Bioorganic & Medicinal Chemistry Letters 25 (2015) 1171–1176

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

BMCL Digest

Conceptual and technical aspects of transfection and gene delivery

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ARTICLE INFO

Article history:
Received 13 October 2014
Revised 30 December 2014
Accepted 9 January 2015
Available online 28 January 2015

Keywords:
Gene transfer
Gene therapy
Transduction
Lipofection
Electroporation

ABSTRACT

Genetically modified animals are state of the art in biomedical research as gene therapy is a promising perspective in the attempt to cure hereditary diseases. Both approaches have in common that modified or corrected genetic information must be transferred into cells in general or into particular cell types of an organism. Here we give an overview of established and emerging methods of transfection and gene delivery and provide conceptual and technical advantages and drawbacks of their particular use. Additionally, based on a flow chart, we compiled a rough guideline to choose a gene transfer method for a particular field of application.

Genetically modified animals are state of the art routine in biomedical research as gene therapy is a promising perspective in the attempt to cure hereditary diseases. Both approaches have in common that modified or corrected genetic information must be inserted into cells in general or into particular cells of an organism.

The DNA needs to be available as a plasmid to be transfected. However, the topic of this Digest is not about cloning strategies but focuses on different methods of transfection and gene transfer and their conceptual application. Transfection can be categorized into physical, chemical and biological methods. In the history of gene delivery into cells, initial successful transfection was achieved with chemical methods.[1,2] The calcium phosphate co-precipitation method was examined by Graham and van der Eb[3] in the early 1970s and became a very popular method, which is still used (see below). An overview of selected transfection and gene-transfer methods is given in Table 1.

Physical transfection approaches include microinjection,[4] optical transfection,[5,6] particle guns (ballistic gene delivery),[7,8] electroporation,[9] sonoporation,[10] magnetofection[11] and electric field-induced molecular vibration.[12] The first two examples are methods where usually only one cell is transfected at a time. The disadvantage is an ultra low throughput, but the certainty that the cell of interest is indeed transfected. Therefore methods like microinjection are popular for gene transfer when only a limited number of cells are available, like a variant in the creation of transgenic animals when the DNA is directly injected into the male pronucleus. After the fusion of the pro-nuclei the diploid zygote nucleus is formed and the zygote is cultivated to the state of the two-cell embryo.

A particular interesting concept of physical transfection is laser-mediated transfection. This can be a direct membrane perforation,[13] methods taking advantage of nanoparticles[14] or techniques using wave-guided optical wave guides (WOW).[15] The idea of the latter is to use μTools that can be moved and navigated by the use of laser beams that are manipulated by programmable diffraction patterns resulting in so called holographic optical tweezers (HOT).[16] The μTool are designed such that their tip can be brought in contact with the cell of interest. The energy of laser-generated photons, which are fed into the tail of the μTool, and that travel through the intrinsic optical waveguides result in a thermoporation at the tip of the μTool (compare Table 1).

A further advantage is that physical transfection does not depend on particular chemical or biological cell properties[17] and therefore cells that are difficult to be transfected by other methods (see below), like cells of the immune system, for example, T-cells, can be successfully manipulated by physical transfection approaches. Methods that can be applied to cell suspensions, for example, electroporation, are particular popular.[17]

Chemical transfection methods are techniques that catalyze DNA cross-membrane transport through the use of Ca^{2+} phosphate,[18] polycations[19] or dendrimers.[20]

Transfection with Ca^{2+} phosphate is one of the least expensive methods and is therefore still applied, whenever large amounts of cells need to be transfected simultaneously, for example, for the production (and later purification) of particular proteins or virus (see below). The method is effective with many different cultured cell types.

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http://dx.doi.org/10.1016/j.bmcl.2015.01.018
0960-894X/

Table 1
Overview of selected popular transfection and transduction methods

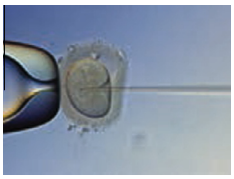
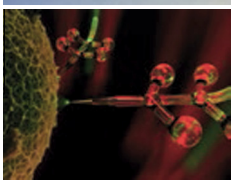
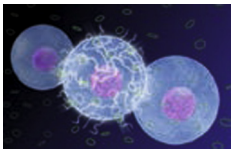
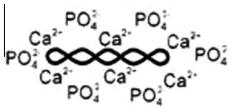
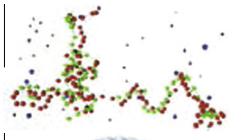
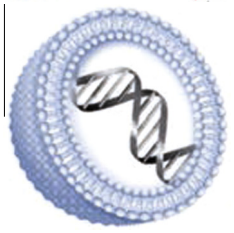
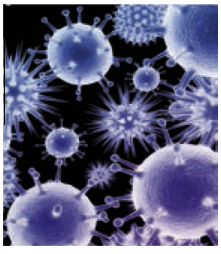
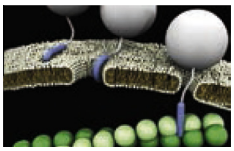
Method	Principle	Scheme	Advantages	Disadvantages	Field of application
Microinjection[4]	Direct injection of the genetic information into the nucleus of the target cell under visual (microscopic) control		Most direct transfection; after initial training high yield of success	Tedious, very limited number of transfected cells, more difficult on small cells	Making transgenic animals, studying RNA trafficking, immuno-cytochemistry
µTool based thermoporation[15]	µTools can be manipulated by laser beams and thermoperforate the cell by laser energy delivered to the tail of the µTool and wave-guided to its tip		Versatile tool with high potential for special requirements	Technical demanding; currently under development and only available in specialized labs	To be determined
Electroporation[9,10,17]	Application of changing electrical potentials to induce the formation of pores in the cell membrane		Established and effective method for otherwise hard to transfect cells in suspension and to some extend in tissue; often used for primary isolated cells	Needs specific adaptation and optimization of parameters for particular cell types; tendency for high level of damaged cells	Wide range, whenever a limited cell viability is tolerable; local transfection in vivo with specialized electrodes (needles)
Calcium phosphate[3,18]	After adsorption of the DNA, calcium phosphate coprecipitates to the cell surface and is taken up by phagocytosis		Cheap and easy to perform; large amounts of cells can be transfected	Relatively low transfection rate; primary cells and cells in suspension can hardly be transfected	Transfection for protein purification and similar approaches
Polycations[19]	The polycations form complexes with the polyanionic DNA molecules and these complexes are taken up by phagocytosis		Large size of DNA can be transfected and it should be useful in gene therapy when viral gene delivery is not useful because of the immune response	Sometimes poor efficiency; cytotoxicity for sensitive cells and high mutation rate of the DNA	Gene therapy together with drug delivery
Lipofection[21–24]	Vesicles of cationic lipids bind to DNA and positively charged complexes bind to the cell surface (negatively charged silica acid residues) followed by uptake into the cells		Simple and fast procedure with high reproducibility	Not suitable for most primary isolated cells	Most popular method in cell biology and related research fields
Dendrimers[20]	Positively charged dendrimers bind with the negatively charged phosphates of the DNA molecule (electrostatic) and the DNA–dendrimer complexes with a positive net charge are taken up by the cell		No or low cytotoxicity; high efficiency in numerous cell lines	Not suitable for most primary isolated cells	Often applied in combination with lipofection, therefore similar field of application
Receptor mediation[7]	Utilises endocytosis for uptake of proteins, DNA is bound to the ligand of the target receptor via a DNA binding moiety (like poly-L-lysin)		Cell specific transfection, very low cytotoxicity and reapplication possible	Effective transfection only possible with cells carrying a high density of the receptor	Cell therapy
Virus[30]	Genetic information is incorporated into a virus and when the virus infects the cell, the protein of interest is transduced in the infected cell		Broad selection of different virus types; high specificity by tissue-specific promoters and tissue tropism (only AAV); fast expression (Semliki-Forrest virus); long constant expression levels (especially Lentivirus); genome integration (only AAV and Lentivirus)	Limited size of DNA; may induce cytopathic effects	Can be used for almost all cell types, except cells of the immune system; preferentially used for terminally differentiated cells like neurons and cardiomyocytes; broad application in vivo

Table 1 (*continued*)

Method	Principle	Scheme	Advantages	Disadvantages	Field of application
Cell penetrating peptides[53]	Design of synthetic peptides that mimic recognition, binding or membrane disruption aspects of viral proteins		No cell specificity	Difficult targeting within an organism	Often used as complement for other non-viral transfection method

References noted next to the method refer to recent reviews or major contributions.

Lipofection is a method that is sometimes classified as a physical method[21] and sometimes as a biological technique.[22] In any case it is a popular transfection method,[23] which when compared with (other) chemical methods leads to a 5- to 100-fold increased transfection rate.[24] Lipofection is well suited in cell lines and many primary neonatal cells. It is based on cationic lipids, which consist of a positively charged head group and one or two hydrocarbon chains. The head group interacts with the negatively charged phosphate backbone of the nucleic acids, thus forming a compact structure. Together with additional neutral helper lipids the transfection complex builds uni-lamellar, liposomal structures with a positive surface charge in aqueous solutions. The interaction with the cell membrane is mediated by this positive charge of the liposomes.

Numerous cationic lipids have been synthesized, including monovalent and polyvalent cationic lipids, cholesterol derivatives or guanidine-containing compounds, some of them being commercially available, such as Lipofectamine (or Lipofectamine 2000), a 3:1 (wt/wt) formulation of the polycationic lipid 2,3-dioleoyloxy-*N*-(2(sperminecarboxamino)ethyl)-*N*,*N*-dimethyl-1-propan-aminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE).[25] Generally speaking, the cationic lipids have three segments: a DNA-interacting headgroup with a net positive charge in physiological conditions or at lower pH such as found in the endosome environment; a hydrophobic lipid anchor group such as cholesterol or fatty acid chains of various lengths and unsaturation states; and a linker group that binds the polar group to the lipidic moiety. The positively charged polar groups are of varying types, including: quaternary ammonium ions, polycations such as spermine, or cationic polymeric compounds such as polyethylenimine, poly-lysine or their derivatives.

Lipofectamine (Life Technologies), FuGene 6 (Promega) or Nanojuice (Merck Millipore) are examples of commercially available transfection formulations or kits, which are commonly used in biomedical research to transfect cell lines. Somewhat problematic is the fact that the user often does not know the real (chemical content) of this composition and the companies are not willing to provide them even when directly asked. For Nanojuice, at least, we know that it is a mixture of polycationic lipids and dendrimers, whereas FuGene 6 just contains 'lipids and other components'.[26] Only indirect information, like the history of an US-patent[27] and a court case[28] about infringement of that patent, supports the speculation that FuGene contains histones.

However, DNA transfer by Lipofection is insufficient in most terminally-differentiated primary cells such as adult cardiac myocytes.[29,30] Then a 'real' biological transfection method such as viral gene transfer is the transduction method of choice.[31–33] In this context, the integration of the indicator DNA into the viral genome can be challenging. However, viral transduction can be performed in isolated cells or even in vivo.[30]

Popular viral systems for transduction include the Semliki-Forest virus,[31] lentivirus,[34] and adenovirus.[35]

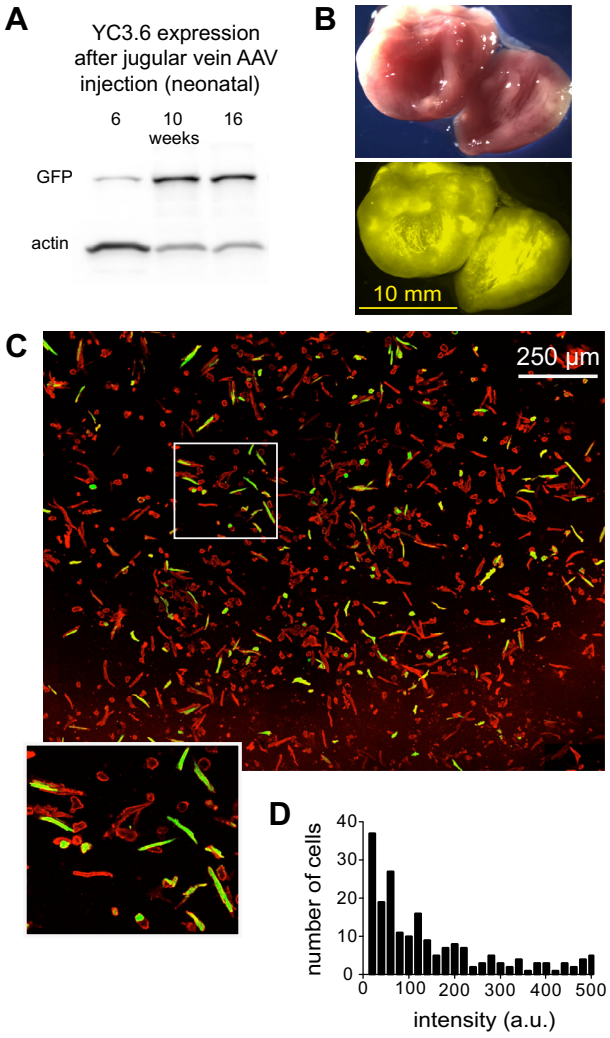


Figure 1. Example of an expression (of the genetically encoded Ca^{2+} indicator YC3.6) after AAV-mediated gene transfer (6×10^{12} vector genomes per ml). (A) Typical Western blots (anti-GFP monoclonal mouse antibody, Roche, Germany) from mouse hearts at different time periods after neonatal jugular vein injection. (B) White light (top) and fluorescence image (bottom) of a cut-open mouse heart 16 weeks after AAV-injection. (C) Single cell expression analysis 16 weeks after AAV-injection by fluorescence imaging of isolated cardiomyocytes of the left atrium. The fluorescent protein is depicted in green, while the membrane staining with CellMask Deep Red is shown in red. The white rectangle indicates the enlarged region replotted in the right bottom corner. (D) Representative histogram of the fluorescence intensity distribution of cells as exemplified in (C). This figure is reproduced from Kaestner et al.[30], with permission from Wolters Kluwer.

Employment of the Semliki-Forest virus leads to a very rapid expression within a very few hours. By contrast, lentiviral gene transfer requires approximately one week reaching detectable levels but typically expression is sustained for several months.

However, third-generation lentivirus vectors with a conditional packaging system are supposed to perform better.[36] When using gene transfer with adenoviruses, expression of the genetically encoded proteins reaches detectable levels within 24 h.[37,38] Adenovirus-mediated expression is rather stable over the time course of at least one week, and adverse effects caused by the viral transduction or induced gene expression during that period are usually not observed.[39,40]

Therefore, adenovirus-mediated gene transfer appears to be the most suitable method for the expression of proteins in cultured primary cells, which show low lipofection transfection rates. Alternatively, Herpes virus-mediated transduction has been successfully used to express proteins in primary cell cultures.[41] For in vivo investigations or gene therapy, the transduction and expression of the gene of interest must be performed not only in isolated cells but also in the living organism. For experimental approaches the production and breeding of transgenic animal lines are a serious undertaking that requires a lot of time, which might be a serious limitation. An alternative approach for tissue specific expression of the gene of interest is to transduce the gene of interest into individual animals, again with the help of a viral vector. Vectors can be applied directly to the tissue of interest by local injection or via the vascular system. While direct injections may lead to a patchy transduction pattern, transvascular or approaches employing systemic application have the advantage of a more homogeneous transduction. However, they face additional obstacles such as neutralizing antibodies, binding to plasma proteins in the circulation, clearance in the liver and ineffective passage through the vascular wall.[42] Furthermore, transduction efficiency depends on successful uptake into the cells of destination. Finally, sustained expression of the transgene depends on the ability of the vector to prevent clearance of its vector genome from the cell, and the lack of an immune response against vector epitopes.

Although there are reports of adenovirus-mediated gene silencing in vivo,[43] expression is usually restricted when injected intravenously most likely due to the strong immune responses of the animals against the adenoviral capsid,[44] triggering/evoking an innate immune response against the vector that rapidly cleared the adenoviral vector. The low number of reports with adenovirus-mediated gene transfer in vivo appears to support this notion. A method that circumvents the immune response is gene painting,[45] in which virus suspensions are mixed with trypsin and directly applied to the organ.[46,47] However, depending on the organ this may require a serious surgery which limits usability and popularity. Therefore, many researchers have focused on adeno-associated viruses (AAVs) as an alternative approach for gene delivery.[32] AAV is a non-pathogenic parvovirus that enters the target cell via receptor-mediated endocytosis. Its low immunogenicity enables a sustained gene transfer.[42] Certain naturally occurring AAV serotypes such as serotype 9 vectors resulted in uniform and extensive cardiac targeting after intravenous injections.[48] However, further modifications of the AAV-vector surface (educated guess or library-based approaches) could extend the tropism of gene transfer, for example, to vessels or increase specificity. Since AAV vectors enable a highly efficient transduction with limited target tissue specificity expression in the tissue of choice can

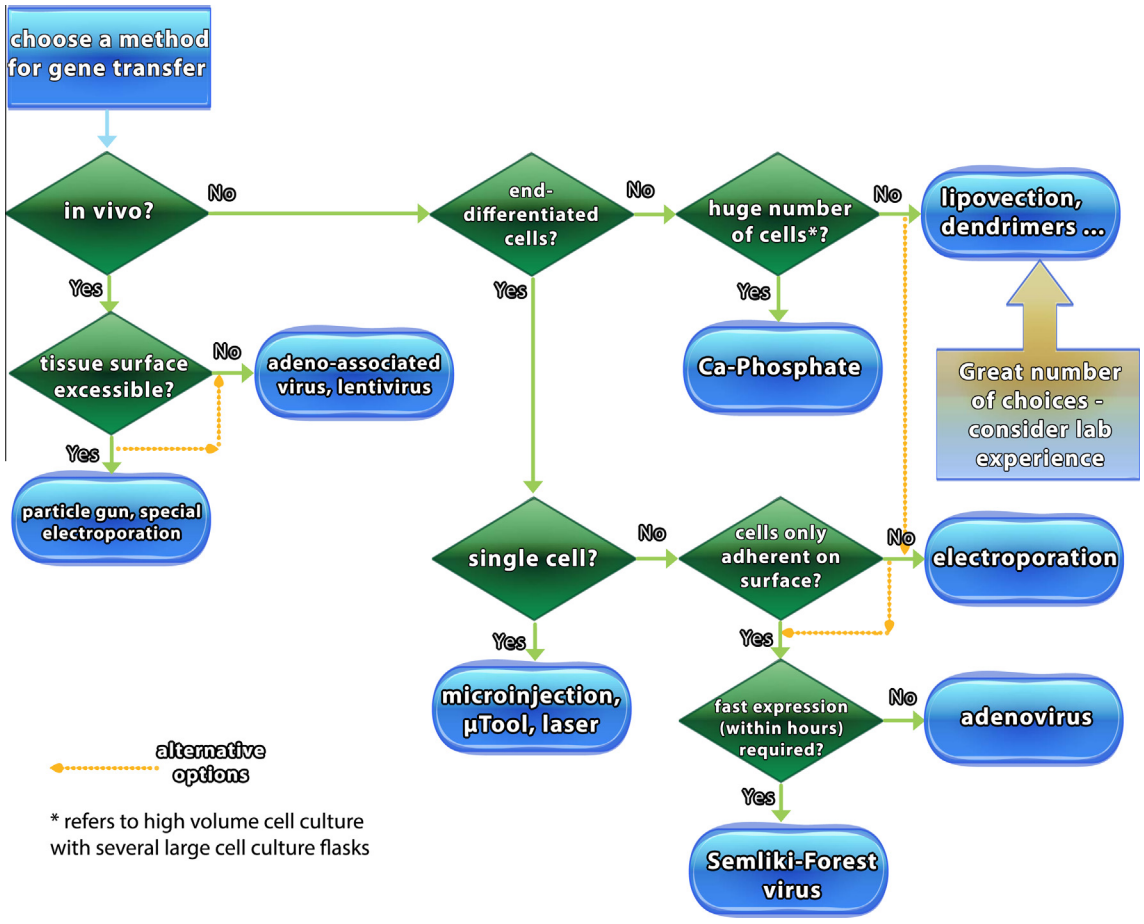


Figure 2. Flow chart of a guideline for selecting a method for gene transfer into eukaryotic cells. The criteria are based on the experience and the judgment of the authors, and they represent an approach to facilitate decision-making but cannot be regarded as an inclusive method.

be augmented by including a tissue-specific promoter or an appropriate microRNA-target site into the AAV-genome.[49]

The role of a tissue-specific promoter is increasingly acknowledged in both, the development of gene therapy and the biomedical research.[50] Figure 1 provides a representative recent example for an in vivo gene transfer towards the expression of the Ca^{2+} sensor YC3.6[51] in cardiac myocytes under control of a cytomegalovirus (CMV)-enhanced myosin light chain 2v promoter after transduction of neonatal mice with AAV serotype 9 vector.

With the success of viral mediated gen transfer, the relationship between structure and function of proteins responsible for these activities lead to studies with synthetic peptides that mimic recognition, binding, or membrane disruption aspects of the viral proteins.[52] These developments resulted in cell penetrating peptides, also known as protein transduction domains, which make up yet another group of transfection agents[53] (compare Table 1).

After an introduction of the different transfection and transduction possibilities one may ask what is the best choice for a particular gene-transfer requirement. Some special cases have already been mentioned above. A general recommendation is difficult, however in Figure 2 we suggest an initial decision tree that might be helpful for two approaches: (i) researchers aiming for a gene transfer may get recommendations to chose their method and (ii) researchers developing new transfection agents may use the guideline in a reverse mode to place their method in the right context of applications.

However, the flow chart in Figure 2 is only a rough classification and a particular application may need deviations or even re-evaluation. An example for a very unusual procedure is the genetic manipulation of erythrocytes. Because these cells have no protein translation mechanism[54], gene transfer needs to be performed in precursor cells (preferably by Lentiviral transduction), which then need to be differentiated into red blood cells.[55]

Novel transfection reagents should be compared to well-established methods, which are mostly commercially available kits or procedures. However, this comparison makes only sense when comparing to known agents. 'Magic' mixtures of unknown coposition of some suppliers (see above) do not qualify as standards and therefore references are limited.

Evaluating new gene transfer methods is usually based on several parameters, starting with the efficiency of the gene transfer (transfection rate; onset and duration of expression) and survival rate of the transfected/transduced cells (viability).

It is worthwhile to mention that in addition to the transfection method itself, there are numerous strategies to increase transfection efficiency and gene expression. One concept is to minimize the lysosomal degradation of the DNA by deactivation of DNAse activity in the lysosomes, for example, by chloroquine.[56] Another option is to favour the active chromatin formation, for example, by sodium butyrate.

When it comes to gene transfer as a therapeutic method additional properties that need to be considered include safety aspects such as the specificity of the gene transfer into the target cells to avoid unwanted expression and adverse effects in other cell types. Another issue is the extent of the expression, because very high levels of expression often cause additional problems such as the occurrence of unspecific cellular responses to the overexpression. Therefore not only the induction of gene expression, but also its termination needs to be solved/adjustable in order to create a successful gene therapy.

Acknowledgments

The research leading to these results has received funding from the European Seventh Framework Program under grant agreement number 602121 and the HOMFOR research program of the Medical Faculty of Saarland University.

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