Preparation of Messenger RNA-Loaded Nanomedicine Applied on Tissue Engineering and Regenerative Medicine

Hsi-Kai Tsou, Cheng-Chung Chang, Tomoji Maeda, and Chin-Yu Lin

Contents

H.-K. Tsou · C.-C. Chang · C.-Y. Lin (✉)
Institute of New Drug Development, College of Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan
e-mail: geant@mail.cmu.edu.tw

H.-K. Tsou
Functional Neurosurgery Division, Neurological Institute, Taichung Veterans General Hospital, Taichung, Taiwan

T. Maeda · C.-Y. Lin
Tsuzuki Institute for Traditional Medicine, College of Pharmacy, China Medical University, Taichung, Taiwan

T. Maeda
Department of Pharmaceutical Sciences, Nihon Pharmaceutical University, Saitama, Japan

H.-K. Tsou
Department of Rehabilitation, Jen-Teh Junior College of Medicine, Miaoli County, Taiwan

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Abstract Tissue engineering holds great promise for regenerative medicine; the multipotent cell lineage, biocompatible tissue-compliant biomaterials, and critical differentiation cues compose the critical factors for successful tissue engineering applied to regenerative medicine. The critical differentiation cues could be provided by recombinant proteins, alternative novel therapeutics, and gene therapy, including viral and non-viral-based methodology. One of the emerged gene therapeutics is messenger RNA (mRNA) administration, which holds critical advantages, such as tentative drug expression, no DNA backbone remaining in the cytosol, and easy to be manufactured as cocktail therapeutics and scaled up in a good manufacture process (GMP) factory. However, the successful mRNA medicine applied to tissue engineering remains challenging, including sophisticated mRNA medicine carriers, endosomal escape capability, targeted delivery, fine-tuned gene expression duration, and subsequent immune responses. This chapter will discuss the up-to-date technologies addressed on mRNA manufacture, sophisticated carrier design, mRNA medicine endosomal escape, nuclease resistance, and sustained expression. Furthermore, combined with tissue engineering, we will also introduce the mRNA medicine selection for therapeutic purposes, applied on the diseased animal model, mRNA activated matrix construction, and subsequent evaluation criteria will also be addressed.

Keywords Messenger RNA · Gene therapy · Regenerative medicine · Polyplex nanomicelle · mRNA-based therapeutics

1 Introduction

RNA therapy has recently gained more attention in new medicine development, especially for an intractable disease eager to find a new therapeutic medication. RNA medicine has many different chemical formulas, structures, and therapeutic mechanisms, such as siRNA, microRNA, and messenger RNA. The most critical function of mRNA is to provide and synthesize the target protein of interest through translation machinery in mammalian cells. Gene therapy using mRNA is a promising alternative with several advantages over that of plasmid DNA (pDNA) and has been extensively investigated in preclinical and clinical studies (Boczkowski et al. 1996; Chan et al. 2019; Lin et al. 2016). The preparation of therapeutic mRNA, loaded in

a drug carrier, endosomal escape scenario, and application in regenerative medicine will be comprehensively depicted.

2 Preparation of Therapeutic mRNA

The most popular and convenient method utilized to synthesize the mRNA is in vitro transcription (IVT), which is entirely proceeded by chemically and enzymatically reaction and is affordable for a general laboratory. The other method is chemically synthesized like a polymerization reaction. Here, we will describe the comprehensive synthesis process of mRNA through IVT in vitro.

2.1 DNA Template

The first step for mRNA synthesis by IVT is to prepare a pure and linearized DNA template for RNA polymerase binding to proceed with the RNA polymerization. The linearized DNA template is often collected from plasmid DNA after particular restriction enzyme digestion, which should be removed or entirely inactivated through heating, EDTA incubation, or proteinase K digestion and column purification. Then, the circular plasmid DNA should be entirely digested by a restriction enzyme, followed by separation through electrophoresis purification. The purity of the DNA template is a human manipulation controllable factor that plays a determinant role in the quality of the subsequently synthesized mRNA. In our experience, after we examined the quality of synthesized mRNA, most of the inferior mRNA quality was attributed to the quality short DNA template. In our experimental data, the gel electrophoresis and DNA bioanalysis show a unique and highly concentrated DNA template prepared for the production of runx1 mRNA (Fig. 1a and b). Besides, the double-strand plasmid DNA contamination in the final mRNA medicine product would also essentially interfere with the outcome of therapeutic efficacy, specifically in the immune activation (Nelson et al. 2020).

2.2 In Vitro Transcription (IVT)

Although the entirely chemical polymerization can be utilized to synthesize the mRNA transcript in an in vitro scenario, the chemically synthesized mRNA length is limited, and the process is complex, usually limited to hundreds of base-pair in a reaction (Nagata et al. 2010) and needs further enzymatic ligation to prepare longer mRNA transcripts (Pradere et al. 2017). Besides, the whole process is expensive and does not yet have sufficient demonstration to examine the feasibility of scaling up the reaction. On the other hand, in vitro transcription is the primary process

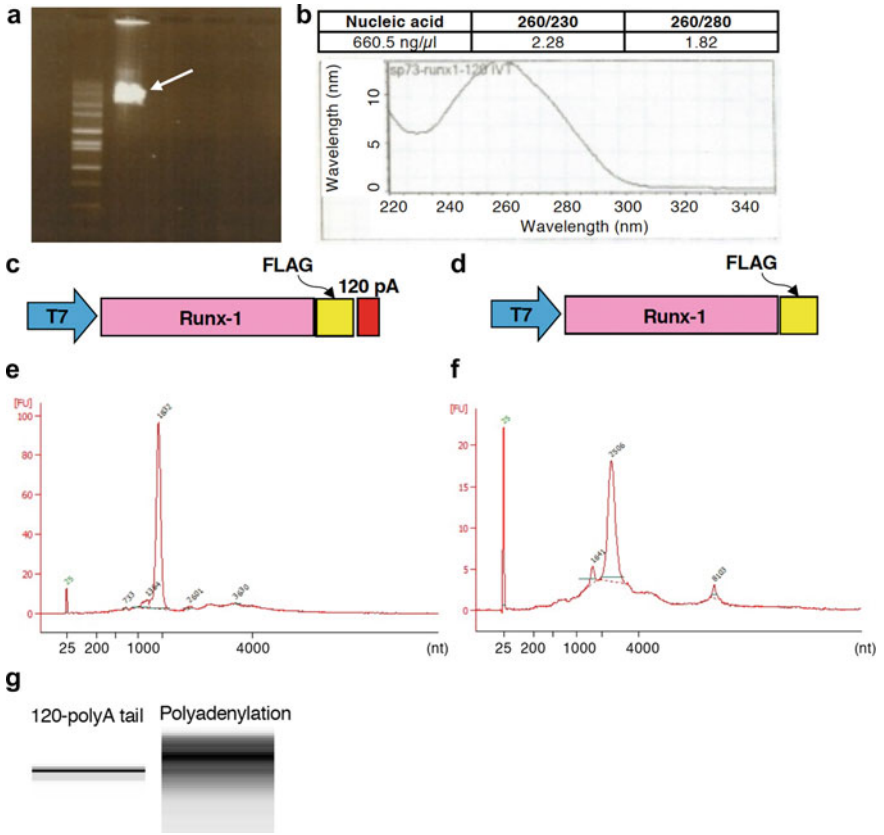

Fig. 1 Preparation of IVT vector template. **a** Plasmid DNA linearized by restriction enzyme, collected by agarose gel electrophoresis. Arrow indicates the linear plasmid with high concentration. **b** Examine the DNA concentration and quality. The O.D. 260/280 ratio higher than 1.8 represents good quality. **c** DNA template vector map with restricted polyA tail. The Runx-1 mRNA fused with the FLAG signal will be encoded under T7 promoter control. **d** DNA template vector map without restricted polyA tail. The Runx-1 mRNA fused with the FLAG signal will proceed with post-transcriptional polyadenylation. **e** Bioanalysis of the Runx-1 mRNA with restricted polyA tail. **f** Bioanalysis of the Runx-1 mRNA with post-transcriptional polyadenylation. **g** Agarose gel electrophoresis of the Runx-1 mRNA with restricted polyA tail and polA tailing through polyadenylation

to manufacture mRNA medicine, particularly facing large-scaled pandemics, such as the current COVID-19 outbreak, which requires billions of doses of vaccines within months. Thanks to the cell-free characteristic of the IVT process, allowing the promptly, large-scaly, and cost-effectively manufacture of the mRNA vaccine combating the COVID-19 pandemic. A 5 L bioreactor can produce almost a million mRNA vaccine doses in a single reaction (Kis et al. 2020). Besides, the mRNA transcript used in the mRNA medicine formulation could be adjusted rapidly to fulfil the required critical proteins combating the different diseases or pathogens

(Chaudhary et al. 2021). The mRNA medicine without DNA backbone in the final formula, attributing the mRNA medicine to be easily prepared as a cocktail to address multi-typed diseases at one shot. Due to the cell-free manufacturing process, the purchasing and maintenance cost of GMP-grade compliant manufacturing apparatus of mRNA medicine is cheaper than that of the plasmid-, virus-related and cell-containing manufacturing apparatus. The IVT process utilizes the recombinant RNA polymerase derived from T7, T3, or SP6 bacteriophages and prepares the synthetic mRNA for clinical use in a completed cell-free scenario, such as application in COVID-19 vaccines. This is one of the critical factors that attributes the Moderna and Pfizer–BioNTech vaccines to be approved by the FDA at an unprecedented pace (Corbett et al. 2020). Furthermore, the chemically modified nucleoside triphosphates (NTPs) are incorporated into the IVT process of mRNA transcript synthesis. For example, replacing 25% of uridine and cytidine in the original mRNA sequence with 2-thiouridine and 5-methyl-cytidine decreased mRNA binding to pattern recognition receptors synergistically, such as TLR3, TLR7, TLR8, and retinoic acid-inducible gene I (RIGI) in human peripheral blood mononuclear cells (PBMCs). This method was utilized to raise the average haematocrit and cure a lethal congenital lung disease in a mouse model using modified murine erythropoietin and surfactant protein B mRNA, respectively (Kormann et al. 2011). Other chemically modified NTPs applied on such as bone regeneration (Balmayor et al. 2016), 5′ cap structure (Wadhwa et al. 2020), alleviate the proinflammatory cytokine expression (Vaidyanathan et al. 2018), and improve translation efficiency (Hajj and Whitehead 2017) especially for the production of prophylactic proteins (Pardi et al. 2018). The Moderna and Pfizer–BioNTech COVID-19 vaccines, which produced > 94% efficacy in phase III clinical trials, contain chemically NTPs-modified mRNAs (Buschmann et al. 2021).

2.3 Capping

The mRNA generated from IVT must be further capped at the 5′ end to expand its full functions in mammalian cells. The 5′-cap of mRNA is a characteristic in eukaryotic cells, involved in numerous interactions required for a normal cellular function. The 5′-cap of mRNA consists of an inverted 7-methylguanosine connected to the head of the eukaryotic mRNA via a 5′–5′ triphosphate bridge, so-called cap 0 and served as quality control for correct mRNA processing and contributes to the stabilization of eukaryotic mRNA, splicing, nuclear export, initiation of translation and mRNA degradation. To initiate a normal translation process, the 5′-cap of mRNA would interact with the cap-binding complex (CBC) in the nucleus for nuclear export and the eukaryotic translation initiation factor 4E (eIF4E) in the cytoplasm for cap-dependent translation. Furthermore, capped RNA is a marker for the innate immune system to distinguish triphosphorylated infected viral RNAs from cellular RNAs. The cytosolic receptor RIG-I would be activated by short single and double-stranded triphosphorylated RNAs and collaborated with MDA-5 to initiate a

successful antiviral response. Moreover, the MDA-5 recognizes long triphosphorylated RNAs and RNAs lacking the 2′-OH methylation at the first nucleotide (cap1), a commonly observed modification in eukaryotes (Muttach et al. 2017).

A correct cap structure would be recognized by a cell to discriminate from pathogen infection. An uncapped mRNA transcript does not adequately represent a eukaryotic circumstance, and the preparation of correctly capped RNAs is essential to assess the function of mRNAs in the cellular context. Furthermore, altering the cap structure bears the potential to increase mRNA stability and translational efficiency. These two properties may dominate the critical factors of utilizing therapeutic mRNA shortly.

In the general lab-scaled mRNA synthesis, we usually have two methods for mRNA capping, including post-transcriptional capping and co-transcriptional capping during the IVT process. For co-transcriptional capping, many chemically synthesized cap analogs could be selected presently. One thing should be mentioned in the direction of mRNA synthesis after using cap analogs, and anti-reverse cap analog (ARCA) was developed to prevent the wrong direction of mRNA synthesis (Kwon et al. 2018). For post-transcriptional capping, the uncapped mRNA transcripts could usually be synthesized in large quantities and cost-efficient. Through Vaccinia virus Capping Enzyme (VCE) adds 7-methylguanylate cap structures (Cap 0) to the 5′ end of RNA generated by IVT, and Cap 0 is sufficient for efficient translation of the RNA in the eukaryotic systems.

Furthermore, the Cap 1 structure could be generated using Cap 0 RNA and 2′-O-methyltransferase. The Cap 1 RNA has been demonstrated to reduce the cellular innate immune response when the RNA is used in vivo. In our practical experience, post-transcriptional capping is convenient, high efficiency, and cost-saving, but the final concentration and weight of eluted mRNA would be slightly lower than that generated by the ARCA kit (Aini et al. 2016; Chan et al. 2019).

2.4 The 5′ and 3′ UTR, Including the PolyA Tail

Another critical factor that influences the mRNA stability and subsequent protein translation is the length of the polyA tail, which approximately 100–300 bp is considered sufficient for the binding of polyA-binding protein to cooperate with translation initiation factor proteins and initiate a successful translation (Linares-Fernandez et al. 2020). Another vital function of the polyA tail is interacting with the 5′ cap to protect the sequence from degradation by exonucleases and decapping enzymes (Wadhwa et al. 2020; Mugridge et al. 2018). Furthermore, the 5′ and 3′ UTRs, including the polyA tail, regulate mRNA translation, half-life, and subcellular localization.

Generally, two methods are used to incorporate the polyA tail into the mRNA sequence generated from the IVT process. They are co-transcriptional and post-transcriptional polyA tailing, respectively. Significant advances in the co-transcriptional polyA tailing have been made to streamline the large quantity of mRNA production. First, clinically used synthetic mRNA is transcribed in vitro

from a DNA plasmid template using the bacteriophage RNA polymerase, such as T7 RNA polymerase (T3 and SP6 RNA polymerases can also be used). Subsequently, co-transcriptionally capped with a 2′-*O*-methylated cap (termed as "CleanCap", developed by TriLink BioTechnologies company) and purified to remove double-stranded RNA (dsRNA) contaminants, reactants, and incomplete transcripts. The polyA tail encodes in the DNA template, eliminating reaction steps and reducing overall production time and material loss. Post-transcriptional polyA tailing using 2′-*O*-methyltransferase enzymes derived from the vaccinia virus.

For example, in our experiment prepared for the production of runx1 mRNA, the runx1 ORF was constructed in an SP73 vector containing a short 5′ UTR under T7 promoter control. For polyA tailing, we constructed both forms for comparison. One, the 3′ polyA tail was constructed in the vector for co-transcriptional polyA tailing (Fig. 1c). The other one, without polyA sequence in vector (Fig. 1d), mRNA generated from IVT synthesis will be further subjected to post-transcriptional polyadenylation through poly(A) polymerase reaction. Therefore, both typed mRNA transcripts were subjected to bioanalysis and gel electrophoresis, showing unique mRNA peaks and tailing-smeared forms, respectively (Fig. 1e, f, and g).

Incorporating the polyA tail in the DNA plasmid template also overcomes the tail length variability from enzymatic polyadenylation using polyA polymerase. PolyA tails of > 100 bp are optimal for therapeutic mRNAs. However, the DNA sequences encoding these long polyA stretches can destabilize the DNA plasmids template used for transcription. A solution to this stability issue is to include a short UGC linker in the polyA tail (Stadler et al. 2017; Eberle et al. 2020). The Pfizer–BioNTech vaccine BNT162b2 against SARS-CoV-2 uses this strategy and contains a ten bp UGC linker to produce the sequence A_{30}(10 bp UGC linker)A_{70}. Together, these innovations have overcome significant manufacturing bottlenecks and facilitated the development of a simple, cost-effective, and scalable one-step mRNA synthesis process.

2.5 Quality Analysis

The mRNA generated from IVT synthesis could be subjected to bioanalysis and gel electrophoresis to examine the quality of the transcript, which largely determines the following gene translation outcome and therapeutic efficacy. In general situation, the polyA sequence constructed in the vector was more suggested due to the higher concentration of mRNA and uniformed polyA length in final purified mRNA medicine.

3 Delivery Carrier Assembled as mRNA Medicine

mRNA medicine is tremendous large molecular therapeutics with negatively charged approximately 10^6 daltons, which cannot be diffused naturally into the cytosol

through the cell membrane with the anionic phospholipid bilayer. Besides, the mRNA medicine would be engulfed by immune cells and degraded by cytosolic nucleases. Many chemical and physical methods are currently developed to deliver mRNA medicine in vitro. However, few reports demonstrate the successful mRNA delivery in an in vivo circumstance, which requires low toxicity, minimizes unwanted immune responses, and controllable immunogenicity. To widely utilize mRNA medicine for clinical therapy requires the development of a safe and effective drug delivery vehicle (Hajj and Whitehead 2017).

The therapeutic mRNA ORF size range is approximately 300–1900 bp (~ 6.6 $\times$ 10^5 daltons) (https://origin-www.qiagen.com), and the mRNA is a single strand with a negative charge due to the phosphate group in the nucleotide chain. Therefore, the cationic polymer or lipids are usually used as the carrier vehicle for delivering therapeutic mRNA.

3.1 Lipid-Based Nanocarrier

Lipid-based nanoparticles are the most popular mRNA delivery vehicle and have gained the most advancement in use clinically. All mRNA-based vaccines against the SARS-CoV-2 in development or approved for clinical use before the end of 2021 employ lipid nanoparticles (LNPs) as delivery vehicles. LNPs possess numerous benefits for mRNA delivery, including simple formulation for manufacturing, modular recipe, biocompatibility, and large capacity for mRNA payload. The RNA medicine loaded and protected in the lipid-based nanoparticle, which typically includes four components: an ionizable lipid, cholesterol, a helper phospholipid, and a PEGylated lipid. All these formulae together encapsulate and protect the fragile mRNA core (Kim et al. 2021).

The lipid nanocarrier possesses a structure similar to the mammalian cell membrane, which would be fused with lipid nanocarrier, endocytosed mRNA-loaded lipid nanocarrier, and released the drug in the cytosol. Pfizer-BioNTech™ and Moderna™ SARS-CoV-2 vaccines utilize lipid-based mRNA carriers (Corbett et al. 2020).

3.2 Polymeric Nanocarrier

In addition to lipid-based nanocarrier, another nanoparticle with less clinical advancement than LNPs is polymeric nanocarrier. These polymers offer similar advantages to lipids that have been broadly applied for mRNA delivery (Kowalski et al. 2019). These polymers with cations condense, absorb nucleic acids into complexes, so-called polyplexes with various shapes and sizes, and can be engulfed into cells through endocytosis. Polyethylenimine (PEI) is the most widely explored polymer for nucleic acid delivery. Although its efficacy is excellent, its application is limited

due to its side-effected toxicity (Moghimi et al. 2005) attributed to its high charge density (Ulkoski et al. 2019). Although charge density is a critical consideration for mRNA complexation, an excessive charge could result in toxicity and serum aggregation (Mintzer and Simanek 2009).

Presently, the use of a low molecular weight form of the cationic polymer and incorporation of PEG into the formulation (Ke et al. 2020), conjugation to cyclodextrin (Li et al. 2016; Tan et al. 2020) and disulphide linkage (Breunig et al. 2007) can mitigate the toxicity of polyethyleneimine. Additionally, several alternative biodegradable polymers have been developed that are less toxic. For example, poly (β-amino ester)s excel at mRNA delivery, especially to the lung (Patel et al. 2019a). Furthermore, because they are easily synthesized by the Michael reaction (Lynn and Langer 2000), large poly(β-amino ester) libraries have been created that facilitate structure–function studies.

Furthermore, similar to the ionizable lipid-based composition in LNPs, pH-responsive polymers have also been examined for mRNA delivery. For example, poly (aspartamide)s conjugated with ionizable aminoethylene through aminolysis modified on the side chains. The mRNA-loaded nanomicelle composed by this block copolymer is protonated at the acidic pH inside endosomes, facilitating RNA delivery. The hydrophobicity and length of the aminoethylene side-chain tremendously influence the protonation and delivery efficacy of poly (aspartamide) (Kim et al. 2019). Furthermore, the pH-responsive charge-altering releasable nanocarriers have gained more attention due to their unique mRNA delivery mechanism. Despite the protonating scenarios after mRNA-loaded nanomicelles are engulfed by endosomes, these charge-altering, releasable nanocarriers self-degrade into neutral, nontoxic by-products at cytosolic pH, and rapid release of the mRNA into the cytoplasm (Haabeth et al. 2018; McKinlay et al. 2017).

One example, polyplex micelle-forming block copolymer modified from polyethylene glycol (PEG)-block-poly(β-benzyl L-aspartate) (PEG-*b*-PBLA) holds great promise for mRNA delivery and various medical applications (Kim et al. 2020) (Kataoka et al. 2000), such as applied on tissue engineering and regenerative medicine in our lab studies. We prepared the polyplex nanomicelles to complex with the Luc2 mRNA. We first synthesized the PEG-*b*-PBLA block copolymer (MW $\approx$ 28,000 with a unimodal molecular weight distribution of MW/Mn (weight-average molecular weight/number-average molecular weight) $\approx$ 1.1) and generated *N*-substituted polyaspartamides (designated as PEG-PAsp(TEP)) bearing four repeating units of tetraethylenepentamine (TEP) in the side chain to increase the positive charge. One exciting improvement upon such a model of mRNA delivery would be incorporating PEG chains to the modified polyaspartamide framework as PEG can help confer "stealth-like" properties to the overall polyplex nanocomposite (Amoozgar and Yeo 2012; Gref et al. 2000; Jokerst et al. 2011). This PEG installation is an essential step in this synergistic model, dominates the immunogenic response after in vivo administration, and may further help improve the overall stability of this mRNA nanomicelle delivery system.

The literature previously reported that the critical function of the polyamine in this complex would condense and adsorb mRNA via electrostatic interaction

and hydrogen bonding. Therefore, under a typical physiological environment, it is hypothesized that these polymeric block copolymers would organize to condense the mRNA load in the interior core, simultaneously exposing the more hydrophilic PEG domains to the peripheral region, ultimately organizing itself into the form of polyplex nanomicelle as nanoarchitecture as shown in Fig. 2a. Furthermore, the addition of PEG was also intended to assist in shielding the interiorly trapped mRNA against potential nuclease attack from physiological circumstances. Therefore, we performed both transmission electron microscopy (TEM) and dynamic light scattering (DLS) to evaluate the precise sizing of these particles in our previous study (Chan et al. 2019), revealed the micelles' sizing ranged between 31 and 35 nm in general (Fig. 2b, c).

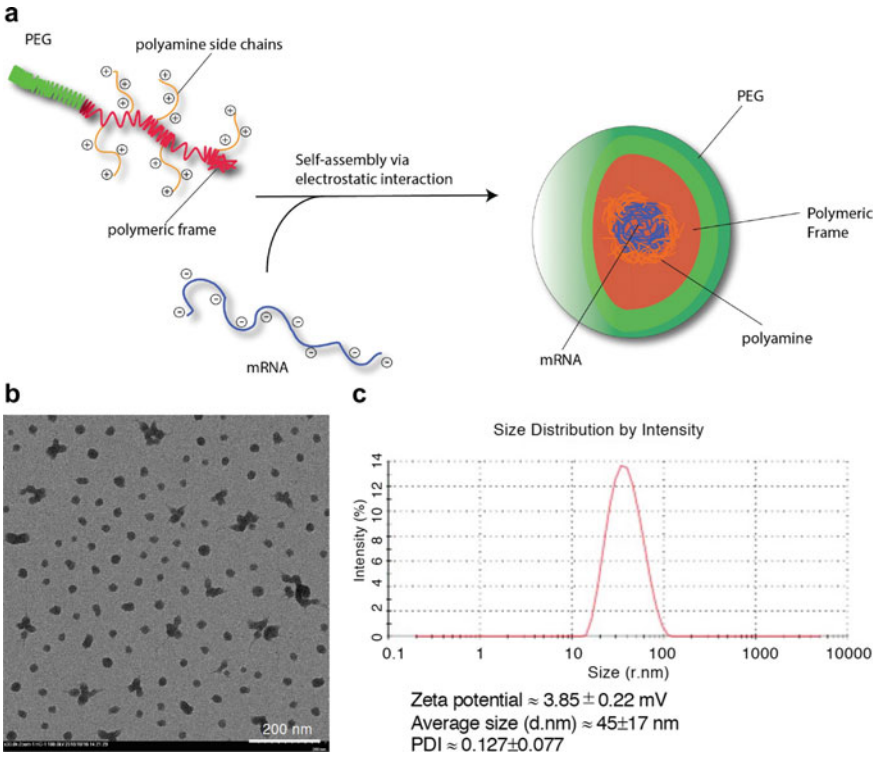

Fig. 2 Self-assembly mRNA-loaded polyplex nanomicelle and physicochemical properties. a Scheme of PEGylated polyamine-based block copolymer electrostatically interacts with mRNA to form self-assembly polyplex nanomicelles. **b** Transmission electron microscopy (TEM) examines the Luc2 mRNA-loaded self-assembly polyplex nanomicelles. **c** Physicochemical properties of Luc2 mRNA-loaded self-assembly polyplex nanomicelles through dynamic light scattering (DLS) measurement. (Figures adapted from Nanomaterials (Basel). 2019 Jan 5:9(1):67 and has gained permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

These PEGylated polyaspartate block copolymer modified with aminoethylene repeats has demonstrated the mRNA delivery capability in the liver (Matsui et al. 2015, 2018), CSF (Uchida et al. 2013; Uchida and Kataoka 2019), pancreatic cancer (Uchida et al. 2015), lung (Yoshinaga et al. 2019), brain (Lin et al. 2016; Federico et al. 2017; Fukushima et al. 2021; Abbasi et al. 2021), spinal cord (Crowley et al. 2019), spinal disc (Lin et al. 2019), knee joint osteoarthritis (Aini et al. 2016), and olfactory nerve malfunction (Baba et al. 2015). All these applications will be discussed in the following sections.

3.3 Peptide-Related Carrier

In addition to the previously described lipid and polymer-based gene carriers, peptides could also be served as gene delivery vehicles of therapeutic mRNA. The peptide sequence comprises the charged amine, either cationic or amphipathic amine groups (for example, arginine) in their backbone and side chains, which could electrostatically bound with mRNA to form the nanocomplexes. For instance, a fusogenic cell-penetrating peptide described in previous publish contains repetitive arginine-alanine-leucine-alanine (RALA) motifs, leading the nanocomplexes to change the conformation at endosomal pH, facilitating pore formation in the endosomal membrane and ameliorating the endosomal escape capability (McCarthy et al. 2014). Another paper demonstrated that the glutamic-alanine-leucine-alanine (EALA) motifs and lysine-alanine-leucine-alanine (KALA) motifs also have the function to interact with nucleic acids and perturb the cell membranes (Li et al. 2004). In addition, RALA was used to deliver mRNA to dendritic cells, particularly antigen-presenting cells (APCs) of the immune system, subsequently eliciting T cell-mediated immunity (Udhayakumar et al. 2017).

Recently, some commercially available cell-penetrating peptides have been launched. One of them, PepFect14, delivers mRNA to an ovarian cancer cell xenografted model established in mice (van den Brand et al. 2019). Another product, arginine-rich protamine peptides (approximately four kDa), turned to cationic property at neutral pH, which could be used to condense, absorb mRNA and facilitate delivery of the peptide-mRNA complexes (Kauffman et al. 2016). In addition, for protamine-based peptides complexed with mRNA, the complexes could activate Toll-like receptor (TLR7, TLR8) pathways that recognize single-stranded mRNA (Kallen et al. 2013). Therefore, the complexes could be served as an adjuvant for vaccine boost or immunotherapy applications. Some well-known applications, CureVac AG company, have been launched trials to evaluate a protamine-containing delivery platform termed as "RNActive" in clinical trials for melanoma (Weide et al. 2009), prostate cancer (Kubler et al. 2015), and non-small-cell lung cancer (Papachristofilou et al. 2019).

3.4 Other Typed Carrier

Other carrier types are used for the mRNA delivery, including the squalene-based cationic nanoemulsions, gene guns (Tavernier et al. 2011), transcript activated matrices (TAMs), or so-called gene activated matrix (GAM). Squalene-based cationic nanoemulsion consists of oil-typed squalene in the nanomicelle core and a lipid shell used to stabilize the squalene and adsorb the therapeutic mRNA on its out shell (Brito et al. 2014). Part of squalene formulations, such as Novartis's MF59, served as adjuvants in a series of FDA-approved influenza vaccines (Tsai 2013). MF59 formulation causes cells surrounding the injection site to secrete chemokines, which recruit antigen-presenting cells (APCs), induce differentiation of monocytes into dendritic cells, and enhance the invaded pathogen-related antigens uptake by APCs (O'Hagan et al. 2012). Another study, human bone morphogenic protein (BMP)-2-encoding mRNA prepared as TAMs, has demonstrated the capability to induce the osteogenic differentiation of MC3T3-E1 cells in vitro and bone regeneration in a non-critical sized femoral bone defect in a rat model (Badieyan et al. 2016) and furthermore combined with stem cells transplantation also induced satisfactory hone healing (Balmayor et al. 2017).

Gold and tungsten are commonly used in gene guns for mRNA delivery because they are readily available and could be coated with nucleic acids. However, numerous other materials have been tested, including platinum, iron, iridium, uranium, and glass. Improvements in the gun itself and the microprojectiles present the potential for this technology to expand in utility. The mRNA-coated microprojectiles were launched into mouse ears with the hand-held gun (Johnston and Tang 1994). Also, a gene gun-mediated gene delivery has effectively transported RNA molecules into several mammalian somatic tissue types. A study using gene gun treatment in mouse epidermis in vivo with messenger RNA expressing the human alpha-1 antitrypsin elicits a robust and consistent antibody response. Furthermore, the gene expression showed an increased titre with subsequent boosts. This study points to a future opportunity of applying RNA delivery methodologies for transgenic studies, genetic vaccination, and gene therapy (Qiu et al. 1996).

3.5 Cation-Free Administration

The last method proposed to deliver mRNA is a cationic material-free administration scenario. The significant toxicity of using cationic polymer to interact with biomolecules with anionic properties such as protein or nucleic acids is derived from the non-specific interaction with the cell membrane. Therefore, an experiment using PEGylated RNA oligonucleotides for mRNA delivery was conducted, showing improved serum nuclease resistance (Yoshinaga et al. 2021). The therapeutic mRNA was hybridized with sophisticatedly designed complementary oligonucleotide RNA, providing a shielding effect. This group used this PEGylated oligonucleotide RNA

to achieve a cationic polymer-free scenario for mRNA delivery. The PEG strands covering the mRNA were proposed to reduce non-specific interaction with charged biomolecules in physiological utilization. Furthermore, to achieve a more efficient mRNA delivery in clinical usages, the PEG strand is easy to modify further to bear other specific ligands, such as tissue or organ-specific homing ligand.

4 Release Profile of mRNA Medicine

4.1 Endosomal Escape

The exact mechanisms of how the polyplex nanomicelle escape from the endosomes is unclear. One popular hypothesis is the "proton sponge theory"; the proton buffering characteristic of the polyplex nanomicelle leads to osmotic swelling and rupture of the endosomes' lipid bilayer membranes (Bus et al. 2018). The other scenario of endosomal escape based on previous data is proposed (Fig. 3) (Chang et al. 2022). The mRNA polyplex nanomicelle comprising N-substituted polyaspartamides with aminoethylene side-chain residues were examined in rat-derived bone

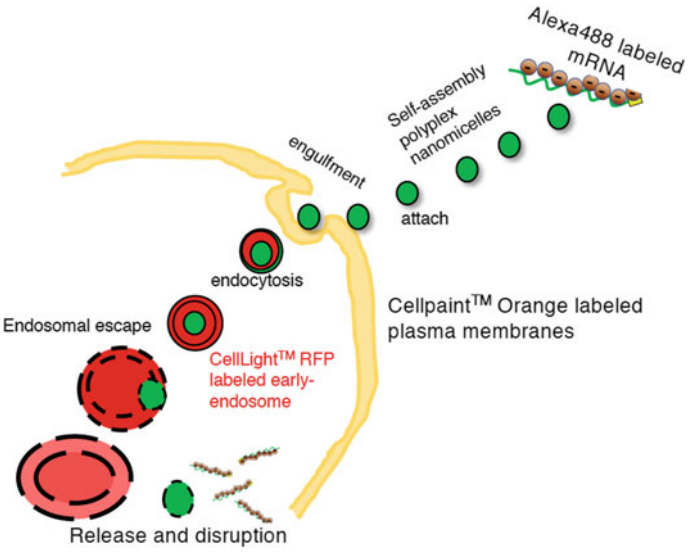

Fig. 3 Endocytosis of Luc2 mRNA-loaded polyplex nanomicelle. The proposed self-assembly polyplex nanomicelle delivers mRNA medicine into cells via endocytosis and the fluorescent dyes labelled mRNA, early endosome, and cytosol for 2-photonic microscopy. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

marrow stem cells. Endosomal escape capability and nuclease resistance capability are correlated with the modulated protonation behaviour of aminoethylene repeats due to different pH circumstances, revealing the critical importance of medicinal chemistry to design polycation structures for promoted mRNA transfection (Uchida et al. 2014). Besides, the odd–even effect also mainly dominates the endosomal escape behaviour of polyplex nanomicelle (Uchida et al. 2014).

In our study, the recombinant therapeutic protein expressed from mRNA delivery is critically attributed to mRNA-loaded nanomicelles' efficient endosomal escape capability at post-endocytosis. TEP N-substituted PEG-polyaspartate block copolymer holds a Gauche conformational transition effect (Tanaka et al. 2020; Cabral and Kataoka 2009), which would be protonated at the acidic circumstance in the endocytosis pathway when the nanomicelles reached late endosome, approximately pH 5.5, attracted apparent anion influx from the cytosol, such as Cl^-, leading to elevated osmotic pressure in the endosome (Uchida et al. 2011; Fröhlich 2012), meanwhile directly interacted with endosomal membrane, resulted in endosomal membrane disruption and nanomicelle escape and consequential mRNA release, achieved high-efficient antigen expression at a short post-transfection time.

4.2 Sustained Expression and Resistance to Nuclease Attack

To achieve the sustained expression of mRNA medicine, one can use a UGC linker between two transcripts, such as the application in manufacturing the Pfizer–BioNTech vaccine BNT162b2 (Stadler et al. 2017; Eberle et al. 2020). Besides, the endosomal escape capability of mRNA-loaded nanoparticles would also influence the mRNA transcript expression scenario and dominate the optimal application fields. For example, the PEG-PAsp(DET) and PEG-PAsp(TEP) held the faster endosomal escaping efficiency and showed superior mRNA medicine therapeutic efficacy in the brain (Lin et al. 2016; Federico et al. 2017; Fukushima et al. 2021; Abbasi et al. 2021). However, PEG-PAsp(TET) polyplex block copolymer encapsulated mRNA medicine expressed superior anabolism altering capability in osteoarthritis animal models (Aini et al. 2016).

An increase in mRNA stability by adding a poly(A) tail of 120 nucleotides in length has been demonstrated (Holtkamp et al. 2006). The optimization UTRs to achieve stability and translational efficiency is also very satisfactory. One strategy combined optimized UTR and chemically modified nucleotide, simultaneously achieving low immunogenicity and higher stability. The uridine and cytidine in the mRNA sequence were replaced by 25% chemically modified 2- thiouridine and 5-methyl-cytidine, respectively (Kormann et al. 2011). This approach was successfully applied for stem cell engineering and bone healing in the rat critical bone defect model (Balmayor et al. 2016).

In addition to the chemically modified nucleotide, stabilizing the mRNA structure, lowering the immunogenicity, and prolonging the mRNA medicine expression

time in vivo was observed. Other approaches directly modified the nucleoside structure, enhancing the resistance to nuclease attack and achieving some milestones. To date, among the 2′-ribose modifications, 2′deoxy-2′-fluoro-RNA (2′-F-RNA) shows remarkable properties for RNA interference (RNAi) applications. It enhanced nuclease resistance and improved siRNA delivery efficacy in vitro and in vivo (Pallan et al. 2011), and RNA oligomers delivery (Shu et al. 2011).

2′-O-methyladenosine-5′-triphosphate increased stability of the resulting RNA strand against nucleases. Thus, this methylation makes it a useful NTP for aptamer synthesis where increased nucleotide composition variation and increased nuclease resistance are desired (Xiao et al. 2012). Another chemically modified nucleotide, 2′,3′-dideoxyguanosine-5′-O-(1-thiotriphosphate), and other ddNTP analog contains a nuclease-resistant phosphorothioate linkage, 1-thiotriphosphate, causes chain termination during polymerase-mediated polymerization. These modified ddNTPs are helpful in antiviral research and many biotechnology applications (Adhikary et al. 2013).

Methylation at cytidine's number 5 atom position is a common, post-transcriptional modification in many RNA species, such as mRNA, miRNA, and tRNA, due to RNA methylation dominating many cellular functions, such as RNA stability, immune response, resistance to antibiotics, mRNA reading frame maintenance, and splicing. One of the modifications, 5-methyl-cytidine-5′-triphosphate (5-Methyl-CTP), is a commonly modified nucleoside triphosphate utilized to impart desirable biotechnical functions, such as increased nuclease stability, increased translation, or reduced interaction of innate immune receptors with in vitro transcribed RNA (Kariko et al. 2011).

Pseudouridine (5-ribosyluracil) was the first modified ribonucleoside discovered and is the most abundant natural modified RNA base, which could be found in structural RNAs, such as transfer, ribosomal and small nuclear RNA. Pseudouridine has been found to enhance base stacking and translation. Pseudouridine-5′-triphosphate (Pseudo-UTP) is used to increase the nuclease stability, translation of mRNA, or alter the interaction of innate immune receptors with in vitro transcribed RNA (Kariko et al. 2008). Totally, 5-Methyl-CTP and Pseudo-UTP and 2-Thio-UTP have been demonstrated to reduce innate immune response, enhance translation, longevity, and improve the therapeutic efficacy of mRNA (Vaidyanathan et al. 2018; Kormann et al. 2011). Another demonstration of 5-Methyl-CTP, Pseudo-UTP, and ARCA was used to prepare in vitro transcribed mRNA, which was successfully applied on inducing pluripotency stem cells (iPSCs) (Warren et al. 2012).

5 Application in Regenerative Medicine

5.1 Brain and CSF

mRNA medicine has been demonstrated in several studies of brain diseases. The feasibility of using polymeric nanomicelle to co-encapsulate mRNA expressing Cas9 nuclease and single-guide RNA (sgRNA) for genomic editing in vivo has been explored (Abbasi et al. 2021). This study revealed that packaging sgRNA and Cas9 expressing mRNA in the identical polymeric nanomicelle particles prevent the sgRNA from being diluted by the body fluid, meanwhile ameliorating the tolerability of sgRNA against enzymatic degradation in physiological conditions. Furthermore, this study demonstrated the sgRNA and Cas9 expressing mRNA co-packaged polymeric nanomicelle-induced genomic editing in parenchymal cells in the mouse brain, including neurons, astrocytes, and microglia using intraparenchymal injection. Totally, the genomic editing and gene expressing efficiency using sgRNA and Cas9 expressing mRNA co-packaged polymeric nanomicelle, apparently superior to that of the sgRNA and Cas9 expressing mRNA packaged in an individual polymeric nanomicelle particle separately. Another study addressed ischaemic neuronal death using mRNA therapeutics. The ischaemic neuronal death causes lifelong severe neurological disorders; currently, no proven effective treatment can prevent ischemia elicited neuronal death. They investigated the brain-derived neurotrophic factor (BDNF) expressing mRNA therapeutics for preventing neuronal death in a rat model of transient global ischemia (TGI) (Fukushima et al. 2021). Their data showed that the BDNF mRNA significantly increased the survival rate of hippocampal neurons after TGI as well as increased the BDNF recombinant protein expression in the rat hippocampus.

Another study addressed mRNA-loaded polymeric nanomicelles administration in CNS through intrathecal injection in the cisterna magna of mice. Successfully observed the gene expression in the brain stem and surrounding neural tissue (Uchida et al. 2013). Furthermore, the same group gets one more step close to the neural disease clinically; Alzheimer's disease (AD) was studied using mRNA medicine (Lin et al. 2016). They studied an mRNA expressing neprilysin (NEP), a protease that degrades Aβ and is a good candidate for this purpose. NEP is a membrane protein composed of ~750 residues (~110 kDa), capable of degrading Aβ monomers and oligomers (Iwata et al. 2001). Principally, the degradation of Aβ by NEP would effectively regulate the initiation and progression of the early stage of AD, resulting in alleviated Aβ deposition and preventing the pathogenic progression in the brain (Iwata et al. 2013; El-Amouri et al. 2008). The strategy of breaking the balance between the monomer or oligomer form of soluble Aβ and the fibril form of insoluble Aβ is potentially to alleviate or prevent the pathogenic change of AD. The exciting characteristic of mRNA medicine is the diffuse scenario in the target tissue. Their study administered GFP-NEP mRNA through intracerebroventricular (i.c.v.) infusion demonstrated that the GFP fused NEP recombinant protein expression broadly diffused in the tissues surrounding the brain ventricle (Fig. 4). This phenomenon

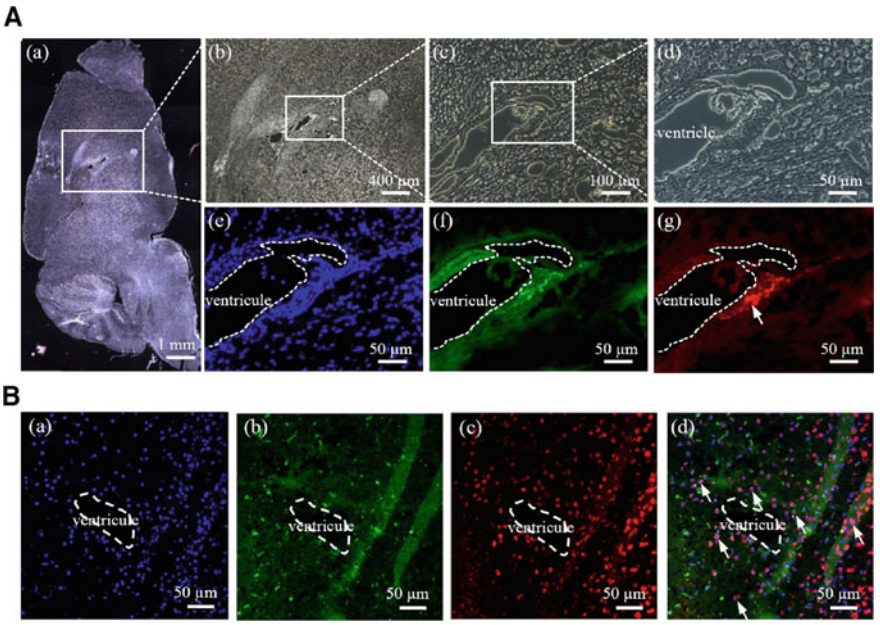

Fig. 4 mRNA-loaded polyplex nanomicelle medicine for brain infusion. Histological evaluation of GFP-neprilysin (GFP-NEP) mRNA-loaded polyplex nanomicelles intracerebroventricular (i.c.v.) infusion in mouse brain. **A** The Cy5 labelled GFP-NEP mRNA was mixed with PEG-PAsp(DET) to form polyplex nanomicelles and injected by i.c.v. infusion. At 24 h post-infusion, the brain was removed for histological examination. **a-d** were bright views of serial magnification showing the infused ventricles, and an identical slide was used for **e** DAPI staining, **f** IHC staining of the GFP-NEP fusion protein, and **g** tracking of Cy5 fluorescence, indicated by a white arrow. **B** Section from GFP-NEP mRNA i.c.v. infusion at 24 h post-infusion further proceeded with **a** DAPI staining, **b** IHC staining of the GFP-NEP fusion protein, and **c** IHC staining of NeuN marker. **d** shows the merged photo, the white arrows indicate widespread expression of both GFP and NeuN in neurons. (Figures adapted from Journal of controlled release. 2016 Aug 10, 235:268 and has gained the permission to reuse through RightsLink®)

was also similarly observed in an mRNA-loaded polymeric nanomicelle hydrodynamically injection in the liver, where the recombinant protein encoded by mRNA administration was detected in almost all liver cells after injection (Matsui et al. 2015). This characteristic is essential for administering a therapeutic protein that is not secreted or membrane-bound; in this case, transfecting more cells becomes necessary.

Furthermore, the gene expression efficiency of mRNA-loaded polymeric nanomicelle administration in the brain was quantitatively compared to the same polymeric nanomicelle loaded with plasmid DNA. A firefly luciferase-expressed mRNA (Luc2 mRNA) and plasmid DNA (Luc2 pDNA)-loaded polymeric nanomicelles were prepared for intracerebroventricular injection, followed by the in vivo imaging system (IVIS) examination. Luc2 mRNA nanomicelle provided apparent luciferase expression in the brain than the Luc2 pDNA or naked Luc2 mRNA (Fig. 5) (Lin et al.

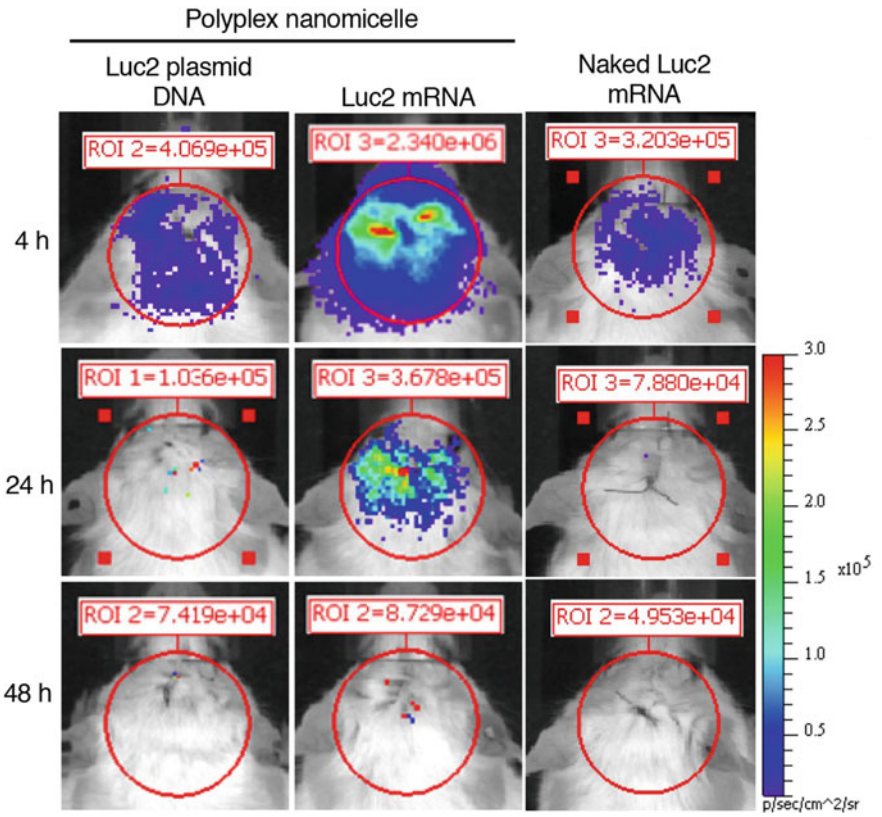

Fig. 5 mRNA-loaded polyplex nanomicelles resulted in superior gene expression in brain tissues after i.c.v. infusion. IVIS images of mice i.c.v. injected with polyplex nanomicelles containing Luc2 plasmid DNA or Luc2 mRNA, compared with infusion using non-protected, naked Luc2 mRNA. (Figures adapted from Journal of controlled release. 2016 Aug 10, 235:268 and has gained the permission to reuse through RightsLink®)

2016). Another study using mRNA encoding Aβ-targeting single-chain variable fragment (scFV) antibody with a secretion signal for passive immunotherapy showed the amyloid burden decreased in an acute amyloidosis model in mice, indicating that the therapeutic scFv encoded by mRNA medicine, an innovative polymeric nanomicelle delivery vehicle, and a suitable secretion signal are necessary for a successful therapeutic formulation (Federico et al. 2017).

5.2 Cartilage

The usage of mRNA medicine in the therapy of cartilage-related disorders, such as osteoarthritis (OA), has been demonstrated. OA is a chronic degenerative joint disease

that broadly happens in the elderly. Unfortunately, presently, no disease-modifying osteoarthritis drug (DMOAD) has been proved for clinical use. A team raised a disease-modifying strategy for OA treatment using anabolic factor mRNA medicine administrated by polymeric nanomicelles (Aini et al. 2016). They developed a polyplex nanomicelle loaded with mRNA expressing runt-related transcription factor 1 (runx1), followed by intraarticular injection in OA knee in a mouse model established by tendon resection. Runx1 is demonstrated to dominate the anabolism effect in the extracellular matrix (ECM) in cartilage, meanwhile playing a direct role in kartogenin-mediated cartilage repair using MSCs (Johnson et al. 2012). Moreover, runx1 overexpression enhanced collagen type II expression and GAG content in OA joint (Yano et al. 2013). Their data demonstrated that the OA progression was significantly alleviated in the runx1 mRNA nanomicelle administration group compared to the non-treatment control (Aini et al. 2016).

5.3 Bone

As previously described, so-called TAM or GAM has been successfully merged with mRNA medicine and applied to critical bone defect healing was demonstrated. TAM loaded with human BMP2 expressing mRNA can efficiently promote bone regeneration in a femoral bone defect model established in the rat (Badieyan et al. 2016). They prepared a collagen sponge pre-loaded with chemically modified mRNA and vacuum-dried to establish a TAM. Subsequently, the sustained delivery, bioavailability, safety, suitability for long-term storage, and efficacy of stimulating bone regeneration in rats' femoral bone defect model were examined. In addition, another matrix manufactured by fibrin to examine the efficacy for stimulating bone regeneration in vivo was also explored (Balmayor et al. 2016). Almost identical experimental procedures, chemically modified human BMP2 encoding mRNA, except for the matrix basal material. Their data showed the improved bio-efficiency of recombinant BMP-2 growth factor in bone tissue repair in a rat model.

Furthermore, the same group manufactured micro–macro biphasic calcium phosphate (MBCP) granules, loaded with chemically modified mRNA medicine, and examined the capability to induce the osteogenesis of stem cells. Their data proved the concept of developing efficient TAMs for bone regeneration by combining chemically modified mRNA (cmRNA) and optimized biomaterials. Besides, the chemically modified mRNA expressing the recombinant human BMP-2 loaded in MBCP granules induced robust collagen I and osteocalcin gene expression in the stem cell culture circumstance (Balmayor et al. 2017). Furthermore, Utzinger et al. developed a bioactive microsphere containing cmRNA bearing lipoplexes to incorporate a therapeutic component to prepare as injectable calcium phosphate types of cement (CPC). Their study demonstrated a methodology to incorporate cmRNA bearing lipoplexes into CPC without loss of function, holding the property to release and transfect immediately after administration in vivo during the cement degradation and cells infiltration (Utzinger et al. 2017). Another study incorporating chemically modified RNA in the

translation initiator of short untranslated regions (TISU) in BMP2 mRNA proposes to extend the mRNA biostability to induce robust osteogenesis. Their study removed some undesirable sequences in BMP2 mRNA, such as the upstream open reading frame in the 5′-untranslated region (UTR) and polyadenylation sequence with an AU-rich tract in the 3′UTR. Subsequently, a translation initiator of short UTRs (TISU) was incorporated with 5-iodo-modified pyrimidine nucleotides. Their data showed superior bone formation, endochondral ossification, and improved vascularization in a critical-sized femoral defect in rats (Zhang et al. 2019).

5.4 Intervertebral Disc (IVD)

We demonstrated mRNA medicine's utilization to mitigate the intervertebral disc (IVD) degeneration scenario (Lin et al. 2019). The IVD is a dynamic tissue, the ECM undergoes continuous remodelling as IVD cells produce new ECM material and proteases degrade old material (Urban and Roberts 2003). Therefore, disc degeneration is not only an ageing disorder but is also related to the shortage of critical factors involved in the homeostasis of the disc matrix (Chan et al. 2006). Since disc degeneration is defined as the disruption of the equilibrium between the anabolism and catabolism of the disc matrix (Fontana et al. 2015). Similarly, disrupted homeostasis exists in chondrocytes in osteoarthritis, and current therapeutic strategies aim to alter this misbalanced homeostasis (Blanco and Ruiz-Romero 2013). Therefore, we hypothesize that similar therapeutic strategies and anabolic factors can be applied to treat disc degeneration, significantly induced by traumatic injury, which needs the excellent and real-time supplement of disc ECM to support the disc structure.

We prove this hypothesis in a rat coccygeal disc model of traumatic injury-induced disc degeneration (Liao 2016; Inoue et al. 2015; Grunert et al. 2014), and utilized a polyethylene glycol (PEG)-polyamino acid nanocarrier to encapsulate a therapeutic mRNA encoding Runx-1, cartilage-anabolic transcription factor (Aini et al. 2016; Baba et al. 2015; Lin et al. 2016; Uchida et al. 2013, 2016).

The PEGylated nanocarrier protection group exhibited superior reporter mRNA expression efficiency than the naked mRNA injection in the rat coccygeal disc injection model and persisted to at least seven days post-injection (Fig. 6). However, the naked mRNA administration only emitted very sparse luminescence and could not be detected by IVIS. Based on this result, then, we triggered traumatic disc degeneration in rat coccygeal discs using a needle puncture and subsequently administrated mRNA therapeutics to examine the regeneration capability.

Magnetic resonance imaging (MRI) data revealed that Runx-1 mRNA delivered by nanocarriers resulted in a higher intensity of MRI-T2 signals, mitigating the loss rate of hydration content inside the disc and slowing the speed of disc degeneration (Fig. 7) for at least four weeks post-injury. This strategy may be helpful to compete with the degeneration process and slow the gradual worsening of disc damage over time. We hypothesize that mucoid materials loss accompanied by decreased hydration content in the acutely traumatized disc eventually leads to complete disc degeneration. Data

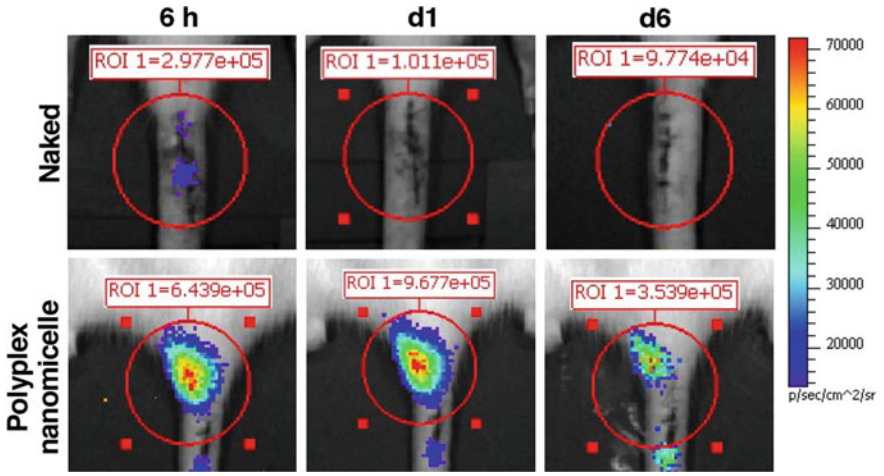

Fig. 6 Luc2 mRNA delivery in the coccygeal disc defect and IVIS observation to detect the mRNA expression duration. Representative images show the luciferase expression in the coccygeal disc in rats. Luc2 mRNA was injected in naked form compared with that loaded in polyplex nanomicelle form. Images were captured by IVIS examination at 6 h to six days post-injection. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

shows the use of nanocarrier developed in our group for mRNA therapeutics delivery, which effectively prevented the inflammation elicited by the naked mRNA administration (Fig. 8) and promoted more apparent collagen, GAG, and aggrecan secretion (Fig. 8) (Lin et al. 2019).

5.5 Olfactory Neuron

mRNA medicine for treating a temporarily olfactory neuron malfunction has been explored in an exciting study (Baba et al. 2015). They temporarily induced an olfactory dysfunction in a mouse model, followed by intranasal administration of polymeric nanomicelle loaded with a therapeutic mRNA, brain-derived neurotrophic factor (BDNF)-expressing mRNA, providing an efficient and persistent recombinant protein expression in the mouse nasal tissues. Remarkably, the recombinant protein expressed mainly in the lamina propria in the nasal cavity primarily contains olfactory nerve fibres, which effectively regulate the immunogenicity of mRNA. Their data showed remarkably ameliorated recovery of olfactory function and repairing the olfactory epithelium to nearly intact tissue. Thus, they showed the therapeutic potential of introducing exogenous mRNA to treat neurological disorders (Baba et al. 2015).

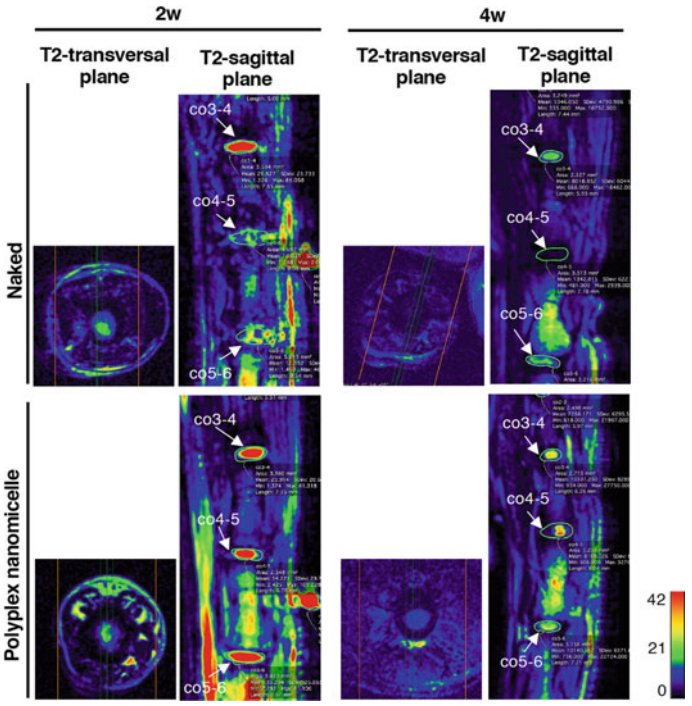

Fig. 7 Runx-1 mRNA delivery in the coccygeal disc defect and MRI examination to detect the hydration change of punctured disc in rats. **a** Runx-1 mRNA was injected in naked form compared with that loaded in polyplex nanomicelle form, and MRI was carried out at 2- and 4-weeks post-injection. Representative images show the T1- and T2-weighted MRIs, which were converted to colour histograms based on the CLUT in the OsiriX MD software. Due to the disc defect being created in the co4-5, the T2-transversal plane shows the T2-weighted image retrieved from the co4-5 in the sagittal plane to directly present the damaged disc's hydration content. Region of interest (ROI) was circled at co3-4, co4-5, and co 5–6 at T2-weighted image from sagittal plane for subsequent CLUT colour intensity calculation. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

5.6 Liver

Fulminant hepatitis in a mouse model treated with B-cell lymphoma-2 (BCL-2) protein-expressing mRNA medicine was described (Matsui et al. 2015). Bcl-2 has been demonstrated to possess the anti-apoptotic effect, which was considered a strategy to combat fulminant hepatitis. The authors manufactured a polymeric nanomicelle loaded with BCL-2 expressing mRNA for hydrodynamically intravenous injection. Their data revealed that the BCL-2 drug delivery in an mRNA form was more effective in reducing fulminant hepatitis-induced apoptosis in the liver than the BCL-2 drug delivery in a plasmid DNA form. They concluded that the

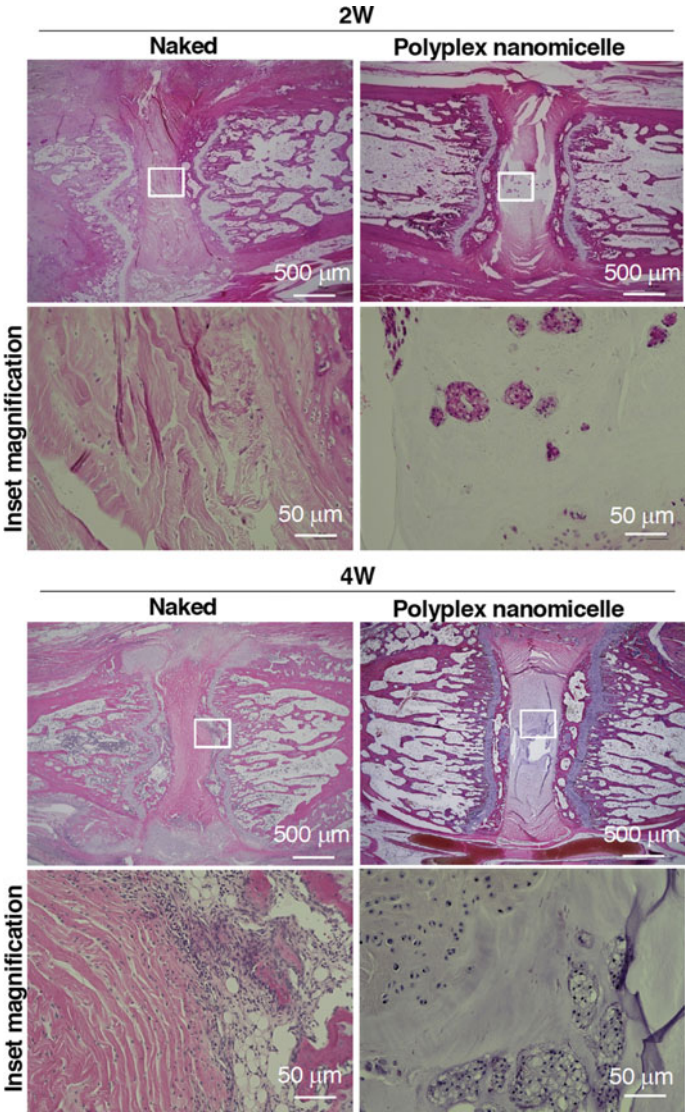

Fig. 8 Hematoxylin and Eosin (H&E) staining to examine the disc's fibrous tissue and jelly-like material. Representative images from the post-punctured animal. Runx-1 mRNA was injected in naked form compared with that loaded in polyplex nanomicelle form. The punctured disc was collected at 2- and 4-weeks post-injection and subjected to H&E staining. The solid line boxed area was magnified to observe the detailed fibrous tissue infiltration or jelly-like material used to represent the hydration content. (Figures adapted from Int J Mol Sci. 2022 Jan 5, 23(1):565 and has gained the permission to reuse. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/)

mRNA-based therapeutics combined with an effective gene delivery carrier could be served as a promising treatment for the intractable disease associated with excessive apoptosis (Matsui et al. 2015).

Furthermore, the same group also applied the BCL-2 mRNA technology to improve the hepatocyte engraftment efficiency (Matsui et al. 2018). Their data revealed that transfection of a BCL-2 expressing mRNA in an ex vivo scenario led to an enhanced survival rate of engrafted hepatocytes in both standard and diseased mouse models with liver malfunction. Meanwhile, the engraftment of BCL-2 mRNA medicine administrated hepatocyte also recovers the liver function from chronic hepatitis. Furthermore, the engrafted hepatocytes that survived for at least one month maintained their hepatic function in the recipient animal. This study demonstrated that even a transient BCL-2 protein expression, sufficient to prevent the transplanted hepatocyte death, is a critical bottleneck and obstacle in the allogeneic cell transplantation. Remarkably, the temporally controlled pro-survival factor protein expression mediated by mRNA administration is a good candidate for a further clinical setting, fitting the maximum demand for safety concerns (Matsui et al. 2018).

5.7 Cardiac and Skeletal Muscle Cells

In the field of cardiac and skeletal muscle gene transfection, fewer studies using mRNA medicine were described, which may be due to the gene transfection in cardiac muscle using naked plasmid DNA showing a surprising effect. Recently, a report described a time- and dose-dependent manner of the therapeutic protein expression in vivo using chemically modified mRNA in an intracardiac and intramuscular injection (Chien et al. 2014). Their data suggested that muscle tissue may also possess a particular property of taking up modified and un-modified mRNA medicine, leading to a tremendous therapeutic protein expression on-site. Even the fully differentiated adult rod-shaped cardiac muscle cells or fused skeletal muscle myotubes could be transfected by mRNA, leading to a high-efficient recombinant protein expression. Besides, this group also developed a time- and dose-dependent, efficient protein expression protocol led by therapeutic mRNA administration in vivo in intracardiac and intramuscular injection, demonstrating that chemically modified mRNA holds a promising capability to direct the expression of any protein of interest.

5.8 Spinal Cord

An mRNA medicine therapy for spinal cord injury treatment was also reported (Crowley et al. 2019). This group developed a polymeric nanomicelle used to carry brain-derived neurotrophic factor (BDNF) mRNA medicine to treat spinal cord injury (SCI) in a mouse model. SCI affects nearly 300,000 people per year globally, especially the young men population, which is the majority of this disease,

usually resulting from sports injuries, car and bike accidents, falls, and violence. Recovery from SCI is tremendously tricky due to the physiological changes and loss of neural functions resulting from the initial spine injury. Besides, the injured tissue emerged as inflammation, apoptosis, loss of myelin, and formation of a glial scar that retards the new axon ingrowth in the days to weeks following SCI, the so-called secondary injury that debilitates the recovery of neural motor function. This group proposed using an mRNA medicine expressing BDNF to prevent secondary injury, improving the long-termed neuron recovery by keeping the neural tissue alive during this critical period.

5.9 Stem Cells Engineering

More and more studies demonstrated that the biological modification of MSCs could be safely engineered by mRNA therapeutics transfection, transiently achieves the intended function, or leads to a targeted differentiation (Andrzejewska et al. 2020; Kim et al. 2018; Van Pham et al. 2014; Zhang et al. 2021). One exciting study using mRNA transfection to engineer MSCs to possess a specific target homing property was shown (Kwon et al. 2018). Due to transfect mRNA into MSCs with a facile, prompt, and transient expression of homing ligand, modified on the surface of MSCs is considered safer than the permanent genetic modification for MSCs. MSCs modified by other cell surface modification methods may disturb the unique differentiation commitment due to the permanent changes of the cellular membrane. Although MSCs possess their intrinsic and unique homing or migration properties, the protein ligands involved in regulating migration are principally expressed on the cellular membrane of MSCs. However, these particular tissue homing-assisted ligands may lose during the MSC expansion in vitro, leading to only a tiny portion of MSCs eventually reaching the target tissue. In principle, applying naïve MSCs for allogenic cell-based regenerative medicine through bolus injection is difficult to achieve the therapeutic effect. They concluded that these unique mRNA transfections, such as homing protein–ligand expression, make MSCs engineered modification safer and more powerful (Kwon et al. 2018).

6 Conclusion

By understanding how the mRNA transcript interacts with the immune system and advancing nucleoside chemistry and manufacturing, scientists have improved the therapeutic and prophylactic application of mRNA medicine for many medical purposes. Primarily, it gained much attention and success in vaccine development combating the urgent and severe COVID-19 pandemic. The mRNA medicine holds many advantages, no chromosome insertion concern, no DNA backbone remnant, facial to produce in large quantity and mix as a therapeutic cocktail, promptly adapt

the transcript to meet the distinct disease requirement, and a bunch of carrier and modified nucleotide choice to alleviate the immune complication and sustain the expression. From the DNA template preparation, IVT, capping, quality analysis to the final of nanoparticle encapsulation or carrier loading, mRNA medicine has successfully demonstrated its therapeutic capability in many fields of regenerative medicine, which would disclose more and more successful examples shortly.

7 Perspective

For the consideration of mRNA medicine design, we propose the carrier should comply with (I) low cytotoxicity, excellent biocompatibility, (II) efficient endosomal escape capability, (III) higher capacity for mRNA payload, and (IV) holds excellent stealth properties in the immunocompetent surveillance. Besides attempting to achieve a satisfactory tissue healing outcome, the utilization of mRNA medicine should combine with optimal scaffolds, holding suitable biomechanical properties in compliance with the grafted site. For specific disease therapy, the utilization of mRNA medicine should be considered more comprehensively, such as the application in vaccine development. Some pathogens may need the attenuated or subunit vaccine rather than the mRNA vaccine. In regenerative medicine, whether the disease scenario needs the recombinant protein administration, viral vector gene delivery, or therapy of mRNA medicine, which needs more thorough studies to demonstrate.

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