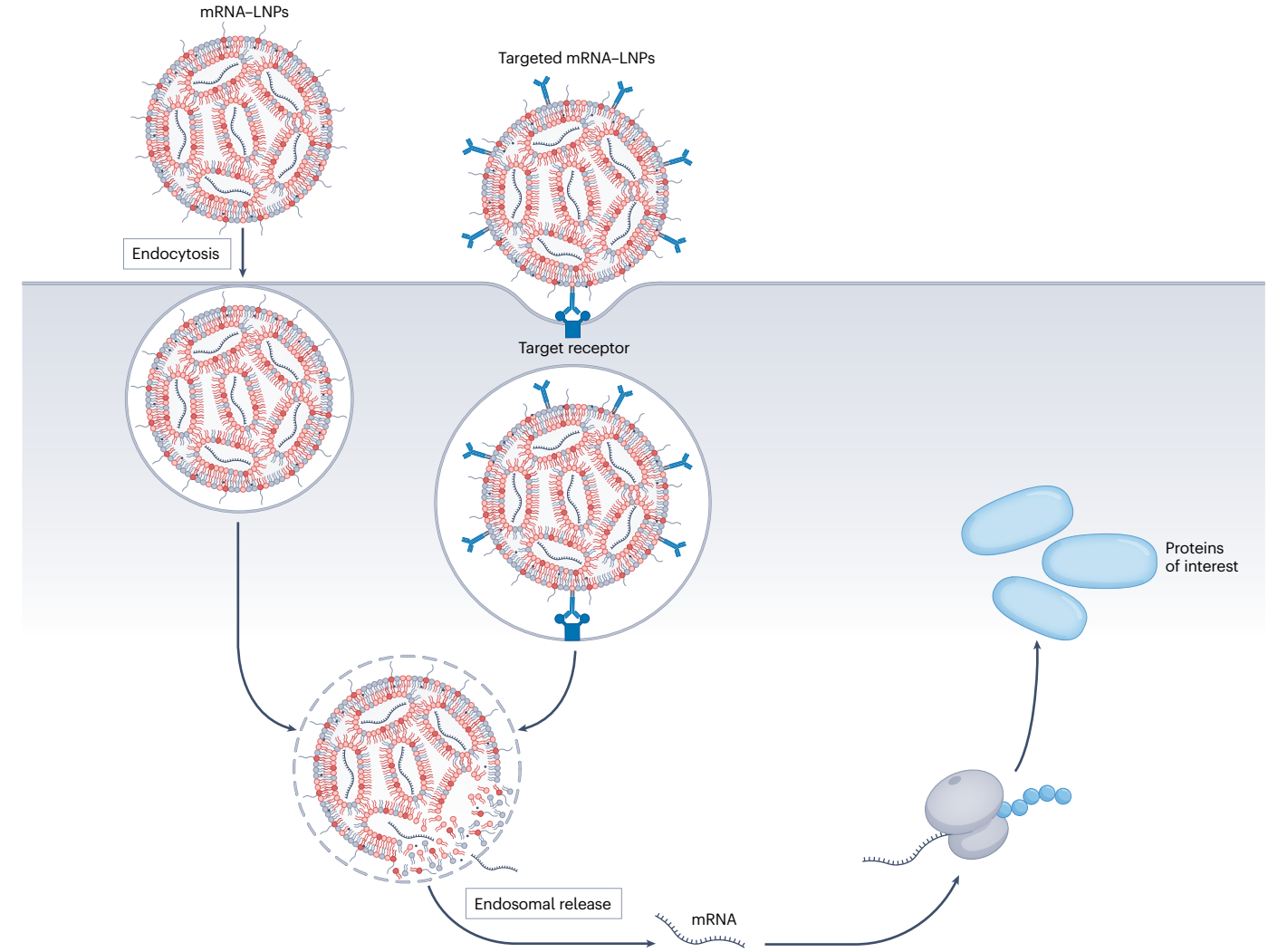


Fig. 1 | mRNA–LNP delivery into cells. mRNA–lipid nanoparticles (LNPs) enter cells through macropinocytosis and clathrin-mediated endocytosis[181]. After internalization, mRNA–LNPs progress through the endosomal pathway. The mRNA payloads 'escape' the endosomal pathway in the early and late endosome stages before they reach the lysosome for degradation, a process known as endosomal escape[181,182]. mRNA–LNP endosomal escape is considered inefficient. Given that this process is not entirely understood, it is a research topic of great interest[181,182]. Once mRNA payloads are released into the cytoplasm, they are translated by the cells' ribosomes into the desired proteins.

vessel leakiness around tumours, which is referred to as the enhanced permeability and retention (EPR) effect[55–57]. Unfortunately, xenograft or syngeneic mouse models do not reflect the true complexity of human cancers, and thus the marked EPR effect and nanoparticle accumulation in tumours observed in studies using these models are probably highly exaggerated[58]. In these mice, tumours tend to be highly vascularized and rapidly proliferating, and present a high degree of immune cell infiltration with a simple stromal architecture[58]. The current estimate is that roughly 0.7% of nanoparticles administered intravenously reach solid tumours in mouse models[59,60].

Interestingly, some investigators have suggested that the extravasation of nanoparticles is not entirely passive and could involve transcytosis mediated by receptors such as neuropilin 1, which has increased activity following nutrient deprivation[57]. Beyond this possibility, considerable heterogeneity in uptake exists between tumour types and between patients, and thus various nanoparticle-based diagnostic tools have been developed to identify patients who are most likely to benefit from nanomedicines[58,61]. Using appropriate imaging techniques, these theranostic approaches could help to monitor tumour accumulation of mRNA–LNPs[61]. One potential pitfall is that these nanoparticles do not always adsorb proteins, and thus their biodistribution, which is mainly determined by size, might not mirror that of therapeutic mRNA–LNPs[62]. Therefore, the therapeutic potential of systemically administered nanomedicines remains to be better understood.

After extravasation to the tumour area, LNPs must penetrate the entire tumour in order to disperse adequately between malignant cells. Studies of tissue penetration have shown that particles >20 nm in diameter barely penetrate the tumour area owing to high cell density and interstitial fluid pressure[61,62]. Theoretically, the minimum diameter of mRNA–LNPs is 20 nm, although they are generally 50–150 nm[63,64].

To facilitate penetration of nanoparticles, matrix-modifying agents (such as enzymes and cytokines) have been tested in preclinical studies[61]. One such approach includes a multiplexed dendrimer that, among other components, incorporates an siRNA against FAK, and thus decreases the anchoring of transfected cells to the ECM[19]. Another approach is a nanoparticle system that incorporates polymers that,

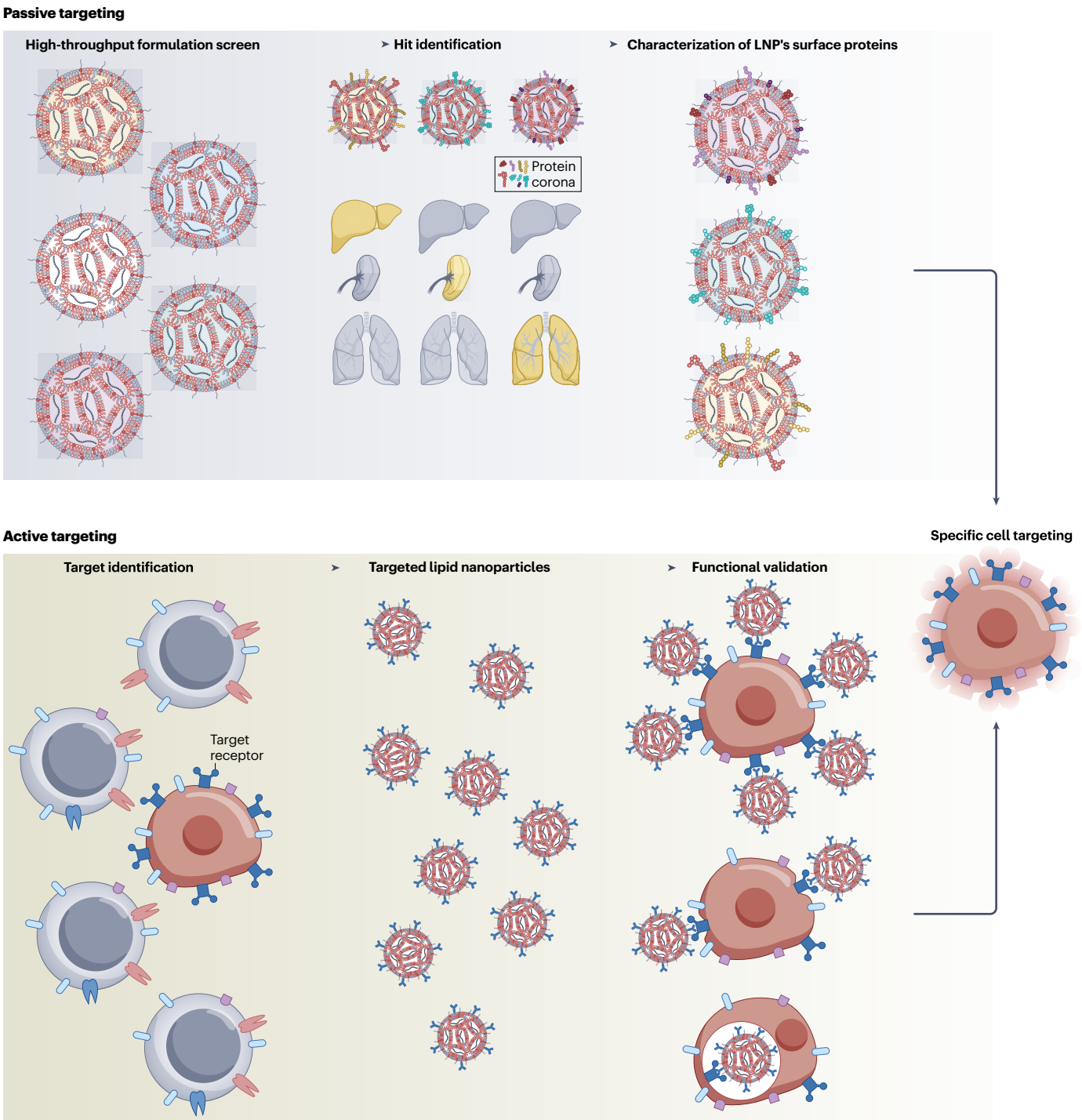


Fig. 2 | Strategies for targeting mRNA–LNPs to tumours. Passive targeting strategies rely on high-throughput screening of multiple formulations in vivo, assessing aspects such as specific tissue distribution (for example, in the liver, spleen and lung). After finding the optimal mRNA–lipid nanoparticle (LNP) formulation for a certain application, reverse engineering is used to discover the mechanisms underlying passive tissue uptake. Active targeting strategies involve identifying a target highly or selectively expressed on the surface of cancer cells. A targeting strategy is then selected and developed as a targeted mRNA–LNP. Finally, the ability of targeted mRNA–LNPs to specifically internalize into target cells and cause a biological effect is examined.

in response to tumour extracellular acidity, break down into smaller dendrimers with notably smaller sizes to enhance tumour penetration[46,65]. Eventually, the use of approaches that regulate tumour fluidics and ECM could be crucial to improve the penetration efficiency of mRNA–LNPs. Interestingly, modifying the ECM with mRNA–LNPs could enhance lymphocyte infiltration[19]. The notion of using mRNA–LNPs to harness the ECM and enhance immune cell infiltration is an exciting avenue for future drug development.

Owing to the aforementioned challenges, direct targeting of solid tumours with systemically administered mRNA–LNPs is hard to achieve. Current efforts using this administration route focus on intramuscular or subcutaneous injections of cancer vaccines that do not attempt to target tumours directly. Cancer vaccines are LNP-based therapeutic agents that do not require cancer tissue enrichment or high levels of protein expression to be effective[66–68]. Some cancer vaccines rely on passive targeting for delivering encoded antigens to DCs[69–71]. These formulations exploit the robust antigen presentation function of DCs and the ability of some subsets of classic DCs to elicit a strong $CD8^+$ T cell-mediated antitumour response[72].

Subsequent efforts will most probably focus on differential delivery of mRNA–LNPs to cancer cells depending on their tissue location, such as the liver, lymphoid organs and bone marrow, that can be endogenously targeted by tweaking formulations[73–75]. Liver expression can be targeted to treat hepatocellular carcinomas or to harness the non-malignant liver to produce antitumour proteins, and leukocytes in lymphoid organs can be manipulated to activate their antitumour function[74,76]. These different cellular biodistributions are attributed to novel ionizable lipid structures and formulation optimization, such as different percentages of PEG-linked lipid incorporated in LNPs[77–79]. However, passive targeting has limited cellular specificity, and the transfection of some cell types with nanoparticles that do not incorporate targeting ligands is challenging.

Active targeting strategies

Active targeting is considered by many to be the holy grail of drug delivery. In the context of mRNA–LNPs, active targeting involves delivery to a specific cell type by modifying the surface of the nanoparticles with targeting moieties that range from small-molecule ligands to monoclonal antibodies. When directed to cancer cells, active targeting approaches have the advantage that they facilitate cellular uptake of mRNA–LNPs, which is crucial for the activity of nucleic acid-based payloads[61]. When directed to non-malignant cells, active targeting approaches can enable the delivery of mRNAs into cells that would otherwise be unlikely to take up LNPs and can be harnessed as living antitumour therapies, such as lymphocytes[80,81]. For example, 30–50% of T cells in mice were transfected using CD4-targeted mRNA–LNPs (as opposed to <10% with other methods)[82]. Here, we discuss several considerations for designing active targeting strategies.

Target selection. For direct targeting of cancer cells, the ideal target is an entirely tumour-specific antigen (TSA) that binds another molecule and is rapidly internalized afterwards (thus, enabling the entry of nucleic acid payloads into cells), and has a controllable biological response upon binding. Despite >400 cell surface proteins having been identified in human cells, this ideal opportunity rarely presents itself. Given that TSAs are rare, many efforts focus on targeting clinically meaningful, highly expressed tumour-associated antigens (TAAs) that are enriched on target cancer cells but are not necessarily exclusive to them.

TAAs, TSAs and immune-regulating receptors can be targeted using antibodies (full antibodies or fragments), peptides, sugars and other small molecules. Reported targets and the molecules that bind them include CD44 and hyaluronic acid, folate receptors and folate, and EGFR, CD29, CD38 and PD-L1 with specific antibodies[4,18,19,46,83–87]. Regarding the choice of targeting moieties, peptides and small molecules are generally easier to manufacture than antibodies, and are usually easier to translate from animal models to humans owing to differences between human and murine TSAs and TAAs regarding receptor structures, and the need to design highly specific antibodies that target them. Examples of peptide-targeting moieties include the iRGD and iNGR peptides, which have been functionalized by lipid conjugation to deliver payloads to bone metastases and solid tumours[88–92]. Nevertheless, antibody-based targeting enables the highest degree of cell specificity. Harnessing antibody derivatives, such as single-chain variable fragments (scFv), is an attractive option that minimizes the risks of host-versus-drug responses associated with the presence of Fc domains.

When considering possible targets, one should address the activity of the targeted ligand and the potential biological effects of the interaction[93]. For example, CD3-targeted mRNA–LNPs used to deliver mRNA payloads to T cells have been shown to have adverse effects, such as the activation and depletion of circulatory and splenic T cell subsets, regardless of payload activity[93]. While T cell activation can be useful, uncontrolled activation can be detrimental owing to potential overactivation and T cell exhaustion. Next, tumoural and patient heterogeneity should be addressed. For example, HER2, a common breast cancer biomarker, is present in only 15–20% of patients with this cancer type and its expression levels can vary between cells within the same tumour[94,95]. Further complicating the matter, target expression can fluctuate at different disease stages; for example, the extracellular domains of certain targets can be cleaved or shed from the membrane during inflammation or they can be modified differently after translation[95–97]. Notably, increased ectodomain shedding of MET, a known cancer target, has been reported to correlate with disease progression in various cancer types[98–100]. Additionally, HER2 expression levels have been reported to change with disease progression and recurrence, and HER2 status significantly changes from negative to low expression in recurrent disease[101,102]. Furthermore, hyperglycosylation is a reported drug resistance mechanism that prevents antibody–target binding and correlates with metastasis formation. For example, MUC proteins are a group of highly glycosylated proteins, and their overexpression has been reported to sterically hinder antibody targeting in cancer disease by forming a mucus boundary layer[103].

Given that TSAs are rare, researchers have devised strategies for improved TAA targeting. This limitation can be addressed by adjusting the affinity and avidity of the targeting approach. High-affinity targeting interactions can result in the binding site barrier phenomenon[61,104], whereby targeted nanomedicines tend to adhere to the first layer of cells expressing the antigen on the exterior of the targeted area. In silico models have predicted that multivalent, lower affinity binding with a flexible linker joining a payload (such as a drug or delivery vehicle) to its targeting moiety can improve the efficiency of targeting of cells highly expressing the antigen[105–108]. Using such a strategy, the individual bond is weaker, yet the overall avidity is higher than that achieved with approaches without a linker. The targeting moiety only weakly binds receptors found scarcely on off-target sites and binds more strongly to the target cells, which have a higher density of target receptors, owing to avidity and not specificity[106]. Interestingly, contrasting reports

exist on the influence of ligand valency and its ability to activate an immune response and change natural internalization pathways[46,109,110]. For example, in an in vitro model, nanoparticles functionalized to folate were internalized upon binding of the folate receptor pathway via clathrin-mediated or caveolae-mediated endocytosis depending on valency[109]. Of note, most models used to study ligand–receptor interactions were developed before the emergence of mRNA–LNPs and thus, although they can be a good starting point to design mRNA–LNP-based strategies, more research is warranted.

Once a target with suitable on-target and limited off-target expression is identified, the next step is to devise an optimal strategy for post-binding internalization. Given that RNA therapeutics must reach the cytoplasm to execute their functions, internalization and endosomal escape are crucial for the activity of mRNA–LNPs (Fig. 2). Most of the data on targeting and receptor internalization comes from studies of antibody–drug conjugates (ADCs). Understanding receptor internalization rates using these data is a good starting point, although the features that apply to ADCs do not necessarily apply to mRNA–LNPs because the latter are generally larger than the former. For both ADCs and mRNA–LNPs, however, the rate of internalization seems to be positively correlated with efficient payload release[81,111,112]. In vivo internalization rates and mechanisms are hard to predict; therefore, many efforts to develop ex vivo systems to best recapitulate this process are underway[113–115]. Close examination of the targeting moiety, target ligand and their interaction is insufficient to predict internalization without considering the interaction in the entire targeting complex context, which can alter the natural interaction. For example, non-natural binding partners, such as targeting antibodies, can be internalized after binding with receptors that do not necessarily internalize upon interaction with their natural ligand[96]. Furthermore, binding can alter the internalization pathway used. For example, EGFR internalization upon binding to the antibody cetuximab can shift from clathrin-mediated internalization to macropinocytosis, based on the modification of the antibody and the subsequent effect of the complex size. Internalization through the latter route can be beneficial for mRNA–LNP delivery because nanoparticles are large and macropinocytosis involves larger membrane areas[112]. Furthermore, EGFR–antibody-mediated internalization is more efficient with antibodies targeting different receptor epitopes than with those targeting a single epitope[112]. Another finding from in vitro models is that, upon binding to ECM ligands, CD44 is internalized more quickly in cancer cells, which can overexpress this protein, than in non-malignant cells, which have lower levels of expression[83,116]. Beyond effective delivery of payloads, if antibodies are not internalized they risk triggering effector functions, such as antibody-dependent cellular cytotoxicity and other Fc region-triggered activities in macrophages[117]. Owing to all these considerations, beyond screening, a deep biological understanding of the targeting interaction, its context in disease, and empirical evidence are crucial for efficient targeting design.

Design of mRNA–LNPs incorporating antibodies. Two main methods can be used to functionalize targeting antibodies on the surface of LNPs. The first involves including an 'anchor' lipid in the LNP formulation to which a functional ligand can be conjugated after particle preparation (Fig. 3a). Frequently used anchor lipids include phosphatidyl ethanolamine, PEG–maleimide and phosphatidyl inositol, which can be chemically conjugated to targeting antibodies[86,118–120]. This method enables controlling the number of targeting moieties present on the LNP via adjustment of the molar mass of the anchoring lipid. However, this method also facilitates the conjugation of incomplete or incorrectly oriented antibodies, such as antibodies with orientations where Fc regions are exposed, which can result in unwanted immune reactions and affects the pharmacokinetic profile and distribution. Furthermore, this strategy is inefficient and results in multiple cleaning stages to remove unbound targeting moieties and large amounts of waste. The second strategy involves preparation of the LNP formulation followed by post-insertion of a hydrophobic antibody derivative (for example, a lipid–ligand conjugate) into the preformed LNPs by optimized co-incubation[18,85,86,117,118,120,121]. This approach does not require a reactive anchor lipid in the LNP formulation and enables better control of the antibody–lipid conjugation[117]. Post-insertion has been proposed to better preserve the original physicochemical properties of LNPs, and methods to optimally post-insert these vesicles have been described[122]. One example is an 'anchored secondary scFv enabling targeting' molecule that can be post-inserted into LNPs and acts as a universal linker by binding the Fc regions of targeting antibodies of choice (Fig. 3b). This system has been successfully harnessed to deliver mRNA payloads to specific immune cell populations and cancer cells in mice[18,84–86].

A wide range of targeted mRNA–LNP-based applications could potentially be used in oncology, of which several are currently being investigated preclinically. While targeting approaches can drive payloads into specific cells that would not necessarily passively take up LNPs, some of the barriers to passive targeting, such as tissue penetration to solid tumours as discussed above, remain unaddressed.

Clinical implementation

Ongoing oncology trials of mRNA–LNP-based therapies focus on local administration of cancer vaccines or intratumoural expression of immunostimulatory cytokine mixtures combined with ICIs[14,123,124] (Table 1; Supplementary Table 1). The approaches tested generally differ in the antigens selected or the cytokines used. To date, most approaches based on systemic administration, such as cancer vaccines, use mRNA lipoplexes. These are lipid-based nanoparticle formulations formed by mixing pre-made cationic-lipid liposomes and RNA, yielding a self-assembled molecular organization[125,126]. LNPs and lipoplexes differ in lipid composition, preparation methods and morphology. mRNA lipoplexes have been reported to elicit pro-inflammatory cytokines in vivo and, owing to potential decomplexation of RNA payloads on their surface, have a greater tendency to aggregate in circulation than mRNA–LNPs[127,128].

Experts developing cancer vaccines are attempting to encode multiple antigens simultaneously and thus provide personalized vaccines. Such vaccines rely on the strengths of the mRNA therapeutic platform: its modularity, ability to incorporate numerous payloads, and speed of manufacture. For example, mRNA-4157 is an mRNA–LNP vaccine that can include up to 34 neoantigen peptides in a single mRNA concatemer encoding the polyepitope, which can be achieved by encoding short epitope concatemers interspersed by cleavage sensitive sites. mRNA-4157 is currently being tested in phase I and II trials. In the phase II KEYNOTE-942 trial, patients with resected high-risk melanoma (stage III–IV) receiving this vaccine in combination with the ICI pembrolizumab had statistically significant improvements in recurrence-free survival and distant metastasis-free survival relative to those receiving pembrolizumab alone.

Personalized cancer vaccines require high speed of product manufacturing. The time from sequencing of patient-derived tumour samples to production and administration of personalized vaccines has been reported to be as short as 30–40 days[129]. This is because, unlike therapies based on small molecules or proteins, most mRNA

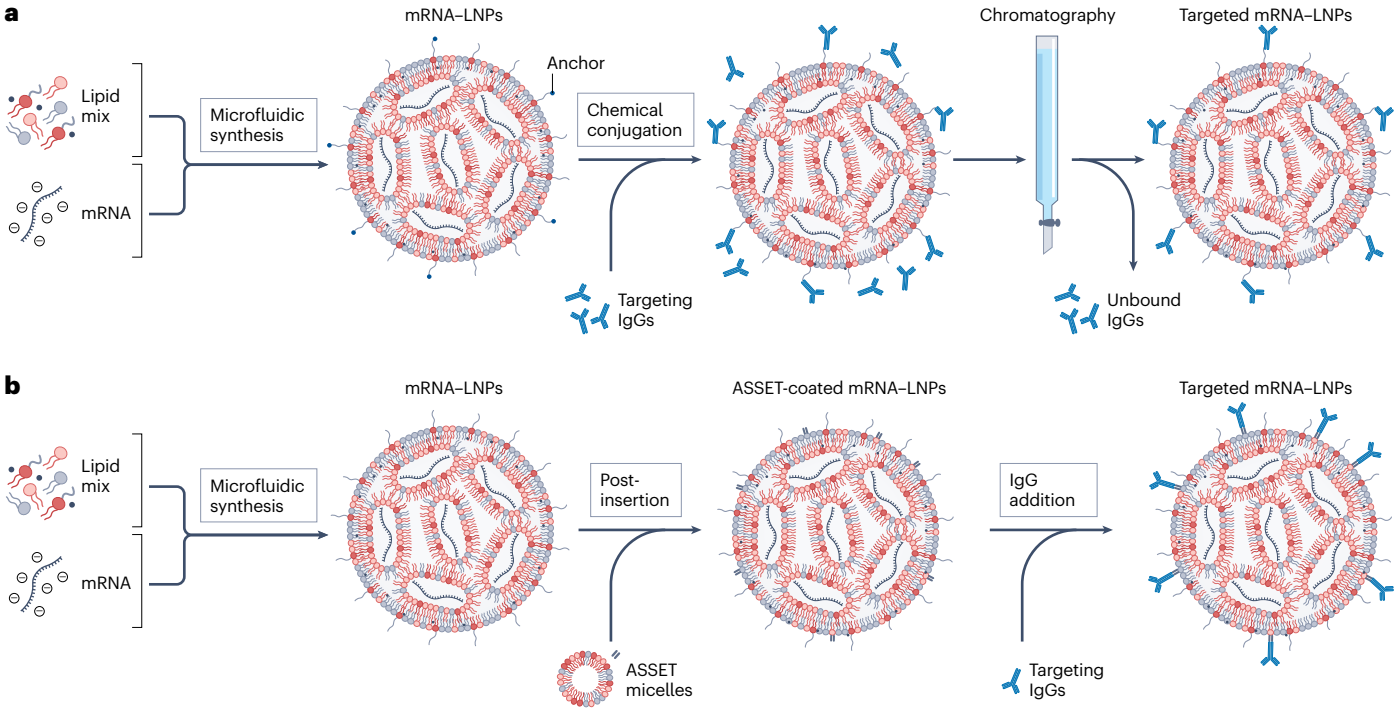


Fig. 3 | Strategies to functionalize antibodies on LNPs. a, Lipid-anchored strategies involve forming lipid nanoparticles (LNPs) with formulations that incorporate lipids with a chemically reactive group ('anchor' lipid). Targeting antibodies are then chemically conjugated to the surface of the particles via the reactive group. Excess antibodies are cleared by chromatography to purify targeted LNPs. **b**, The anchored secondary scFv enabling targeting (ASSET) strategy involves forming mRNA–LNPs followed by post-insertion of ASSET protein purified in micelles into the LNPs The ASSET–LNPs are then coated with the targeting IgGs through interaction of their Fc region with the scFv domain in the ASSET to preserve their active conformation and control their orientation on the LNP surface.

payloads differ only in nucleotide sequence, and therefore individualized manufacturing can be carried out rapidly[130]. Regarding versatility, mRNA–LNPs can be administered via various routes beyond those traditionally used, such as inguinal lymph injections guided by ultrasound imaging[131]. In a first-in-human study involving patients with stage III–IV melanoma, ultrasound-guided intranodal injection of individualized cancer vaccines resulted in all patients developing T cell responses against vaccine neoepitopes. The study demonstrated a decrease in metastatic events in all patients[131]. This variability further expands the accessibility and adaptability of mRNA–LNPs to different tumour locations and properties.

Treatment regimens generally involve multiple injections; for example, in a phase II trial BNT111 is being administered six to eight times in combination with the anti-PD-1 antibody cemiplimab[124] (Table 1). Another example is a clinical trial of personalized cancer vaccines for pancreatic cancer. In this study, the treatment regimen involved administration of an ICI and one to eight doses of personalized cancer vaccine followed by 12 bi-weekly doses of fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX)[126]. These multiple injection regimens can result in LNP reactogenicity, lipid accumulation in tissues over time and clearance by anti-drug antibodies. Nevertheless, BNT111 received Fast Track designation from the FDA for the treatment of metastatic melanoma, indicating that at the current development stage, the benefits in these patients are considered to outweigh the potential deficits. While cancer vaccines are the most clinically advanced application of mRNA–LNPs in oncology, other attempts include expression of pro-inflammatory cytokine mixtures, of bispecific antibodies for target specific stimulation, of chimeric antigen receptors (CAR) in T cells in patients with solid tumours and even of epigenetic regulators. Examples of studies testing these approaches include a phase I trial of intravenously administered mRNA–LNPs encoding bispecific antibodies targeting claudin 6 and CD3, early clinical trials assessing intravenously administered mRNA–LNPs encoding IL-2 and IL-7 (ref. 132) or intratumoural injections of mRNA–LNPs encoding OX40L, IL-23 and IL-36γ[133], and a phase I trial testing CAR antigen vaccines (CARVac) intended to enhance the efficacy of CAR T cells in the challenging setting of solid tumours[134] (Table 1).

One potential aspect that can be improved to further advance new clinical opportunities in the field is the 'gap' between mouse models and patients, which is well known in oncology and is becoming increasingly recognized in the LNP field as well. For example, cancer vaccine doses typically used clinically are in the microgram per kilogram range, whereas in mice they can reach ≥0.25 mg/kg[67,130,135]. Further complicating matters, studies have demonstrated that mRNA–LNP vaccines induce secretion of IL-1 in humans, whereas in mice they upregulate IL-1 receptor antagonist protein. This differential activation might explain why mice can tolerate doses of LNPs up to 1,000-fold higher than those tolerated by humans[136]. Data from several studies indicate that the ionizable lipid component induces inflammatory responses independently of administration route, causing rapid neutrophil infiltration[137]. Beyond immunogenicity, other studies have claimed that mRNA–LNP delivery is different among mice, non-human primates and humans[138,139]. For example, formulations optimized for expression in rodent liver tissue are not necessarily optimized for expression in

larger animals or humans[138,139]. These discrepancies between mice and humans could reflect other yet-to-be-revealed variations in activity and should be further explored in future studies.

Outlook: design considerations

The use of mRNA–LNPs faces some challenges, beyond improving targeting strategies. One of these challenges is the instability of mRNA–LNPs at refrigerator temperature, which is considered to be driven by the inherent instability of the mRNA payload rather than the instability of the LNP vehicle[2,140,141]. Clinical trials testing non-frozen and lyophilized preparations are underway; nonetheless, this challenge is more relevant to the distribution of prophylactic vaccines for infectious agents (NCT05137236, NCT05085366, NCT04816669) than for oncology applications[142–144]. Next, LNPs are not inert and can increase cytokine levels in the host, causing immediate inflammation. This property might be beneficial for immunotherapy purposes; however, complement activation-related pseudoallergy responses following mRNA–LNP administration are frequently attributed to the PEG–lipid components of the LNP[145,146]. Furthermore, anti-PEG antibodies have been detected in individuals who have received an mRNA–LNP-based COVID-19 vaccine[147,148]. For regimens requiring repeated dosing, such as those used with mRNA–LNP-based cancer therapies, these anti-PEG antibodies might lead to rapid clearance of LNPs, impeding treatment efficacy. To this end, researchers are seeking alternatives to PEG–lipids, although, to date, PEG remains the stealth molecule most commonly used in formulations. Other studies are attempting to improve the

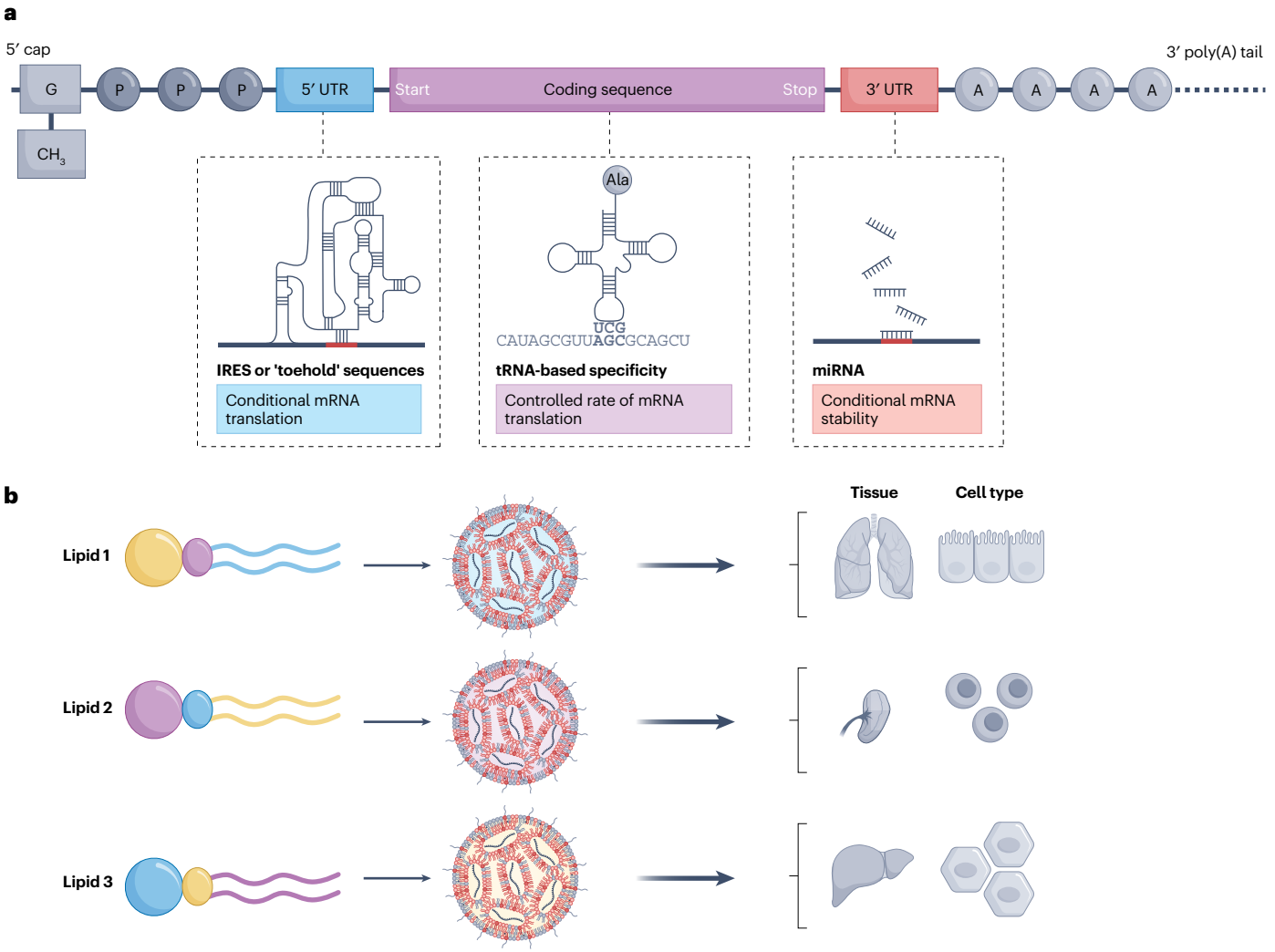


Fig. 4 | Future directions for improved targeting strategies. a, Conditional mRNA payload expression could be regulated by specific sequences encoded on the mRNA payload itself. Some approaches utilize reactive 3D structures in the mRNA 5′ untranslated region (UTR) that can activate or prevent translation initiation in the presence of molecules and/or sequences differentially present in target cells. IRES regulation of translation initiation is highly suitable for these approaches because it obliges initiation to depend on the 3D structure. Additionally, distinctive tRNA expression patterns between cell types can be used to translate sequences at different rates in different cellular populations and/or tissues. Finally, binding sequences for cell-specific microRNAs (miRNAs) could be incorporated into the UTRs to affect degradation rates in different cellular populations. **b**, Another approach to improving mRNA–LNP targeting would rely on a better understanding of the factors affecting the biodistribution of different LNP formulations. Specifically, the structure–activity relationship between different ionizable lipid structures and certain organs and cellular distribution tendency requires a better mechanistic characterization.

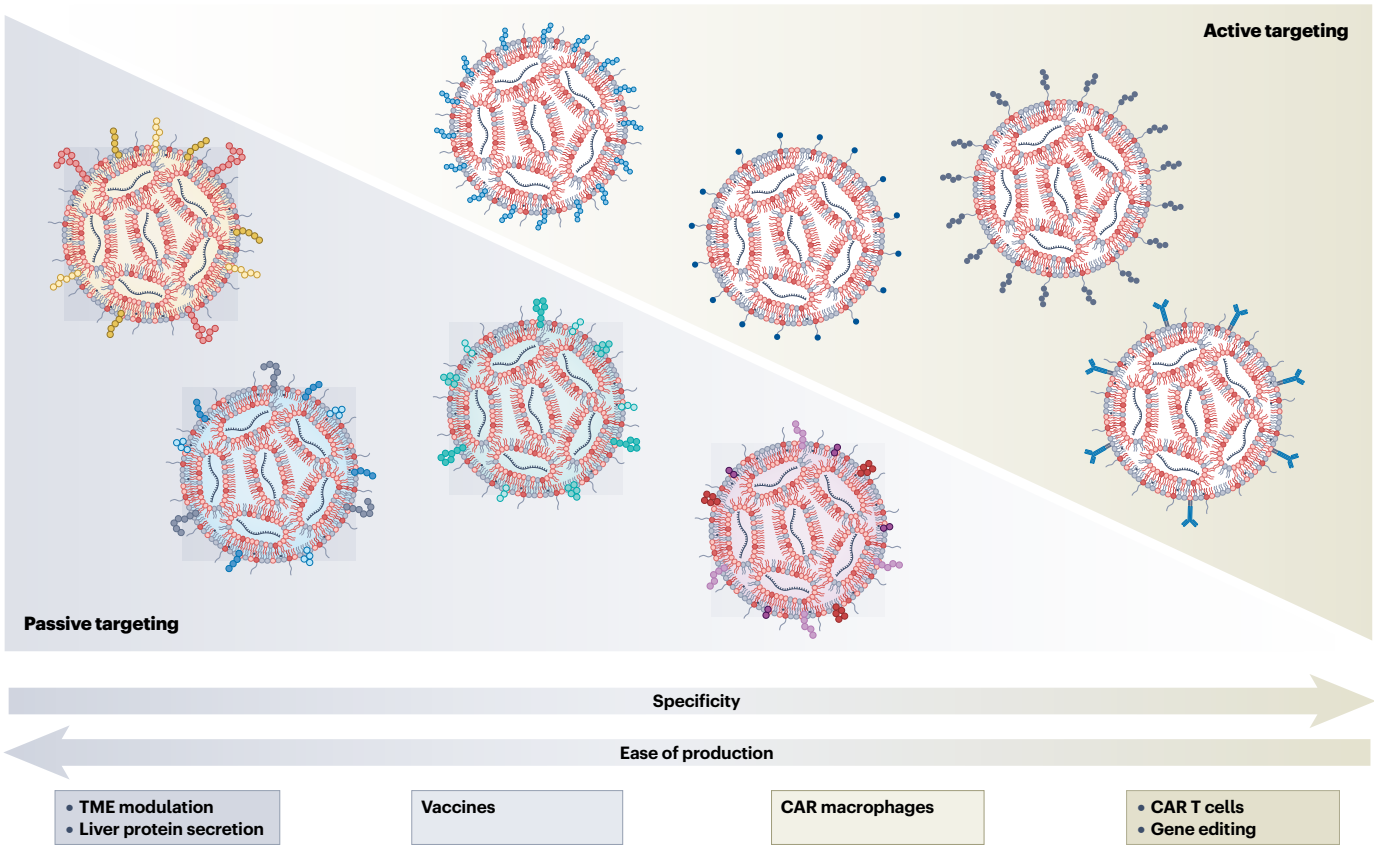


Fig. 5 | Considerations and potential applications of passive and active strategies for targeting tumours with mRNA–LNPs. The choice between active and passive targeting strategies for targeting tumours using mRNA–lipid nanoparticles (LNPs) involves a compromise. Compared with passive approaches, active approaches enable higher specificity in cell targeting but their manufacturing is more complex. An important aspect to consider is that, as depicted, each targeting strategy is better suited for certain therapeutic applications. CAR, chimeric antigen receptor; TME, tumour microenvironment.

clearance of ionizable lipids, resulting in increased biocompatibility and reduced adverse effects. For example, DLin-MC3-DMA, the ionizable lipid in the first RNA–LNP approved, has a long liver tissue half-life[149,150]. Pharmacokinetic analyses from a phase II trial of patisiran in patients with hATTR amyloidosis demonstrated that siRNA–LNPs can reach the liver within 1 h of intravenous injection, yet elimination from the liver took 3 days[151]. To improve clearance, next-generation, biodegradable lipids frequently incorporate labile ester bonds in their hydrophobic tails[152].

To increase mRNA–LNP specificity and reduce off-target expression, many fascinating attempts to drive differential payload expression based on specific sequences encoded by the mRNA payload itself have been made. From the manufacturing perspective, this strategy is feasible because it relies on encoding programmable sequences rather than on chemical modification of proteins or lipids. Furthermore, this strategy can be harnessed to target cancer cells that lack TSAs. Programmable sequences could either enhance or inhibit expression of targets in specific cell types. For example, downregulation of target expression has been achieved in preclinical models by incorporating miRNA binding sites on the payload to inhibit expression in common sites of mRNA–LNP accumulation following systemic administration, such as miR-122 in the liver[153–155]. Interestingly, programmable sequences have also been used for cell-specific translation. Translation activation can be programmed by manipulating the structural dynamics of the mRNA molecule; for example, 'toehold' sequences in 5′ untranslated regions of mRNAs hinder translation unless they are bound by a transcript specific to the target cell. Furthermore, when inducible IRES sequences are used to control the initiation of translation, expression can be more effectively regulated by mRNA structural shifts[156,157]. Payload-specific differential expression can also be based on the selection of codons in the open reading frame, taking advantage of the proposed non-random distribution of tRNAs expressed in different cell types and tissues[158–160]. Therefore, cell specificity can also be achieved at the level of expression of the mRNA payload (Fig. 4a).

Many efforts are currently being invested in the expansion of the potential array of uses for mRNA–LNPs in oncology, beyond the applications that have advanced to clinical trials. Firstly, mRNA–LNPs are being used for T cell transfection in vivo to generate CAR T cells in situ. Compared with autologous CAR T cells, the proposed advantages of this approach are the lack of genomic integration, reduced production costs and minimal toxicities owing to the transient nature of the mRNA payload, which could limit the adverse systemic immune response and toxicities derived from the sustained activity of CAR T cells[161]. Active targeting of mRNA–LNPs to T cells has been achieved using antibodies against CD4, CD3 or CD5 (refs. 82,162,163). Another possibility is

creating CAR macrophages, a cell population highly prone to LNP uptake and considered a promising therapy in patients with solid tumours[164]. These applications of mRNA–LNPs, as well as many others, could benefit from sustained protein expression, now limited primarily by the stability of mRNA molecules in vivo. To address this issue, circular RNA platforms have been gaining attention in the past few years[165].

Another emerging application in oncology is the use of mRNA–LNPs for in vivo genome editing of target cells using tools such as CRISPR–Cas9. In this context, the advantage of using mRNA-encoded genome-editing proteins over the synthetic proteins is the transient expression achieved with mRNA, which limits adverse events[166]. In cancer research, CRISPR–Cas9 editing has been used in preclinical studies and is appealing because of its ability to cause permanent specific changes[18]. CRISPR-based targeting approaches, however, are highly dependent on the ability to specifically direct payloads to target cells. We hope to see this powerful tool used against intracellular cancer targets, as it would provide a novel window of opportunity for genetic modification of targets typically considered undruggable. As with other approaches discussed, the key to the success of these strategies will be the specificity resulting from irreversible genome engineering.

Future improvements in mRNA–LNP-based therapies, in our view, will mostly be aimed at improving cell-specific expression to reach cells more specifically and decrease off-target expression, thus broadening potential applications (Fig. 4). Regarding passive targeting, efforts are underway to characterize the relationships between formulation, structure and activity using artificial intelligence tools[167]. However, the field is in the early stages and thus, large open-access databases that meticulously document LNP formulations and their biodistribution results are lacking. Yet, we expect super-selective cell expression to be mostly achieved only with mRNA–LNPs incorporating active targeting moieties. Finally, to be successful, the clinical translation of active targeting approaches requires novel efforts to reduce the complexity of manufacturing.

Conclusions

In the past few years, promising advances have been made in targeting mRNA–LNPs to tumours using passive or active approaches. The choice of either approach usually depends on a compromise between ease of production and specificity (Fig. 5). The choice of a targeting strategy for a particular malignancy involves considerations such as whether targetable characteristics have been identified, the feasibility of intratumoural injection, and the tendency to metastasize. This decision should also take into account the different payloads available, their off-target toxicity and the importance of specific expression for therapeutic effect treatment efficacy. Manufacturing limitations, although sometimes overlooked during treatment design and postponed to the later stages of the drug development process, should preferably be addressed early on. Indeed, manufacturing considerations could become crucial for a drug to be feasibly produced at the large scales required for clinical implementation. In the past few years, mRNA–LNPs have become a major tool in the prophylactic vaccine space; now, they are being tested in oncology clinical trials. We expect to see increasing cell-specific expression using various targeting methods that will enable even more new therapeutic applications in oncology with this versatile, powerful platform.

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E.K. and N.A.-E. researched data for this article. All authors contributed substantially to the discussion of the content, wrote the article, and reviewed and/or edited the manuscript before submission.

Competing interests

D.P. receives licensing fees (to patents on which he was an inventor), has invested, has consulted, has been on the scientific advisory boards or boards of directors, given paid lectures or conducted research at Tel Aviv University sponsored by ART Biosciences, BioNTech, Earli, Kernal Biologics, Merck, NeoVac, Newphase, Roche, SirTLabs Corporation and Teva Pharmaceuticals. All other authors declare no competing financial interests.

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