Journal of Controlled Release 240 (2016) 227–234

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Materials for non-viral intracellular delivery of messenger RNA therapeutics

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ARTICLE INFO

Article history:
Received 3 September 2015
Received in revised form 14 December 2015
Accepted 18 December 2015
Available online 21 December 2015

Keywords:
mRNA
Drug delivery
Gene therapy
Endosomal escape
Immunoengineering
Genome editing

ABSTRACT

Though therapeutics based on messenger RNA (mRNA) have broad potential in applications such as protein replacement therapy, cancer immunotherapy, and genomic engineering, their effective intracellular delivery remains a challenge. A chemically diverse suite of delivery materials with origins as materials for cellular transfection of DNA and small interfering RNAs (siRNAs) has recently been reported to have promise as non-viral delivery agents for mRNA. These materials include covalent conjugates, protamine complexes, nanoparticles based on lipids or polymers, and hybrid formulations. This review will highlight the use of delivery materials for mRNA, with a specific focus on their mechanisms of action, routes of administration, and dosages. Additionally, strategies in which these materials can be adapted and optimized to address challenges specific to mRNA delivery are also discussed. The technologies included have shown varying promise for therapeutic use, specifically having been used to deliver mRNA *in vivo* or exhibiting characteristics that could make *in vivo* use a possibility. In so doing, it is the intention of this review to provide a comprehensive look at the progress and possibilities in applying nucleic acid delivery technology specifically toward the emerging area of mRNA therapeutics.

1. Introduction

Aberrant protein expression is a frequent hallmark of many diseases, ranging from genetic disorders to cancer. Thus, the ability to control protein expression *in vivo* has broad therapeutic potential. Nucleic acids endogenously control and regulate intracellular protein expression *via* a number of established mechanisms. DNA can be transcribed to produce messenger RNA (mRNA), which in turn can be translated into specific proteins [1]. Alternatively, various RNA interference (RNAi) pathways exist in which oligonucleotides in the form of small interfering RNAs (siRNAs) or other types of RNAs silence protein expression [2]. In using nucleic acids therapeutically, a number of barriers must be overcome in the delivery of exogenous molecules [3,4]. Among these, barriers of entry into the cell in order to realize efficient intracellular delivery of nucleic acid present perhaps the most formidable challenge. As nucleic acids are large, hydrophilic, anionic molecules, they cannot readily traverse the hydrophobic lipid membrane of the cell [5]. Furthermore, the association of free nucleic acids with invading pathogens has resulted in the evolution of a number of innate defense mechanisms that include circulating nucleases, which degrade nucleic acids, and pattern recognition mechanisms that serve as activators of the innate immune system [6,7]. For these reasons, efficient delivery is often mediated by a vector to entrap, protect, and shuttle the nucleic acid payload across the cell membrane to enable access to the cytosol (for siRNAs and mRNAs) or the nucleus (for DNAs) in order for the nucleic acid to elicit its function. These delivery vectors for nucleic acids are made from diverse synthetic or natural materials (lipids, polymers, peptides, antibodies, small molecules, metals, *etc.*) and come in a variety of geometric architectures (nanoparticles, microparticles, conjugates, solid devices, hydrogels, *etc.*). The delivery material is crucial in realizing efficient and efficacious nucleic acid therapy.

Historically, early efforts to transfect cells led to the observation that positively-charged molecules, typically rich in protonating amine groups, could readily form electrostatic complexes with negatively-charged DNA, and further that the resulting nanocomplexes could enter cells and facilitate protein expression [8]. The evolution of these cationic delivery materials, beginning with initial efforts to use naturally-occurring molecules like protamine [9] and poly(L-lysine) [10] and extending to more recent examples of synthetic molecules specifically designed for gene delivery like poly(β-amino esters) [11] or DLinDMA [12] lipids, is the focus of this review. Following the discovery of siRNA in the late 1990s [13], these and other delivery materials often proved useful in applications for siRNA delivery. In addition, a number of novel delivery materials were specifically designed for

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http://dx.doi.org/10.1016/j.jconrel.2015.12.032
0168-3659/

siRNA delivery, such as ionizable lipid-like molecules [14,15], and these will be discussed in this review as well.

Recently, there has been great interest in the therapeutic use of mRNA [16–19], which also requires effective delivery materials to reach its target. From a delivery perspective, there are inherent benefits to using mRNA instead of DNA. mRNA must only be delivered to the cytoplasm where cellular translation machinery is located; conversely, DNA requires transfection to the nucleus, which introduces the added physical barrier of the nuclear membrane. From a therapeutic perspective, protein expression arising from mRNA is more transient than that from DNA. From a safety perspective, mRNA does not carry the risk of genomic integration associated with DNA insertional mutagenesis [20]. Delivery of mRNA also offers many therapeutic directions beyond protein replacement/supplementation therapies. For example, the inherent immune-activating adjuvant properties of foreign RNAs can be leveraged for the intracellular delivery of mRNAs coding for specific antigens, which could be broadly applied in cancer immunotherapy [21], prophylactic vaccines [22], and allergy tolerization [23]; this particular use of mRNA is the most clinically advanced and thus constitutes the majority of the applications discussed here. Additionally, mRNAs have potential for use in the emerging field of genome editing and genomic engineering [24,25].

In this review, the various classes of materials that have been used for nucleic acid delivery will be highlighted along with perspective on the use of these materials specifically for the therapeutic delivery of mRNA. Of note, viral vectors, which can also successfully deliver nucleic acids, have been extensively reviewed elsewhere [26,27] and are not discussed as part of the present contribution. A recent review [18] extensively focused on the therapeutic potential and challenges surrounding mRNA as a drug, but did not describe in detail the specific delivery materials that would facilitate its use. Here, we focus primarily on mRNA delivery materials, along with their mechanisms of action, routes of administration, and dosages for *in vivo* applications. The use of materials for mRNA delivery will be framed in the context of the history of their use in the delivery of other classes of nucleic acids, as many of these materials were initially designed for siRNA and DNA delivery applications. Materials are discussed in order of increasing complexity, starting with the delivery of naked mRNA, followed by protamine-based delivery systems, then lipid and polymer materials, and concluding with hybrid formulations.

2. Delivery of naked oligonucleotides

Naked nucleic acids cannot readily cross cell membranes as a result of their size, charge, hydrophilicity, and degradability [5]. However, there exist some native mechanisms by which mRNA can translocate across the cell membrane without the use of a delivery material: studies performed *in vitro* have demonstrated the capability of cells to uptake naked mRNA *via* scavenger-receptor mediated endocytosis, and though most mRNA accumulates and degrades in lysosomal compartments, some intact mRNA is able to access the cytoplasm and express protein [28]. Common routes of transient membrane permeabilization, such as electroporation, provide another method to transfect cells with naked mRNA and are commonly used to study mRNA activity or immunogenicity *in vitro* [29] and *in vivo* [30]. Microinjection, in which mRNA is directly injected into the cell using a micropipette, is useful for efficient delivery of mRNA to single cells *in vitro* [31]. Other physical methods such as hydrostatic pressure transfection, sonoporation, and laser irradiation, have been used *in vitro* for DNA delivery [32] but are less commonly used for mRNA delivery. The use of a gene gun, in which naked mRNA is coated onto the surface of gold microparticles and pneumatically shot into a target cell at high speed, has also been described for mRNA transfection [33].

Intravenous injection of unmodified mRNA without a delivery material leads to rapid degradation by ribonucleases and can activate the innate immune system [7]. Direct local injections of naked mRNA, *e.g.* subcutaneously [34], intramuscularly [35], or intranodally [36], have shown some utility in applications in which an immune response is desired and relatively low levels of generated protein are required, such as for vaccination [18]. Some techniques have been demonstrated to improve naked mRNA potency; significant enhancements in both protein expression and duration were observed following intranodal injection of naked mRNA when dissolved in buffers containing calcium ions [36]. As of this writing, there have been dozens of preclinical studies and clinical trials in which the direct injection of mRNA was evaluated for cancer (melanoma, renal cell carcinoma), infectious diseases (influenza, tuberculosis), allergy tolerization (peanut, egg white), and protein replacement (anemia, asthma); these trials and others have been reviewed extensively elsewhere [18]. Thus, in some specific circumstances, the direct local injection of naked mRNA may have therapeutic utility.

Systemic delivery of naked siRNA to the liver, on the other hand, has been mediated through direct conjugation of targeting ligands to the siRNA molecule. In a report from 2004, Soutschek *et al.* [37] conjugated cholesterol to siRNA *via* the 3′ end of the sense strand so as not to interfere with the antisense/RISC binding required for RNAi. Intravenous injection of the siRNA–cholesterol conjugate into mice at a high dose (50 mg/kg) resulted in silencing of an endogenous gene coding for apolipoprotein B and transfection was found to be mediated by lipoprotein trafficking [38]. Current state-of-the-art for liver-targeted siRNA conjugates uses direct conjugation of a triantennary GalNAc small molecule (Fig. 1a), a derivative of galactose which has remarkably high affinity for the asialoglycoprotein receptor found on heaptocytes [39–41]. GalNAc-conjugated siRNAs exhibit potent silencing in hepatocytes *in vivo* following subcutaneous administration at 5 mg/kg and are being evaluated in ongoing human clinical trials [42,43].

Despite the success demonstrated for siRNA conjugates, to our knowledge direct small molecule conjugation approaches for mRNA delivery have not yet been reported. Differences in size, stability, and function between siRNA and mRNA contribute to making mRNA delivery *via* direct conjugation a more challenging endeavor. The molecular weight of therapeutically-relevant mRNA can be orders-of-magnitude larger than siRNA, and thus the relative targeting ligand to RNA size ratio is much smaller for mRNA conjugates, and the secondary structure of the mRNA may obscure the ligand from its cognate receptor. Another important difference is the existence of the siRNA sense strand, which offers a convenient route of chemical conjugation through terminal modification without impeding function, as only the antisense strand binds to the RISC complex [44]. Also, siRNA can be made routinely using established oligonucleotide synthesis techniques, enabling more control over orthogonal synthetic modification than is possible for mRNA which, owing to its length, is primarily produced *via in vitro* transcription [18] and thus presents significant challenges in controlling the location of potential modifications.

Furthermore, while siRNA can be protected against degradation by ribonucleases through chemical modifications to the RNA nucleotides and/or phosphodiester bonds [7,45] without significantly hindering the ability of siRNA to participate in the RNAi pathway, modifications to the bases of mRNA can be more challenging and alter ribosomal translation [46,47]. It should, however, be noted that several base modifications, such as pseudouridine (ψ) and 5-methylcytidine (5mC) (Fig. 1b), have been reported to be tolerated with decreased immunogenicity and may even increase net protein production compared to unmodified mRNAs in some settings [48–50]. There are a number of other design considerations in preparing mRNA for eventual therapeutic use. Typically, a 5′ cap and polyadenylated 3′ tail are used to increase the translation efficiency and also to protect against nuclease degradation [51,52]. Furthermore, the sequence of the untranslated regions (UTRs) of mRNA (which flank the coding region) can facilitate translation and stability [53,54]. A recent report describes sequence-engineered mRNA, in which both the coding region and UTRs are optimized through iterative screening, resulting in significantly increased