



Dace Avotiņa

**INFLUENCE OF THE TIME-PHASE
VARIED MAGNETIC FIELD ON THE
NUCLEIC ACIDS DELIVERY
INTO THE CANCER CELLS**

Summary of Doctoral Thesis
for obtaining the degree of a Doctor of Medicine
Speciality – Theoretical Medicine

Riga, 2016

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Doctoral Thesis was carried out at the Rīga Stradiņš University August Kirhenšteins Institute of Microbiology and Virology

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ABBREVIATIONS

ABBREVIATION	EXPLANATION
AO/EB	Acridine Orange/Ethidium Bromide staining
CM	CombiMAG magnetic nanoparticles
Cos7	African green monkey kidney fibroblast-like cell line
DNA	deoxyribonucleic acid
E. coli	<i>Escherichia coli</i>
ECFP-ERp29pDNA	plasmid DNA containing ECFP-ERp29 gene
Fe²⁺	ferrum (II) ion
HeLa	human cervix adenocarcinoma cell line
HEPG2	human hepatocellular adenocarcinoma cell line
L	lipofection
L siRNA	co-lipofection with siRNA
LacZpDNS	plasmid DNA containing LacZ gene
LIP	Lipofectamine2000 transfection reagent
LM	liposomal magnetofection
LM LFV	liposomal magnetofection in time-varied magnetic field
LM LFV siRNA	co-liposomal magnetofection in time-varied magnetic field with siRNA
LM siRNA	co-liposomal magnetofection with siRNA
NdFeB	neodymium-iron-boron
PB	Prussian blue staining
PC3	human prostate adenocarcinoma cell line
pDNA	plasmid DNA
PEI	polyethylenimine
siRNA	small interference ribonucleic acid
SPION	superparamagnetic iron oxide nanoparticle

INTRODUCTION

Cancer is uncontrolled cell growth resulting from somatic mutations and epigenetic changes of several genes.

Gene therapy is a type of therapy based on the introduction of one or more genes encoding normal, functional proteins into the patient's cells genetic material in order to replace a mutated gene (-s) (Mehier-Humbert and Guy, 2005; Dick et al., 2015).

Nowadays, there are several hundred different genes (> 1% of the human genome), whose mutations are associated with the development of cancer (Futreal et al., 2004; Wishhart, 2015), nevertheless the gene therapy is highly an experimental method of treatment and only few gene therapy products are applied for the clinic.

Gene delivery is one of the most problematic steps in gene therapy, since it includes not only the delivery of the therapeutic gene to the cells, but also an effective attachment to the cell membrane with the subsequent transfer across the membrane into the cell to the nucleus. In addition, the gene delivery process is interfered by the therapeutic agents (nucleic acids) properties – poor ability to diffuse through the cell membrane due to their size, negative charge and hydrophilic nature (Jafari et al., 2012). The outcome of the gene delivery is directly dependent on the selected vector system – it should be effective, specific and safe, but the ideal gene delivery vector is characterized by the number of properties (Somia and Verma, 2000; Ibraheem et al., 2014):

- attaches to the therapeutic genes of different size and shape in an effective manner;
- protects the therapeutic gene against the serum, extracellular and intracellular endonucleases degradation;

- provides the delivery of therapeutic genes to the specified dividing and non-dividing target cells regardless of their localization site and integrity to the surrounding tissues;
- is non-immunogenic;
- is non-toxic.

Viruses containing vector systems are the most common type of the gene delivery. Viruses' inherent natural properties – the ability to easily overcome the cell membrane barrier by stimulating endocytosis processes and expressing viral genes using a host biosynthetic mechanism makes them as efficient gene delivery vectors (Mulligan, 1993). Despite this, they have a number of significant deficiencies – virus proteins immunogenicity, which can provoke a strong body's immune response and elevated cytotoxicity at high viral loads (Teramoto et al., 2000; Anson, 2004). There is also a known risk of insertion mutagenesis, which can lead to the oncogenic activation, as well as random formation of infectious virus particles (Lachmann and Davies, 1997; Thrasher et al., 2006; Bushman, 2007). Non-viral – chemical and physical vectors have several advantages compared to viral vectors. They are easily synthesized and exploitable, capable of delivering the unlimited length and amount of coding sequences and have low immunogenicity (Ruß and Wagner, 2007). Most of the chemical vectors are cationic compounds. Thus, they can effectively bind both to negatively charged phosphates of the nucleic acids backbone forming a stable complex and the negatively charged cell membrane. For instance, cationic lipids form spherical hydrophobic structures – liposomes, wherein the therapeutic gene is encapsulated in the lipid bilayer (Fraleley et al., 1980). In the process of liposomes formation, a positively charged hydrophilic part of the lipid binds along the nucleic acid chain backbone, but the mutually interacting hydrophobic parts delay the separation of hydrophilic parts and at the same time serves as a coating providing protection of the nucleic acid

(Kennedy et al., 2000; Oberle et al., 2000). This mechanism makes the liposome-mediated gene delivery or lipofection as one of the most effective and most widely used methods for the transfection of all types of nucleic acids in different cell lines. High cytotoxicity is the main disadvantage of chemical gene delivery vectors (Moghimi et al., 2005; Hunter; Lv et al., 2006), they are very sensitive to even small changes in pH, temperature and salt concentration, so the use of them for *in vivo* studies is problematic. The main advantage of physical gene delivery is the ability to overcome a number of intra-cellular and extracellular barriers bypassing one or more passive gene delivery stages (Brunner et al., 2002). This ensures a direct access to the cell cytoplasm or even the nucleus, which means that in theory, any membrane impermeable molecule can be delivered into the cells and this approach increases simultaneously the velocity of the gene delivery, as well as efficiency. Magnetofection is one of the most effective physical gene delivery methods. It is based on the superparamagnetic nanoparticles (SPION) coupled nucleic acids accelerated concentration onto the cell surface and delivery into the cells under the influence of the external magnetic field. Compared with other physical gene delivery methods, magnetofection has several advantages. In the case of magnetofection, natural attachment and internalization mechanisms are used, thus avoiding the damage of the cell membrane, which results in a substantial decrease of method's cytotoxicity (Laurent et al., 2011; Sapet et al., 2011). The influence of the magnetic field in tissues ranges from 10 to 15 cm deep, so that the magnetic nanoparticles accumulate evenly not only in a surface, but also in deep tissue layers (Goudy et al., 2008). Magnetofection efficiency is dependent on both physio-chemical properties of SPION, as well as the magnetic field parameters – the magnetic field intensity and magnetic field gradient.

Permanent magnets create their own continuous static magnetic field where direction of the magnetic field gradient is vertical along the axis Z (Hofmann-Antenbrink et al., 2009). Thus, the SPION motion in solution against

the magnet in the static magnetic field is axial. Several publications describe an alternative magnetic fields involving complex action of magnetic forces on SPION along the axis X-Y-Z. As a result of the influence of such magnetic fields the SPION motion in solution is changed – it is not only axial, but can also be lateral, oscillating and rotating. It is believed that in the result of the altered SPION movement the transfer of them through the cell membrane is promoted, but the exact mechanism of this phenomenon has not been completely elucidated. The time-phase varied magnetic field is generated with the help of the magnetofection device DynaFECTOR (Figure 1).

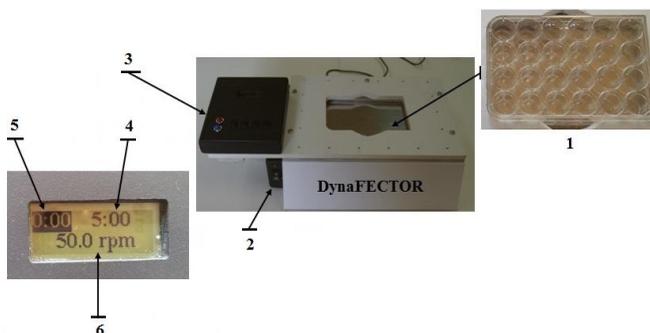


Figure 1. The magnetofection device DynaFECTOR: 1 – position of the 24 well plate, 2 – power switch, 3 – control panel, 4 – exposure duration in time-phase varied magnetic field, 5 – exposure duration in static magnetic field, 6 – magnets rotation frequency

The time-phase magnetic field is based on a permanent magnets plate orbital rotation in a plane parallel to the cell culture plate (Figure 2).

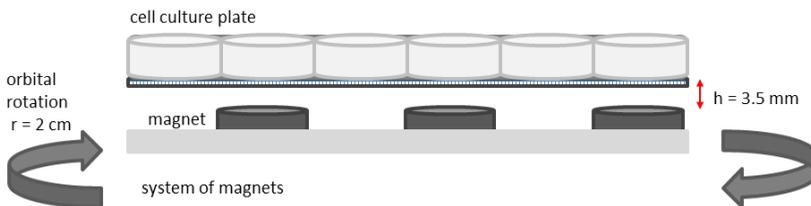


Figure 2. **Rotation plane of the magnets array against the plane of the 24 well cell culture plate**

In the rotating magnet array the influence of specific magnetic forces on the SPION is characterized by the magnets positions in the orbit, phases 1, 2 and 3, respectively (Figure 3).

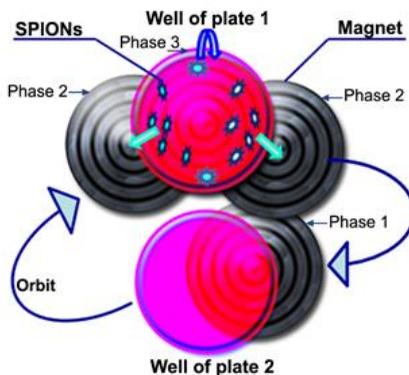


Figure 3. **Principle of the rotating magnet array: schematic illustration of the magnet array for one magnet and two wells of the cell culture plate; Phases 1., 2., 3. – magnet positions under the wells in the process of an orbital rotation (Adapted from Karpov *et al.*, 2014)**

In the case of magnet absent under the well of the plate in phase 1, the action of magnetic force is zero. In the phase 2, the lateral motion of SPION occurs, because the radial component becomes dominant, but in the phase 3 the motion of SPION occurs in the axial direction, because the magnet is positioned straight below the well.

As a result of computer modelling it was found that in the result of the axially lateral movement of SPION during the sedimentation process, they distribute both in the central part, as well as at the external border of the magnet, which contributes to more uniform sedimentation onto the surface, too. The influence of the time-phase varied magnetic field on the delivery efficiency of the SPION-nucleic acids complexes into the cells is not clarified.

Aim of the study

To investigate the influence of the time-phase varied magnetic field on the delivery efficiency of nucleic acids into the cancer cells.

Objectives of the study

1. To compare the sedimentation of superparamagnetic iron oxide nanoparticles in solution in the static and time-phase varied magnetic field using magnetofection device DynaFECTOR.

2. To perform the experimental optimization of the reaction conditions in the static and time-phase varied magnetic field using magnetofection device DynaFECTOR in order to achieve the maximum efficiency of nucleic acids delivery into the cancer cells.

3. To compare the delivery efficiency of nucleic acids into the cancer cells among different gene delivery methods.

4. To compare the cytotoxic effect of the application of different gene delivery methods into the cancer cells.

5. To compare the internalization efficiency of superparamagnetic iron oxide nanoparticles in the static and time-phase varied magnetic field using magnetofection device DynaFECTOR.

Hypothesis of the study

Under the influence of the time-phase varied magnetic field, the axially lateral motion of superparamagnetic iron oxide nanoparticles occurs, which stimulate the uptake of them thus also increasing the delivery efficiency of nucleic acids coupled with particles.

Scientific novelty of the study

In this study influence of the time-phase varied magnetic field to the nucleic acids delivery efficiency into the cancer cells *in vitro* will be evaluated experimentally and thereby enhanced gene delivery method – the liposomal magnetofection in time-phase varied magnetic field will be described. The method could be used for more effective gene delivery into the different monolayer cell lines and in perspective for the therapeutic gene transfer into the cancer cells *in vivo*.

2. METHODS

Complex methodological approach was used in the study. Methods and their use is summarized below.

Determination of the **sedimentation profile** of CM:

- sedimentation of CM in cell culture medium in the static and time-phase varied magnetic field;
- microscopy with obtaining microphotographs.

LacZ and **ECFP-ERp29** pDNA acquisition:

- pDNA transformation in *E. coli* and cultivation of transformed bacteria;
- pDNA purification from *E. coli* culture using Plasmid Midi Kit;
- concentration measurements of the purified pDNA.

Optimisation

Determination of the optimal pDNA:LIP and pDNA:LIP:CM **relation**:

- PC3 and HEPG2 cells transfection in the static and time-phase varied magnetic field using different LacZpDNA:LIP and LacZpDNA:LIP:CM amount/mutual relation;
- staining of transfected cells using β -Gal Staining Kit;
- microscopy with obtaining microphotographs;
- determination of transfection efficiency/cytotoxic effect.

Determination of the optimal **exposure duration**:

- PC3 and HEPG2 cells transfection with 2.5; 5; 10; 20 min exposure in the static and time-phase varied magnetic field using optimal LacZpDNA:LIP:CM;
- staining of transfected cells using β -Gal Staining Kit;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program, transfection efficiency calculations.

Determination of the optimal **magnets rotation frequency**:

- PC3 and HEPG2 cells transfection in the time-phase varied magnetic field with frequency of 5; 25; 50; 100 rpm using optimal LacZpDNA:LIP:CM;
- staining of transfected cells using β -Gal Staining Kit;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program, transfection efficiency calculations.

Determination of the optimal **magnetic field intensity**:

- PC3 cells transfection in the static and time-phase varied magnetic field with an optimal exposure duration and magnets rotation frequency using optimal LacZpDNA:LIP:CM;

- staining of transfected cells using β -Gal Staining Kit;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program, transfection efficiency calculations.

Determination of the efficiency of different transfection methods

Determination of **LacZpDNA:LIP:CM** delivery efficiency:

- PC3 and HEPG2 cells transfection with **L**, **LM** and **LM LFV** methods;
- staining of transfected cells using β -Gal Staining Kit;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program, transfection efficiency calculations.

Determination of **ECFP-ERp29pDNA:LIP:CM** delivery efficiency:

- PC3 and HEPG2 cells transfection with **L**, **LM** and **LM LFV** methods;
- the analysis of transfected cells using Western blot;
- densitometry analysis using *Image Reader LAS-1000* program.

Determination of **LacZpDNA:LIP:CM :siRNA** delivery efficiency:

- PC3 cells transfection with **L/L siRNA**, **LM/LM siRNA** and **LM LFV/LM LFV siRNA** methods;
- staining of transfected cells using β -Gal Staining Kit;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program, transfection efficiency calculations.

Determination of the cytotoxic effect

Determination of cytotoxicity caused by **LacZpDNA:LIP:CM** delivery:

- PC3 cells transfection **L**, **LM** and **LM LFV** methods;
- staining of transfected cells using AO/EB method;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program, dead cells percentage calculations.

Determination of the iron content in cells

Determination of **internalized Fe²⁺** by **cell counts**:

- PC3 cells magnetic labelling with CM in the static and time-phase varied magnetic field;
- staining of labelled cells using PB staining method;
- microscopy with obtaining microphotographs;
- cell counts using *ImageJ* program.

Determination of **internalized Fe²⁺** by **amount/cell**:

- PC3 cells magnetic labelling with CM in the static and time-phase varied magnetic field;
- spectrophotometric analysis of the labelled cells;
- calculations of the amount of internalized ferum.

3. RESULTS

3.1. Influence of the time-phase varied magnetic field on the sedimentation of SPION

Results obtained from computer modelling showed, that under the influence of magnetic field generated by the rotating magnet array the motion of SPION occurs both in axial and lateral direction, thus promoting more uniform distribution of SPION and forming characteristic pattern of sedimented SPION onto the surface. To verify experimentally whether there are differences in the sedimentation of SPION between the static and time-phase varied magnetic field, the CM sedimentation onto the surface of the cell culture plate well in typical environment of real transfection conditions, OptiMEM cell culture medium was performed (Figure 3.1).

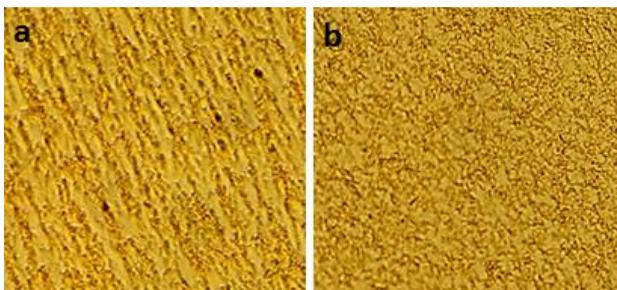


Figure 3.1. **Comparative analysis of the SPION sedimentation profile; the characteristic pattern of SPION dispersion in the static magnetic field (a) and time-phase varied magnetic field (b), $\times 400$**

In case when the sedimentation was carried out in the static magnetic field, SPION formed a pronounced line-like pattern (Figure 3.1 (a)). In the time-phase varied magnetic field the distribution of SPION was more uniform

(Figure 3.1 (b)), forming of pronounced shape structures wasn't observed. Obtained results support the assumption that axially lateral motion of SPION in solution caused by the time-phase varied magnetic field phase, leads to a more uniform SPION distribution onto the surface of the cell culture plate well.

3.2. Influence of the time-phase varied magnetic field on the gene delivery into the cancer cells

3.2.1. LM LFV – optimal reaction conditions

As the result of optimization it was found that the highest transfection efficiency rates with minimal cytotoxic effect can be achieved at the mutual relation of LacZpDNA:LIP:CM 1:2:1 (Figure 3.2). The liposomal magnetofection at the mutual relation of LacZpDNA:LIP:CM 1:1:0.5; 1:2:0.5; 1:1:1; 1:1:2 leads to lower efficiency. While at the mutual relation of LacZpDNA:LIP:CM 1:3:0.5; 1:3:1; 1:2:2; 1:3:2 we found the strong cytotoxic effect (a large amount of damaged cells).

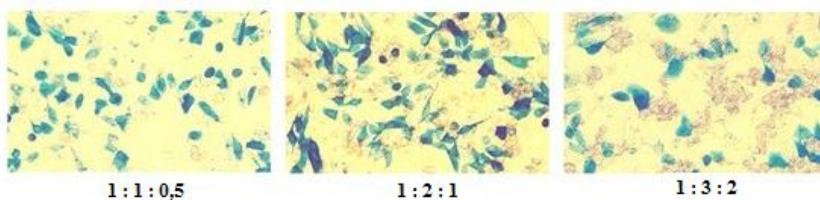


Figure 3.2. Variances of the transfection efficiency and cytotoxic effect depending on the mutual relation of LacZpDNA:LIP:CM (by the β -galactosidase expression in PC3 cells in the time-phase varied magnetic field), $\times 200$

The highest number of β -galactosidase expressing cells without the simultaneous cytotoxic effect in the static magnetic field both in PC3 and HEPG2 cells can be obtained at the mutual relation of LacZpDNA:LIP:CM 1:2:1. The same mutual relation of LacZpDNA:LIP:CM 1:2:1, respectively, allows to obtain the maximum number of β -galactosidase expressing cells without the simultaneous cytotoxic effect also in the time-phase varied magnetic field both in PC3 and HEPG2 cells.

The highest number of β -galactosidase expressing cells without the simultaneous cytotoxic effect both in PC3 and HEPG2 cells can be obtained at the mutual relation of LacZpDNA:LIP 1:2.

The maximum siRNA inhibition effect by the number of β -galactosidase expressing cells was achieved using 50 nM siRNA against the β -galactosidase.

Obtained data were further used for the detection of optimal parameters of the magnetic field – exposure duration, magnets rotation frequency and magnetic field intensity.

The highest number of β -galactosidase expressing PC3 and HEPG2 cells was observed at the 5 min exposure in the static magnetic field (Figure 3.3). With an increase of the exposure duration in the magnetic field, the number of β -galactosidase expressing PC3 and HEPG2 cells decreased, although results of the exposure of 5 min and 10 min are equivalent, especially in HEPG2 cells (Figure 3.3 (b)). Optimal exposure duration in the static magnetic field – 5 min was used in further experiments.

An analogous result was obtained by the determination of the optimal exposure duration in the time-phase varied magnetic field. The highest number of β -galactosidase expressing PC3 and HEPG2 cells was observed at the 5 min exposure in the time-phase varied magnetic field (Figure 3.4). Similar to that in case of static magnetic field with an increase of the exposure duration > 5 min the stable decrease of the transfection efficiency both in PC3 and HEPG2 cells was observed.

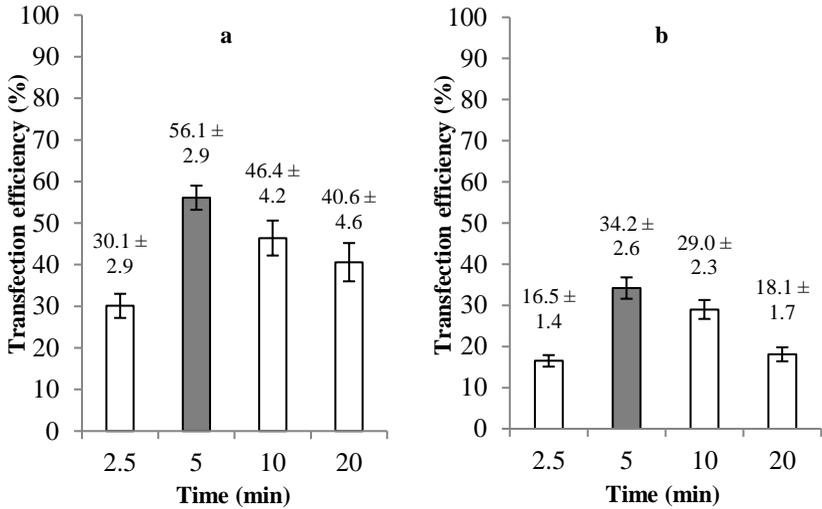


Figure 3.3. Transfection efficiency in PC3 cells (a) and HEPG2 cells (b) by the number of β -galactosidase expressing cells with variable exposure duration in the static magnetic field (n = 3)

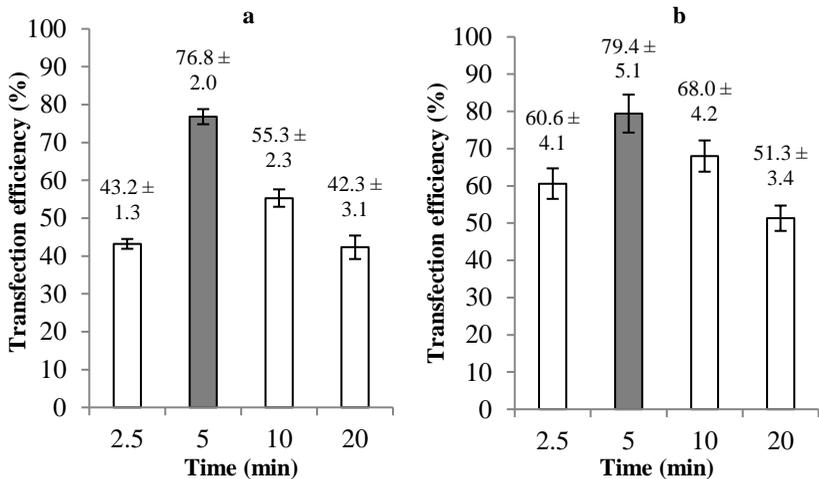


Figure 3.4. Transfection efficiency in PC3 cells (a) and HEPG2 cells (b) by the number of β -galactosidase expressing cells with variable exposure duration in the time-phase varied magnetic field (n = 3)

In further experimental work the effect of time-phase varied magnetic field characterizing parameters – magnets rotation frequency and magnetic field gradient on the LacZ gene expression was estimated.

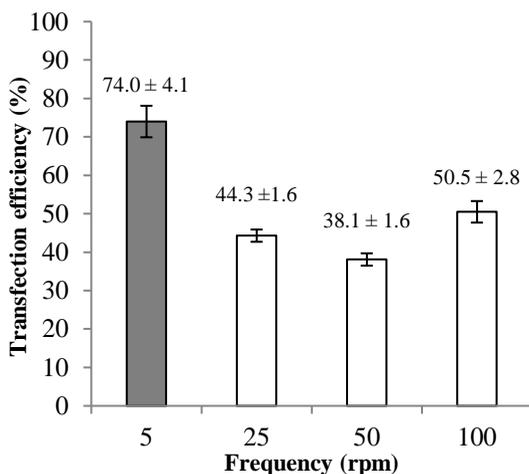


Figure 3.5. Transfection efficiency in PC3 cells by the number of β -galactosidase expressing cells with variable magnets rotation frequency at 5 min exposure duration in the time-phase varied magnetic field (n = 3)

When analysing the transfection efficiency by the number of β -galactosidase expressing PC3 cells at different magnets rotation frequencies of the rotational magnets array, it was found that the 5 min exposure with 5 rpm frequency in the time-phase varied magnetic field is the most effective (Figure 3.5). With an increase of the magnets rotation frequency (25 and 50 rpm) the rapid decrease in the transfection efficiency was observed, but at the 100 rpm the transfection efficiency tended to increase again.

The highest transfection efficiency rates in HEPG2 cells by the number of β -galactosidase expressing cells were observed at the 50 rpm magnets rotation frequency with the 5 min exposure in the magnetic field (Figure 3.6). Obvious,

that high transfection efficiency also persists at the 100 rpm magnets rotation frequency.

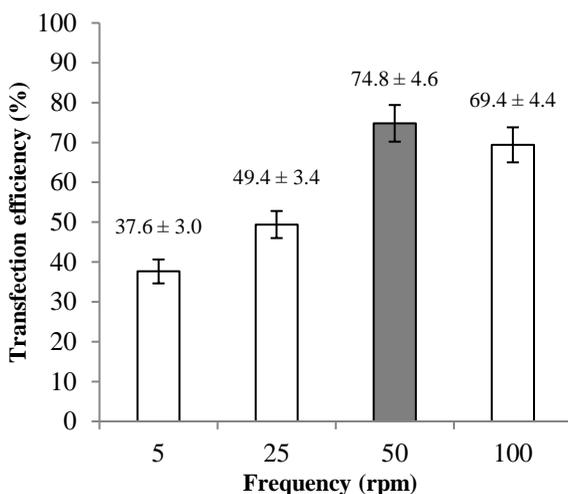


Figure 3.6. Transfection efficiency in HEPG2 cells by the number of β -galactosidase expressing cells with variable magnets rotation frequency at 5 min exposure duration in the time-phase varied magnetic field (n = 3)

When analysing the changes in transfection efficiency depending on the magnetic field intensity, the highest LacZ expression in PC3 cells both in the static and time-phase varied magnetic field was observed at the maximum magnetic field intensity used – 0.35 T (Figure 3.7).

With a decrease of the magnetic field intensity the stable decrease of the transfection efficiency in both cases was observed, though decrease of the transfection efficiency was more rapid in the static magnetic field (Figure 3.7. (a)) than in the time-phase varied magnetic field (Figure 3.7. (b)).

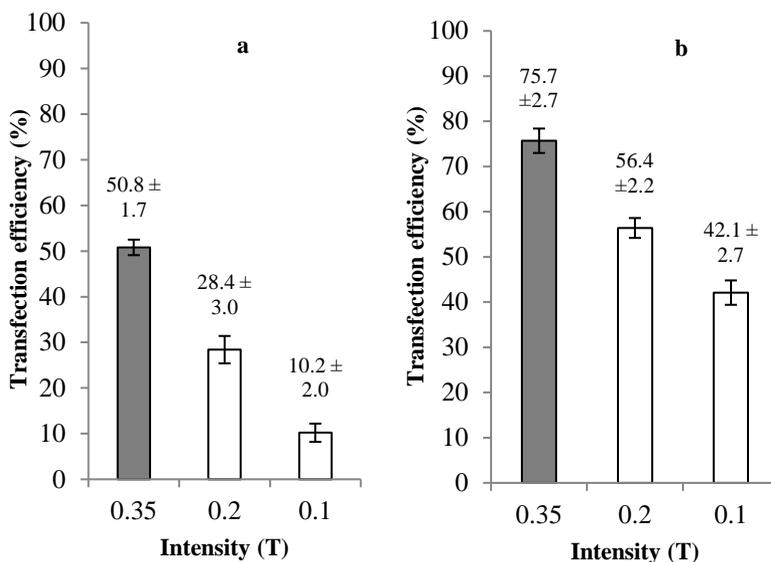


Figure 3.7. **Transfection efficiency in PC3 cells by the number of β -galactosidase expressing cells with an optimal exposure duration/frequency and variable magnetic field intensity in the static magnetic field (a) and time-phase varied magnetic field (b) (n = 3)**

Obtained rates by the number of β -galactosidase expressing cells, reflecting the optimal reaction conditions were used to evaluate the influence of the time-phase varied magnetic field on the expression of LacZ and ECFP-ERp29 genes in PC3 and HEPG2 cells in comparison with two widely used conventional transfection methods – liposomal magnetofection and lipofection.

3.2.2. Influence of the time-phase varied magnetic field on the nucleic acids delivery into the cancer cells

Figure 3.8. represents results obtained by the transfection of PC3 cells with pDNA containing LacZ gene using different gene delivery methods –

lipofection, when cells are not exposed to magnetic field, liposomal magnetofection in the static magnetic field and time-phase varied magnetic field in optimal reaction conditions.

The significant increase ($p < 0.001$) of LacZ expression in PC3 cells was found under the influence of LM LFV in comparison with other two methods – liposomal magnetofection and lipofection.

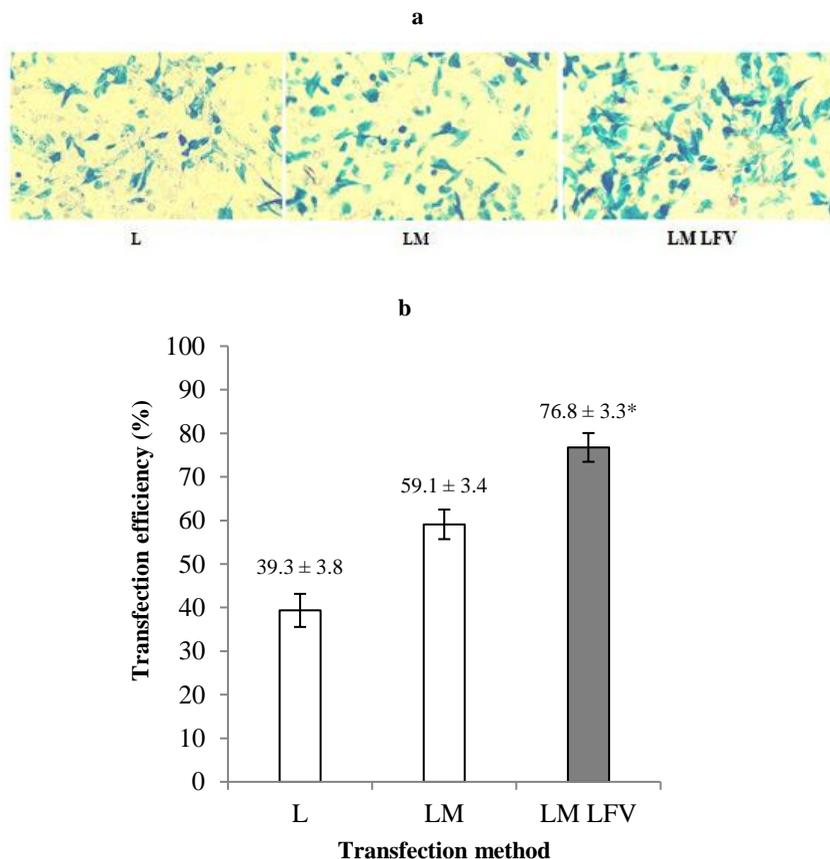


Figure 3.8. **LacZ gene delivery into PC3 cells using three different gene delivery methods: (a) β -galactosidase expression in PC3 cells ($\times 200$), (b) transfection efficiency by the number of β -galactosidase expressing cells ($n = 9$; * $p < 0.05$ compared with L and LM, Tukey test)**

With an application of time-phase varied magnetic field, the highest transfection efficiency by the number of β -galactosidase expressing cells can be obtained – 79.6% of transfected cells, which is for 21% more in comparison with LM and for 42% more in comparison with L. Furthermore, it was observed, that in stained PC3 cells transfected with LM LFV method, the characteristic colouring intensity is higher when compared with LM and L (Figure 3.8 (a)). It indirectly indicates that with an application of LM LFV not only a rise of the number of β -galactosidase expressing cells can be observed, but also an increase of the β -galactosidase expression level in transfected cells.

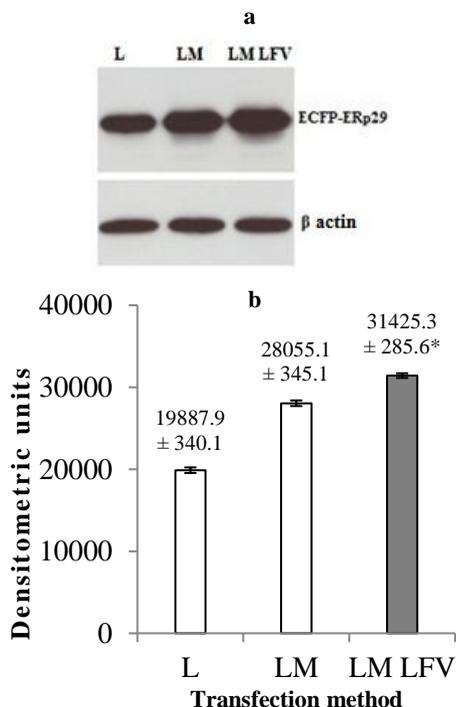


Figure 3.9. ECFP-ERp29 gene delivery into PC3 cells using three different gene delivery methods: (a) ECFP-ERp29 expression in PC3 cells (Western blot analysis), (b) transfection efficiency by the expression level of ECFP-ERp29 (n = 9; * p < 0.05 compared with L and LM, Tukey test)

Similar results were obtained by the analysis of the influence of time-phase varied magnetic field on the ECFP-ERp29pDNA delivery into the PC3 cells (Figure 3.9).

When analysing the transfection efficiency (in densitometric units), reflecting protein expression level, it was found, that by the use of LM LFV also the delivery efficiency of ECFP-ERp29 gene into PC3 cells can be significantly elevated ($p < 0.001$) in comparison with other two methods – L and LM. In case of LM LFV the total ECFP-ERp29 expression level is for 6% higher when compared with LM and for 22% higher in comparison with L.

Figure 3.10. represents results obtained by the transfection of HEPG2 cells with pDNA containing LacZ gene using different gene delivery methods – lipofection, when cells are not exposed to magnetic field, liposomal magnetofection in the static magnetic field and time-phase varied magnetic field in optimal reaction conditions.

The significant increase ($p < 0.001$) of LacZ gene expression also in HEPG2 cells was found under the influence of LM LFV in comparison with other two methods – liposomal magnetofection and lipofection.

With an application of the time-phase varied magnetic field the highest transfection efficiency by the number of β -galactosidase expressing cells can be obtained – 87.7% of transfected cells, which is for 51% more when compared with LM and for 56% more when compared with L. As visible in Figure 3.10 (a) also in stained HEPG2 cells transfected with LM LFV method, the characteristic colouring intensity is higher when compared with LM and L.

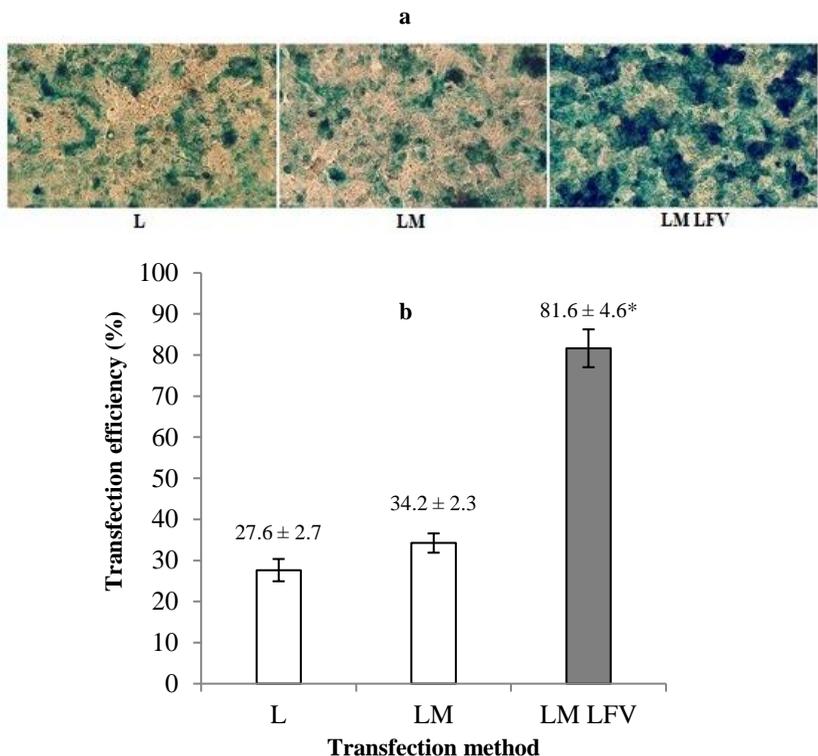


Figure 3.10. **LacZ** gene delivery into HEPG2 cells using three different gene delivery methods. (a) β -galactosidase expression in HEPG2 cells ($\times 200$); (b) transfection efficiency by the number of β -galactosidase expressing cells ($n = 9$; * $p < 0.05$ compared with L and LM, Tukey test)

Data reflecting ECFP-ERp29 expression in HEPG2 cells are represented in Figure 3.11.

It was found, that by the use of LM L FV the delivery efficiency of ECFP-ERp29 gene into HEPG2 cells can be significantly elevated ($p < 0.001$) in comparison with other two methods – L and LM. The total ECFP-ERp29 expression level growth under the influence of LM L FV is for 9% higher in comparison with LM and for 15% higher in comparison with L.

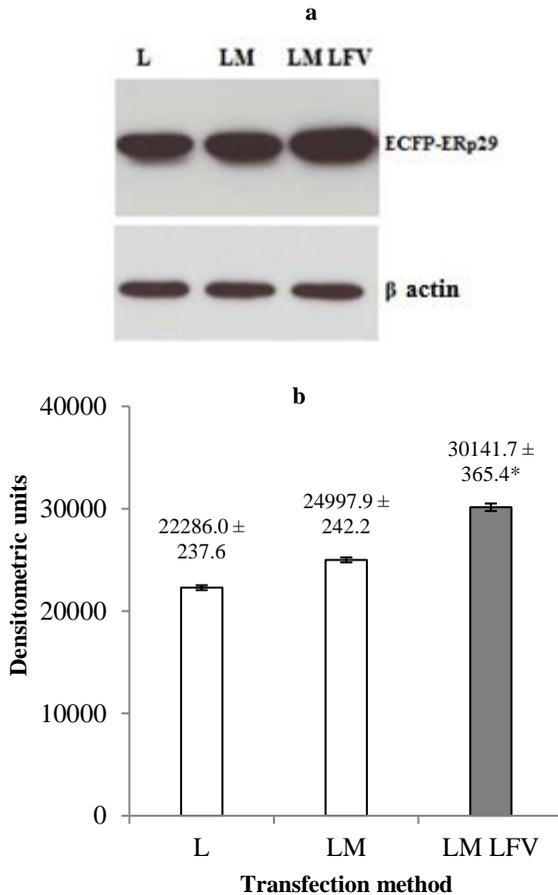


Figure 3.11. **ECFP-ERp29 gene delivery into HEPG2 cells using three different gene delivery methods. (a) ECFP-ERp29 expression in HEPG2 cells (Western blot analysis), (b) transfection efficiency by the expression level of ECFP-ERp29 (n = 9; * p < 0.05 compared with L and LM, Tukey test)**

Obtained results indicate, that among the three different gene delivery methods, the use of LM LFV leads to the highest number of reporter gene expressing cells, as well the highest total protein level in transfected cells.

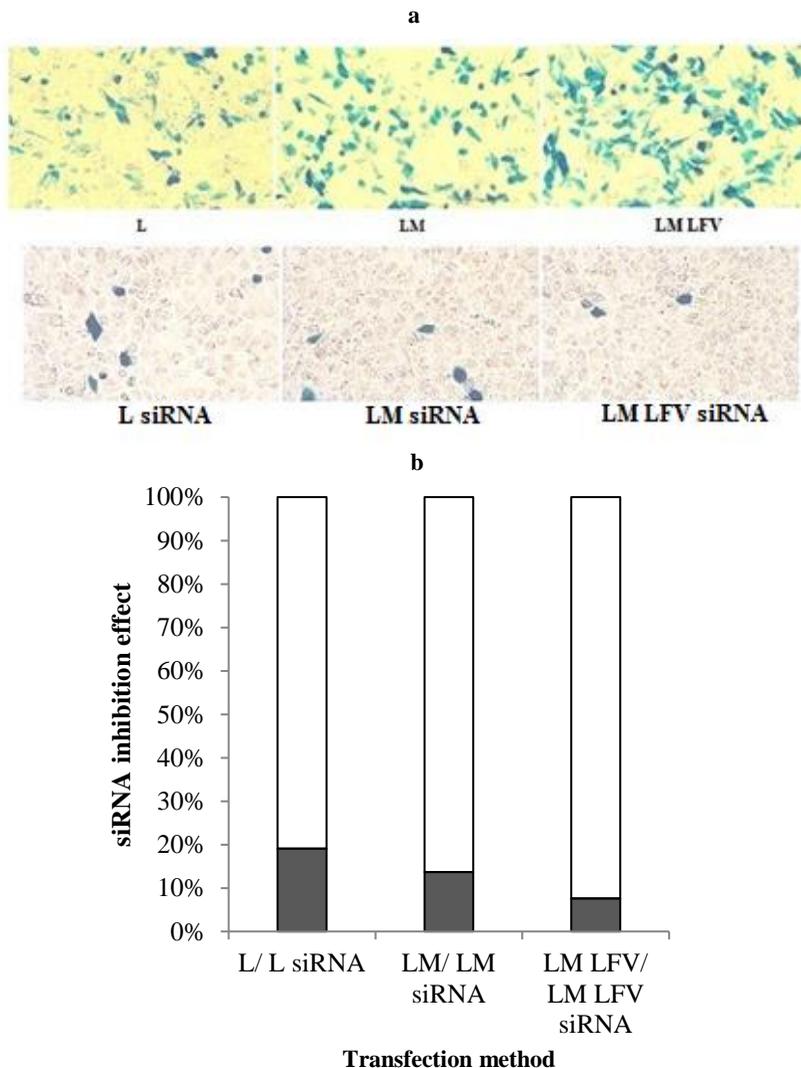


Figure 3.12. LacZ gene and siRNA against the β -galactosidase delivery into PC3 cells using three different gene delivery methods: (a) LacZ gene expression in PC3 cells ($\times 200$), (b) transfection with LacZ (white) and co-transfection with LacZ and siRNA against β -galactosidase (gray) ($n = 9$; $p < 0.05$ compared with L/L siRNA un LM/LM siRNA, Tukey test)

In series of co-transfection experiments the inhibition effect of siRNA to the expression of β -galactosidase in PC3 cells using different gene delivery methods was estimated. Results are represented in Figure 3.12.

The significant siRNA inhibition effect ($p < 0.001$) on the β -galactosidase expression in PC3 cells was achieved using all three gene delivery methods (Figure. 3.12 (a)). However, when assessing the siRNA inhibition effect against the transfection without siRNA – L, LM and LM LFV, respectively, it was found, that under the influence of LM LFV the highest β -galactosidase expression inhibition effect was achieved – 92.4% β -galactosidase non-expressing cells, respectively, which is significantly more ($p < 0.001$) when compared to LM (86.3%) and L (80.9%).

3.2.3. Cytotoxic effect of the use of the time-phase varied magnetic field

Parameters characterizing the viability of PC3 cells transfected with LacZpDNA are represented in Figure 3.13.

Visually the highest cytotoxic effect was observed in PC3 cells samples transfected using LM (Figure 3.13 (a)). At the result of calculations, it was found, that under the influence of LM LFV the number of apoptotic and dead cells in samples significantly decreases when compared to other two methods – LM and L (Figure 3.13 (b)). The percentage of live PC3 cells under the influence of LM LFV achieved 94%, while in the case of LM – 82% and L – 90% ($p < 0.001$).

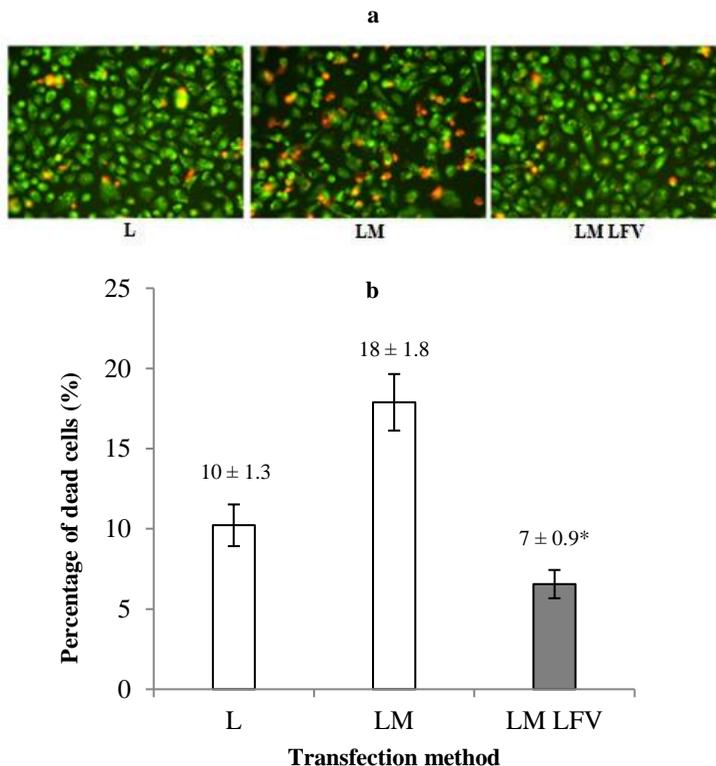


Figure 3.13. **The cytotoxic effect of the use of different gene delivery methods: (a) stained (AO/EB method) PC3 cells transfected with three different gene delivery methods ($\times 200$), (b) cytotoxicity ($n = 9$; * $p < 0.05$ compared with Land LM, Tukey test)**

3.3. Influence of the time-phase varied magnetic field to the SPION delivery efficiency into the cancer cells

To confirm results obtained in previous series of experiments, that the increase of the number of β -galactosidase expressing cells and total ECFP-ERp29 expression level under the influence of time-phase varied magnetic field is induced by the increased amount of SPION and

SPION-nucleic acids-liposomal component complexes sedimented and internalized into PC3 cells, the qualitative analysis of internalized SPION was performed. By performing a magnetic labelling of cells with 10 μg SPION/cell culture plate well and using PB staining, the presence of SPION in the cytoplasm of magnetically labelled cells was clearly approved.

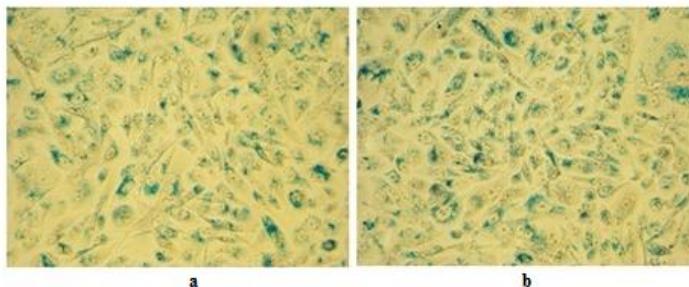


Figure 3.14. Magnetically labelled stained PC3 cells (PB method) ($\times 200$): (a) – magnetic labelling performed in the static magnetic field, (b) – magnetic labelling performed in the time-phase varied magnetic field; the blue colouring of cells indicates the presence of internalized SPION

As seen in Figure 3.14 the magnetic labelling efficiency of PC3 cells after the exposure in both static magnetic field (Figure 3.14 (a)) and time-phase varied magnetic field (Figure 3.14 (b)) was close to 100%. Visually, between both methods used for labelling, the difference in the colouring intensity was observed, thus indirectly indicating about the difference in the amount of internalized iron.

To confirm results obtained by the PB staining, the amount of internalized SPION was quantitatively detected, by performing the spectrophotometric analysis of the iron content of magnetically labelled cells.

It was found that the amount of an internalized iron in PC3 cells under the influence of time-phase varied magnetic field significantly increases – twice when compared to the static magnetic field (Figure 3.15). The obtained results confirm the connection between the internalized SPION and SPION-nucleic

acids complexes, respectively with increased gene expression in cancer cells under the influence of the time-phase varied magnetic field.

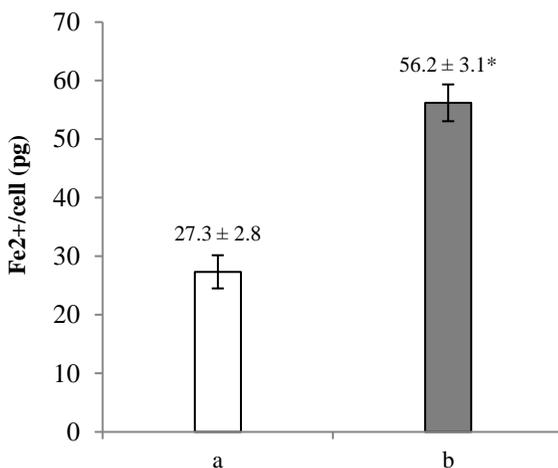


Figure 3.15. The amount of iron in magnetically labelled PC3 cells: (a) – magnetic labelling performed in the static magnetic field, (b) – magnetic labelling performed in the time-phase varied magnetic field (n = 9; * p < 0.05 compared to a, t-test)

In this study it was demonstrated experimentally, that under the influence of time-phase varied magnetic field more uniform SPION distribution onto the surface of the cell culture plate well can be achieved compared to SPION distribution onto the surface in the static magnetic field. Under the influence of time-phase varied magnetic field the amount of internalized SPION, as well as both the number of transfected cells and total level of protein expressed in transfected cell increases. Under the influence of time-phase varied magnetic field the cytotoxic effect caused by the gene delivery decreases.

DISCUSSION

The effectiveness of the gene therapy is characterized by the delivery of adequate dose of a therapeutic gene to the target cells without causing significant cytotoxic effect. Magnetofection is one of the most efficient gene delivery methods based on the SPION coupled nucleic acids delivery to the cells with an assistance of external static magnetic field. Magnetic field allows a rapid concentration of nucleic acids onto the surface of cells thus increasing the amount of nucleic acids transferred into the cell.

Literature data analysis showed that the application of a variety of alternative (non-statistic) magnetic fields improves the magnetofection efficiency. It is believed that the complex influence of the magnetic field causes oscillations of SPION-nucleic acids complexes in different directions thus stimulating their transfer through the cell membrane, but the mechanism of this phenomenon has not been completely clarified. Our interdisciplinary group has described the new – time-phase varied magnetic field, which is based on the orbital rotation of permanent magnets in the plane parallel to the cell culture plate. Results obtained from the computer modelling showed that, under the influence of the time-phase varied magnetic field the sedimentation of SPION occurs alternately moving them in axial and lateral directions resulting in the uniform SPION distribution onto the surface.

In the Thesis it is practically proved that the use of the time-phase varied magnetic field can significantly increase the efficiency of nucleic acids delivery into the cancer cells.

To confirm the results of the computer modelling, first the distribution of sedimented SPION in the static and time-phase varied magnetic field was mutually compared. Obtained results showed the sharp difference in distribution of SPION onto the surface of the cell culture plate well – in the static magnetic

field SPION formed pronounced line-like pattern with relatively large areas without SPION located between those line-like structures, while in case of LM LFV the distribution of SPION was more uniform and areas without SPION wasn't practically observed. Such SPION sedimentation profile could be explained by the specific magnets rotation program of the magnetofection device DynaFECTOR, that provides a continuous variability of the magnetic field forces associated with the three different phases (magnets position below the well) in the magnets rotation process. Obtained results could indicate, that during the orbital motion of magnets the continuous displacement of SPION in the axially lateral direction occurs, thus providing a stepwise sedimentation "step by step" so, probably, inhibiting the formation of linear chain-like structures. Diverse effect of different magnetic fields on the motion of magnetic nanoparticles in the solution was also observed in other studies. In the study of Gravel's group significant differences in the magnetic nanoparticles displacement were observed after the exposure in the magnetic field generated by the tubular bipolar three-phase electromagnet. In the *rotating* magnetic field magnetic nanoparticles formed round shaped swirl structures, but in case of *oscillating* and *static* magnetic fields – linear structures, which corresponds with the observed in this study regarding the static magnetic field.

If during the process of SPION sedimentation in the time-phase varied magnetic field displacement of particles occurs both in the axial and lateral direction, it is expected that also the motion of SPION-nucleic acids-liposomal component complexes will take place not only in an axial, but also in lateral direction. That could possibly facilitate the internalization of these complexes into cells, resulting in increased transfection efficiency, respectively.

The optimal mutual relation of pDNA-LIP-SPION for magnetofection in the static and time-phase varied magnetic field for both cells lines was identical: 1:2:1. At the mutual relation $x < \text{pDNA-LIP-SPION}$ the decrease of the nucleic

acids delivery efficiency was observed, while at the mutual relation $x > \text{pDNA-LIP-SPION}$ the cytotoxic effect increased. Despite the identical mutual relation of pDNA-LIP-SPION , used for the magnetofection in the static and time-phase varied magnetic field, the transfection efficiency in case of time-phase varied magnetic field was higher, than that in the static magnetic field. This could indicate, that under the influence of time-phase varied magnetic field the amount of internalized nucleic acids increases and it is associated with the rotation induced lateral movement of $\text{SPION-nucleic acids-liposomal component complexes}$ as all other parameters (magnetic field intensity, duration of exposure) compared with the static magnetic field are identical.

Magnetofection efficiency varies depending on the duration of exposure to the magnetic field. It was observed that the most efficient LacZ gene delivery into the PC3 and HEPG2 cells (determined by the number of cells expressing β -galactosidase) occurs at 5 min exposure, both in the static and time-phase varied magnetic field. Shorter exposure leads to reduced gene delivery efficiency, indicating that the exposure duration of < 5 min is insufficient for concentration of $\text{SPION-nucleic acids-liposomal component complexes}$ onto the cell surface.

The results correspond with findings of the Huth group – in studies with HeLa cell line SPION concentration around the cell membrane was observed already after 5 min of exposure in the magnetic field. In our previous studies we detected the transgene expression in cancer cells transfected with magnetofection method already after 4 hours, compared with lipofection method – 8 hours, thus confirming findings of studies regarding magnetofection kinetics in comparison with other methods, such as lipofection (Mykhaylyk et al., 2009b, 2010).

Changes in magnetofection efficiency depending on of exposure to the magnetic field previously observed Kamau with co-authors. Comparing reporter gene expression in several cell lines after 5 and 20 minutes of exposure to the combined static/oscillating magnetic field, using two types of SPION , it was found 20 min exposure to a magnetic field to be more effective. Unlike, our

obtained results indicate that using only 5 min exposure in both static and time-phase varied magnetic field it is possible to achieve high transfection efficiency; however, differences in study design should be noted. In the study of Kamau's group the gene delivery into the Cos7 and HeLa cells were provided with magnetofection method using commercial *polyMAG* and PEI coated SPION (synthesized by authors). Any of these factors – SPION, as well as reporter gene coding plasmid DNA, selected cell line and finally the transfection method can affect the gene delivery efficiency.

The maximum short exposure duration could be a critical factor in clinical trials, where in order to achieve the desired effect it's necessary to use the high-intensity magnetic field. It is demonstrated that exposure to moderate intensity (0.5 to 2 T) magnetic field does not cause any side effects (Leszczynski, 2005), but exposure to high intensity (200 T) magnetic field can cause the degradation of DNA (Li and Chow, 2001). In this study, as the result of specific magnets displacement the moderate (0.35 T) magnetic field is generated; however, it is sufficient also for an effective use *in vivo* (Chertok et al., 2011).

Interesting results were obtained when assessing changes in magnetofection efficiency depending on the magnets rotation frequency – the most important characterizing parameter of the time-phase varied magnetic field. Computer modelling demonstrated that sedimentation of SPION onto the surface depends on the magnets rotation frequency – when increasing magnets rotation frequency ω to the specified value ($\omega = 40$) the sedimentation of SPION onto the surface becomes more uniform and does not change significantly with a further increase in frequency. Experimentally it was found that magnetofection efficiency varies depending on the magnets rotation frequency, but differently in different cell lines. The highest number of β -galactosidase expressing PC3 cells was obtained at 5 rpm with a 5 min exposure to the magnetic field, while the highest number of β -galactosidase expressing HEPG2 cells – with 5 min exposure to the magnetic field at the magnets rotation frequency of 50 rpm.

Differences between results obtained from computer modelling and experimental part of the study may indicate a role of the cell membrane in the internalisation outcome, however in order to determine it precisely, additional studies are needed. Experimental results could be explained primarily by different membrane properties of PC3 and HEPG2 cells, because it is known that the internalisation success of the SPION is directly dependent on the cell type (Kamau et al., 2006; Cromer Berman et al.; Schwarz et al., 2012). PC3 cells ($d = 23 \mu\text{m}$) are more than two times bigger than HEPG2 cells ($d = 10 \mu\text{m}$). With an increase of the speed of magnets rotation, the SPION-nucleic acids-liposomal component complexes motion in the lateral direction along the cell surface is speeded up, thus, possibly giving the additional stimulus to endocytosis processes, that could be a decisive factor for the smaller cells. The results also indicate that the rotating magnets system allows customizing the magnetofection protocol for the transfection of different cell types with an appropriate adjustment of magnets rotation frequency.

Data obtained by assessing the influence of magnetic field intensity changes on the β -galactosidase expression are logical, because it is known that with the increase in distance from the magnet, the magnetic field intensity decreases and this consecutively affects the efficiency. In the Fouriki study there were no statistically significant differences observed in transgene expression rates depending on the distance from the magnet – 3 mm (0.1 T) 4 (0.08 T) and 5 mm (0.06 T). In our study, the change in distance is much higher – 9 and 6 mm in relation to the control distance of 0 mm, which means the greater difference in magnetic intensities – 0.1 T and 0.2 T against the 0.35 T, respectively.

Magnetofection efficiency at 5 min exposure to the magnetic field with the magnets rotation frequency of 5 rpm in PC3 and 50 rpm in HEPG2 cells by the number of β -galactosidase expressing cells reaches 79.6% and 87.7%. These are very high rates of magnetofection efficiency, taking into account that both

PC3 and HEPG2 cell lines belong to hard to transfect cell lines with characteristic transfection efficiency range of 30 to 40%.

When analysing the efficiency of the gene delivery it is essential to clarify both in how many cells and how much in one/all cells the successful gene delivery have been occurred. From literature, in such kind of studies in order to reflect the efficiency of gene delivery, basically the quantitative luciferase activity detection assay is used; however, it's not possible to determine the number of transfected cells by this method. In this study both indicators were analysed.

Comparative data obtained by the transfection of cancer cells using three different methods showed that under the influence of the time-phase varied magnetic field the delivery efficiency of nucleic acids significantly increases:

a) Both by the number of reporter gene expressing cells – by 21% in comparison with LM and 42% compared to L in PC3 cells and by 51% in comparison with LM and 56% compared to L in HEPG2 cells;

b) And the total protein amount – by 6% in comparison with LM and 22% compared to L in PC3 cells and by 9% in comparison with LM and 15% compared to L in HEPG2 cells.

Significantly, that the improved efficiency of the gene delivery in case of LM LFV in comparison with the LM and L were observed in cases when the expression of the reporter gene did not exceed 50%, which confirms the prevalent effect of the time-phase magnetic field over the other transfection efficiency influencing factors.

Convincing results were obtained when testing the inhibition effect of siRNA on the expression of β -galactosidase using co-transfection method. When determining the inhibition effect of siRNA against the magnetofection efficiency (without siRNA) the significant advantage of LM LFV (92.4%) compared with both the LM (86.3%) and L (80.9%) was observed, indicating a potential of method for the use of therapeutic siRNA delivery into the cancer cells.

Compared to the both widely used conventional gene delivery methods (L and LM), the use of the novel gene delivery method (LM LFV) cause less cytotoxic effect, which has been demonstrated using the PC3 cell line. The observation regarding the motion of SPION during the sedimentation in time-phase varied magnetic field indicates, that as the result of magnets rotation SPION distributes evenly and the formation of large-sized structures in the solution and thus onto the cell membranes is prevented. This is likely to reduce the cell damage and thus associated with less cytotoxic effect compared with the LM. By contrast, observations relating to specific distribution of SPION under the influence of static magnetic field explain the high LM cytotoxicity rates. The cytotoxic effect of SPION under the influence of static magnetic field is widely analysed by Bae and co-authors. Authors demonstrated that under the influence of the moderate static magnetic field (mean 0.4 T) SPION is forming aggregates, which is then concentrated on the surface of NCTC 1469 (normal mouse hepatocytes cells) cells. Due to the concentration of such aggregates onto the surface of the cell, but not to the internalisation in cells the cell viability is significantly affected, depending on both the initial concentration of SPION and duration of exposure to a magnetic field.

The distribution of SPION is probably the prevalent factor regarding cytotoxicity rates in this study, taking into account that the SPION is relatively non-toxic and the use of them as components of various compounds by the literature data can even reduce the overall cytotoxicity (Leung et al., 2013).

Magnetic labelling with SPION and subsequent detection of the amount of Fe^{2+} in magnetically labelled cells confirmed that under the influence of the time-phase varied magnetic field more SPION are transferred into the cells. Thus, it is reasonable to assume that under the influence of time-phase varied magnetic field also more SPION-coupled nucleic acids are transferred into the cells.

Until now, in this type of studies, where the influence of different non-static magnetic fields has been analysed, an increased delivery of nucleic acids into the cells is associated only with the influence of lateral motion of SPION as the stimulating factor of endocytosis. Dobson's group (Fouriki et al., 2010) has been developed the oscillating magnets system *magnefect-nano*TM. It is based on the generation of 2 Hz lateral oscillations in 200 μm range by the NdFeB magnet system. With this system the SPION-complexes sedimentation onto the cell surface is provided followed by a lateral motion along the surface of the cell to stimulate the internalisation of SPION-complexes. Kamau with co-authors described the use of the dynamic field generator *Dynamic Marker* for the gene delivery into the cells. This system is based on the generation of 50 Hz magnetic field by electromagnets acting along the axis Z and an additional 0.75 Hz magnetic field acting along the axis X with an amplitude of 1.5 cm, thereby causing SPION-complexes variations in perpendicular and parallel direction against the cell culture plane with a corresponding amplitude of 50 and 0.75 Hz. These lateral oscillations together with a possible rolling movement on the cell surface stimulate the transfer of SPION-complexes through the cell membrane.

Results obtained from this study indicate that more likely the increased SPION-nucleic acids-liposomal component complexes internalization into the cells could occur in the result of the action of multiple factors. One of the key factors, which have been proven experimentally, is the even distribution of SPION onto the surface of the cell culture plate well in the time-phase varied magnetic field due to the axially lateral motion of SPION in the solution. In the static magnetic field many SPION-nucleic acids-liposomal component complexes are concentrated on the cell surface in a short period and can't be internalized into the cells simultaneously, which can lead to a formation of chainlike structures. Sedimentation "step by step" in the axially lateral direction in the time-phase varied magnetic field impedes the simultaneous SPION-nucleic

acids-liposomal component complexes sedimentation onto the cell surface and the formation of chainlike structures, respectively, resulting in smoothing internalization of them.

It cannot be excluded that also the lateral motion of SPION-nucleic acids-liposomal component complexes on the surface of cells promotes the internalization – in the result of motion more complexes interact with the cell surface – the cell membrane is more stimulated mechanically. Jenkins and co-authors observed that in neurospheres cultures exposed to oscillating lateral fluctuations, the plasma membrane of cells has more pronounced relief. In studies of other authors, it has been demonstrated that external mechanical force induced membrane stimulation promotes both the endocytosis and exocytosis (Apodaca, 2002) and the enhanced penetration of the cell is primary caused by mechanical stimulation of endocytosis processes (Fouriki et al., 2010). While this mechanical stimulation can be caused by the fluctuating SPION-nucleic acids complexes motion along the surface of the cell membrane under the influence of an alternating magnetic field (Lim et al., 2012) as well as the formation of temporary pores in the cell membrane due to the vibrations induced by the alternating magnetic field (Dahmani et al., 2013).

CONCLUSIONS

1. Differences on a distribution of SPION sedimented onto the surface in the static and time-phase magnetic field indicates a different motion of SPION in a solution under the influence of these magnetic fields. Probably, it is the axially lateral SPION motion, resulting from the action of time-phase magnetic field.

2. Magnets rotation frequency (rpm) is one of the most important characteristics of the time-phase varied magnetic field. Different optimal magnets rotation frequency for PC3 cells (5 rpm) and HEPG2 cells (50 rpm) at the same duration of exposure in the magnetic field indicates that the magnets rotation frequency plays an important role in increasing the efficiency of gene delivery.

3. Significant increase of the number of transfected PC3 and HEPG2 cells with the overall increase in the total level of protein expressed in transfected PC3 and HEPG2 cells as well as the relevant increase in the gene delivery inhibition effect and the decrease in the cytotoxic effect in PC3 cells confirm the positive effect of the time-phase varied magnetic field on the delivery of SPION-nucleic acids-liposomal component complexes into the cancer cells. Liposomal magnetofection in the time-phase varied magnetic field is more efficient nucleic acids delivery method for cancer cells than lipofection and liposomal magnetofection.

4. In general, by the analysis of the obtained results it can be concluded that under the influence of the time-phase varied magnetic field the axially lateral motion of SPION-nucleic acids-liposomal component complexes in

solution occurs. As a result → more uniform sedimentation of SPION onto the cell surface occurs → amount of internalized SPION increases → amount of internalized SPION-nucleic acids-liposomal component complexes increases → both the number of magnetofected cells and gene expression level in magnetofected cells increases → cytotoxicity decreases.

PUBLICATIONS

PUBLICATIONS

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ABSTRACTS

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PATENT

Kozlovs V., Karpovs A., Priedīte V., **Avotiņa D.**, Stradiņš P., Kalējs M., Kasjanovs V., Mironovs V. Telpiski sakārtotas šūnu struktūras izveidošanas paņēmiens no dzīvotspējīgām pieauguša cilvēka šūnām. LV-14595B, 20.02.2013.

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