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REVIEW

Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems

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Because of its safe and effective protein expression profile, *in vitro* transcribed messenger RNA (IVT-mRNA) represents a promising candidate in the development of novel therapeutics for genetic diseases, vaccines or gene editing strategies, especially when its inherent shortcomings (for example, instability and immunogenicity) have been partially addressed via structural modifications. However, numerous unsolved technical difficulties in successful *in vivo* delivery of IVT-mRNA have greatly hindered the applications of IVT-mRNA in clinical development. Recent advances in nanotechnology and material science have yielded many promising nonviral delivery systems, some of which were able to efficiently facilitate targeted *in vivo* delivery of IVT-mRNA in safe and noninvasive manners. The diversity and flexibility of these delivery systems highlight the recent progress of IVT-mRNA-based therapy using nonviral vectors. In this review, we summarize recent advances of existing and emerging nonviral vector-based nanotechnologies for IVT-mRNA delivery and briefly summarize the interesting but rarely discussed applications on simultaneous delivery of IVT-mRNA with DNA.

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INTRODUCTION

As a rapidly emerging class of nucleic acid therapeutics, *in vitro* transcribed messenger RNA (IVT-mRNA)-mediated transfection offers an attractive alternative to plasmid DNA (pDNA) or viral vector-mediated gene therapy. Once IVT-mRNA has reached the cytoplasm, translation from the IVT-mRNA starts instantly without the need to enter the nucleus to be functional (Figure 1). This feature makes IVT-mRNA not only much more effective in quiescent and hard-to-transfect cells, but also excludes the potential risk of insertional mutagenesis compared with DNA transfection. For a long time, the widespread applications of IVT-mRNA have been limited because of its short half-life and the fact that unmodified IVT-mRNA activates various Toll-like receptors and triggers an increase in cytokine levels and its associated toxicity.[1–4] Great efforts have been invested in modifying different structural elements of IVT-mRNA to systematically improve its intracellular stability and translational efficiency. These advances ultimately lead to the significant increase of protein expression from IVT-mRNA over several orders of magnitudes.[5–7] Meanwhile, the incorporation of naturally occurring modified nucleosides such as pseudouridine, 2-thiouridine, 5-methyluridine, 5-methylcytidine or N6-methyladenosine in IVT-mRNA has been shown to substantially reduce immune stimulation and stabilize the molecule against RNase cleavage.[1,3,8,9] Such stabilized non-immunogenic IVT-mRNA has demonstrated improved cytosolic persistence and protein expression, resulting in therapeutic effects in animal models of genetic diseases.[1,10] Therefore, because of its improvement in stability and reduced immunogenicity, IVT-mRNA could be used for clinical applications in many therapeutic applications in the near future.

Among all the possible clinical applications for IVT-mRNA, immunotherapeutic application is currently the only field in which clinical experience is already available.[11,12] The intrinsic advantages of mRNA-based immunotherapy lie in the self-adjuvant activity of IVT-mRNA and the fact that relatively small amounts of encoded antigen are sufficient to obtain robust efficacy signals.[13] However, the challenge is different in protein-replacement therapies where higher amounts of deficient or defective intracellular proteins are to be substituted through *in vivo* administration of IVT-mRNA.[14] The challenge is particularly difficult when precise targeting of certain cell types and organs is required.[14] For such applications, the absence of safe and effective delivery systems becomes a significant barrier especially for the clinical use in diseases where no activation of immune response is desired. The ideal IVT-mRNA *in vivo* delivery systems are expected to protect their payloads against ubiquitous endonucleases, avoid immune detection, prevent nonspecific interactions with proteins or nontarget cells, allow targeted delivery to tissues of interest and promote cell entry efficiency (Figure 1).[15] Indeed, it has become increasingly clear that the success of gene therapy critically depends on the effectiveness of gene delivery that is largely determined by the delivery systems or carriers.[16–19] In general, three main strategies for IVT-mRNA delivery can be distinguished so far, namely (1) physical methods that transiently disrupt the barrier function of the cell membrane; (2) viral-based approaches that utilize the naturally occurring biological modes of uptake; and (3) nonviral vectors that utilize rationally designed and easily formulated chemical nanocarriers.

Physical methods have been intensively investigated for IVT-mRNA delivery and are to some extent efficient. Electroporation, one prominent example, is now established as a favored method for IVT-mRNA transfection of hematopoietic cell types,[20] and the development of novel devices for electroporation of large numbers of cells in sterile conditions has enabled the

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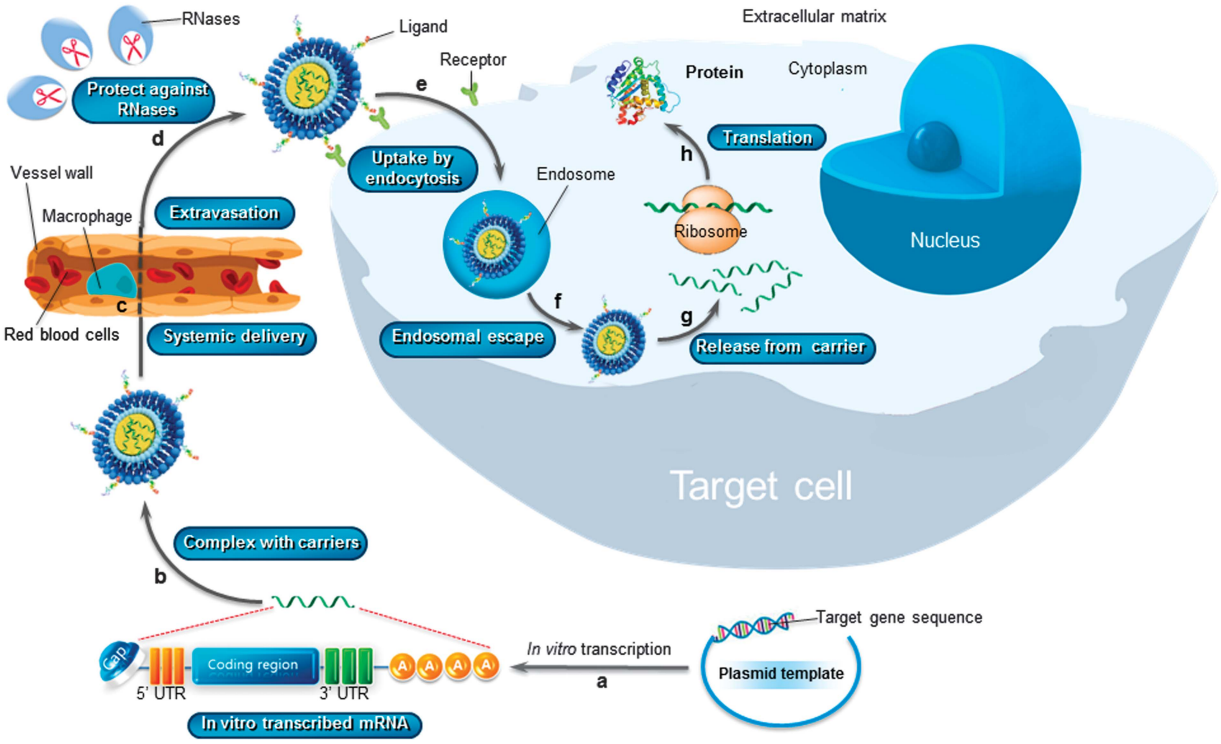

Figure 1. Schematic illustration of the production and successful systemic delivery of IVT-mRNA using nonviral vector approach. (**a**) A linearized pDNA template with target gene sequence is used for the production of IVT-mRNA via *in vitro* transcription in a cell-free system. The structure of IVT-mRNA consists of the cap, 5′ untranslated regions, the coding region (also regarded as the open reading frame), 3′ untranslated regions and the poly(A) tail. All these components can influence the translational activity and stability of IVT-mRNA within cells. (**b**) IVT-mRNA spontaneously forms nanosized complexes with cationic materials via electrostatic interaction. (**c**) After systemic administration of formulations containing IVT-mRNA, they should successfully accumulate to target organ or tissues without the nonspecific interactions with nontarget cells or proteins and should avoid immune detection. (**d**) The delivery system should protect IVT-mRNA against ubiquitous endonucleases within extracellular matrix and (**e**) promote target cell entry efficiency possibly via the interactions between targeting ligand modified on the surface of delivery system and corresponding receptors expressed on the target cell surface. (**f**) Endocytosed formulations will be located in the endosome where numerous enzymes exist, and the carrier of IVT-mRNA should facilitate endosmal escape via endosome disruption. (**g**) IVT-mRNA should be released from cytoplasmic located formulations and transport to ribosome. (**h**) Finally, protein of interest can be translated from IVT-mRNA by using protein synthesis machinery of the target cell.

development of a rapid clinical-grade protocol for a broad range of IVT-mRNA-based cell therapy applications.[21] Nevertheless, physical methods are often harmful to the cells and are overall ill-suited for *in vivo* applications.[22–24] Recombinant viruses have been commonly studied as DNA delivery vector, and there are some reports available where viral vectors have been used as mRNA carriers.[25–27] However, viral vectors may be associated with inherent shortcomings, such as potential reverse genome insertional risks, difficulties to control the gene expression, vector-size limitations as well as strong immunologic side effects.[15,28]

Indeed, an increasing number of investigations on *in vivo* IVT-mRNA transfection have adopted nonviral vectors that have already demonstrated their huge potential for the delivery of various nucleic acids, for example, pDNA and short interfering RNA (siRNA).[15,29] Because of the biocompatible and diversified properties of nonviral vectors, they can be easily formulated with IVT-mRNA and their delivery kinetics adapted to achieve controlled release of therapeutics.[1,30,31] In this review, we focus on the features, drawbacks and prospects of the state-of-the-art nonviral vectors for IVT-mRNA delivery. More information on above-mentioned physical methods and viral vectors can be found in other well-written reviews.[32–35] Finally, we briefly summarize the potential of combined delivery of IVT-mRNA and DNA for future therapeutic applications.

LIPID-BASED IVT-mRNA DELIVERY SYSTEMS

Cationic lipids have become the most ubiquitously utilized and studied nonviral vectors for IVT-mRNA transfection so far. They can spontaneously form lipoplexes with IVT-mRNA through electrostatic interaction (Figure 2a). Numerous studies have reported significantly higher transfection rates using cationic lipids as carriers as compared with other nonviral vectors.[36,37] As a result, a large number of different lipid-based materials have been investigated for IVT-mRNA delivery. Some of those were originally developed for IVT-mRNA delivery, whereas others were reformulated from the other fields, for example, siRNA delivery. We artificially classify and review these lipid-based materials into three categories, namely DOTAP (1,2-dioleoyl-3-trimethylammonium-propane)-based formulations, commercially available liposomal formulations and recently emerged lipid nanoparticles (LNPs) developed from combinatorial synthetic methodology and library screening.

Historically, DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride) was the first synthetic cationic lipid used to condense and deliver IVT-mRNA encoding luciferase into different cell lines *in vitro*.[38] Its derivative, DOTAP, is relatively cheap to prepare and possesses superior IVT-mRNA delivery efficiency, therefore making it a extensively studied cationic lipid in this field.[39,40] Several studies aimed at further enhancing DOTAP-mediated IVT-mRNA transfection have been performed. Zohra *et al.*[41] modified DOTAP-based liposomal carriers with

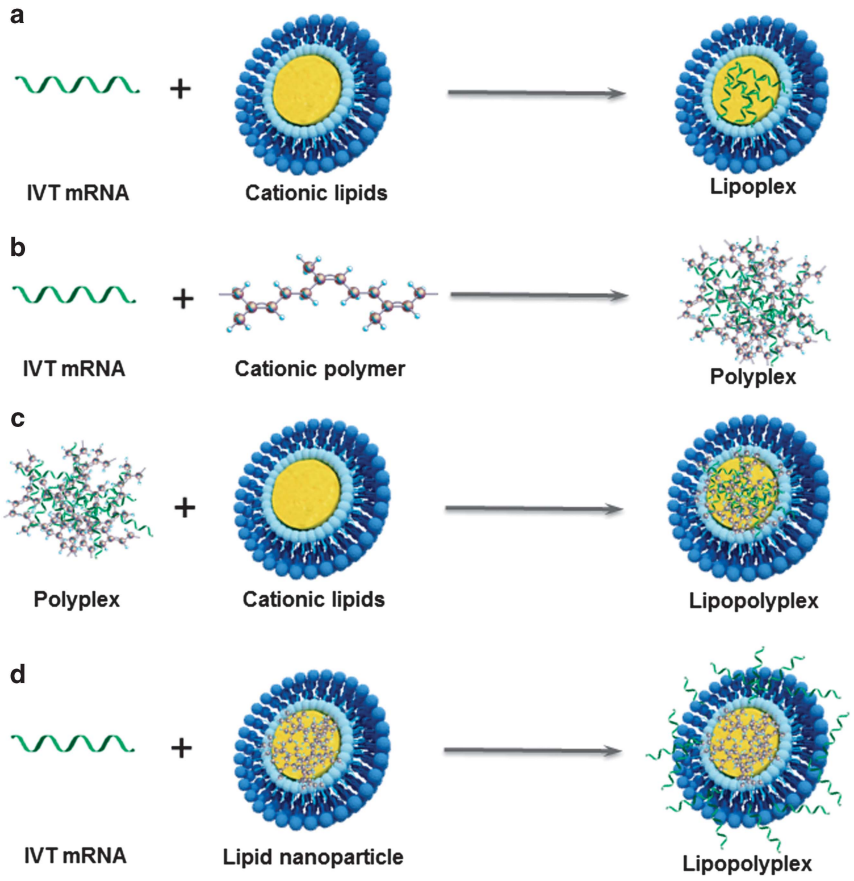

Figure 2. Representative scheme of the structure and preparation of different nonviral vectors for IVT-mRNA delivery. (**a**) Negatively charged IVT-mRNA spontaneously interact with cationic lipids to form lipoplexes. (**b**) Cationic polymers condense IVT-mRNA into nanoparticles and result in stable polyplexes. (**c**) After being complexed with cationic polymers, IVT-mRNA containing polyplexes could be further enveloped by a lipid 'shell'. (**d**) Lipopolyplex formed by a polymer 'core' and a lipid 'shell' with IVT-mRNA absorbed on the nanoparticle surface.

carbonate apatite (an inorganic crystal that has a strong affinity for nucleic acids), and reported a 5–15-fold higher transfection compared with control group (only DOTAP). They further identified enhanced IVT-mRNA cellular uptake with the help of inorganic additives through effective endocytosis as the major contributor to the observed higher transfection efficiency.[42] In a recent study, the same authors demonstrated that incorporating fibronectin, a recognition motif to target $\alpha_5\beta_1$ integrins, into DOTAP-apatite particles loaded with IVT-mRNA enhanced transgene expression of delivered IVT-mRNA (both qualitatively and quantitatively) in HeLa cells compared with nontargeted particles.[43] All these studies highlighted the important role of cellular uptake in the delivery of IVT-mRNA. Another strategy to improve DOTAP-mediated IVT-mRNA transfection is the utilization of neutral lipids to improve endosomal escape. DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) is one of the most commonly used candidate that shows enhancement on gene expression when coapplied with cationic lipids.[44] This effect of DOPE is attributed to its ability to facilitate liposome forming and its tendency to transit lipoplexes from a bilayer structure to a hexagonal arrangement under acidic pH at the endosomal level that may facilitate the fusion or the destabilization of endosomal membranes.[45] Besides endosomal escape function, DOPE could also act as a helper lipid for DOTAP-based cationic lipids to reduce aggregation of the lipid systems.[46]

In addition to promoting cellular uptake, positive charges of cationic lipids promote nonspecific interactions with nontarget cells and extracellular components such as negatively charged serum proteins, thereby resulting in the rapid clearance of such formed aggregates.[47,48] The advantage of positive charges from cationic lipids *in vitro*, however, hampers their *in vivo* application and clinical development. The most common way to partially overcome this hurdle is to shield the nanoparticle surface with hydrophilic, uncharged polymers such as polyethylene glycol (PEG). This strategy has been widely used in delivery of pDNA and siRNA payloads to improve the *in vivo* transfection profile, reduce nonspecific binding and increase the circulation half-life.[49,50] Besides, the terminal of PEG structure could be further modified with certain ligand(s) to endow the formulation with active targeting ability that is a crucial parameter influencing the delivery efficacy of many delivery systems. With the help of targeting ligand(s), not only the amount of complexes that localize to target organ/tissue could be greatly enhanced, but also the nonspecific absorption by unwanted cells could be minimized. Moreover, the effects of a targeting ligand go well beyond cell-specific uptake. Properties like the extent and kinetics of nanoparticle uptake by endocytosis as well as their intracellular fate can be potentially modulated by the conjugated ligands presented on the carriers. A successful example based on these strategies could be demonstrated by Wang *et al.*[51] who applied PEGylation and ligand incorporation to a lipid-based IVT-mRNA delivery system for systemic delivery to tumors. In this study, DOTAP/cholesterol liposomes were coated with DSPE-PEG (1,2-distearoyl-phosphatidylethanolamine-polyethylene glycol) and DSPE-PEG-anisamide. The hydrophilic PEG molecules were expected to screen the positive charge by DOTAP/cholesterol lipid bilayer and anisamide could facilitate receptor-mediated internalization of the nanoparticles into the sigma receptor overexpressing cancer cells.[51] These

formulations demonstrated stability, high *in vitro* transfection ability, low cytotoxicity, specific targeting to tumor site and efficient *in vivo* anticancer action.[51]

It is worth mentioning that some commercially available liposomal formulation, such as Lipofectamine and Dreamfect Gold, proved to be potent carriers for IVT-mRNA transfection.[52] In our recent study, complexes formed by Lipofectamine 2000 and IVT-mRNA encoding green fluorescent protein transfected $>50\%$ of human bronchial epithelial cells, whereas the number of green fluorescent protein-positive cells obtained by polymer-based formulations was 3%.[53] However, these reagents can have limited utility *in vivo* due in part to their toxicity and poor transfection potency.[54] Another promising lipid-based formulation that should be emphasized is GL67A that is considered as the gold standard in nonviral respiratory gene transfer.[55,56] Previous studies have extensively demonstrated their therapeutic potential, the low toxicity and safety profile in many preclinical and clinical trials.[57,58] Andries *et al.*[59] suggested that GL67A formulations also facilitate pulmonary delivery of IVT-mRNA. However, more investigations, especially *in vivo* studies, should be performed to screen and validate the IVT-mRNA delivery potency of GL67A-based formulations.

Taking advantage of the recent innovations in systemic delivery of siRNA using LNPs, a growing number of reports have suggested the potential of various LNPs in IVT-mRNA-based therapy. LNPs are composed of cholesterol (aids in stability), naturally occurring phospholipids (support lipid bilayer structure), a PEG derivative (decreases aggregation and nonspecific uptake) and an ionizable lipid (complexes negatively charged RNA and enhances endosomal escape).[29] Evidence within the siRNA delivery community has regarded the ionizable lipid as the most pivotal component for efficacy. A ionizable lipid, DLinDMA (1,2-dilinoleyloxy-3-dimethylaminopropane), which is highly effective at delivering siRNA systemically in rodents and non-human primates,[60] was used as the main component of LNPs for IVT-mRNA vaccination by researchers from Novartis institutes.[61,62] Ethanol dilution was used to produce small uniform lipid particles in size range of 79–121 nm with a high degree of IVT-mRNA encapsulation.[61] This unique vaccine technology was found to elicit broad, potent and protective immune responses in mice that were comparable to a viral delivery technology, illustrating the huge potential of this type of lipid-based delivery system.[61] Because of the inherent structural differences between IVT-mRNA and siRNA, improving IVT-mRNA delivery will definitely require further optimization in the materials used for delivery. Rational design has been made to increase the potency of DLinDMA by systematically varying structural elements in different regions of the lipid. Several new structural analogs have been synthesized through this approach. These analogs, though having only minor chemical differences, have demonstrated remarkable improvement in potency.[54] These include synthesis of DLin-KC2-DMA, DLin-MC3-DMA and L319.[63] Recently, Thess *et al.*[64] used DLin-MC3-DMA to encapsulate and deliver IVT-mRNA coding for erythropoietin in mice, pigs and non-human primates. Therapeutically relevant concentrations of erythropoietin were achieved following intraperitoneal or intravenous injection of sequence-optimized IVT-mRNA.[64]

Another class of lipid-like materials, characterized by polyamine cores and multiple hydrophobic tail moieties, has also been developed for siRNA delivery through the use of combinatorial synthetic methodology and library screening.[65,66] This class of lipids, including C12–200, cKK-E12, and 503O13, constitutes some of the most potent, state-of-the-art materials for siRNA delivery.[54] Recently, Anderson and colleagues[44,67] have shown that C12–200 LNPs originally developed for siRNA delivery can be optimized specifically for IVT-mRNA delivery. The potency of IVT-mRNA-loaded C12–200 LNPs *in vivo* increased over sevenfold compared with the original formulation by varying the LNP formulation parameters including lipid weight ratios, phospholipid identity and excipient composition.[44] Increased ionizable lipid/mRNA weight ratios and the incorporation of DOPE were considered as key features of the optimized formulation. Interestingly, this optimized lipid nanoparticle formulation did not improve siRNA delivery, indicating differences in optimized formulation parameter design spaces for siRNA and IVT-mRNA.[44] Another recent study supporting the notion that lipid-like materials originally synthesized for siRNA might contribute to advancements in IVT-mRNA delivery is performed by Turnbull *et al.*[68] They successfully demonstrated that C14–113 LNP-based formulation represents an effective nonviral agent for efficient cardiac delivery of IVT-mRNA in small and large animals, via multiple routes of administration, in healthy and disease hearts, using IVT-mRNA doses that are orders of magnitude lower than previous studies.[4,68]

This optimization of lipid-like materials for IVT-mRNA delivery entails not only reformulating existing materials but also developing new materials. The Anderson group[69] sought to design and synthesize novel LNP components capable of delivering IVT-mRNA with unprecedented levels of *in vivo* efficacy. They recently synthesized a series of ionizable lipids based upon alkenyl amino alcohols. The first four members of this series of materials, named OF-00 through OF-03, were formulated with cholesterol, DOPE, C14-PEG-2000 and IVT-mRNA coding for erythropoietin into mRNA LNPs.[69] OF-02 LNPs significantly outperformed benchmark LNPs including C12-200 cKK-E12 and 503O13 in terms of *in vivo* erythropoietin production across a broad linear dose–response window.[69] The authors further concluded two structure/function relationships of interest within these novel ionizable lipids, namely (1) only alkenes with a *cis* geometry promote *in vivo* efficacy and (2) the optimal number and placement of two *cis* alkenes per tail matches those observed in optimized siRNA LNPs.[69,70] The importance of local structural transformations could also be illustrated by a recent study performed by Li *et al.*[71] They designed and synthesized a series of lipid-like 1,3,5-triazinane-2,4,6-trione (TNT) derivatives consisting of a six-membered ring and three lipid tails for IVT-mRNA delivery, named TNT-b8 to TNT-b14.[71] These novel derivatives exchanged the positions of hydroxyl and amino groups compared with the structure of a previously reported lipid-like compound for efficient siRNA delivery (TNT-a10).[72] According to an *in vitro* luciferase assay, TNT-b10-containing IVT-mRNA LNPs were 10-fold more efficient than TNT-a10 formulation under the same condition, demonstrating the position change of functional groups on lipid-like materials can dramatically improve delivery efficiency for IVT-mRNA.[71] Although these structure/function relationships are empirical at the moment, they may potentially shape subsequent generations of novel lipid-like materials for IVT-mRNA delivery.

The mechanism for the intracellular delivery of nucleic acids mediated by LNPs is composed of several distinct steps, and the key endosomal escape step 'provides the major opportunity to improve the delivery system' as suggested in a study from Zerial and co-workers.[73] Both endocytosis and potency of LNPs are highly dependent on the specific ionizable lipid used. It is postulated that ionizable lipids, which are neutral at physiological pH but positively charged in acidic endosomes, facilitate quick release of the RNA cargo from maturing endosomes through disruption of the endosomal membrane and finally result in efficient transfection.[74] Several studies implied that the key parameter determining efficient endosome disrupting ability of ionizable lipids is an acid dissociation constant (pKa) in a narrow pH range of 6.2 to 6.5.[32,75] Based on this hypothesis, Dohmen and colleagues[76] synthesized a diverse set of ionizable lipids from a series of oligoalkylamines and subsequently evaluated their IVT-mRNA delivery capability. Results show that a lipid containing tetramine with alternating ethyl-propyl-ethyl spacers showed highest transfection efficiency that was linked to a high buffering capacity in a narrow range of pH 6.2 to 6.5.[76] A recent publication authored by Pitard and colleagues[77] further confirmed the

importance of 'endosome escape' process. They synthesized a new series of ionizable lipids based on the aminoglycoside tobramycin and evaluated their corresponding transfection efficiency in different cell types using IVT-mRNA, DNA and siRNA. All sets of evaluation led to the identification of a lead molecule named compound 30.[77] The presence of ester bonds makes compound 30 endogenous to natural lipids that may result in better hydrophobic interactions with the natural anionic phospholipids when compared with the other molecules of the series.[77] The better transfection potency of compound 30 may be a consequence of these improved interactions that favor the 'lipid mixing mechanism' (the formation of new bilayer structures obtained from the association of the anionic lipids from the membrane and the cationic lipids from the lipoplexes) and trigger a more efficient endosomal escape of all types of nucleic acids.[77]

POLYMER-BASED IVT-mRNA DELIVERY SYSTEMS

Cationic polymers can be regarded as prominent noncovalent interaction partners for nucleic acids, providing satisfactory *in vivo* transfection. They are easy to generate and offer huge flexibilities in terms of structure modifications. Some sequence-defined polymers are beneficial for establishing precise structure/activity relationships. As a result, cationic polymers have attracted much attention in the area of gene therapy. Over the past few decades, plenty of cationic polymers have been synthesized, evaluated and applied as effective delivery vehicles for nucleic acids. Delivery of pDNA and siRNA by cationic polymer has been extensively reviewed elsewhere.[78] Even though the use of cationic polymers for IVT-mRNA delivery is not so well studied when compared with pDNA and siRNA, they hold great potential and have developed to rival the status of many well-studied lipid systems. The complexation of negatively charged IVT-mRNA with cationic polymers occurs spontaneously through charge–charge interaction (Figure 2b), forming polyplexes that normally show higher stability than lipoplexes.[34] Cationic polymers not only bind but also condense IVT-mRNA into nanostructures that are capable of improving IVT-mRNA uptake via endocytosis, protecting IVT-mRNA from nuclease degradation and facilitating endosome escape.[34]

The first trial of polymeric IVT-mRNA delivery dates back to 1973 when diethylaminoethyldextran was used to complex IVT-mRNA for *in vitro* transfection.[79] More extensive investigations started from 2001 when Bettinger *et al.*[80] performed a comprehensive study with a variety of well-known cationic polymers including linear and branched polyethyleneimine (PEI), poly-L-lysine and polyamidoamine dendrimer. In their study, efficient cationic polymers used for pDNA delivery demonstrated a very low potency for IVT-mRNA delivery. More investigations revealed that high molecular weight polymers bound IVT-mRNA too tightly for it to be released and thus resulted in poor gene expression.[80] When lower molecular weight PEI 2 kDa were used, the binding strength with IVT-mRNA was weaker, resulting in a better expression in the presence of endosomolytic agents.[80] All these observations indicate that IVT-mRNA binding to cationic polymers is generally stronger than pDNA binding and the binding strength represents a critical parameter in IVT-mRNA expression. Tailor-made design of cationic polymers has to be considered individually for each type of nucleic acid to identify and generate optimized candidates for efficient delivery.[32]

The substantial importance of specific design on polycation structures for promoted IVT-mRNA transfection could be illustrated by another study that focused on the transfection efficiency of IVT-mRNA polyplexes comprising *N*-substituted polyaspartamides with varied numbers of side chain aminoethylene repeats.[81] Polyplexes with odd number aminoethylene repeats (PA-Os) produced sustained increase in IVT-mRNA transfection efficacy compared with their counterparts containing even number repeats (PA-Es). However, this phenomenon was contradictory to that of pDNA polyplexes prepared from the same *N*-substituted polyaspartamides.[82] Further investigations via flow cytometry and microscopy studies revealed that overall transfection efficacy was determined through the balance between endosomal escaping capability and stability of translocated IVT-mRNA in the cytoplasm.[81] PA-Es efficiently transported IVT-mRNA into the cytoplasm, but their poor cytoplasmic stability led to facile degradation of IVT-mRNA, resulting in a less durable pattern of transfection.[81] Alternatively, PA-Os with limited capability of endosomal escape eventually protect IVT-mRNA in the cytoplasm to induce sustainable protein expression.[81] Higher cytoplasmic stability of pDNA compared with IVT-mRNA may shift the limiting step in transfection from cytoplasmic stability to endosomal escape capacity, thereby giving an opposite transfection profile.[81] These data highlighted the importance of fine-tuning the polymer chemistry to regulate each key step involved in IVT-mRNA transfection to achieve maximum efficacy.

Based on these findings, the same research group established polyplex nanomicelles prepared by self-assembly of PEGylated poly(amino acid) block copolymer. The nanomicelles have a core–shell structure surrounded by a PEG outer layer, with the inner core of a functionalized PAsp(DET) (poly(*N*′-(*N*-(2-aminoethyl)-2-aminoethyl)aspartamide)). PAsp(DET) has enhanced endosomal escape potential because of pH-sensitive membrane destabilization as well as the unique characteristic of rapidly degrading into nontoxic forms under physiological conditions.[83] These nanomicelles were shown to allow *in vivo* IVT-mRNA transfection into the central nervous system and to provide a sustained protein expression.[84] Because of the shielding effect of PEG that avoids IVT-mRNA recognition by Toll-like receptors on host immune cells, the formulations exhibited a pronounced effect for suppressing immune responses that could be induced by exogenous IVT-mRNA.[84,85] In a latest study, the research group further found that polyaspartamide with more aminoethylene repeats in its side chain (PAsp(TEP)) were more effective in preventing IVT-mRNA degradation in serum as compared with PAsp(DET).[86] This increased stability in PAsp(TEP) is presumably attributed to the increased cationic charge at the end of the side chain, as they found that the stability of the polyplexes comprising polycations and IVT-mRNA increased with higher protonation of the terminal primary amino group in the side chains.[82] They additionally improved the stability of IVT-mRNA-loaded nanomicelles composed of PAsp(TEP) by attaching a hydrophobic cholesterol moiety. Animal studies revealed that cholesterol-modified nanomicelles generated efficient protein expression from the delivered IVT-mRNA, resulting in remarkable therapeutic effect, whereas nanomicelles without cholesterol failed to show a detectable effect.[86] Because cholesterol moiety can stabilize the nanomicelle through additional coagulation property to the core of the nanomicelle, these results clearly demonstrate that stabilization of IVT-mRNA carriers is pivotal for systemic IVT-mRNA delivery.

The rapid developments of controlled living radical polymerization methodologies allow for the synthesis of previously unattainable polymeric gene delivery systems with predetermined architectures, well-defined functional segments and low material heterogeneity.[87] Variety of block polymers based on p(DMAEMA) (poly(*N*,*N*-diethylaminoethyl methacrylate)) have been synthesized and applied to IVT-mRNA delivery. Our group has previously investigated how PEGylation may affect the physiochemical and biological activity of p(DMAEMA)-based polymers for IVT-mRNA delivery and revealed that PEGylation increased the ability of p(DMAEMA) to complex IVT-mRNA and the tendency to form monodispersed particles as compared with unmodified control.[88] The increased binding between PEGylated p(DMAEMA) and IVT-mRNA may help to improve the stability of the complexes and thereby decreases the chances of IVT-mRNA degradation. Most interestingly, we observed that PEGylation of p(DMAEMA) increased IVT-mRNA transfection threefold, whereas the same

PEGylation reduced pDNA transfection efficiency at ~100-fold.[88] Further evaluations demonstrated that PEGylation potentially improved endosomal release of the IVT-mRNA payloads into the cytoplasm and thus resulted in improved transfection.[88] Recently, Cheng *et al.*[89] developed a series of p(DMAEMA)-based triblock copolymers designed to enhance the intracellular delivery of IVT-mRNA. Apart from the p(DMAEMA) and PEG containing building block (PEGMA), a copolymer of diethylaminoethyl methacrylate (DEAEMA) and butyl methacrylate was incorporated into the backbone of triblock copolymers to facilitate cytosolic entry.[89] These polymers exhibited pH-dependent hemolytic activity, favorable size and charge when complexed with IVT-mRNA, and low polyplex cytotoxicity.[89] Subsequently, the blocking order and PEGMA segment length were systematically varied to further investigate the effect of different polymer architectures on IVT-mRNA delivery efficacy. They found that IVT-mRNA polyplexes formed by polymers with the PEGMA segment in the center of the polymer chain demonstrated enhanced polyplex stability against heparin displacement relative to other configurations and were associated with the highest *in vitro* transfection efficiencies, with longer PEGMA block lengths enhancing IVT-mRNA binding and significantly improving delivery efficacy.[89] This study indicated that IVT-mRNA transfection was potentially linked to polyplex stability that is an important factor that needs to be carefully modulated in order to achieve optimal outcomes.

Similar to lipid-based delivery systems, a number of both synthetic and naturally derived polymers, in recent years, have transitioned from use in siRNA/DNA delivery to IVT-mRNA delivery. A very potent and commonly used synthetic polymer in this case is PEI, as its transfection efficiency is relatively high both *in vitro* and *in vivo* at dosages where the polymer displays only moderate cytotoxicity.[90] PEI is only partly protonated at neutral extracellular pH and would still bind nucleic acid by interelectrolyte interactions, but it would increase its protonation and charge density within acidifying endosome.[78] This process should destabilize the polyplex-containing endosomal vesicle and subsequently facilitate the endosome escape of nucleic acid molecule, thereby offering PEI a favorable status in nucleic acid delivery field. Apart from the original form of PEI, optimized versions of PEI and derivatives have also been applied in the delivery of IVT-mRNA. Li *et al.*[91] developed a potent PEI-based intranasal vaccination system using cationic cyclodextrin low molecular weight PEI conjugate complexed with IVT-mRNA to overcome biological barrier in the nasal epithelium by reversibly opening the tight junctions, and the delivery vehicle enhanced the paracellular delivery of IVT-mRNA and consequently minimized absorption of toxins in the nasal cavity. Strong immune responses were achieved via the high mucosal affinity of cyclodextrin and the good adjuvanticity of the cationic PEI polymer.[91] In terms of naturally derived polymers, a prominent example would be chitosan that is found in the exoskeleton of crustaceans. Chitosan is composed of repeating D-glucosamine and *N*-acetyl-D-glucosamine units. The primary amine group on the D-glucosamines can become positively charged at acidic pH and complex with negatively charged nucleic acids to form polyplexes.[92] Chitosan and its derivatives have been most prevalent in the field of DNA and siRNA delivery,[93] and have also recently been applied for the delivery of IVT-mRNA.[94]

HYBRID IVT-mRNA DELIVERY SYSTEMS

IVT-mRNA can be loaded into hybrid nanoparticles that comprise various kinds of materials including lipids, polymer and peptide in one structure for more potent transfection. Hybrid nanoparticles normally integrate several potential advantages of their components and thus provide more flexibility when compared with nonhybrid systems for the delivery of therapeutic compounds. Specifically, these hybrid nanoparticles normally share a core–shell structure, the properties of the 'core' can be tuned in order to respond to external stimuli that can help to facilitate endosomal escape;[95–97] meanwhile, the 'shell' could enhance the nanoparticle stability and pharmacokinetics and confer surface tunable properties.[98,99] IVT-mRNA could be condensed within the 'core' (Figure 2c) or be absorbed to the surface of the 'shell' (Figure 2d) for efficient delivery, whereas release profiles may vary in a way that surface adsorbed IVT-mRNA release faster.[95]

Ternary complexes formed by complexing nucleic acids with cationic polymers and liposomes, namely lipopolyplexes, were originally developed for DNA and siRNA transfection.[100,101] Initial works on IVT-mRNA transfection via lipopolyplexes were performed by Hoerr *et al.*[102] who condensed IVT-mRNA encoding galactosidase with polycationic peptide protamine and then encapsulated the complexes with liposome. The resulting lipopolyplexes protected the IVT-mRNA payload *in vitro* for a longer period of time and demonstrated *in vivo* protein expression and subsequent immune responses.[102] Numerous pH-responsive polymers that show endosome-disrupting function due to the pH changes upon endocytosis have been explored for hybrid IVT-mRNA delivery. Mockey *et al.*[103] developed PEGylated derivatives of histidylated polylysine and L-histidine-(*N*,*N*-di-n-hexadecylamine) ethylamide liposomes (histidylated lipopolyplexes) for the delivery of IVT-mRNA vaccine against melanoma. In this formulation, IVT-mRNA encoding melanoma-associated antigen was condensed by histidylated polylysine, and the imidazole group on the side chain of histidine endowed the system with charge switching ability at endosomal pH condition.[103] Systemic injections of this histidylated IVT-mRNA lipopolyplexes demonstrated specific and significant protection against B16F10 melanoma tumor progression in mice.[103] Based on these results, the same group then formulated mannosylated and histidylated lipopolyplexes (Man11-LPR100) that were obtained by adding mannosylated and histidylated liposomes to IVT-mRNA containing PEGylated histidylated polylysine polyplexes.[96,104] The mannosylation enabled targeted IVT-mRNA delivery of the system to dendritic cells through interaction with the mannose receptor.[104] A greater inhibition of B16F10 melanoma growth was obtained after mice were intravenously immunized with tumor antigen IVT-mRNA loaded Man11-LPR100, indicating that Man11-LPR100 is an efficient system for the delivery of IVT-mRNA in dendritic cells.[96] In another study, Su *et al.*[95] developed biodegradable core–shell structured nanoparticles with a poly(β-amino ester) core enveloped by a phospholipid bilayer shell for *in vivo* delivery of IVT-mRNA-based vaccines. The pH-responsive poly(β-amino ester) component was chosen to promote endosome disruption, whereas the DOTAP containing lipid surface layer was selected to minimize toxicity of the polycation core and to efficiently adsorb IVT-mRNA onto the surface of these net positively charged nanoparticles.[95] Such IVT-mRNA-loaded particles showed both efficient *in vitro* cellular uptake and cytosol location with low cytotoxicity as well as expression of the reporter protein *in vivo* as soon as 6 h after administration, whereas naked IVT-mRNA counterpart showed no signal.[95] Apart from protamine and pH-responsive cationic polymers, positively charged short peptide could also be used as 'core' component in hybrid IVT-mRNA delivery system. For example, efficient IVT-mRNA transfection in cultured mouse cardiac fibroblasts was achieved via a heart-targeting sequence fused to a nine arginine containing peptide (CRPPR-R9) and Lipofectamine containing ternary complexes. Results demonstrated the partial direct reprogramming of cardiac fibroblasts toward cardiomyocyte cells because of its efficient transfection with low toxicity.[105] CRPPR-R9 was designed to electrostatically condense IVT-mRNA because of its nine positively charged arginine residues, and then trigger endocytosis and endosome escape of the complexed IVT-mRNA in cardiac fibroblasts.[105] Similarly, the lipid 'shell' component in the hybrid IVT-mRNA delivery system may potentially be substituted by polymer-based materials. An attractive candidate among them is

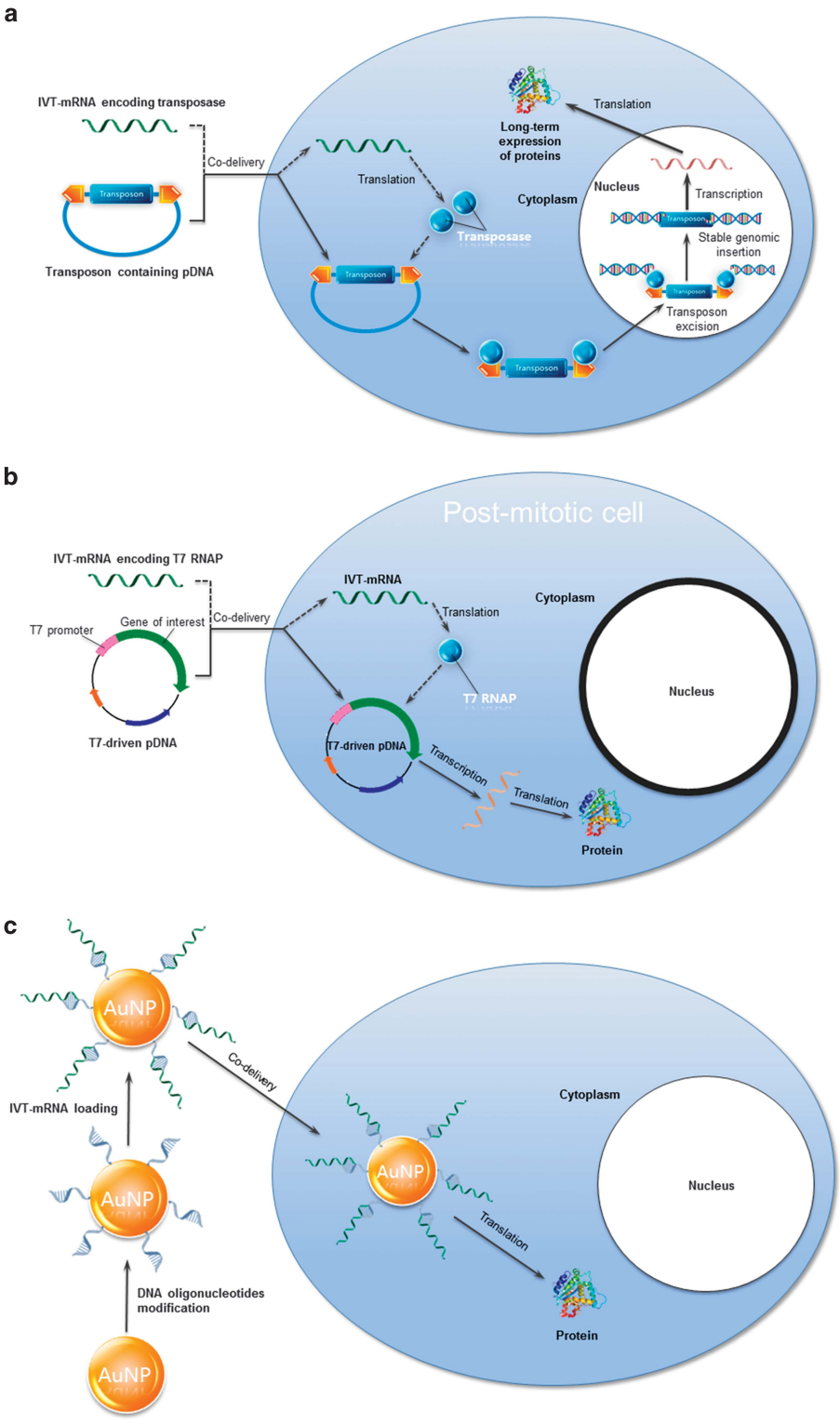

Figure 3. The schematic presentation of applications on simultaneously delivery of IVT-mRNA and DNA. (**a**) IVT-mRNA as a source of transposase for transposon-mediated long-term transgene expression. IVT-mRNA encoding transposase and transposable DNA containing the gene of interest were codelivered into the target cells. Upon entry to the cytoplasm, IVT-mRNA locates to the endosome and expresses the transposase. The transposase is able to specifically excise the gene of interest from donor plasmid (transposon) and then efficiently insert such genetic cargo into chromosomes for safe and stable long-term gene expression. (**b**) IVT-mRNA as a source of RNA polymerase (RNAP) for cytoplasmic expression of pDNA containing gene of interest in post-mitotic cells. In order to bypass the nuclear barrier, T7 promoter containing pDNA can facilitate efficient gene expression within the cytoplasm of quiescent cells with the help of T7 RNAP that is encoded by codelivered IVT-mRNA. The advantage of using T7 RNAP encoding IVT-mRNA instead of T7 RNAP protein exists in enhanced amount of intracellular T7 RNAP and reduced immune responses. (**c**) IVT-mRNA delivered by DNA oligonucleotide functionalized AuNP. The modification of DNA oligonucleotide enables AuNP to efficiently load and deliver IVT-mRNA payloads into eukaryotic cells.

poly(ε-caprolactone) (PCL), approved by the FDA (Food and Drug Administration). Nanoparticles composed of PCL are promising for their high colloidal stability in a biological fluid, facile cellular uptake by endocytosis, low toxicity *in vitro* and *in vivo* and controlled release of their cargo.[106] PCL nanoparticles were proposed in a latest study for the intracellular delivery of IVT-mRNA molecules. The hybrid nanoparticles have a core–shell structure with IVT-mRNA and protamine containing inner core surrounded by PCL layers, providing high stability and stealth properties to the nanoparticles, thereby addressing the issue of IVT-mRNA instability.[107]

Hybrid IVT-mRNA delivery systems can also be formulated with more than three components. Recently, the research team from Novartis institutes reported an IVT-mRNA-based vaccination formulation using a hybrid cationic nanoemulsion.[108] Cationic nanoemulsion was prepared by mixing and heating squalene, DOTAP and sorbitan trioleate to 37 °C, and the resulting oil phase was then combined with an aqueous phase consisting of Tween-80 in citrate buffer at pH 6.5.[108] The final emulsion had a small size below 100 nm and *in vivo* delivery of antigen encoding IVT-mRNA elicited potent immune responses in a variety of animal models including mice, rats, rabbits and non-human primates comparable to a viral delivery technology.[108] One advantage of cationic nanoemulsion is that its chemical components are already used in previous clinical trials.[31] Recently, the Anderson group[109] developed a new class of multi-component nanoparticle formulation containing polymer-brush materials based on poly(glycoamidoamine)s Three different poly(glycoamidoamine) polymers containing tartarate, galactarate or glucarate sugars combined with three different amine-containing monomers were synthesized based on a modular design strategy. They found that particles formed by complexation of poly(glycoamidoamine) polymers and IVT-mRNA were too large to be suitable for *in vivo* evaluation.[109] In order to reduce particle size and improve polydispersity, DSPC (1,2-distearoylsn-glycero-3-phosphocholine), mPEG2000-DMG (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy-(polyethylene glycol)-2000]) and cholesterol were incorporated in the system to formulate compact nanoparticles.[109] Alkyl tail brushes were added along the polymer backbone to facilitate interactions of lipid-based materials with the polymers. The authors evaluated these formulations and concluded several structure/activity relationships. (1) Transfection efficiency is increased as the number of amino groups is increased.[109] This might result from a higher *zeta* potential of the nanoparticles facilitating an enhanced cellular uptake when more amino groups are present in the structure. (2) The tartarate sugar improves efficiency relative to the galactarate or glucarate sugar, indicating that the number of hydroxyl groups is important for efficient delivery.[109] Appropriate number of hydroxyl groups may potentially balance the hydrophlicity/hydrophobicity of the nanoparticles that may in turn improve the stability of the complexes. (3) Shorter alkyl tails improve transfection performance,[109] as they may result in smaller size of nanoparticles when interacting with lipid components.

EXPLORATIONS ON SIMULTANEOUS DELIVERY OF IVT-mRNA AND DNA

An interesting but scarcely addressed topic on gene transfer is the codelivery of IVT-mRNA and DNA. The protein expression profile of these two nucleic acids is completely different; wherein the onset of protein expression is faster with IVT-mRNA but the expression lasts for a very limited time, but transfection with DNA leads to much higher protein and persistent expression but starts at a later time point.[37] These properties can be integrated into one system to take numerous advantages. According to the function of IVT-mRNA or DNA component, the existing studies could be summarized into following categories.

IVT-mRNA as a transposase source (Figure 3a): the short-term expression of IVT-mRNA could be one of the major limitations for its application in long-term treatment of inherited diseases owing to the currently unknown knowledge about application frequencies of repetitive IVT-mRNA administrations. However, a feasible solution could be offered by, for example, *Sleeping Beauty* transposon system that offers stable and long-term transgene expression via the potential integration of exogenous genes into the host cell genome in the presence of transposase.[110] In most early studies, the transposase component has been provided by expression of DNA molecules. It would be problematic if transposase-encoding DNA sequences integrate into the host genome, and the long-term expression of transposase may lead to transposon remobilization and reintegration. To address these problems, transposase-encoding IVT-mRNA has been utilized as an effective alternative to transposase-encoding pDNA. The delivery of IVT-mRNA encoding for transposases of the *Sleeping Beauty*, piggyBac or Tol2 transposon systems and corresponding pDNA-based transposon vectors resulted in stable genomic transposition in mammalian cells and *in vivo* in rodents.[111–115] The use of IVT-mRNA rather than plasmids for the expression of the transposases drastically increased the survival rate of the injected cells because injection in the cytoplasm is easier and safer than pronuclear injection. Transgenic efficiency when creating transgenic mice increased from just 3% to a level of $>20\%$.[114] Improved control with respect to the duration of transposase expression reduces the probability of remobilization of transgenes.[116] This codelivery strategy holds potential not only for the long-term correction of genetic disorders but also in the field of regenerative medicine and stem cell technology.

IVT-mRNA as a polymerase source (Figure 3b): a major factor restricting nonviral pDNA-mediated transgene expression is the inefficient transfer of pDNA from the cytoplasm to the nucleus. Nuclear entry is particularly challenging in post-mitotic or quiescent cells that represent the majority of cells *in vivo* as there is limited breakdown of the nuclear envelope.[117] On the other hand, IVT-mRNA has the intrinsic advantage of successful expressing proteins in slowly dividing cells because of the bypassing of the nuclear barrier. An increasingly attractive idea to improve the pDNA-based transfection is the hybrid mRNA/DNA cytoplasmic expression system. Codelivery of IVT-mRNA encoding T7 RNA polymerase with a T7-driven plasmid produced 10–2200-fold greater transfection in primary cells compared with a cytomegalovirus-driven plasmid, and 30-fold higher expression than enhanced T7-based autogene plasmid,[118] indicating that codelivery of IVT-mRNA is a promising strategy to yield increased expression with pDNA. This system represents an important step towards improving the capability of nonviral vectors mediated gene transfer in cells where the nuclear membrane is a significant barrier.

DNA oligonucleotide as a linker (Figure 3c): gold nanoparticles (AuNPs) have several attractive properties including bioinert, nonpathogenic and ease of synthesis as a platform for gene delivery systems.[119] However, the unmodified AuNPs could only deliver covalently crosslinked antisense DNA. Single-stranded DNA functionalized AuNP (AuNP-DNA) could serve as a universal platform for loading and delivering nucleic acids containing a sequence complementary to the cargo DNA that is covalently linked with AuNP and a target gene expression sequence.[120] AuNP-DNA conjugates have been reported to efficiently load and deliver IVT-mRNA into human cells *in vitro*, and the direct injection of IVT-mRNA nanoparticles into xenograft tumors in mice resulted in the higher transfection efficiency compared with IVT-mRNA containing lipoplex counterpart.[121] Another advantage conferred by AuNP-DNA conjugate is that it can efficiently mediated IVT-mRNA translation without a cap structure, and this is likely because the AuNP-DNA conjugate serves as a functional substitute

for the cap structure by tethering translational factors to the IVT-mRNA.[121]

CONCLUSION AND FUTURE PERSPECTIVE

IVT-mRNA-based therapeutics have broad potential to treat a range of important diseases that currently have no satisfied solution, and the further development of delivery systems will be a key to the success of these approaches. In the past decades, substantial advances have been achieved in the development of nonviral-based delivery system for IVT-mRNA. However, the vector design and mechanism understanding in this field are still limited. Continuous work is still necessary to find more potent delivery vehicles that can provide high transfection activity and biocompatibility with minimal toxicity, high selectivity and specificity. As of now, it is difficult to postulate which is the most efficient delivery system for IVT-mRNA as there is lack of sufficient studies to compare state-of-the-art carriers and determine the optimum one. Based on our knowledge, there are some key aspects that significantly improve IVT-mRNA delivery efficiency: (1) the delivery system could form stabilized complexes with IVT-mRNA in order to protect its payloads from degradation; (2) moderate binding strength that allows the delivery system to release IVT-mRNA at target destination; (3) efficient cellular uptake by target cell populations; (4) efficient endosomal escape of the carrier complexes into cytoplasm. The overall balance between all these aspects of a certain carrier might determine its final delivery efficiency. Only the delivery systems that fulfill all these criterial may show a promising IVT-mRNA delivery profile. The difficulties lie in the fact that the structure/function relationship of IVT-mRNA delivery systems seems to work in a quite subtle way where even a slight change within the structure of the delivery systems could overwhelmingly influence their delivery efficacy.[71,76,82] With the studies going wider and deeper, we will finally figure out the underlying mechanisms. However, at the current stage, the accumulated empirical knowledge on carrier design may help us to develop subsequent generations of novel IVT-mRNA delivery systems, just like those promising LNPs derived from combinatorial synthetic methodology and library screening. The exploration on IVT-mRNA-based therapeutics may not be limited to the search of delivery systems, but the combinatorial use of IVT-mRNA with DNA also holds huge potential in the area of gene therapy as well as next-generation genome editing techniques.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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