Non-viral Vectors and Drug Delivery: In Vitro Assessment

Dragos Peptanariu, Marc J. M. Abadie, and Mariana Pinteala

Abstract Advances in nanotechnology, chemistry and molecular biology are making possible the transformation of conventional medicine into a personalized one where we are increasingly talking about intelligent drug carriers and targeted delivery of drugs and genes. Gene therapy emerges as a new field in biomedical research and seeks to establish a roadmap to the clinic and the market by promising solutions to incurable diseases. Various approaches are being tried for the implementation of gene therapy, on the one hand viral transporters and on the other hand non-viral solutions. Along with non-viral transfection vector design and synthesis, *in vitro* biological evaluation is required to investigate both the ability of vectors to transfect, and their good biocompatibility. For accurate and reliable outcomes, the choice of the correct method is essential.

Keywords Gene therapy · Drug delivery · Non-viral vectors · Polyethylenimine · Bioassays · Transfection · Cytotoxicity

Abbreviations

AM	Acetoxymethyl
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CRISPR	Clustered regularly interspaced short palindromic repeats
DMSO	Dimethyl sulfoxide
GFP	Green fluorescent protein
GR	GelRed

D. Peptanariu (✉) · M. Pinteala
"Petru Poni" Institute of Macromolecular Chemistry, 700487 Iasi, Romania
e-mail: peptanariu.dragos@icmpp.ro

M. J. M. Abadie
Aggregates, Interfaces and Materials Energy (ICGM-AIME, UMR CNRS 5253), Institute "Charles Gerhardt" of Montpelier, Montpellier, France

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MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
PEI	Polyethylenimine
PEG	Polyethylene glycol
PLL	Poly-L-lysine
TALEs	Transcription activator-like effectors
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
ZFPs	Zinc-finger proteins

1 Introduction

The advancement in scientific fields like biochemistry and molecular biology has contributed to a greater understanding of the causes of disease and its genetic basis. It has intensified the drive to pursue new and more successful therapies, and it has gradually introduced the idea of gene therapy as a promising choice in modern medicine.

Severe inherited or acquired genetic diseases such as leukemia, multiple myeloma, Parkinson's disease cystic fibrosis, muscular dystrophy, hemophilia those are some of the conditions whose patients are unsatisfied with traditional therapies and are currently seeking a therapeutic remedy.

Gene therapy promises the prospect of genetic disease curative treatment by inserting genes into the cells of patients, rather than using traditional medications that only offer symptomatic care.

Gene therapy is becoming more and more common, as of 2017 almost 2600 clinical trials have been performed and although viral therapies dominate the market for approved products, there is a growing interest in non-viral technologies (Ginn et al. 2018).

2 Gene Therapy Overview

With the advent of genetic engineering in the early 1970s a new avenue for gene therapy opened up. Two main instruments were necessary for the technique: a method of cloning unique genes for diseases and an efficient gene transfer tool.

The US scientists Theodore Friedmann and Richard Roblin demonstrated the promise of the gene therapy technology. In 1972 they published an article in Science (Friedmann and Roblin 1972) indicating that genetically engineered tumor viruses

could be used to move the genetic material required to treat patients with genetic disorders.

This system was first evaluated in the case of beta-thalassemia which is a severe blood condition caused by a genetic mutation in beta-globin gene. In 1976, scientists from the Cold Spring Harbor Laboratory and Harvard University cloned the beta-globin gene (Maniatis et al. 1976). This was the first gene to cause disease ever cloned.

In 1980, Dr. Martin Cline from the University of California in Los Angeles made the first attempt to use gene therapy in humans (Sun 1982). Cline and his team were unsuccessful in the treatment of two patients with beta-thalassemia by injecting the recombinant gene into their bone marrow and then reinfusing the cells. The findings of the experiment were inconclusive, unpublished and posed legal questions and public debate.

Later on, in the 1980s, retroviruses paved the way in developing more efficient carriers for gene therapy. Richard Charles Mulligan from Massachusetts Institute of Technology, along with his collaborators, managed to genetically alter a mouse leukemia retrovirus so that it could transport and deliver DNA sequences without reproducing in humans (Mann et al. 1983).

Benefiting from these new advances, the first approved gene therapy trial in humans is carried out by Michael Blaese, French Anderson and colleagues at NIH in 1990. It used a retroviral-mediated delivery of adenosine deaminase gene (ADA) to the T cells of two children affected by serious combined immunodeficiency disease (SCID) (Blaese et al. 1995; Sheridan 2011). That proved to be effective in one of the patients and encouraged the launch of numerous gene therapy trials in the following decades. The technique expanded by introducing new vectors, such as adenoviruses.

Eventually, the first commercial gene therapies appeared on the market. In 2003, Geneticin, a recombinant adenovirus treatment for squamous cell carcinoma in the head and neck (Pearson et al. 2004) was approved in China. In 2012, Glybera is licensed, the first for Europe, as a gene therapy suggested in the treatment of lipoprotein lipase deficiency (Burnett and Hooper 2009; Miller 2012).

The journey of gene therapy was not without issues, with the death of some patients or side effects including leukemia caused by viral vectors requiring legislative and ethical reconsideration.

Motivated by the problems encountered by viral vectors, research on non-viral vectors began to develop in parallel. In 1980 it was first demonstrated how phospholipid phosphatidylserin-based liposomes could transport DNA from SV40 virus to monkey kidney cells (Fraley et al. 1980).

Vectors based on cationic polymers represent a very wide class due to their chemical diversity; they are very versatile and can have countless functional groups and targeting elements. Within them, the poly-L-lysine polypeptide (PLL) was the first such vector to demonstrate the ability to bind DNA (Olins et al. 1967; Laemmli 1975). PLL is followed by PEI, a cationic polymer rich in amino groups, which has demonstrated the ability to bind and transfect DNA since the 1990s (Boussif et al. 1995).

Then, non-viral vectors began to diversify, comprising a wide range of liposomes, cationic polymers, dendrimers, peptides, vectors to which a number of chemical modifications were made to improve transfection and targeting properties (Yin et al. 2014; Lostalé-Seijo and Montenegro 2018).

In recent years, genomic editing techniques involving zinc-finger proteins (ZFPs), transcription activator-like effectors (TALEs) and clustered regularly interspaced short palindromic repeats (CRISPR)—Cas systems have gained increasing interest (Cong et al. 2013; Gaj et al. 2013; Mali et al. 2013; Urnov et al. 2010). They aim to accurately edit a genomic sequence and are an important step towards personalized medicine.

3 Viral or Non-viral

Among the viral ones, adenoviral vectors are probably of the most common. These vectors have the advantage that they function well in non-dividing cells, which is something hard to achieve by non-viral vectors. From the retroviruses, lentiviral vectors are also very popular, especially human immunodeficiency virus and the herpes simplex virus. Besides the fact that they do operate in non-dividing cells, these carriers could pack big DNA molecules, making them suitable for larger genes. Unfortunately it is hard to control the place of insertion into genome, raising safety concerns. They are better used *in vitro* in difficult to transduce cells.

Both viral and non-viral gene carriers have drawbacks and benefits which are summarized in Table 1.

Non-viral methods of introducing genetic material into cells, also called transfection methods, are numerous and comprise physical methods like electroporation, magnetofection, microinjection, gene gun, sonication, optical transfection and chemical methods which rely mostly on liposomes, dendrimers and cationic polymers. Among the first non-viral agents invented were cationic lipids, PLL and PEI.

PEI is perhaps the most studied cationic polymer used in transfection. Our research group has developed several vectors made of low molecular weight PEI stable attached through covalent bonds (Uritu et al. 2015a; Uritu et al. 2015b; Dascalu et al. 2017; Ardeleanu et al. 2018) to different hydrophobic cores (Fig. 1), as well as vectors based on dynamic chemistry where components where linked through reversible imine bonds (Clima et al. 2015; Turin-Moleavin et al. 2015; Ailincai et al. 2019; Craciun et al. 2019).

In general, high molecular weight PEI (15–25 kDa) is very effective in transfection, but this comes with a price, increased cytotoxicity. At the opposite pole is low molecular weight PEI, which has a good biocompatibility but also a low efficiency in transfection. Our approach was to combine low mass PEI molecules for good biocompatibility and the use of hydrophobic cores to improve transfection.

Table 1 Risks and benefits of viral and non-viral vectors

Viral vectors	
Benefits	Risks
Very high efficiency Could be applied to different human cell types	Limited carrier capacity Usually infect more than one type of cell, not always a desirable aspect Random genomic integration Risk of Weismann barrier breach and germline modification Overexpression of transgene Strong immune reaction Risk of transmission from the patient to other individuals or into the environment High costs
Non-viral vectors	
Benefits	Risks
Low costs Large scale production Reduced safety concerns Low immunogenic potential Increased cargo capacity Flexibility	Lower efficiency Increased toxicity at efficient concentrations

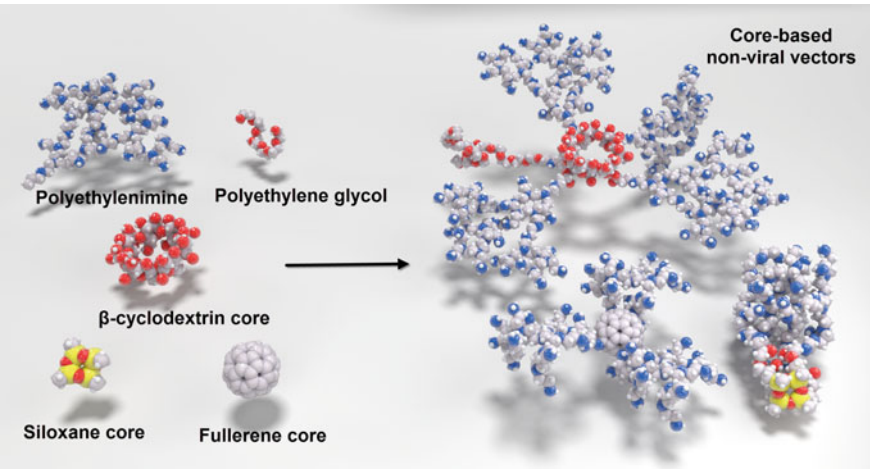

Fig. 1 Non-viral vectors. Their synthesis was done by attaching PEI and PEG molecules to hydrophobic cores such as fullerene, siloxane, β-cyclodextrin

4 Polyethylenimine Vectors, Mechanism of Action

The process of DNA packaging by PEI is due to the electrostatic forces between the positive charges of the polymer and the negative ones of the nucleic acids. The result is the formation of polyplexes, which are particles with a certain degree of polydispersity. The nitrogen/phosphorus ratio (N/P ratio) which depends on the polymer/DNA ratio has a very important role in the transfection efficiency, particles with electric positive surface being more efficient in the interaction with the negatively charged surface of the cell membrane. The interaction process between polyplexes and cells requires endocytosis (Bieber et al. 2002; Rejman et al. 2005) and is facilitated by syndecans and the catabolism of the extracellular matrix (Demeneix and Behr 2005; Kopatz et al. 2004; Christianson and Belting 2014). Polyplexes make contact with the cell membrane through syndecans, transmembrane proteins that present in their distal extracellular portion heparan sulfate, anionic components that interact with the extracellular matrix and cationic particles. The process is continued with the agglomeration of several syndecan molecules and the activation of cytoskeletal proteins with which syndecans react through the intracytoplasmic domain. Eventually the action of the cytoskeleton will pull the cell membrane inward, resulting in its invagination and sequestration of polyplexes into endosomal vesicles (Fig. 2).

The endosomal sequestration of PEI/DNA complexes can be problematic because the physiological maturation of endosomes results in either fusion with lysosomes

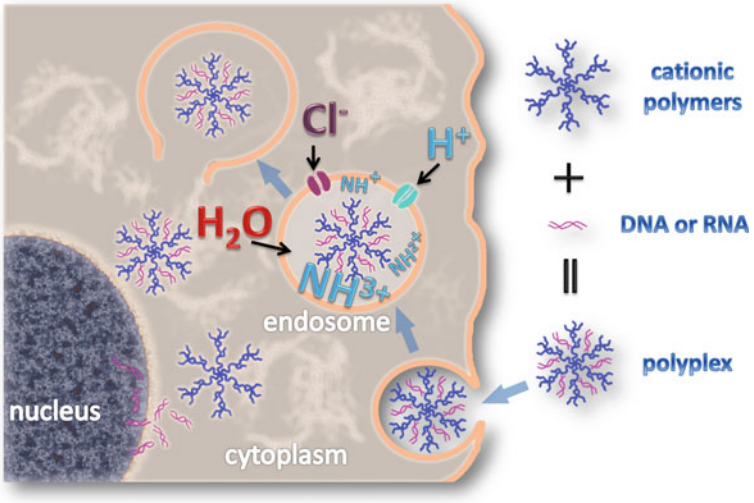

Fig. 2 Endosomal escape. First, the polyplexes interact with the cell membrane and enter the cell through endocytosis. In the endosome ATPase proton pumps actively translocate protons from the cytosol into the endosomes but PEI based vectors with 'proton sponge' property will become protonated and will resist the acidification. The protons influx is followed by passive entry of chloride ions leading to water influx, osmotic pressure and possible rupture of the endosome.

or direct transformation into lysosomes. The lysosomal acidic environment, rich in degradable enzymes, would not be a friendly space for any drug.

But here PEI emphasizes its feature of proton sponge by using amino groups capable of protonation, which can absorb much of the protons pumped into the endosomes by proton pumps, thus resisting the tendency to acidify. The active inflow of protons will be followed by the passive influx of anions, such as chlorine, and the increased ionic concentration will facilitate the penetration of water by osmotic pressure and the swelling and rupture of the endosome with the release of polyplexes in the cytosol.

The exact mechanism of endosomal escape is controversial and not fully understood (Won et al. 2009).

Some researchers argue that there is no change in lysosomal pH due to the presence of PEI and V-ATPases proton pumps could overcome the buffering capacity of PEI (Benjaminsen et al. 2013). Moreover, the bursting of endosome could actually be detrimental for cell health due to the release of apoptosis inducing enzymes (Roberg et al. 2002).

Once released into the cytosol, the journey of polyplexes does not end here, for gene expression to take place, DNA must reach the nucleus. The cytosol is not a safe place for DNA, on the one hand it is a very crowded space, through which it diffuses hard and at the same time it contains nucleases that can quickly degrade nucleic acids. This is why it is important that DNA does not break down from polyplexes yet. The most advantageous time for polyplexes to reach the nucleus is during mitosis.

5 Addressing Efficiency and Biocompatibility Using *in vitro* Assays

In order to support the research of transfection vectors, regardless of whether their destination is *in vivo* or *in vitro* use, it is necessary first of all to test them *in vitro.* The tests must tackle, on the one hand, the transfection potential and, on the other hand, their cytotoxicity.

5.1 Gel Retardation Assay and Dye Exclusion Assay

Before transfection experiments begin, the ability of vectors to bind DNA must be investigated first. There is no reason to make transfections on cells with molecules that do not prove the ability to pack nucleic acids.

One of the most common and oldest tests to evaluate the packaging capacity of non-viral vectors is gel retardation assay. This technique is actually nothing else but a variation of affinity electrophoresis procedure used to test protein-nucleic acids

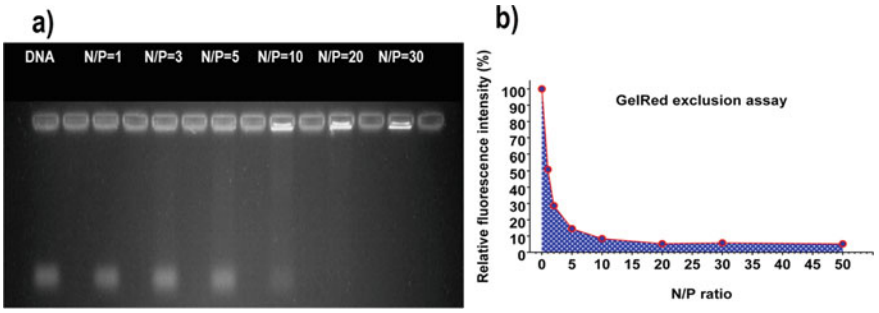

Fig. 3 Binding capacity of vectors. A vector based on PEI and fullerene core was the subject of testing using a) gel retardation assay and b) dye exclusion assay

interactions which can identify if a protein or a protein mixture will attach to a specific DNA or RNA sequence (Scott et al. 1994).

Unlike the classic use of this test in protein-nucleic acid interactions, here the place of the protein is taken by our vector, and the specificity of the interaction with certain nucleic acid sequences interests us less, being more important the ability to bind DNA and RNA in general.

The assay is usually performed as electrophoresis on an agarose gel. Free DNA is placed in one well of the gel, and polyplexes in increasing N/P ratios are placed in the next wells. As expected, once electric current is applied, DNA being an electronegative molecule will begin to migrate into the electric field. In the case of polyplexes, however, this migration will be slowed down with the increase of the N/P ratio, being completely abolished over a certain N/P. At the same time, the bands corresponding to polyplexes will be less bright due to the displacement of the staining agent by the polymeric vector (Fig. 3a).

A test called GelRed (GR) dye exclusion assay is another one that we have used in our work (Vasiliu et al. 2018; Craciun et al. 2019) to investigate the binding capacity of vectors. GR is a sensitive dye used to stain nucleic acids and is safer to use than ethidium bromide because it is impermeable to the cell membrane and therefore cannot penetrate living cells.

GR fluorescence increases significantly as it interacts and binds to double-stranded DNA molecules. Due to this fact, it can also be used to investigate the ability of vectors to bind DNA. Thus, for this test, samples with polyplexes DNA-vector are first prepared individually at various N/P ratios, after which GR is added to each of them. As the N/P ratio increases, more and more GR will be excluded by the vector from the DNA complex resulting in a decrease in fluorescence (Fig. 3b). The result is read with the help of a plate reader. This test is more sensitive than gel retardation assay, requires much smaller amounts of reagents and provides a quantitative result, allowing comparison between the binding powers of various vectors.

5.2 *Evaluation of Transfection Efficiency and Biocompatibility*

Once the ability to bind nucleic acids by vectors has been proven, we can move on to testing the efficiency of transfection, i.e. the purpose for which we created these chemical structures.

In general, such studies generate libraries of chemical compounds that must be tested in different concentrations, ultimately raising the number of samples a lot. Thus, it is very important that the chosen assays meet a number of characteristics such as: efficiency in simultaneous running on a large number of samples, the ability to generate reproducible data, and last but not least to be economically feasible.

To help us get results more easily, polyplexes are prepared using test vectors and a plasmid containing a reporter gene such as luciferase, β-galactosidase or a fluorescent protein like green fluorescent protein (GFP) (Bono et al. 2020). The purpose of these reporter genes is to generate an easily detectable result in the case of a successful transfection (Fig. 4).

Performing a cell transfection experiment must take into account several aspects.

Cellular mitosis is important for the success of transfection, for this reason it is good for the cells to be in the exponential growth phase and their confluence to be between 70 and 90% for adherent cells. The optimal cell density must be found to obtain the best possible transfection result (Malloggi et al. 2015).

Cell lines are preferable in transfection experiments precisely because they have a high rate of division, in contrast to aging primary cells, which rarely replicate and are very difficult to transfect (Pezzoli et al. 2017). The health of the cells is

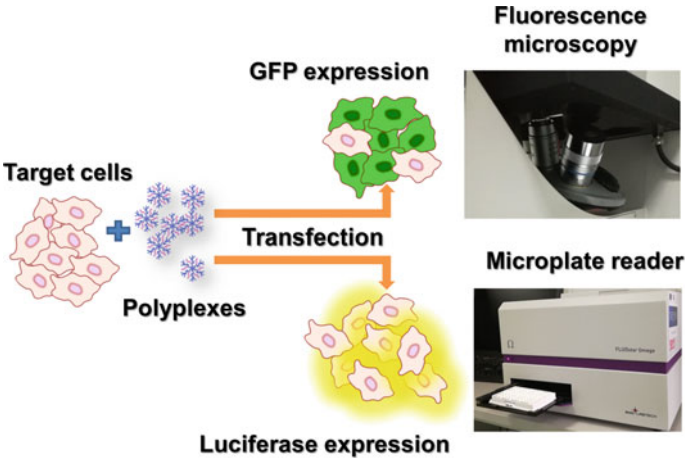

Fig. 4 Evaluation of transfection efficiency. Cells are transfected with polyplexes containing reporter genes. Efficient transfection results in cells expressing either a fluorescent protein which can be seen in fluorescence microscopy, or luciferase which could be quantified after reaction with luciferin by reading light emitted with a luminometer

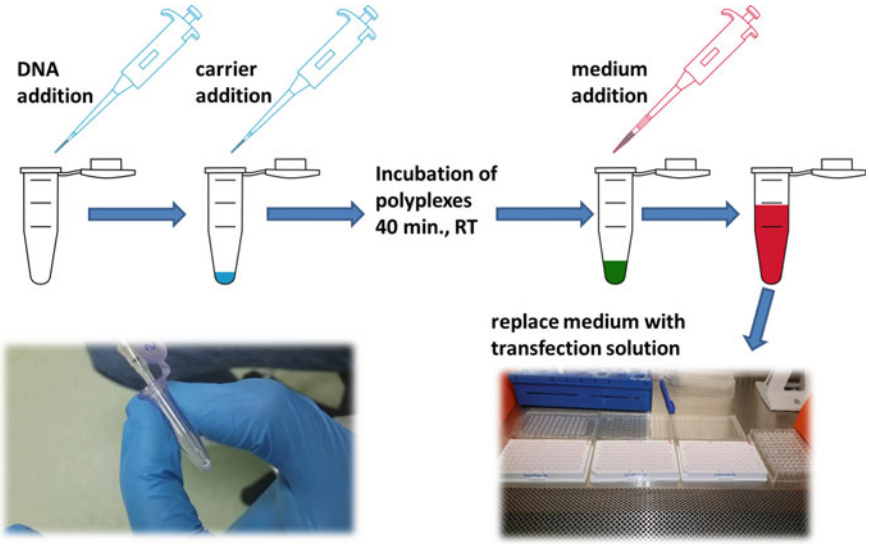

Fig. 5 Preparation of transfection solution. Polyplexes are created by mixing the vector with plasmid DNA, after which the cell culture medium is added. The final solution is used to treat the cells.

ensured by the culture conditions, among them their nutritional environment being very important. Thus, the cells cannot be bathed directly in a polyplex solution and expected to maintain their full viability. For this reason, it is preferable for the transfection to take place in a culture medium in which polyplexes are also present. The experiment begins by forming a polyplex solution by mixing vectors with plasmid DNA in the absence of the culture medium, after which the latter is added (Fig. 5).

It is important that things are done in this order because the presence of the culture medium during the formation of polyplexes can interfere with the process.

The preparation of polyplexes in a salt environment leads to their aggregation over time, which facilitates sedimentation and improves transfection by increasing the concentration in the vicinity of adherent cells (Jones et al. 2013; Hill et al. 2016).

For the reason mentioned above related to good cell viability, the polyplex solution is good to represent a small fraction of the final volume that will be used for the treatment of cells (van Gaal et al. 2011).

Serum can be present, although serum proteins can interact with polyplexes, the serum culture medium improves the quality of transfection because it ensures better cellular health.

Because cationic polymers destabilize the cell membrane (Hong et al. 2006; Leroueil et al. 2008), it is preferable not to use antibiotics in the transfection medium since they could penetrate cells more easily and induce an increase in toxicity.

Several N/P ratios will be tested to find those optimal for transfection. To establish the range of N/P concentrations, the data from the DNA binding experiment will be

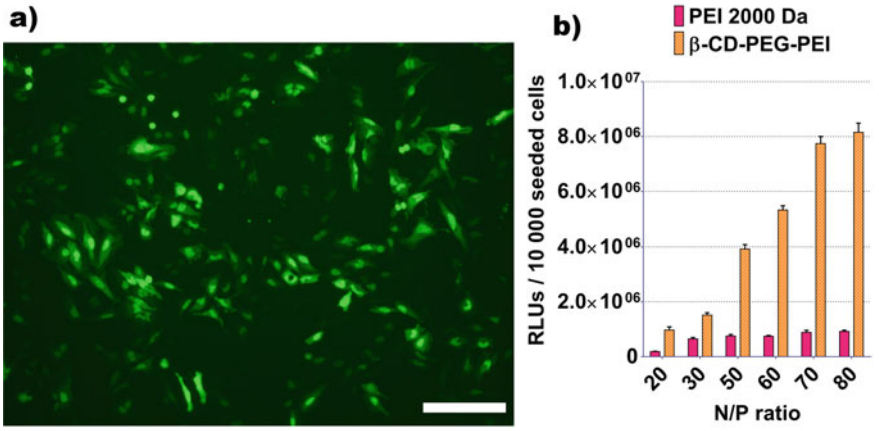

Fig. 6 Typical transfection results. a) Fluorescence microscopy of Hela cells 48 h after transfection with GFP and vector based on polyethylenimine attached to a rotaxane core. Scale bar 200 μm (Ardeleanu et al. 2018). b) Luciferase assay on HeLa cells 48 h after transfection with luciferase gene carried by a vector made of β-ciclodextrine core with PEI and PEG. Results expressed in RLUs ± S.E.M (standard error of the mean). RLUs = relative light units (Dascalu et al. 2017).

used, in general the N/P values at which the maximum binding capacity begins to be reached are the ones where an optimal transfection takes place.

Transfection with the GFP reporter gene gives us a qualitative result by visualizing the transfected cells under a microscope. A plasmid carrying the GFP gene fused to the gene of a nuclear localization peptide will lead to the accumulation of the GFP protein in the cell nucleus, allowing an easier view of the results (Fig. 6a).

Fluorescence microscopy will bring a qualitative result, but it may be subjective, transfection with the reporter gene for luciferase (Fig. 6b) is a more sensitive test that provides a valuable quantitative result that allows the detection of more subtle transfections and permits comparison between the efficiency of various vectors or at various N/P ratios. In the case of this assay, a global response, mediated on the entire amount of cells in the sample is given.

Luciferase is an oxidative enzyme present in certain animal species that manifest bioluminescence.

Light is released in the luciferase reaction as luciferase acts on its specific substrate, luciferin, in the presence of ATP (Fig. 7).

Luciferase assay is very reliable: because the luciferase bioluminescence does not require light excitation, there is limited auto-fluorescence and thus practically background-free fluorescence.

Light sensitive devices such as luminometers or microplate readers equipped with luminescence module can measure light emission.

In the case of β-galactosidase gene transfection, the successfully transfected cells will express the β-galactosidase enzyme and after treatment with the substrate X-gal,

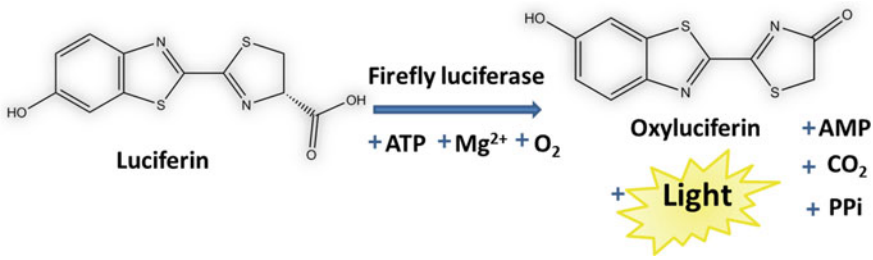

Fig. 7 Luciferase reaction. Luciferin protein is converted in the presence of ATP by the enzyme luciferase into oxyluciferin. The reaction occurs with photon emission which can be quantified by a luminometer.

an intensely blue-colored product is obtained. Thus, the transfection can be quantified by reading the absorbance to a microplate reader.

Flow cytometry is another method to quantitatively evaluate transfection performed with reporter genes for fluorescent proteins, such as GFP. However, it should be noted that unlike the luciferase assay, larger amounts of cells are required for flow cytometry. Also, the method involves a longer time to prepare the samples for reading, with the risk of introducing differences between the first and last samples read. However, this method is very useful to find out the percentage of cells that have been transfected.

The purpose of non-viral vectors is to transfect cells; however it is important that this process takes place without harmful effects on cell viability. Therefore, *in vitro* testing of vectors should also include the analysis of their biocompatibility. Microplate assays are of choice to analyze the large number of samples required by a library of compounds at different concentrations.

Metabolic colorimetric assays are often used for their simplicity; they do not directly determine viability and proliferation, but do so indirectly by measuring the cell's ability to convert a tetrazolium salt (MTT, MTS, XTT) to formazan.

MTT is a colorimetric test described by Mosmann (Mosmann 1983) to quantify the proliferation and survival of mammalian cells. This test is based on the ability of cellular oxidoreductase enzymes in living cells to reduce a tetrazolium salt into a water-insoluble purple formazan.

In a typical MTT experiment, the cells of interest are cultured in the presence of the test substance for a certain amount of time (1–2 days) after which the cell cultures are treated with the MTT agent for several hours. At the end a formazan is obtained which will be dissolved by the addition of DMSO and the absorbance at 560 nm will be read on a microplate reader. The absorbance value of formazan is directly proportional to the number of healthy cells.

Very similar, in the case of MTS it is another tetrazolium salt that produces a water-soluble formazan, thus eliminating the solubilization stage at the end of the MTT test (Fig. 8).

These tests cannot discriminate between reduced proliferative activity or slowed cellular metabolic rate, but they could be complemented with other viability tests such

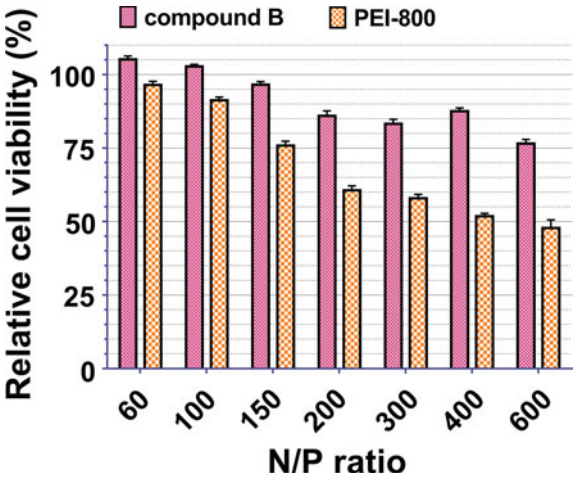

Fig. 8 MTS assay. Relative viability of HeLa cells 48 h after transfection with compound B, a non-viral vector constructed with a linear siloxane core with low molecular mass PEI attached through imine linkage. Results expressed as percentage relative to viability of untreated cells ± S.D. (standard error of the mean). The results show a better biocompatibility of compound B compared to the PEI from which it was synthesized (Ailincai et al. 2019).

as live-dead staining. One such familiar test is the staining with propidium iodide and calcein AM (acetoxymethyl). Calcein AM stains living cells in fluorescent green, while propidium iodide, impermeable to the living cell membrane, stains dead cells in fluorescent red by binding to nucleic acids.

Flow cytometry can be used both to quantify transfection and to evaluate cell viability. But as we mentioned, care must be taken in experiments involving a large number of samples and in the time elapsed between the acquisitions of various samples.

6 Final Notes

Medicine is evolving and although not perfect, gene therapy is here to stay, enhancing its methods each year. Improved synthesis techniques in the field of non-viral vectors have made new molecular structures possible. The number of transfection compounds has become impressive and there is an increasing demand for bioassay screening.

In-vitro biological assays for non-viral vectors must be carefully selected for their sensitivity, specificity, reproducibility and the option to be easily applied. As none of the transfection assessment tests provide all the information, it is good practice to combine them. For example, transfection with GFP and luciferase provides a comprehensive picture of transfection efficiency by providing both qualitative and quantitative information.

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