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Methods of Transfection with Messenger RNA Gene Vectors

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Abstract

Non-viral gene delivery vectors with messenger RNA (mRNA) as a carrier of genetic information are among the staple gene transfer vectors for research in gene therapy, gene vaccination and cell fate reprogramming. As no passage of genetic cargo in and out of the nucleus is required, mRNA-based vectors typically offer the following five advantages: 1) fast start of transgene expression; 2) ability to express genes in non-dividing cells with an intact nuclear envelope; 3) insensitivity to the major gene silencing mechanisms, which operate in the nucleus; 4) absence of potentially mutagenic genomic insertions; 5) high cell survival rate after transfection procedures, which do not need to disturb nuclear envelope. In addition, mRNA-based vectors offer a simple combination of various transgenes through mixing of several mRNAs in a single multi-gene cocktail or expression of a number of proteins from a single mRNA molecule using internal ribosome entry sites (IRESes), ribosome skipping sequences and proteolytic signals. However, on the downside, uncontrolled extracellular and intracellular decay of mRNA can be a substantial hurdle for mRNA-mediated gene transfer. Procedures for mRNA delivery are analogous to DNA transfer methods, which are well-established. In general, there are three actors in the gene delivery play, namely, the vector, the cell and the transfer environment. The desired outcome, that is, the efficient delivery of a gene to a target cell population, depends on the efficient interaction of all three parties. Thus, the vector should be customised for the target cell population and presented in a form that is resistant to the aggressive factors in the delivery *milieu*. At the same time, the delivery environment should be adjusted to be more vector-friendly and more cell-friendly. The recipient cells should be subjected to a specific regimen or artificially modified to become receptive to gene transfer with a particular vector and resistant to the environment. As a rule, barriers outside tissues (e.g. mucus) and an aggressive

intercellular environment complicate gene delivery *in vivo*, which, therefore, requires more complex gene transfer procedures than transfection of tissue culture cells. This review is focused on transfection methods for mRNA vectors, which rely either on the forceful propulsion of mRNA inside the target cells (e.g. by electroporation or gene gun) or on the complexing of mRNA with other substances (e.g. polycationic transfection reagents) for delivery via endocytic pathways.

Keywords: Gene therapy, epigenetic reprogramming, gene vaccination, mRNA gene vectors, transfection methods, gene transfer

1. Introduction

1.1. Why messenger-RNA-based vectors are used in gene delivery?

DNA or RNA fragments of choice can be amplified in bacteria and eukaryotic cells by piggybacking on replicating episomes, called 'cloning vectors'. In contrast to 'cloning vectors', 'gene vectors' are the vehicles that transfer genes into cells. All gene vectors contain nucleic acids or their analogues (e.g. Peptide Nucleic Acid – PNA) as the carriers of genetic information. The complexity of gene vectors ranges from naked DNA or RNA to multi-component nano-devices with a finely ordered internal structure, which can be either virus-derived or purely synthetic. The aim of gene delivery is often the presence of specific proteins in the target cells. One way to achieve this is to transfer an immediate information source for protein biosynthesis, that is, messenger RNA (mRNA), into target cells. Presently, mRNA-based vectors are established multipurpose gene vectors applicable to a diverse range of tasks in gene therapy, gene immunisation and transgene-mediated cell-fate reprogramming [1-4].

The long-term storage of genetic information in cells is mediated by DNA, while short-term cellular memory is stored in RNA. So, if a permanent genetic change in the target cells is desirable, either DNAs or RNA-templates for reverse transcription into DNA are used as carriers of genetic information within the gene vectors. If only a non-permanent genetic change is wanted, then gene vectors containing a translatable 'sense' RNA strand ('positive strand') seem to be particularly suitable. Such vectors, whether based on mRNA generated *in vitro* or cellular mRNAs (including cellular mRNAs isolated through packaging into viral capsids), can reach ribosomes and express genes in the cytosol, without nuclear entry. There are five important implications of the extra-nuclear status of mRNA vectors.

Firstly, as mRNA does not require transfer to nucleosol for expression, mRNA-based vectors can be used in applications where a rapid and transient effect is required, e.g. wound healing or antigen-presenting. The transgene expression is fast because mRNA vectors, as opposed to DNA vectors, do not need to pass through the barrier of the nuclear envelope, which confines the nucleosol in non-dividing cells and do not need to enter the nucleus and then to exit it. In addition, no time is wasted on intra-nuclear transcription in both dividing and non-dividing cells.

Secondly, the fact that gene delivery with mRNA vectors is capable of attaining transgene expression in non-mitotic cells with a closed nuclear envelope is remarkable *per se*. Thus, mRNA vectors can be more efficient than DNA vectors for the transfection of clinically important post-mitotic cells like neurons and cardiomyocytes [5]. In fact, in non-dividing cells there is no dilution of externally delivered mRNAs and their protein products in cell divisions; this circumstance can contribute to longer persistence of mRNA-vector-mediated transgene expression in these cells in comparison to dividing cells.

Thirdly, the major mechanisms of transgene silencing, e.g. chromatin remodelling and genomic DNA methylation [6], are entirely intra-nuclear and, thus, are irrelevant for the desired expression of exogenous mRNA.

Fourthly, for the successful implementation of many therapeutic strategies, it is important that gene delivery with mRNA vectors cannot cause potentially deleterious mutations via insertional gene inactivation or undesired position effects like gene activation in the neighbourhood of a chromosomally integrated transgene. Indeed, in many cases the full long-term consequences of the genomic insertions are difficult to predict and so any permanent genetic change is often unwanted. As any gene delivery with mRNA-based vectors does not leave an undesired genomic trace, gene transfer with mRNA vectors *in vivo* benefits from the absence of the safety risks and ethical controversies of vector elements being incorporated into the human germ line and subsequently being transmitted vertically through future generations.

Fifthly, as only extra-nuclear localisation of externally delivered mRNA is required for transgene expression, 'milder' transfection conditions (e.g. shorter electric field pulses during electroporation) might be sufficient for delivery of mRNA into its 'expression milieu'. Indeed, 'milder' conditions increase the cell survival rate and, hence, offer higher transfection efficiency with mRNA vectors in comparison to DNA vectors [7].

Another advantage of mRNA-based vectors is the flexibility to combine several mRNAs into a single multi-gene cocktail. In addition, a number of proteins can be expressed from a single mRNA using internal ribosome entry sites (IRESes), ribosome skipping sequences or *bona fide* proteolytic signals. The ease of transgene reshuffling makes mRNA-based vectors particularly convenient in the complex tasks of epigenetic engineering, where multiple combinations of transgenes need to be screened to assess their cell-fate reprogramming effectiveness. However, on the downside, uncontrolled extracellular and intracellular decay of mRNA can be a substantial hurdle for mRNA-mediated gene transfer.

1.2. What strategies are used to deliver mRNA-based vectors to cells?

Methods for mRNA delivery are similar to DNA transfer procedures, which are well-established. Overall, there are three actors in the gene delivery play, namely, the vector, the cell and the transfer environment. The desired outcome, the efficient transfer of a gene to a target cell population and its installation as a functional transgene depends on the productive interaction of all three parties. Thus, the vector should ideally be targeted to reach the desired cells selectively and efficiently and also presented in a form that is resistant to the aggressive factors in the delivery *milieu*. At the same time, the delivery environment should be adapted to be

better vector-accommodating and better cell-accommodating. The recipient cells should be subjected to a specific set of treatment procedures or artificially modified to become receptive to gene transfer with a particular vector and resistant to the environment.

In general, mRNA-based vectors can be delivered to cells in tissue culture (*in vitro*) and intracorporeally (*in vivo*). *In vitro* gene delivery is a necessary step in *ex vivo* strategies of gene immunisation [8, 9], gene therapy [10] and therapeutic cell-fate reprogramming [4]. As a rule, barriers outside tissues (e.g. mucus) and an aggressive intercellular environment complicate gene delivery *in vivo*, which, therefore, requires more complex transfection procedures than transfection *in vitro*. The standard transfection routes rely either on the forceful propulsion of mRNA inside the target cells (e.g. by electroporation or gene gun) or on the complexing of mRNA with other substances (e.g. polycationic transfection reagents) for delivery via endocytic pathways.

2. Gene delivery with mRNA-based vectors

The key parameter describing gene delivery is 'efficiency of gene transfer'. This variable can be defined as a ratio of a number of cells, which successfully received the intended genetic cargo, to the number of all cells, into which delivery of the genetic cargo was attempted. 'Successful' gene transfer normally implies not only the delivery of the genetic material *per se* but also its adequate expression in the recipient cells. Overall, 'efficiency of gene transfer' unavoidably depends on the hierarchy of multiple factors, which includes the efficiency of passage of the genetic cargo into the target cells and the efficiency of transgene expression. Instability of mRNA is a critical factor limiting the efficiency of gene transfer with mRNA-based vectors because extracellular degradation of mRNA precludes its entry into the cell and intracellular degradation of mRNA silences expression of mRNA-borne transgenes. Relative instability of mRNA is part and parcel of the general design of the living cell, as it allows the cell to change its gene expression profile depending on external stimuli and internal differentiation program. Thus, all methods of mRNA transfer into mammalian cells need to address instability as an inherent feature of mRNA. Thus, we prelude the description of the current methods of mRNA-based gene delivery with an in-depth overview of the technical means to increase the stability of mRNA.

2.1. Increasing the stability of mRNA in extracellular and intracellular environments

Comparative instability of RNA is determined by its molecular structure. In general, DNA and RNA have similar structures; they are polymers of nucleotides, composed from nucleobases, pentose sugar components and phosphate group residues. Three of the nucleobases – cytosine, adenine and guanine – are identical in RNA and DNA; the fourth nucleobase is uracil in RNA and its methylated analogue, thymine, in DNA. The sugar component in RNA is ribose and the sugar component of DNA is deoxyribose. An extra negative charge of the hydroxyl group in the RNA's ribose repels the negatively charged phosphate group and, so, makes RNA less amenable to folding into a double helix in comparison to DNA. The formation of hydrogen

bonds between two neighbouring RNA strands is less efficient than between comparative DNA strands, contributing to the transiency of the existence of double-stranded RNA. Abundance of single-stranded segments in mRNA makes it poorly suitable for faithful repair, while stability of double-stranded genomic DNA in living cells is upheld through template-mediated enzyme-mediated repair. As a result, mRNA is vulnerable to strand breaks and spontaneous mutations.

In general, mRNA can dissipate in the form of an *in vitro* preparation, in the intercellular setting or within living cells. There are several types of degradation.

Firstly, as a substantially thread-like molecule, mRNA is susceptible to 'mechanical' degradation due to strand breaks caused by fluid shear stress or surface tension forces.

Secondly, mRNA degradation can be catalytically mediated by enzymes leading to RNA decomposition via 3'-terminal deadenylation, 5'-terminal decapping or endonucleolytic degradation. Cellular RNases are either proteins or ribozymes; the latter group is exemplified by RNase MRP and RNase P [11]. Important co-factors in enzymatic mRNA decay can be small metal ions, e.g. Mg^{2+} .

Thirdly, RNA decomposition can be catalysed by metal ions only, without enzymes. Thus, Mg^{2+} and lanthanides ions are known to accelerate RNA decay [12]. In practical terms, it is difficult to exclude a role for a trace amount of protein-based or RNA-based ribonucleases, even in ostensibly ion-led RNA degradation.

Fourthly, similarly to DNA, RNA is vulnerable to degradation in acidic conditions via 'depurination'. Depurination is the loss of adenine and guanine from the nucleic acids due to hydrolysis of their N-glycosyl linkages to ribose. Sensitivity of mRNA to low pH can compromise the transfer of mRNA vectors that are delivered to cells via endocytosis because mRNA faces degradation in acidified endosomes.

Kinetics of mRNA degradation is often described with exponential models of decay, with the 'half-life' of mRNA molecules being used as a parameter. Indeed, typical features of exponential decay can be observed during the extinction of expression of transgenes delivered to mammalian cells with mRNA vectors. Thus, the expression of transferred EGF-FLAG mRNA had reached the maximum level between 12 and 72 hours post-delivery and was very small but still detectable after 14 days [13].

While appropriate stability of many natural mRNAs was honed by natural selection, artificial mRNA vectors, e.g. new chimeric mRNAs, might be more vulnerable to attack by RNases. The mechanism of such instability can rely on the formation of double-stranded segments within mRNA. It is thought that the co-evolution of eukaryotic cells and their viruses resulted in cellular 'friend or foe recognition systems' perceiving specific regions of double-stranded RNA as hostile. Thus, it is not unusual for double-stranded RNA to be a target for an RNase attack, e.g. by the Dicer endoribonuclease, which is normally a part of RNAi silencing machinery. Double-stranded RNA is also a known inducer of the TLR3-mediated innate immune response, which can potentiate RNA degradation. Therefore, the emergence of non-desired segments of double-stranded RNA within molecules of mRNA gene vectors or between different molecules in mRNA vector mixtures should be considered in the design of

mRNA vectors and their cocktails. Clearly, the longer the individual mRNA vector molecules and the higher the number of individual mRNA species in the vector cocktails, the higher the chance for the appearance of double-stranded RNA segments through spurious sequence homologies. Thus, the design of mRNA vectors is bound to include the search of such homologies *in silico* and minimisation of any potential unwanted double-stranded-RNA-forming regions through appropriate nucleotide changes. Some short regions of double-stranded RNA, e.g. 'stem' segments in tRNAs, do not induce adverse cell responses. Such short RNA-duplex-forming regions could be intentionally introduced into mRNA vector molecules to achieve high compactness with ensuing increased resistance to shear stress. Such compacting could be particularly relevant for long RNA molecules. In addition, extra compactness of mRNA vectors might result in their beneficial resistance to RNase-mediated degradation because of reduced RNA access to the catalytic centres of RNases. Suitable segments for RNA self-compression can be provided not only by short duplex-forming regions but also by G-quadruplex-forming sequences [14]. Furthermore, compacting of mRNA vectors can be achieved using peptides and proteins. For example, poly(A)-binding protein (PABP) has roles in nuclear export, enhancement of translation and mRNA stability and can be complexed with mRNA vectors to achieve their condensation. Another mRNA compacting option is the employment of cytoplasmic polyadenylation element binding protein (CPEB). Clearly, in addition to their employment in mRNA compacting, peptides and protein ligands can be used to link mRNA to elements required for efficient cell penetration and localisation within intracellular compartments capable of efficient support of protein synthesis.

Mechanical instability of mRNA vectors is more critical in the transfer systems such as gene gun, aerosol, electroporation and sonoporation, where potentially powerful shearing forces can emerge. However, encouragingly, mRNA, which is 'shaved off' along the trajectory of the gene gun particle in tissue, was shown to be functionally active [13]. Clearly, more studies of mRNA stability in extreme conditions are required.

In laboratory environment, mRNA is normally stored at -80 °C as autodegradation and other forms of degradation are substantially limited at this temperature. Freeze-thaw cycles, which can cause breaks in RNA during phase transitions, are better avoided, e.g. by splitting mRNA preparations into smaller aliquots. As many RNA degradation pathways are active only in solutions, lyophilisation (drying from the frozen state) is often used to improve storage stability of mRNA. Precautions to prevent the degradation of RNA *in vitro* by RNases are important. RNaseZap™, a proprietary mixture of three RNase inhibitors, can be used to inactivate RNases on various surfaces. A common RNA protection agent is RNasin, a commercial version of a placental protein with RNase A inhibitor activity. Vials with RNA are often packaged into antistatic bags to avoid the adsorption of RNase-contaminated dust due to electrostatic attraction. Suitable 'protective packaging' of mRNA vectors can also be applied at the molecular level with mRNAs being coated in a suitable biodegradable or soluble material within micro-droplets. Another protection option offered by nanotechnology is mRNA packaging in pleated sheets of hairpin RNA, which form 'micro-sponges' [15].

The stabilisation of mRNA is particularly important when the mRNA populations are extracted from cells; in this case, mRNA preservation with cell-permeable RNase inhibitors can be accomplished using proprietary tissue storage and RNA stabilization RNAlater™

solution (Life Technologies). Another approach for the protection of cellular mRNAs from RNAses is to use Proteinase K to destroy all the proteins in the crude preparation including RNAses. Furthermore, it is possible that defence against RNAses by RNAsin could be extended to the intracellular environment via the development of cell-permeable versions of RNAsin. Indeed, cell-permeable versions of various proteins, which were furnished with 'protein transduction domains' (PTDs), were reported in the literature [16, 17].

2.2. Methods of transfection with mRNA vectors

Procedures for mRNA delivery are analogous to DNA transfer methods, which are well-established. In general, there are three actors in the gene delivery play, namely, the vector, the cell and the transfer environment. The desired outcome, that is, the efficient delivery of a gene to a target cell population, depends on the efficient interaction of all three parties. Thus, the vector should be customised for the target cell population and presented in a form that is resistant to the aggressive factors in the delivery *milieu*. At the same time, the delivery environment should be adjusted to be more vector-friendly and more cell-friendly. The recipient cells should be subjected to a specific regimen or artificially modified to become receptive to gene transfer with a particular vector and resistant to the environment.

2.2.1. Delivery of mRNA using transfection reagents

Transfection of cells using specialised chemicals, called 'transfection reagents', is a well-established approach for the delivery of DNA to cells [18]; it is also proven to be suitable for mRNA transfer. For example, this transfection strategy was used by Weide and co-authors, who bound mRNA to a polycation protamine and used the resultant transfection-competent complexes for mRNA-mediated gene delivery [19].

In general, this type of transfection technology relies on the abrogation of the electrostatic repulsion of the negative charge of RNA and the negative charge of the cell surface. This is accomplished through complexing the RNA and the cell surface with polycations to neutralise the negative charges or, alternatively, through 'positive overcharging' of RNA-polycation complexes to cause attraction between positively charged complexes and the negatively charged cell surface. Thus, the electrostatic interaction between negatively charged phosphate groups of RNA and positively charged groups residing in the polycation 'carrier' polymer (typically amines or amidines) results in the compaction of RNA into globular complexes called polyplexes. The ensuing condensation is useful to protect mRNA from hostile factors in the transfer environment (e.g. RNAses and sheer stress). Condensation is also useful to enhance mRNA penetration into the recipient cells through the endocytosis route. Essential factors that control mRNA condensation are the local concentrations of mRNA and other components of the complexes, which can be substantially increased through 'molecular crowding' in water solutions using hydrophilic polymers like poly (N-vinyl) pyrrolidone. The important parameter of the resultant colloid is the 'electrokinetic potential' (also known as 'zeta potential') of the particles, which determines the likelihood of the undesired coagulation and flocculation of the complexes. Low colloidal stability of lipoplexes and polyplexes (aggregation of the vector particles) can be a particular problem upon systemic administration *in vivo*. Stability of

the complexes can be enhanced through furnishing the surface of the vector particles with a layer of an additional polymer (e.g. a derivative of polyethylene glycol), which can also mask the particles from immune surveillance *in vivo*. Various extra-elements can be covalently attached to the polycation polymer backbone or incorporated into polyplexes through non-covalent bonds. These mRNA vector elements can include moieties promoting gene transfer and expression, such as cell-targeting peptides, membrane-penetration-enhancing PTDs, cytoskeleton attachment elements and other agents for targeted intracellular localisation. The architecture of the vector particles can be quite intricate. For example, mRNA can be contained within bubble-like 'neutral' liposomes. The liposomes can be encrusted with targeting ligands and viral membrane-fusion proteins resulting in 'virosomes' [20, 21].

Undesired binding of the transfection complexes to elements of the environment with subsequent loss of transfection activity should always be taken into account [22]. In particular, binding of vector particles to serum proteins is a known issue. Transfection reagents vary in their affinity to serum. Thus, Lipofectamine-based transfection is inhibited by serum, while FugeneHD-based transfection is not.

Many transfection reagents are toxic. For example, the toxicity of DEAE-dextran is well-known; therefore, normally transfection with DEAE-dextran requires repeated cell washing steps. Less toxic reagents can be obtained through optimised chemistry. An example of a less toxic polycation is the polysaccharide chitosan, which is produced from the shells of crustaceans or fungi. The toxicity of transfection reagents is cell-specific and should be carefully evaluated for the relevant target cell types.

A common problem that arises in mRNA delivery with transfection reagents is the degradation of mRNA, primarily through depurination, in acidified intracellular compartments such as acidified endosomes. Methods to avoid this degradation include: 1) buffering of endosomes with an externally added compound, e.g. chloroquine; 2) employment of endosome-disruption agents to enhance and accelerate mRNA escape from the endosomes. Both endosome buffering and membrane disruption can be performed by the transfection reagent itself. One example of such a multifunctional transfection reagent is polyethylenimine (PEI), a polymer, which contains protonable amines and, therefore, acts as both the acidity regulator and the agent providing the vector with an efficient escape route from endosomes into the cytosol through the 'proton-sponge' mechanism [23]. As ruptured endosomes could release aggressive proteolytic enzymes, excessive disruption of endosomes is detrimental for cell survival. Thus, transfection reagents falling into the 'golden mean' in terms of their endosome-disruption properties should be sought. Peptide elements, such as Listeriolysin O (LLO, encoded by the *hly* gene of *Listeria monocytogenes*) [24], which can produce pores in endosomes and, thus, can allow mRNA to avoid degradation in the acidic environment, are potentially useful tools to achieve high efficiency of transfection with mRNA-based vectors. Ideally, the endosomolytic activity of such peptides should be reversible, with pores forming in weak acidic conditions and then sealing back after the release of mRNA.

A number of techniques known to enhance DNA transfection are likely to be suitable for the improvement of RNA transfection. Thus, the employment of 'smart' thermo- and pH-sensitive polymers can augment transfection [25]. The candidate procedures also include transfection

enhancement with laser light treatment [26], glycerol cell shock and dimethylsulphoxide (DMSO) cell shock. Another possible transfection enhancement method is magnet-assisted gene delivery. As RNA cannot move in the magnetic field by itself, in this scenario mRNA vectors need to be complexed with paramagnetic particles, which move in the magnetic field but do not become magnetized themselves. Extra-strong permanent rare-earth-metal-based magnets (samarium-cobalt or neodymium-iron-cobalt types) are currently standard tools in DNA transfection. Alternatively, devices generating a vibrating magnetic field (1Hz – 5Hz), e.g. manufactured by *Nanotherics*, can be employed [27]. One of the advantages of magnetic gene delivery is the ability to focus it and, thus, to target only selected cell populations *in vivo* [28].

2.2.2. Transfer of mRNA into cells after their treatment with a high-strength electric field

One of the common methods of mRNA-mediated gene transfer is electroporation [7, 29], which relies on the ability of a high-strength electric field to induce the formation of transient pores in cellular lipid membranes. As mRNA is negatively charged, it can be moved through these pores via electrophoresis driven by the electric field. Electroporation can be performed in carefully controlled conditions *in vitro* and also *in vivo* for the gene transfer *in situ*, including a single cell format [30]. Both adhesive and suspension cell types can be transfected using electroporation. Adhesive cells often require detachment, loading into a cuvette and re-attachment after electroporation; this procedure might reduce the cell survival rate and, consequently, reduce electroporation efficiency for adhesive cells in comparison with suspension cells.

The removal of ionic conductors prior to electroporation is important to avoid the ion-mediated electric current with the associated overheating and arcing. In addition, the purification of RNA from macromolecular substances prior to electroporation, e.g. the removal of RNA-binding proteins, is conducive for unimpeded RNA electrophoresis through the pores.

The choice of cell medium is critical for the success of mRNA delivery by electroporation *in vitro*. Electroporation buffer composition is determined by the requirements for: 1) minimal electrical conductivity; 2) optimal conditions (including pH and osmotic strength) for cell survival in a high-voltage and high-temperature environment; 3) safe *milieu* for the stability of mRNA vectors; 4) optimal parameters for mRNA transfer into the cells [31].

Buffer components should be chosen to avoid their degradation in a strong electric field and elevated temperature with the undesired release of ionic conductors. Cell-survival-supporting osmotic pressure in electroporation buffers can be achieved with non-ionic substances such as glycerol and also sugars such as sorbitol. Negatively charged cells are the subjects of electrophoresis, with some cell death occurring at the positive electrode. Cell death at the positive electrode might adversely change the electric parameters within the electroporated mixture. Therefore, minimisation of cell electrophoresis through the neutralisation of the cell surface charge by electroporation buffer components is desirable. An important parameter affecting cell survival is cell density. The presence of anti-apoptotic factors in the cell pre-treatment medium, electroporation mixture and/or cell after-treatment (recovery) medium might be useful [32]. Thus, anti-apoptotic ROCK II-kinase (Rho-kinase) inhibitors could be particularly

valuable for maintaining the viability of stem cells, as some of these inhibitors (e.g. Y-27632) were shown not to affect their totipotency or pluripotency status. Alternatively, mRNA coding for electric-field-resistance, heat-resistance and/or anti-apoptotic factors could be included in the mRNA vector cocktail.

Pure mRNA or mRNA vector complexes can also be adversely affected by mRNA degradation, in particular because of overheating and mechanical shear stress due to the high strength electric field. Thus, electroporation with mRNA, if compared to electroporation with DNA, is likely to benefit from the fact that treatment by a less intense electric field for a shorter time seems to be required for mRNA vectors to reach their expression *milieu* within the cells in comparison to DNA vectors. This is because the pore formation in the plasma membrane is a sufficient membrane opening event for the successful transgene expression with mRNA vectors, while transgene expression using DNA vectors, particularly in non-dividing cells, appears also to depend on electric-field-induced pore formation in the nuclear envelope [33].

Efficient electroporation requires the optimisation of a number of electrical parameters. The fundamental variable in electroporation is the electric field strength, the magnitude of which is often measured as the number of volts applied to a centimetre of an electric circuit's length. The high strength field is often delivered as a voltage pulse with the voltage decaying through 'natural' kinetics of exponential extinction. Either resistance or capacitance parameters in the electroporation circuit can be used to control the rate of voltage drop in the 'exponential decay' protocols. The greater the resistance, the longer the decay and the larger its 'time constant', which is registered by electroporation equipment. Similarly, the greater the capacitance, the larger the 'time constant' of the decay. However, the capacitance parameter is the only one available to regulate the rate of exponential decay, when resistance is 'set to infinity' (that is, when the voltage output circuit is left open). Exponential decay transfection is thought to take advantage of the 'post-electroporation' stage when mRNA is driven by an electric field into the not-yet-closed pores in the membrane. Alternatives to the exponential decay electroporation protocols are offered by 'square' voltage pulse regimens where the voltage drops momentarily after being constant during a specified time. Both 'exponential decay' and 'square' voltage electroporation procedures could be optimised in terms of the number of pulses and duration of a pause between the pulses. Electric field strength can vary between different pulses. Thus, the initial low voltage step could be used for the preliminary 'loading' of the recipient cells' surface with mRNA. Clearly, the composition of the electroporation medium and the electrostatic details of the vectors also play an important role in the desired pre-electroporation adsorption of gene vectors on recipient cells.

Electroporation *in vitro* is accomplished either in cuvettes or in a multielectrode array format with a defined distance between the electrode plates [32]. Some commercial electroporators like BioRad *GenePulser XCell* provide a voltage versus time plot. However, ideally, current should also be recorded by an oscilloscope, providing a diagnostic signature of the electroporation experiment. Indeed, it was observed that the occurrence of rapid spontaneous current undulations during treatment of bacterial cell specimens by the electric field correlated with the efficiency of DNA delivery into the cells [34].

2.2.3. *Ultrasound-assisted delivery of mRNA*

In addition to electroporation, the formation of membrane pores can also be achieved via microcavitation induced by ultrasound in the presence of microbubbles (e.g. lipid-encased octafluoropropane gas). The transfection technique, which exploits these pores, has become known as 'microbubble-assisted sonoporation' [35] and was successfully used with mRNA vectors [36]. Sonoporation was discovered when it turned out that the microbubbles, which were used as contrast agents for ultrasound-imaging, were, in fact, potent gene transfer enhancers. Microbubbles are extremely flexible transfection tools. They can be prepared in cationic, anionic or neutral forms and can be PEGylated for increased stability *in vivo*. Microbubbles can be made more effective through their covalent attachment to the recipient cells' surface [37]. Sonoporation can be combined with chemical methods of transfection [38]. Directing the transfer of the vector particles by focusing the ultrasonic waves to the target tissue is a practical option [39, 40].

2.2.4. *Gene gun delivery of mRNA*

Delivery with a gene gun, also called bio-ballistic or biolistic gene delivery, is one of the established methods for mRNA transfer *in vivo* [13, 41]. Typically, for biolistic delivery, mRNA is precipitated with ethanol, isopropanol or CaCl₂/spermidine on the surfaces of the metal projectiles. The obtained mRNA-coated projectiles are then propelled by gas pressure pulse into the recipient cells. Normally, an inert gas, such as helium, is employed. The metal core of a projectile is composed of tungsten or gold. Bombardment *in vivo* normally reaches the outer 1 mm of the target tissue, which defines the range of amenable tissues. The penetration depth also depends on the gas pressure, the particle size and the type of tissue. Gas delivery pressure is an important parameter to optimise in order to achieve maximal efficiency of delivery. Gene transfer can be focused to the target area with straightforward mechanical protection of the surrounding tissue from bombardment. Some mRNA can be lost extracellularly prior to the projectiles reaching the desired recipient cells because stripping of mRNA can occur as the particles pierce the tissue [13].

2.2.5. *Delivery by injection*

Direct intracellular microinjection into cells is tedious and limits the experiment to *modus operandi* with only one cell at a time. However, microinjection continues to be a commonly used method of gene delivery with mRNA vectors to large cells like oocytes [42].

In contrast to DNA vectors, no intra-nuclear transfer of mRNA vectors is desired. This circumstance is likely to simplify the use of the multi-needle/multi-cell format for mRNA delivery. Indeed, multi-cell microinjection of nucleic acids was successfully performed with silicon microneedles [43] and carbon nanotubes [44]. Technology for manufacturing dissolvable and biodegradable microneedles is available [45, 46] and is likely to be used for gene delivery with mRNA vectors in the future.

2.2.6. *'Bactofection' and 'mycofection' for mRNA delivery*

A well-known method to deliver an mRNA–DNA mixture into cells' interior is 'bactofection', which capitalizes on the phagocytic properties of the target cells and/or the cell-invasive

properties of some pathogenic bacteria. Thus, a self-destructing invasive *Listeria monocytogenes* strain was employed to deliver mRNA directly into the cytoplasm of macrophages, dendritic cells and epithelial cells [47]. In this study, the powerful T7 RNA polymerase transcription system was used to over-express mRNAs coding for EGFP protein and ovalbumin in bacterial cells, with mRNAs being adapted for translation in eukaryotic cells through the insertion of an IRES element into its 5' untranslated portion. In order to benefit from *bona fide* caps and poly(A) tails on the delivered mRNA, which are expected to direct its efficient translation in the recipient eukaryotic cells, a yeast-based modification of bactofection was devised, which was called 'mycofection' [48]. 'Mycofection' involves the biosynthesis of the desired translation-competent mRNA in yeast and its delivery into the target cells via internalisation of mRNA-delivering yeast by the target cells. In the above study, baker's yeast, a non-invasive microorganism, was used for mRNA delivery, so the spectrum of recipient cells was restricted to the cells capable of phagocytosis. It should be noted that while, on the one hand, this restriction presents a limit to the usefulness of the yeast-based mRNA delivery system, on the other hand, it can be used for selective targeting of specific cell populations, e.g. human dendritic cells.

2.3. Methods of detection of mRNA post-delivery

The efficiency of transfection using mRNA can be evaluated, with the mRNA introduced to cells being detected both in the form of mRNA *per se* and as mRNA-encoded protein.

Methods of mRNA detection should focus on the intracellular mRNA because of the background of undelivered mRNA, which is difficult to avoid. A number of approaches are available. Total cellular RNA or poly(A)⁺ mRNA can be fractionated using electrophoresis in an agarose or polyacrylamide gel supplemented with a chaotropic agent to remove the heterogeneity in the RNA's gel-mobility due to variations in the secondary structure of RNA molecules. Analytical approaches based on hybridization of homologous strands of nucleic acids include both classical Northern blotting and modern microarray formats. In addition, microscopy methods, such as *in situ* hybridization, can provide important information on the intracellular localisation of the detected mRNA. An efficient strategy for highly sensitive and intracellular-location-specific detection of mRNA is the insertion of the bacteriophage MS2 stem-loop region into particular mRNAs for their recognition by the fusion protein composed of MS2 coat protein and GFP protein domains [49]. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative 'real time' RT-PCR can detect and quantify very small amounts of mRNA, including in the single-cell mRNA analysis scenario.

If the proteins, encoded by mRNA vectors, have an easily tested function, the successful delivery of mRNA can be confirmed by measurement of that function. However, many therapeutically important mRNAs, such as those used in epigenetic reprogramming, code for proteins with regulatory activities, which cannot be straightforwardly tested. Antibody-based detection methods, e.g., immunostaining and Western blotting, can be used, provided a specific antibody is available. Alternatively, fusions of the target proteins with a moiety from a fluorescent protein, such as EGFP or dsRed, can be employed. Other easily detected protein domains include enzymes and antibody-tags. Commonly used short tags include FLAG, V5,

Myc, HA peptide sequences, for which antibodies are commercially available. An antibody-independent tag, HaloTag[®] peptide, which can be bound to fluorescent labels attached to HaloLink[™] adapter, was developed by Promega. Strong ligands, including glutathione S-transferase (GST), maltose binding protein (MBP), protein A, protein G, streptavidin and His-tag moieties can be used both for the detection and preparative isolation of proteins expressed from mRNA vectors. The mRNA sequence can be designed to encode a testing domain on the N-terminus of the protein, on the C-terminus of the protein or on both the termini. As many native eukaryotic proteins are secreted into the endoplasmic reticulum with coincidental cleavage of the 'signal peptide', the placement of a testing domain on the N-terminus is often more challenging, because, firstly, it should not be cleaved off by the signal peptidase and, secondly, it should not interfere with the secretion process. Detection of proteins, 'proteomics', is currently a rapidly progressing field both in terms of improvement of its sensitivity and in terms of its expansion to the analysis of multiple samples [50].

2.4. Combined delivery of multiple gene messages using mRNA vectors

Combined delivery of multiple gene messages is often desired. Thus, future advances in gene therapy are likely to require simultaneous delivery of several curative genes. The simultaneous delivery of several messages is strictly required in current cell-fate reprogramming procedures. Immunisation with several genes for antigens is also common. Another typical gene co-transfer scenario is the combined delivery of a target gene and a marker gene. Conveniently, mRNA vectors also possess properties facilitating the delivery of several transgenes in one go. In general, the strategies for the mingling of several messages include the assembly of mRNA cocktails, construction of polycistronic mRNA vectors and engineering of mRNAs coding for fusion proteins or fusion proteins 'split-up' using the ribosome skipping mechanism.

Indeed, mRNA-based vectors are straightforward to assemble into multicomponent cocktails including bouquets of total mRNA extracted from specific cell populations. Admixture of mRNA capable of expressing a marker protein GFP was successfully used to monitor the delivery of a target gene mRNA [51]. Clearly, the same 'internal control' co-delivery strategy can be applied to the transfer of cocktails of curative, cell fate reprogramming or antigen-encoding mRNAs, which can be spiked with a marker gene mRNA.

Alternatively, several transgenes can be expressed from a single mRNA. The construction of polycistronic mRNA is often a convenient strategy to combine several reasonably short messages. The translation of the downstream cistrons in eukaryotes can occur only after re-initiation at IRES sequences positioned between the cistrons. As a rule, an IRES element (e.g. borrowed from Encephalomyocarditis Virus) is embedded within a sequence of about 500 bases. The efficiency of the IRES elements can be regulated by changing the length of the intercistronic sequence [52]. Multiple transgenes were previously successfully assembled into a single and efficient IRES-joined transcription unit [53]. Again, a common scenario is a single transcript co-delivery of a target gene and an easily detected marker as an internal control used to gauge the level of expression of the target gene. However, one should be aware that in

polycistronic gene co-delivery, the expression level of the downstream transgene is not necessarily equal to the expression level of the upstream transgene.

Another possible strategy to combine several messages for the simultaneous expression of several functions is to generate mRNA for a fusion protein, e.g. the target protein fused with a readily detectable moiety. Fusion proteins can be conveniently 'split up' into individual polypeptide chains using viral 'ribosome skipping' sequences like 2A or, alternatively, using *bona fide* proteolytic signals for resident proteases in recipient cells.

3. Future perspectives of mRNA-based gene delivery methods

There is a considerable scope for improvements of the procedures for gene delivery with synthetic mRNA. Further advancements in the field of mRNA-based gene delivery are likely to include the development of more stable, easily deliverable and gene-expression-efficient forms of mRNA vectors, incorporating specialised ligands for cell-specific targeting, cell penetration and intracellular targeting. The vector improvements, the use of potent stimulators of targeted cells' receptivity, the refinement of the mRNA cellular entry procedures and also the employment of optimised modulators of the intercellular environment are expected to increase the efficiency of gene transfer and the efficiency of body-locus-targeting, especially in clinical applications.

The potential for advancement of gene delivery with synthetic mRNAs looks strong in comparison to alternative rapid delivery methods. Indeed, high speed and other benefits of non-viral synthetic mRNA vectors are shared by: 1) virally encapsidated RNAs; 2) cell-permeable proteins. Thus, the packaging of mRNA (in positive-strand RNA viral vectors) or a template for mRNA (in vectors containing negative-strand RNA, e.g. Sendai virus-based vectors) within viral capsids is an attractive method of condensing and protecting RNA cargo destined for delivery into the cytosol of target cells. However, there are two unavoidable downsides of viral packaging in RNA transfer. Firstly, viral capsids dictate rigid size constraints for vector RNA. Secondly, this strategy tends to be tedious because the encapsidation of each RNA species requires the laborious insertion of an appropriate viral packaging sequence. Similarly, if compared to protein delivery by protein transduction into cells, gene transfer mediated by synthetic mRNAs is more advantageous since each protein to be delivered through direct cell entry requires time-consuming insertion of an effective PTD sequence. Clearly, various delivery methods can be combined. For example, as protein transduction is a very fast method of increasing the concentration of specific proteins in target cells, so, it can, in principle, be used to augment mRNA transfer. So, as cell viability currently appears to be a critical hurdle in mRNA-based gene delivery, it is possible that most significant future advances in mRNA-mediated gene transfer will be achieved through extensive employment of cell penetrating proteins capable of supporting the viability of cells before, during and after mRNA delivery procedures. The refined mRNA-based transfection techniques can also be applied to delivery of other medicinally important species of RNA, such as siRNA.

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