

TOXICITY & TRANSFECTION

Guidelines



This application note will focus on how we deal with toxicity among the various methods of transfection and the following points will be addressed:

1. the careful design of our cationic lipid-based TR to be as biocompatible as possible;
2. the new patented class of polymer that bypasses cellular activation and stress to enhance viability;
3. how an efficient optimization can reduce TR-induced toxicity;
4. the Magnetofection™ technology that allows to lower NA acid doses for a more efficient transfection with reduced toxicity;
5. the benefit of using Magnetofection™ as a complement to TR to increase efficiency while lowering toxicity of classic delivery vehicles and appears as an alternative to other physical methods of transfection such as electroporation.

TOXICITY & TRANSFECTION

State of the Art, How to lower it, Helix-In, Reduction of Oxidative Stress & Magnetofection™

The detection of foreign nucleic acid (NA) invasion is a fundamental and essential mechanism of host defense. The presence of an exogenous NA such as DNA, mRNA or siRNA in the cytosol of mammalian cells is a danger signal, indicating for example, that a DNA-containing virus has infected the cell. This signal triggers a cascade of host immune response, which involves the expression of type I interferon that helps to clear the “what is sensed to be an” infection either by getting rid of the foreign NA (degradation) or engaging apoptosis pathways to contain the exogenous NA expression¹. With this postulate, we thus can approach the limit of an efficient transfection reagent (TR). While demonstrating high NA delivery rate into cell, **the TR has to compact and protect its cargo against numerous extracellular barriers**. When finally the NA enters the cell it triggers an activation signal: aiming at delivering the greatest quantity of nucleic acid, the TR thus induces an opposite cellular answer that limits its own efficiency. Moreover, the composition of the delivery vehicle itself that was engineered to be the most efficient as possible might have a dramatic impact on cellular viability. **Cruel dilemma**: should a delivery vehicle be powerful in transfection with the risk of toxicity for the cells or be totally compatible



Specialized in delivery vehicles for transfection and transduction, here at **OZ Biosciences (OZB)**, we have spent lot of efforts in developing, synthesizing and formulating delivery carriers, aiming at transfecting cells with any kind of nucleic acids in an effective way while preserving viability. To do so, we propose both classic chemical transfection reagents based on biodegradable cationic lipids and a new class of polymer (**cationic hydroxylated amphiphilic multi-block polymer - CHAMP**) biocompatible, ionizable and biodegradable.

In order to address also physical methods for transfection, more than 15 years we have developed **Magnetofection™** that uses a magnetic field to attract and cluster magnetized vectors onto cell surface, allowing to reduce NA dose and to address sensitive and hard-to-transfect cells.

1. Li, X.-D. et al. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. Science 341, 1390–1394 (2013).

CATIONIC LIPID, TRANSFECTION EFFICIENCY AND TOXICITY

Since several years now, **cationic lipids-DNA complexes** (lipoplexes) have been extensively investigated and widely used as gene carrier in gene therapy to deliver DNA into mammalian cells owing their potential advantages over viral vectors such as safety, versatility and low immunogenicity². However, **their transfection efficiency both in vivo and in vitro** depends in a rather complex way on different interconnected parameters from **chemical composition of the lipid components** (cationic lipid structure and co-lipid), **size** and **distribution of the complexes** formed with the nucleic acids to the nucleic acid dose.

Each cationic-lipid based delivery vehicle thus presents its own capacity to transfect cells that relies on its intrinsic properties; the number of charges or structure for example directly influences nucleic acid binding and overall efficiency. Paradoxically, one would expect that increasing the amount of nucleic acid or ratio (volume of cationic lipid to NA) would be sufficient to enhance transfection efficiency. But for a given NA amount, **increasing ratio induces indeed a raise in transfection efficiency** but only up to a certain extent; the same observation is noticed for a given volume while increasing NA amount.

As demonstrated in the **figure 1** below for DNA transfection, it is generally observed that for a cationic lipid-based transfection reagent **there is no direct relationship between transfection efficiency and DNA dose or ratio of lipid-to-DNA**. On the contrary it appears that for a given dose transfection increases, reach a plato then decreases as if some conditions were not able to correctly transfect cells. Moreover based on this matrix, low DNA amounts and/or ratio meet some difficulty to be efficient whereas on the opposite, high DNA dose and/or ratio fail to be effective due to excessive toxicity.

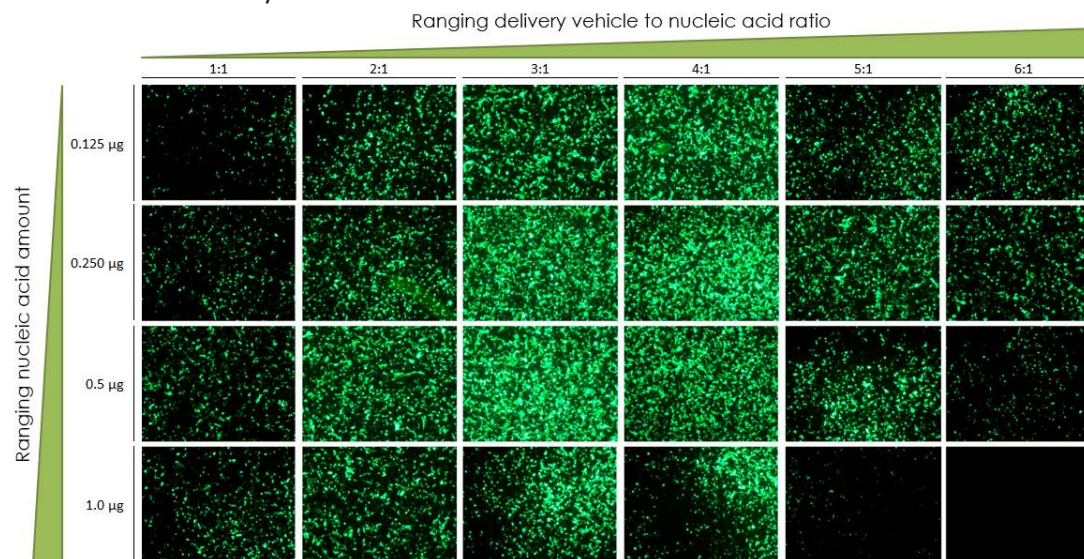
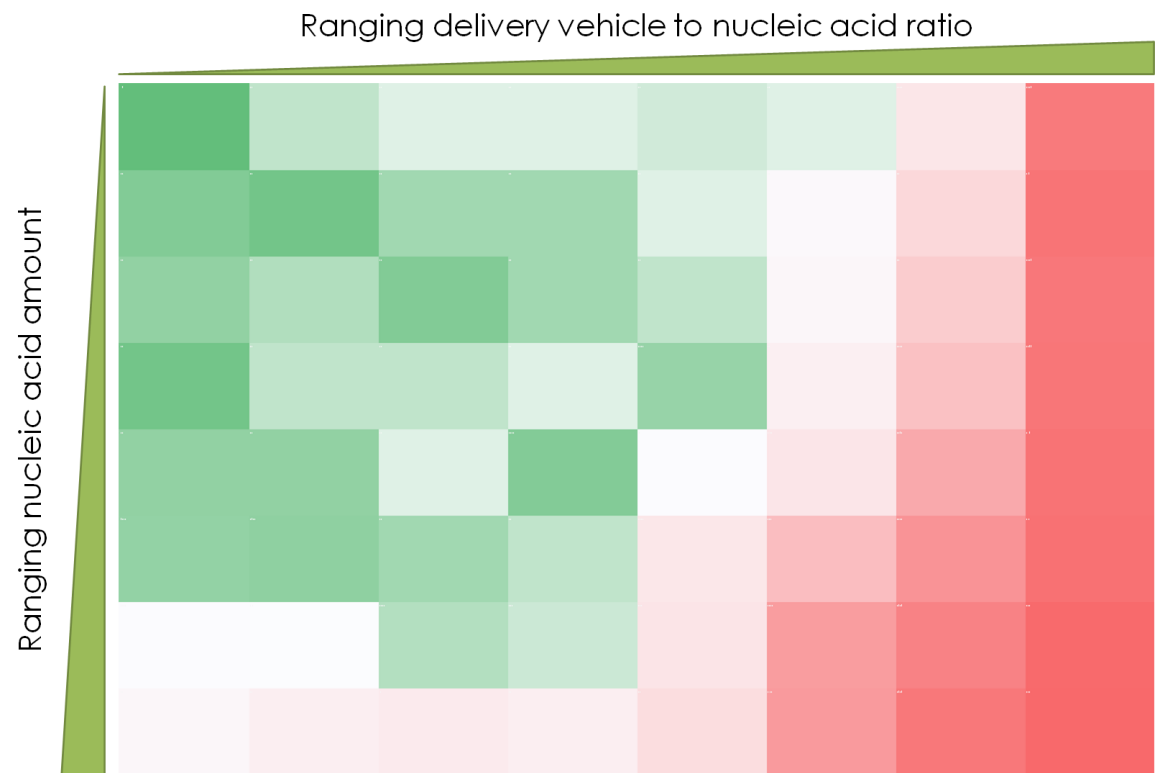


Figure 1. Transfection efficiency matrix. COS7 cells were transfected with ranging doses of DNA (y Y axis) and increasing ratio of lipid-to-DNA (X axis). DNA amounts and ratios are given as indicative values. 48 H after, Green Fluorescent Protein (GFP) expression was monitored by fluorescence microscopy.

Figure 2: Matrix representing toxicity depending on nucleic acid amount and ratio. As observed in this matrix toxicity (red color) increases with nucleic acid amount and/or lipid-to-nucleic acid ratio whereas high viability index (green color) corresponds to lower nucleic acid quantities or lower ratios.



These results are observed whatever the cationic lipid used; of course, depending on the later (composition, molarity, co-lipid...) the efficiency would vary accordingly and it is not rare to observe patterns of transfection as depicted above. As a corollary to this, toxicity begins to appear when transfection efficiency increases and reaches the plateau.

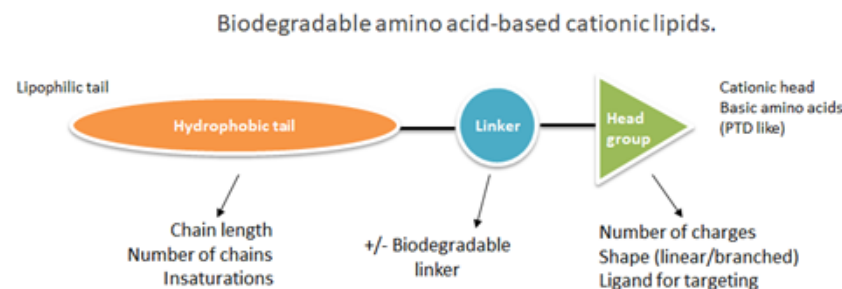
It is generally reported for any lipid, that **the viability decreases** with the **with high quantities of nucleic acid and/or the volume of lipid used** as represented in **figure 2**. By superposing two matrices of efficacy and viability, we can thus easily spot a window where an efficient transfection only results in no- or at least -a mild toxicity.

What OZ Biosciences proposes to reduce toxicity of lipid-based transfection?

OZ Biosciences catalogue of cationic lipids

In order to decrease the intrinsic toxicity of the delivery vehicles, the most evident solution was to work on cationic lipids, ideally, with harmless nature for the cells while keeping intact their capacity to compact nucleic acid, to deliver it up to the nucleus and to efficiently induce transfection. To this end, we can rely on the patented structures of our catalogue of lipids that are mostly all based on the same model (**figure 3**) including a hydrophobic tail, a linker and a cationic head group³.

Figure 3: representative schematics of OZ Biosciences biodegradable cationic lipids. The biodegradability property of OZB's lipids comes from their patented composition and structure. The lipophilic hydrophobic tail can be engineered so that chain length or number of insaturations might be adjusted. A biodegradable linker can also be added to increase the viability and the cationic head group can be changed to increase or decrease number of charges conferring various degrees of nucleic acid complexation.



The hydrophobic domain is generally either a single, double or triple hydrocarbon chain or a cholesterol derivative. The aliphatic chains are not necessarily symmetrical. They are usually composed of 12 to 18 carbon units, that can be either completely saturated or contain double bonds (e.g. oleyl group). **The most common linkers** used are **ethers** and **esters**, although **amides** and **carbamates** are also employed. The head group often consists of primary, secondary or tertiary amines, but also quaternary ammonium salts, guanidino or imidazole groups for example.

**Lullaby™ is the ideal siRNA
transfection reagent
for gene silencing**

The difficulty in designing an efficient lipid-based non-viral vector is that even relatively small structural changes to these domains are known to affect transfection efficiency and/or cellular viability, sometimes drastically. Moreover, even if cationic lipids can be formulated into liposomes alone, they are generally mixed with a neutral co-phospholipid such as dioleoyl phosphatidylethanolamine (DOPE). By finely tuning the structure, composition and dosage of each component of the mere lipids and especially the linker part that confers biodegradability and/or by adjusting co-lipid content and concentration, we have set up a range of commercially available TR for the delivery of any nucleic acid. They present a low toxicity index with a high efficiency when used at the recommended concentrations due to their biodegradable and biocompatible characteristics. Indeed, this depends on many other parameters such as the application (gene expression, silencing ...) or the nucleic acid quality (method of purification, size, quantity...) and of course the cell line used (sensitive primary cell, cell line...).

Among the numerous publications mentioning our TRs to transfect various nucleic acids in any cell type, (we recommend the reader to refer to our online citation database: <https://www.ozbiosciences.com/module/citationfinder/default>) the most representative one is the **article by Emma J Shanks from the Cancer Research UK Beatson Institute, Glasgow, (UK) that collated a library of 26 transfection reagents and chose Lullaby™ among them for its lack of toxicity and high efficiency to silence gene expression and screen siRNA library.** *"We have used this reagent in over 20 cell lines and have found it essential in enabling siRNA screens in hard to transfect cell lines such as those derived from GEMM pancreatic tumors and AML suspension cell lines, with minimal toxicity" as cited by the author⁴, de-monstrating that the design of cationic lipids with a special care given to the linker greatly impact the overall experiment with an improved viability.*

Helix-IN™ DNA Transfection Reagent

Preserve Viability, Reduce Cellular Stress
& Experience High Efficiency

www.ozbiosciences.com

THE NEW CLASS OF POLYMERS: HELIX-IN™

Beside lipid-based TR, **cationic polymers** are the other class of **non-viral delivery vehicles** routinely used for **genetic modification of cells**. Even if cationic lipids have gained increasing attention since several decades as they are **safer than viral vectors**⁵, these synthetic carriers are generally unsatisfactory because they lack of one or several functions needed for optimal performance and become rapidly toxic under unfavorable conditions. Synthetic polymers were extensively studied as cationic polymers play a crucial role for the development of gene transfer agents due to their **extraordinarily good potential to condense nucleic acids and their chemical versatility** that “easily” allows generating, modifying and synthesizing linear, branched or dendritic polymeric structures with multiple functions⁶.

Polyethylenimine (PEI) is one of **the most used**⁷ but **numerous drawbacks have limited its application** and many alternatives (polylysine, polyamidoamine, dendrimer, polyallylamine and methacrylate/ methacrylamide polymers) have been synthesized gaining ground on efficacy and reducing toxicity without however reaching all the promises.

The main issue comes from the fact that low cationic charge density inhibits DNA condensation capabilities while polymers with high cationic density condense DNA into structures amenable to cellular internalization but high charges contribute to their cytotoxicity. Given as less toxic than cationic lipids, one of the major issues still remains the activation of innate immune response induced by the gene delivery system⁸.

In order to lower the cellular answer (innate response, oxidative stress, apoptotic pathway...), **we have designed and developed a novel patented Cationic Hydroxylated Amphipilic Multi-block Polymer (CHAMP)** which is **biocompatible, cleavable, pH responsive and bi-functional**.

This new class of cationic polymeric transfection reagent **Helix-IN™** (OZ Biosciences, #HX11000) comprises 3 moieties each bearing different characteristics and functions, and combines three synergistic notions:

5. Xiang, Y., Oo, N. N. L., Lee, J. P., Li, Z. & Loh, X. J. Recent development of synthetic nonviral systems for sustained gene delivery. Drug Discov Today 22, 1318–1335 (2017).

6. Ruponen, M., Ylä-Herttuala, S. & Urtti, A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. Biochim Biophys Acta 1415, 331–341 (1999).

7. Boussif, O. et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci U S A 92, 7297–7301 (1995).

8. Sakurai, H., Kawabata, K., Sakurai, F., Nakagawa, S. & Mizuguchi, H. Innate immune response induced by gene delivery vectors. Int J Pharm 354, 9–15 (2008).

Figure 4: Comparison of **Helix-IN** transfection reagent to competitors in term of Secreted Embryonic Alkaline Phosphatase (SEAP) production and Viability in different cell types. Different cell types were transfected using **Helix-IN™** and different other transfection reagents according to their respective protocol 48H after transfection, (A) SEAP production in cellular supernatant was measured using the **SEAP Assay Kit (OZ Biosciences, #SP00500)** and viability was determined using **MTT cell proliferation Kit (OZ Bioscience, #MT01000)**.

- 1- the concept of “passing through the membranes barriers” due to its charge, pH-sensitive and hydrophobic properties,
- 2- the idea of “stealth transfection” where DNA is protected, masked and supported all the way to its nuclear uptake and
- 3- the notion of biocompatibility due to biodegradable and cleavable moieties.

Thanks to these features, **Helix-IN™** allows to reach **high yields of transfection** and **protein production** compared to other transfection reagents with attenuated toxicity index as demonstrated in the **figure 4** below. Whereas SEAP production was one of the highest compared to 6 other TR, the viability index was comparable to others. Remarkably when looking at competitors, we can observe for one TR that when the efficiency reaches high yields (for example Tit), the viability index was the lowest. On the other hand, TR inducing low toxicity such as xF or tF failed at producing high quantities of proteins. Only **Helix-IN™** was able to have it both ways, inducing a **high yield of protein production while maintaining high viability**.

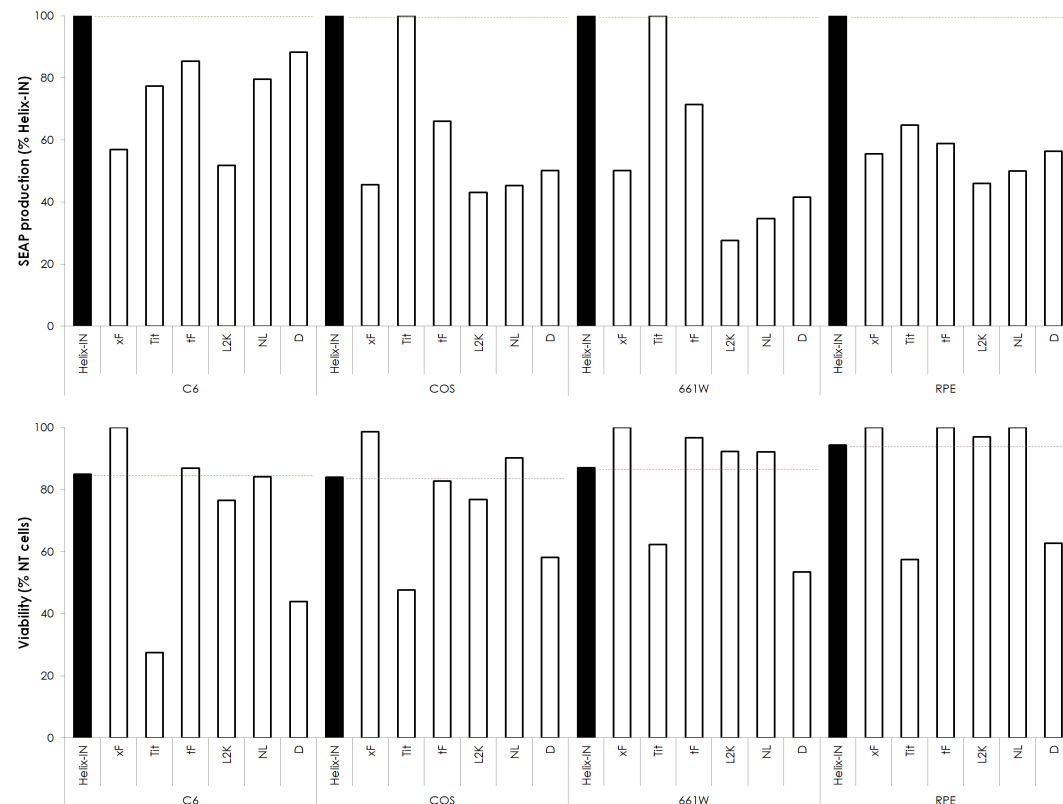


Figure 5: The new class of polymer promotes transfection while lowering cellular stress. Different cell types were transfected using Helix-IN and different other transfection reagents according to their respective protocol. 48H after transfection, (A) SEAP production in cellular supernatant was measured using the **SEAP Assay Kit** (OZ Biosciences #SP00500) and ROS activity was determined using **ROS Detection Assay Kit** (OZ Biosciences #ROS0300).

Most importantly, with **this new polymer technology**, that makes the DNA “invisible” inside the cytosol, we were able to **lower the cellular stress in response to transfection** as demonstrated in the **figure 5** below. Everything happened as if the cell could not detect the presence of an exogenous nucleic acid until it has reached the nucleus. As experienced, while protein production was higher than other TR, the generation of reactive oxygen species (ROS) was reduced compared to others, demonstrating the capacity of **Helix-IN™** to efficiently transfect cells while lowering the cellular stress and thus cell activation.

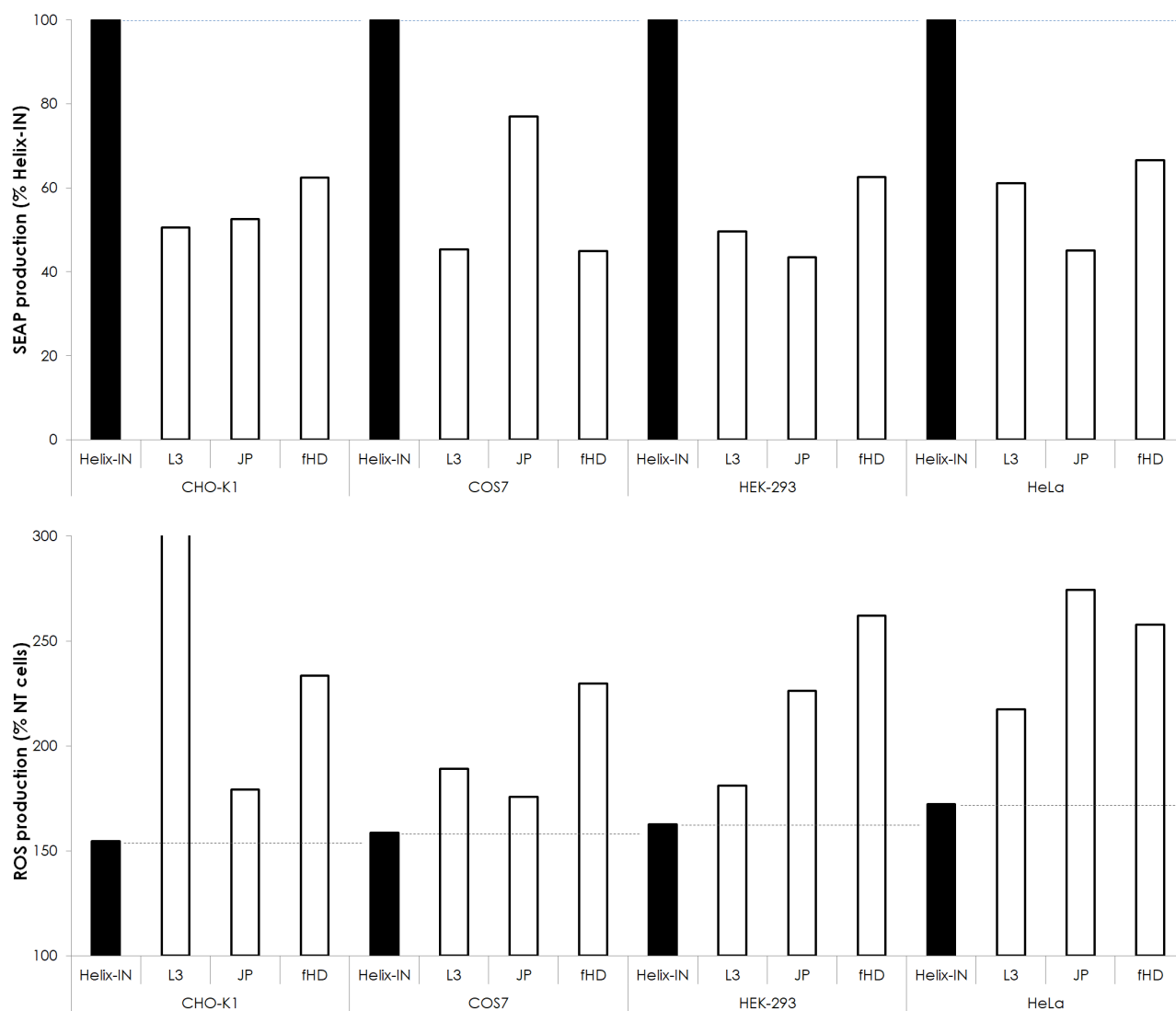
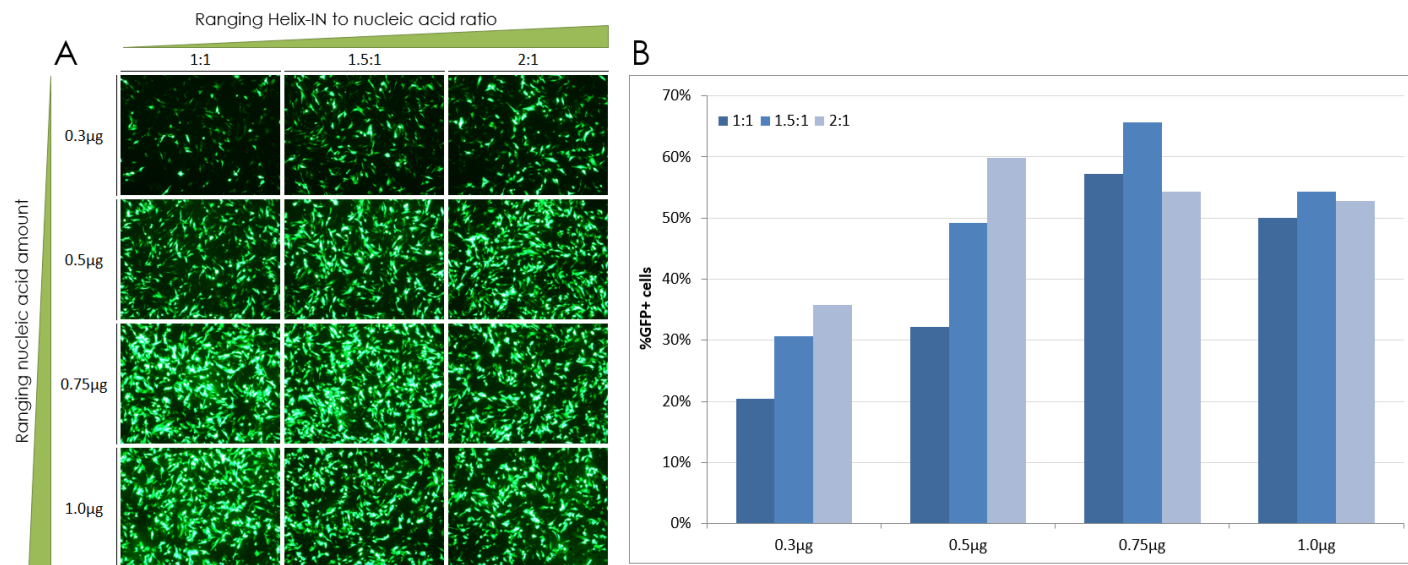


Figure 6: C2C12 transfection with **Helix-IN**. C2C12 cells were transfected with **pVec-tOZ-GFP** and **Helix-IN** using 0.3 to 1 µg per well of DNA in a 24-well plate and ratio of Helix-IN to DNA from 1:1 to 2:1. Transfection efficiency was monitored 48H after by fluorescence microscopy (A) and percentage of cells expressing GFP was measured by flow cytometry (B).

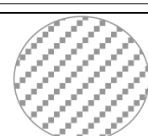
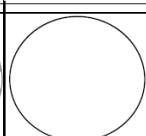
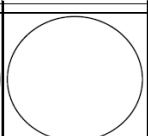
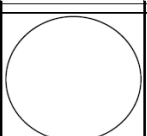
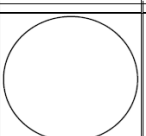
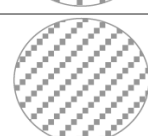
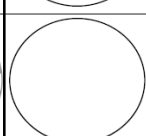
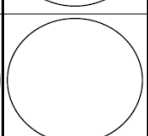
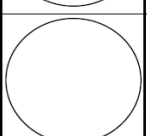
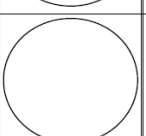
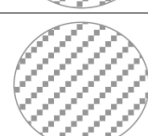
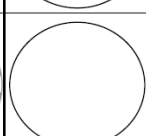
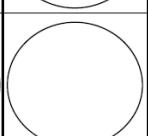
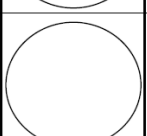
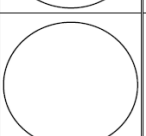
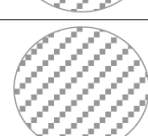
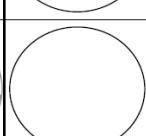
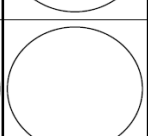
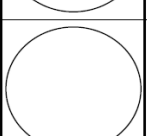
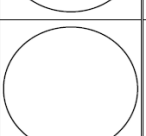


Altogether, the results confirm that this new class of transfection reagent not only outperforms other chemical ones in term of efficiency but also and above all, preserve the cells from activation while maintaining the production of ROS at a “close to basal” level. This has an impacting and direct effect on the viability and thus on the overall transfection level.

OPTIMIZATION PROCEDURE FOR ANY OZ BIOSCIENCES TRANSFECTION REAGENTS: FOLLOW THE “OPTIMIZATION MATRIX”

Generally, all the TR formulations alone display low toxicity, however it increases with the augmentation of the lipid/polymer–NA molar charge ratio; the transfection efficiency markedly depends on the lipid or polymer composition and on the lipoplex or polyplex size, in a rather correlated way. Therefore, it is important to find the best transfection conditions for each cellular model as all the parameters mentioned above might have a dramatic influence on efficiency and/or viability depending on the cell lines; one cell type maybe more sensitive to one transfection condition than another and a couple [NA dose/ratio] could be efficient for one cell model and toxic for another one. To get rid of these cell-to-cell and TR-to-TR variations, we generally recommend performing an “optimization procedure” based on the following model prior to any transfection experiment (**figure 7**). The example below is given for a lipid-based TR for DNA delivery but can be applied to any other chemical type with any nucleic acid. The idea of an optimization matrix is to test at least 4 ratios of TR, each one with different NA quantities; this results in a 4 by 4 matrix allowing testing 16 transfection conditions. Within these conditions it is thus easier to find the best parameters that genetically modify the cells efficiently with a minimal toxicity index. As a matter of fact this methodology should be applied for every cell type and every TR tested to ensure a correct yield with a maximal biocompatibility.

Figure 7: Optimization plate layout – example given for lipid based DNA transfection. Cells are seeded in a 24-well plate and 4 DNA amounts are complexed to 4 rRatios of transfection reagent. After experiment evaluation, pattern of efficiency appears and allows finding the most compatible conditions.

		Ratio of cationic lipid to DNA				
		1:1	2:1	3:1	4:1	
DNA amount (µg)	0,125					
	0,25					
	0,5					
	1					

OZ Biosciences also proposes controls for transfection to monitor TR-induced toxicity.

****NOTE:** it is important to take into consideration that even if green fluorescent protein has become the most popular control gene to be used as a living marker for positively transfected cells and provides researchers with a valuable method of measuring gene expression and cell tracking, it is known since many years that excessive or prolonged GFP expression may induce immune response and apoptosis⁹. In consequence high yield of transfection using GFP may induce a decrease in viability but only because the gene expression is toxic to the cell and not due to the transfection conditions or the delivery vehicle. This should be taken into consideration also when high yields of transfection are reached.*

Associated to transfection reagents, the nucleic acids to deliver are a key component of efficiency and toxicity following genetic modification. Size, design, resuspension buffer, encoded or targeted gene, method of purification... are some of the critical parameters that greatly influence transfection and thus may have a mild to dramatic impact on viability. This is why, beside the design of delivery vehicles, we have spent some effort in the development of nucleic acids to be used as controls for transfection with the production of plasmid DNA or mRNA. Respectively, **pVectOZ transfection plasmids** are engineered in an optimized backbone where sequences affecting transgene expression levels were eliminated; they **encode for the 5 most popular reporter genes** (CAT, GFP*, LacZ, Luciferase and SEAP). The same care has been taken in the synthesis of reporter gene mRNA that are modified with 5-methoxyuridine (5moU) to reduce innate immune response and have been optimized to yield improved stability and performance; they encode for mCherry, Tomato, GFP* and F-Luc. These controls are essential when setting up transfection experiments as they remove the unknown part linked to the NA of interest and this is why we recommend to use them during the optimization procedure. As this nucleic material is well established and qualified, if a lack of efficiency or any toxicity is monitored then it would be due to a non-optimized condition or to an elevated susceptibility for a cell line to a TR. A simple optimization as described previously or a change in TR composition would thus correct this.

One aspect we have chosen in order to reduce the toxicity of our lipid-based transfection reagent is to **work directly on the structure and composition of cationic lipids** as previously mentioned. In parallel we **have developed new class of polymers** that have **demonstrated high efficiency correlated to a reduced cellular activation and thus toxicity**.

Even if they are powerful for many cell types and especially cell lines, these two chemical methods find their limit when addressing the genetic modification of hard-to-transfect or sensitive cells, generally primary cells. Commonly, primary cells are more reluctant to lipoplexes- or polyplexes-based transfection and get activated or enter into apoptosis more rapidly than immortalized cells or cell lines. Therefore, physical methods are generally employed to transfect them as they are more powerful even if they showed higher toxicity.

In an effort to keep intact the capacity of physical approach to genetically modify cells while maintaining viability, we have developed **the Magnetofection™ technology that borrows the best of chemical and physical methods as an alternative to (1) increase the yield of genetic modification while (2) lowering cellular stress and/or toxicity**.

9. Liu, H. S., Jan, M. S., Chou, C. K., Chen, P. H. & Ke, N. J. Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 260, 712–717 (1999).

MAGNETOFECTION™: EFFICIENT AND NON-TOXIC, REDUCE TOXICITY OF LIPID-BASED TRANSFECTION REAGENTS

This application note will not focus on **the Magnetofection™** technology *per se* as other talented authors have published impacting reviews on this matter (**we recommend for example the review by the inventor of the methods published in 2011**¹¹) but rather on how the toxicity is reduced while using this technics and how it can be associated with other chemical methods to increase their efficiency and lower the toxic effect of transfection.

In the early 2000's, Christian Plank and colleagues defined the term **Magnetofection™** as “nucleic acid delivery under the influence of a magnetic field acting on nucleic acid vectors that are associated with magnetic nanoparticles”¹⁰. Among the various benefits of **Magnetofection™** we can cite for example the improvement of the dose–response relationship in nucleic acid delivery, a strong improvement of the kinetics of the delivery process and the possibility to localize nucleic acid delivery to an area, which is under magnetic field guidance. **This method, available both in vivo and in vitro**, can be applied to **non-viral as well as viral vectors** and since then, **OZ Biosciences** has developed and commercialized various **Magnetofection-based reagents** that contributes to **an exponentially increasing number of papers** in the field.

To be successful in **Magnetofection™**, magnetic particles need to possess some functionalities that allow them to be associated with a gene delivery vector into a magnetic vector. The associations are mainly mediated through electrostatic and hydrophobic interactions and the vector can be nucleic acid either alone or in combination with an enhancer (nonviral lipoplex or polyplex) or viral vector.

The protocol is easy to execute: the vector (nucleic acid or lipoplex/polyplex) is put into contact with magnetic nanoparticles and incubated at room temperature for 15 to 20 min. After the magnetic complexes are formed, they are added to the cells and the cell culture plate is positioned onto a magnetic plate. Finally, after **Magnetofection™** procedure, the culture plate is placed back under standard culture conditions until evaluation of the experiment, aka gene expression or silencing (**figure 8**). The postulate here is that the magnetic properties of the particles have to be sufficient to concentrate the vector at the targeted cells under magnetic force.

10. Scherer, F. et al. Magnetofection: enhancing and targeting gene delivery by magnetic force in vitro and in vivo. *Gene Therapy* 9, 102–109 (2002).

11. Plank, C., Zelphati, O. & Mykhaylyk, O. Magnetically enhanced nucleic acid delivery. Ten years of magnetofection-progress and prospects. *Adv Drug Deliv Rev* 63, 1300–1331 (2011). 260, 712–717 (1999).

Figure 8: Magnetofection™ protocol with suspension of nucleic acids or lipoplexes. Naked nucleic acids suspension or lipoplexes formed by the incubation of NA and lipid-based transfection reagent are added to specific magnetic nanoparticles; the specificity being dictated by the application. After incubation time at room temperature (RT), the magnetic complexes or the magnetized lipoplexes are added into the cell supernatant. The cell culture plate is then incubated for 20 min at RT on a magnetic plate delivering a specific magnetic field to attract and concentrate magnetic vector onto the cell surface [Magnetofection™]. Cells are finally incubated under standard culture conditions until evaluation of the experiment.

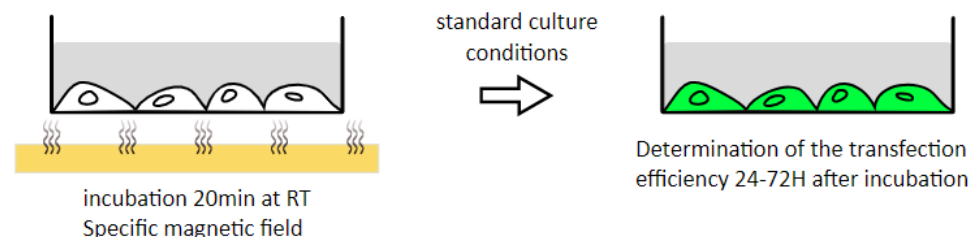
Suspension of nucleic acids or lipoplexes are added to magnetic nanoparticles



Magnetic complexes or magnetized lipoplexes are formed by incubation at RT



Cell plate is placed onto a specific magnetic field to perform Magnetofection™



Magnetic devices composed of neodymium-iron-boron (Nd-Fe-B) permanent magnets deliver a magnetic gradient field strong enough to sediment the applied paramagnetic vectors onto cells within a few minutes. This is now obvious how the advantages of **Magnetofection™**, in terms of toxicity and efficiency, naturally result from the properties mentioned above. First, the largest fraction of the vector dose is available to the cells within minutes of incubation, which strongly accelerates transfection kinetics. Cells are thus in contact with the vector at the same time therefore reducing the inhibitory effect mediated by signal dangers that one cell could send to its close neighborhood alerting surrounding cells that would then become reluctant to transfection. Secondly, the high increased concentration of vectors at the cell surface warrants a dramatically improved dose-response profile. Finally, due to the previous points altogether, lower nucleic acid dose is necessary to induce an improved transfection effect thus reducing toxicity as lower doses of nucleic acid material enters the cells.

Magnetofection™ allows to use lower doses of nucleic acid thus reducing toxicity

Since almost 20 years now, **Magnetofection™** has been used in a variety of cellular models to transfect both **viral and non-viral vectors** and among the latter ones, **any kind of nucleic acids**. As explained previously, the capacity to rapidly concentrate vectors onto the cell surface and the reduced amount of NA used has made **Magnetofection™** **the transfection method of choice for primary and hard-to-transfect cells**. These cells are moreover generally sensitive to genetic modification to which they easily respond by initiating apoptosis program leading to cell death. Totally biodegradable because they are made of a superparamagnetic iron core surrounded by a life-compatible coating, the magnetic nanoparticles (MNPs) are obviously degraded and do not interfere with cellular pathway (except iron degradation pathway of course due to their nature) or with following experiments. As a matter of fact, it was previously demonstrated that preloading cells with up to 50 pg Fe/cell in the form of core-shell iron oxide MNPs increased the efficiency of subsequent **Magnetofection™** without causing toxicity¹². And since only low doses of NA are necessary for an improved transfection effect, **Magnetofection™** **remains one of the most efficient methods presenting the lowest rate of toxicity**. This has been validated over the years by thousands of publications that illustrated the fact that **Magnetofection™** **can be applied to any kind of vectors (DNA, siRNA, mRNA, ODN, LNA, miRNA, ... adenovirus, lentivirus...)** **to transfect every kind of cell types (from cell lines to primary cells as well as in vivo)**. Among them, we can highlight the transfection of primary neurons that are truly representative of the capacity of **Magnetofection™** methods.

Actually, these cells are one of the most sensitive and difficult cells to transfect and using a specific **Magnetofection™-based TR: NeuroMag™** (OZ Biosciences, #NM51000), **all sort of neurons have been transfected no matter the assessed DIV (DIV = Day in vitro, from 0 to 21) ; this is of high importance when knowing the fragile nature of early and aged neurons particularly**. Cortical neurons¹³, dopamine neurons derived from iPSc¹⁴, dorsal root ganglion neurons¹⁵, hippocampal¹⁶, or motor neurons¹⁷ are only a few examples of the type of neurons to which **NeuroMag™** can be applied to in vitro**.

****NOTE:** these are only few examples of different cell types efficiently transfected with Magnetofection™. More than a thousand publications are now available and we thus invite the reader to have a look to our citation database for an exhaustive list of references mentioning the use of our magnetic-based transfection reagents at: <https://www.ozbiosciences.com/module/citationfinder/default>

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12. Mykhaylyk, O. et al. Nucleic acid delivery to magnetically-labeled cells in a 2D array and at the luminal surface of cell culture tube and their detection by MRI. J Biomed Nanotechnol 5, 692–706 (2009).
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OZ Biosciences has designed several optimized Magnetofection™ Transfection Reagents according to defined applications:

NON VIRAL APPLICATIONS

- PolyMag™ | PolyMag Neo™
- CombiMag™
- Magnetofectamine O2 Kit
- NeuroMag™
- Glial-Mag™
- SilenceMag™
- FluoMag™
- SelfMag™

VIRAL APPLICATIONS

- ViroMag™
- ViroMag R/L
- AdenoMag™
- Mag4C-LV / Mag4C-AD

IN VIVO APPLICATIONS

- In vivo PolyMag™ & DogtorMag™
- In vivo ViroMag™
- In vivo SilenceMag™
- XPMag™

Extending its use *ex vivo*, the **Magnetofection™** was also demonstrated to be highly efficient to silence gene expression without causing any toxicity. In retinal explant, the quantification of apoptotic nuclei in each nuclear layer of the retina showed the absence of toxicity when retina explants received **XPMag™** (OZ Biosciences, #XP00500) complexed with siRNA¹⁸.

Finally it is *in vivo* that **Magnetofection™** has proven its total harmlessness for many years since 2007 and the first work by Jahnke A et al that applied **Magnetofection™** to the immunogen therapy of feline fibrosarcoma¹⁹. Plasmid DNA coding for a cytokine gene associated with magnetic nanoparticles was injected directly in the vicinity of the tumor under application of a permanent magnet localized on the outside of the animal. During that study more than 150 animals have been treated. The treatment was very well tolerated and drastically increased the percentage of long-term relapse-free animals. Since then, numerous works *in vivo* have confirmed the total safety of **Magnetofection™** whether after injection of magnetized siRNA with **in vivo SilenceMag™** (OZ Biosciences, #IV-SM30500) in the rat sciatic nerve and subarachnoid space between L5 and L6²⁰ or by the *in vivo* delivery of mitochondrial mRNA into inguinal adipose tissue of mice²¹.

Beside toxicity, an important parameter to monitor is the cellular activation that could result in behavioral or phenotypical modification without necessarily inducing apoptosis. The changes induced by cellular stress can although have a dramatic impact on the experiments without even being noticed by the user. Another strong point of **Magnetofection™** is not only viability is not impacted during transfection but also, this method keeps the cellular activation to a basal level. In this way, even cells that are sensitive/reluctant to transfection due to activation such as microglial cells showing upregulation of protein involved in the inflammatory response such as TNFα and TLR2 upon transfection with classic transfection reagents²² can be thus genetically modified using **Magnetofection™**. To illustrate this, we can cite Carrillo-Jimez A. et al. that stated the following conclusion in their paper published in Front Cell Neurosci 12, 313 (2018): *“Here, we describe an **easy**, and **effective method** based on the **Glial-Mag™ method** (OZ Biosciences) using magnetic nanoparticles and a magnet to **successfully transfect primary microglia cells with different siRNAs**. This method does not require specialist facilities or specific training and does not induce cell toxicity or inflammatory activation”*²³.

18. Bassetto, M. et al. New Method for Efficient siRNA Delivery in Retina Explants: Reverse Magnetofection. *Bioconjug Chem* 32, 1078–1093 (2021).

19. Jahnke, A. et al. Intra-tumoral gene delivery of fIL-2, fIFN-γ and fGM-CSF using magnetofection as a neoadjuvant treatment option for feline fibrosarcomas: a phase-I study. *J Vet Med A Physiol Pathol Clin Med* 54, 599–606 (2007).

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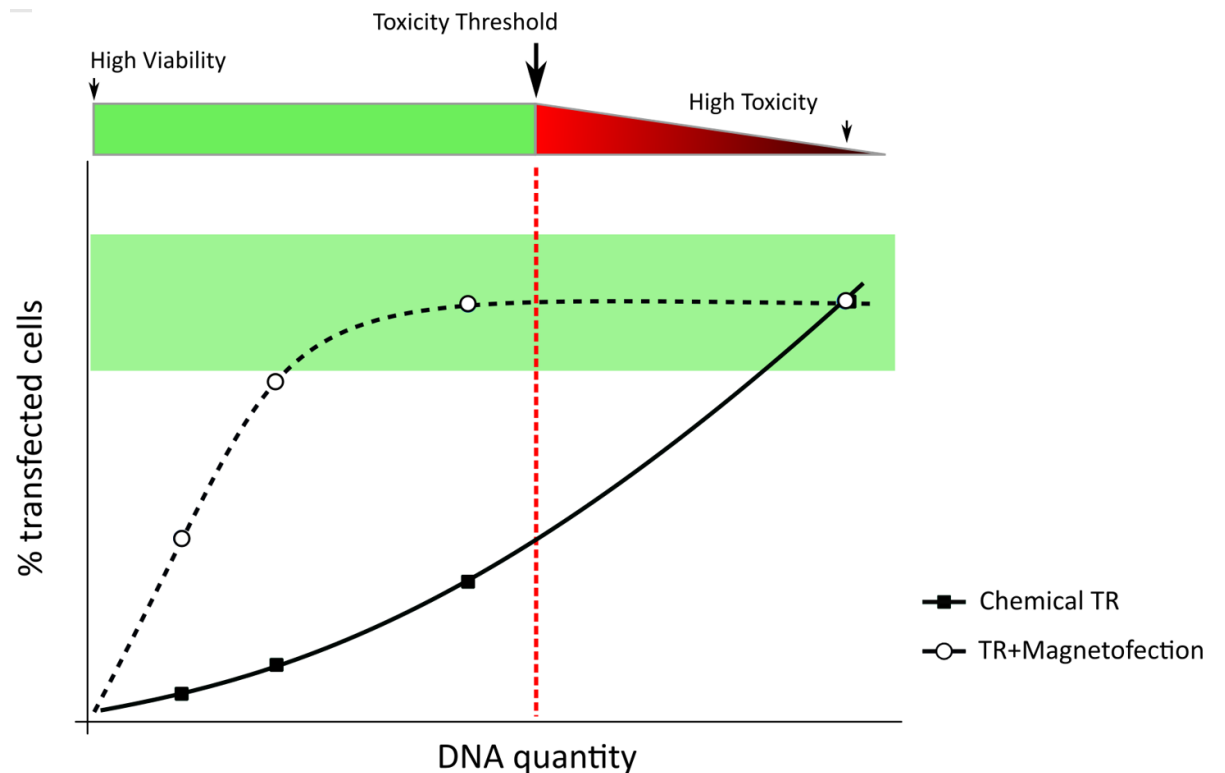
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Magnetofection™ : Increases chemical TR efficiency for a reduced toxicity

The best illustration of the **Magnetofection™** capacity to lower toxicity comes from its association with chemical transfection reagents such as lipid-based ones. As illustrated in the **figure 9**, the efficiency of a classic chemical TR raises with the increase of DNA quantity (black line). As previously mentioned, every transfection condition met a “toxicity threshold” represented by the red dot line, at which viability begins to decline until high toxicity begins to appear. Therefore, considering chemical TR, the condition leading to maximum transfection obviously takes place in a zone where toxicity balances efficiency. With the addition of **Magnetofection™** featuring **CombiMag™ magnetic nanoparticles (OZ Biosciences, #CM21000)** to chemical TR, the % of transfected cells increases rapidly (black dot line) as lower nucleic acid doses are required and reaches maximum of efficiency before hitting the toxicity threshold. For the same efficiency, less nucleic acid is used with **Magnetofection™** implying a lowered cellular answer leading to activation, stress and/or apoptosis.

Figure 9: Magnetofection™ protocol with suspension of nucleic acids or lipoplexes. As the magnetic force drives the gene vector towards the target cells, **Magnetofection™** allows the vector dose to concentrate into the cell very rapidly and triggers delivery via endocytosis. Consequently, as opposed to a classic chemical TR, high transfection efficiencies can be achieved with less nucleic acid amount and maximum efficiency is reached before hitting the toxicity threshold.



OZ BIOSCIENCES DELIVERY VEHICLES VERSUS ELECTROPORATION & NUCLEOFECTION

Known as one of the most efficient method to deliver nucleic acids into cells and to induce genetic modification and since its introduction in the early 1980s, **the Electroporation technics** has become a routine method. Briefly, **electroporation** uses high electric fields to transiently permeabilize biomembranes in a reversible ways and allowing introduction of nucleic acids inside cells. This method which is preferentially **chosen to transfect cells in suspension and mainly immune cells with a quite high efficiency** has however **shown very early a strong toxicity**²⁴. Moreover previous reports have indicated that **electroporation** induces the **production of reactive oxygen species (ROS), mainly O₂**, which are generated on the permeabilized part of the cell membrane only when cells are reversibly permeabilized. **These ROS can damage lipids, proteins and nucleic acids and, consequently effect long-term cell survival**²⁵. Even in vivo where this method induces high protein expression and is often used for veterinary purposes, **the electroporation procedure on its own, caused severe muscle damage consisting of infiltration and degradation of skeletal muscle**²⁶.

A refinement of this method, **Nucleofection** uses the same physical principles to induce direct probe transfer into the cell nucleus. **Nucleofection** is nowadays getting more and more attention due to its high efficiency and **reported lower toxicity compared to electroporation**. However, previous data suggest that applying low or high-intensity electric fields can **induce intracellular calcium release, damage of nucleic acids, inhibition of cell cycle progression by affecting potassium channels and interference with the formation of mitotic spindles**. Moreover, as stated and demonstrated in their paper by **Mello de Queiroz et al.**, even if **Nucleofection** does not induce massive cell death, it still **induces non-specific modifications in the metabolic activity of transfected cells that can have dramatic impact on cell behavior**²⁷.

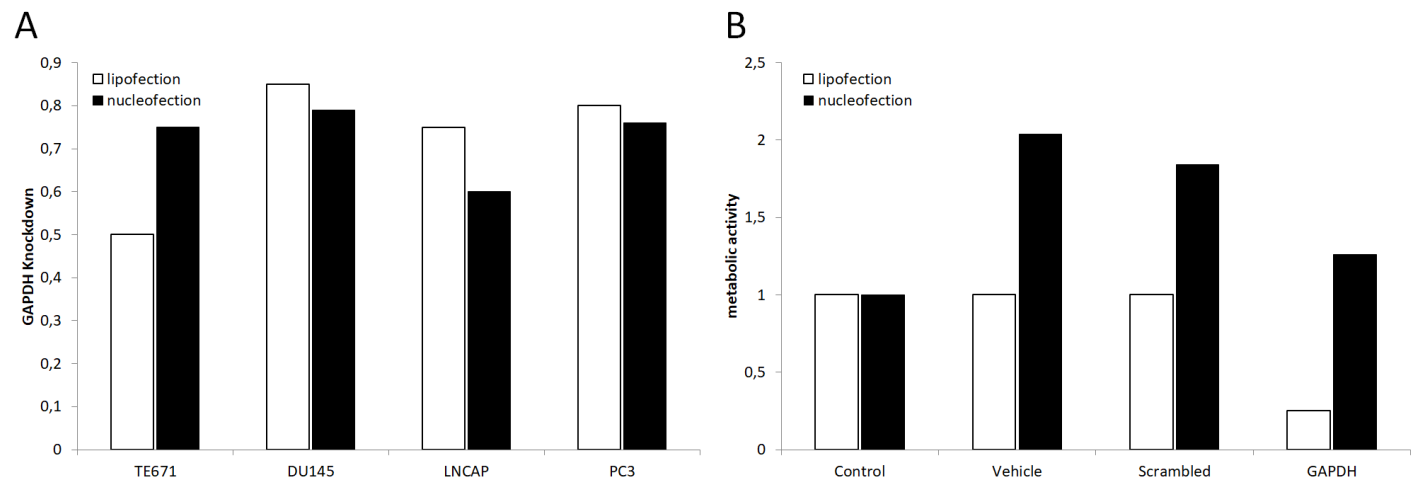
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Figure 10: comparison between nucleofection (black bars) and lipofection (white bars) in terms of efficiency and metabolic activity. (A) GAPDH knockdown of tumor cells and (B) metabolic activity of DU145 cell line. Control= no treatment, vehicle= mock transfection.

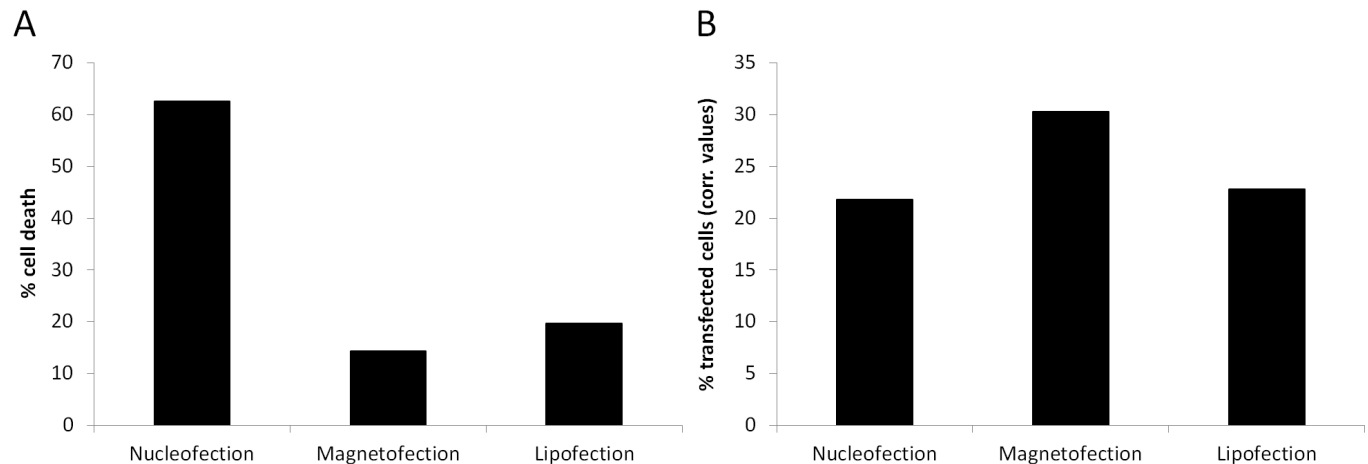


In a second set of experiments, the authors showed that **nucleofection also changed the subcellular distribution of the transfected protein**: whereas lipofection of a fluorescent protein-encoding DNA in NIH-3T3 resulted in a diffuse pattern throughout the whole cell, **nucleofection induced most of the fluorescent signal to be concentrated inside the nucleus**. Once again, such effect proved cell type-specific, as no differences in protein distribution between lipofection and nucleofection were observed in HEK293.

Nucleofection & Electroporation are said to be Superior to Lipofection & Magnetofection: Not Totally True...

Beside the numerous successes of **electroporation** with respect to the uptake efficiency, transfection rates (most specifically for hard-to-transfect suspension and/or immune cells) and high-throughput yield for numerous cell applications in association with nanoparticles, this technique has to face a major problem regarding cell survival. As disclaimed by the various manufacturers, electroporation is said to be superior to other methods such as chemical ones (**lipofection**) or even other physical ones (**Magnetofection™**) and it is true that the percentage of transfected cells is very high: it is frequent to reach up to 80-100% of transfected cells depending on the cell type. And this is a real improvement for cells that appear reluctant to transfection. However, this apparent high efficiency has to be put in correlation with the toxicity index. Actually, the numbers that are reached after electroporation are based on the remaining living cells and not on the overall starting cell number and it is thus not rare to face a survival rate of 20% after transfection by electroporation. It is important to keep in mind that there is a bias in the final results as they take only in consideration the living cells after genetic modification and not the whole cell population; the true overall transfection yield becomes now 20% if 100% of the remaining cells are transfected.

Figure 11: Comparison of toxicity and transfection efficiency between the 3 methods. (A). Toxicity was compared between the three methods of transfection using MTT. (B) Transfection efficiency after correction by taking into account toxicity.

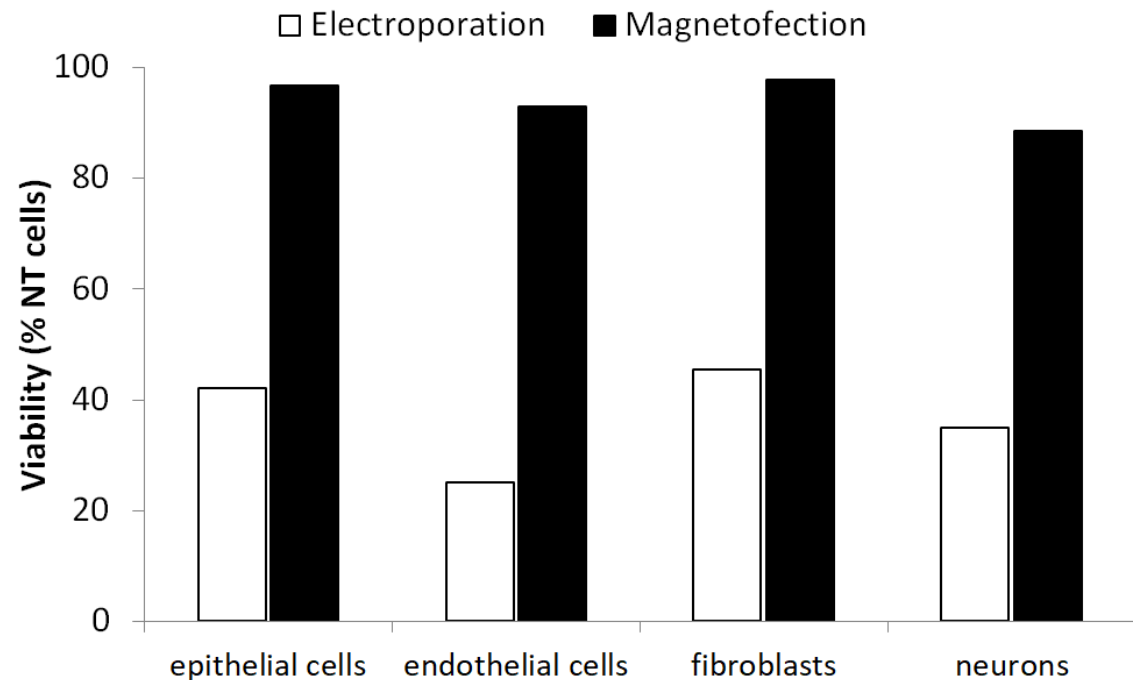


One of the best illustrations to this can be found in the work by **Scheibe F et al. in 2012** that investigated the **transfection efficiency of mouse bone-marrow** derived mesenchymal stem cells (MSCs) with three different techniques: **Nucleofection**, **Magnetofection™** and **Lipofection**. **Using disconcerting technical approaches, they promptly conclude that Nucleofection outperformed the two other methods and disregard cytotoxicity.**

Actually, when taking a closer look to the methodology and the results some pitfalls begin to appear. Briefly, the culture and transfection conditions totally vary between the three protocols and do not follow the manufacturer's recommendation. The authors concluded that the latter gives the best percentage of transfected cells (60%) compared to **Magnetofection™** (30%) or **lipofection** (23%) without taking into consideration the cell survival rate. Indeed, **nucleofection induced a massive cell death** as only 37% of MSC survived whereas the two other technics resulted in less than 20% toxicity (**figure 11-A**). As a result, if we relate the efficacy values to the survival rate, we find completely different results from those presented in the paper: **Magnetofection™ allowed to transfect more than 30% of the total cell population with the highest viability index (figure 11-B)**. These results confirm that **Magnetofection™ remains the less toxic method to transfect cells with the highest efficiency.**

Moreover, when comparing the two transfection methods for viability over four cell types, we observe that **Magnetofection™ does not result in a massive loss of viability compared to electroporation that induces a dramatic toxic effect on every cell type tested: between 60% and 80% of toxicity are reached after electroporation (Figure 12).**

Figure 12: Viability comparison between electroporation and Magnetofection in various cell types. Different cell types were transfected using electroporation and Magnetofection™ according to the respective manufacturer's instructions. Viability was further analyzed using MTT.



Altogether, these observations revealed that electroporation and nucleofection not only induce massive cell death but also result in dramatic changes in cell behavior compared to other transfection methods. These effects are cell-type dependent and difficult to predict; therefore, proper standards and controls are mandatory during nucleofection experiments, especially when correlating cell proliferation with metabolic activity or assessing the subcellular distribution of transfected proteins.

As a matter of fact, thanks to its properties and the observation that magnetized complexes allow to use less nucleic acid amounts for an increased efficiency, the **Magnetofection™ methods appears the best alternative and solution to genetically modify numerous cell type**. Based on its technology, this technic is more suited for primary adherent cells onto which the magnetic complexes are guided and concentrated by a specific magnetic field. However, at this point it is important to keep in mind that electroporation and nucleofection remain the best non-viral solutions so far to genetically modify suspension and

CONCLUSION

Over the years we have developed multiple transfection reagents based on chemical or physical properties respectively cationic lipids, polymers and **Magnetofection™** with respect to cell viability. Of course, the major goal motivating the design of a new TR is the efficiency of the delivery system: cargo has to be delivered inside the cell and be expressed to achieve the highest yield of transfection as possible. However, this is not the only goal that drives us: genetic modifications have no perspective if the cell is to die. This is why our delivery systems have been developed to preserve the best they can cellular integrity and cell survival. Whether it is our lipid reagents, our polymers or the **Magnetofection™**, **they all present a high efficiency associated to significant viability when used at the optimized conditions.**

Our catalog of patented lipids are built around the same structure that allows us to finely tune their properties depending on the application (**nucleic acid to deliver, cell type...**) and find the right balance between toxicity and efficiency: thanks to their biodegradable properties conferred by the cleavable linker, the transfection occurs without too much damages for the cell. Recently, we have developed a new class of cationic polymers based on the **CHAMP™ technology** to lower cellular answer and stress to basal level. Highly efficient and almost undetectable by the cell, this new class of chemicals spearheaded by **Helix-IN™** is shaping the future of transfection reagents with stealth capacities for a maximal viability index. Lastly, the **Magnetofection™ technology aiming at transfecting primary and hard-to-transfect cells has proven over the years its compatibility with any cell type, its highest efficiency and its total harmlessness for in vitro, ex vivo and in vivo applications.**

Finally, even if we have developed efficient and biodegradable delivery systems, the bring-to-home message is that, **whatever the transfection reagent, nucleic acid or application, always perform an optimization procedure prior to any experiment to find the best balance between toxicity and efficiency!**

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Europe / Asia OFFICE

OZ Biosciences SAS
 Parc Scientifique de Luminy, zone entre-
 prise, case 922, 13288 Marseille Cedex 09,
 FRANCE
 Ph : +33 486 94 85 16
 Fax : +33 486 94 85 16
 E-mail: contact@ozbiosciences.com



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US OFFICE

OZ Biosciences Inc.
 4901 Morena Boulevard, Suite 901
 San Diego, CA 92117,
 USA
 Ph : +1 858 246 7840
 Fax : +1 855 631 0626
 E-mail : contactusa@ozbiosciences.com

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Europe / Asia OFFICE

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 Parc Scientifique de Luminy, zone entre-
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 FRANCE
 Ph : +33 486 94 85 16
 Fax : +33 486 94 85 16
 E-mail: contact@ozbiosciences.com



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US OFFICE

OZ Biosciences Inc.
 4901 Morena Boulevard, Suite 901
 San Diego, CA 92117,
 USA
 Ph : +1 858 246 7840
 Fax : +1 855 631 0626
 E-mail : contactusa@ozbiosciences.com