LATVIJAS UNIVERSITĀTE
BIOLOĢIJAS FAKULTĀTE
MOLEKULĀRĀS BIOLOĢIJAS KATEDRA

DENDRIMĒRI KĀ LUCIFERĀZI SATUROŠAS PLAZMĪDAS TRANSFEKCIJAS VEKTORI, *IN VITRO* UN *IN VIVO* PĒTĪJUMI

Maģistra darbs

Autors: Anita Bērziņa
Stud. apl. nr. ab16155
Darba vadītāji: Dr. hab. Maksim Ionov; PhD Mārtiņš Kālis
Konsultants: Dr. Maria Isaguliants
Recenzents: Dr. med. Linda Gailīte

RĪGA 2018

UNIVERSITY OF LATVIA
FACULTY OF BIOLOGY
DEPARTMENT OF MOLECULAR BIOLOGY

DENDRIMERS AS TRANSFECTANTS FOR LUCIFERASE-ENCODING PLASMID, *IN VITRO* AND *IN VIVO* STUDIES

Master's thesis

Author: Anita Bērziņa
Stud. ID nr. ab16155
Supervisors: Dr. hab. Maksim Ionov; PhD Mārtiņš Kālis
Consultant: Dr. Maria Isaguliants
Reviewer: Dr. med. Linda Gailīte

RIGA 2018

Table of contents

Abbreviations

AE2 G4	piperidine terminated generation four phosphorous dendrimer
CBD-CS	cationic carbosilane dendrimer
CD	circular dichroism
CPD G4	generation four cationic phosphorous dendrimer
DLS	dynamic light scattering
DLVO theory	Derjaguin, Landau, Verwey and Overbeek theory
DOTMA	(N-[1-(2,3,-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride)
EB	ethidium bromide
EPR effect	enhanced permeability and retention effect
G1, G2 … G_n	dendrimer generations
HUVEC cell line	human umbilical vein endothelial cell line
IM injections	intramuscular injections
PALS	phase analysis light scattering;
PAMAM G4	generation four poly(amidoamine) dendrimer
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pDNA	plasmid DNA
PEG	polyethylene glycol
pVax-Luc	pVax1 plasmid encoding luciferase reporter gene
ROS	reactive oxygen species
w/w	weight/weight ratio

Summary

Gene therapy is a powerful method for treating various genetic diseases and DNA vaccine development, yet, lack of safe and efficient DNA delivery methods still remains the limiting obstacle to human gene therapy. Various synthetic gene delivery agents that are safer than recombinant viruses have been developed, however, they do not possess the required efficacy.

Dendrimers are type of synthetic delivery vehicles that exhibit several advantages over other delivery agents, and these nanoparticles have been investigated over past few years for *in vitro* and *in vivo* genetic material delivery. In this study, four different dendrimers – generation four poly(amidoamine) dendrimer PAMAM G4, generation four cationic phosphorous dendrimer CPD G4, generation four cationic phosphorous dendrimer AE2 G4 and generation two cationic carbosilane dendrimer CBD-CS G2, were studied to characterize their ability to interact with plasmid DNA (pDNA) and form dendrimer-pDNA complexes. Furthermore, *in vitro* transfection efficiency and cytotoxicity experiments were performed. Also, the ability of two dendrimers – CPD G4 and CBD-CS G2 – to introduce DNA *in vivo* in murine animal model was examined using bioluminescence imaging.

The results of the biophysical characterization studies indicate that all four dendrimers efficiently bind pDNA and form stable complexes. Dendrimer CBD-CS G2 was the most efficient for cell transfection, and no significant cytotoxicity was observed. *In vivo* bioluminescence studies showed that CBD-CS G2 dendrimer is not an efficient genetic material delivery vehicle. In contrast, mice treated with pDNA complexed to increasing concentration of CPD G4 dendrimer, displayed bioluminescence in concentration dependent manner, and the highest photon flux was obtained in mice treated with 50 μg of CPD G4. These results indicate that phosphorous dendrimer CPD G4 is the most suitable one of the four investigated dendrimers for *in vivo* gene delivery, however, increase of transfection efficacy requires further optimization of DNA-dendrimer formulations.

Key words: gene delivery, transfection, electroporation, dendrimers, bioluminescence imaging, nanoparticles.

Kopsavilkums

Gēnu terapija ir nozīmīga dažādu ģenētisku slimību ārstēšanai, kā arī DNS vakcīnu attīstībai, taču vēljoprojām limitējošais faktors gēnu terapijas pielietošanai klīnikā ir drošas un efektīvas metodes trūkums, lai DNS ievadītu šūnās. Ir izveidoti dažādi sintētiski vektori, kas ir drošāki par rekombinanto vīrusu metodi, taču tie nav pietiekami efektīvi.

Dendrimēri ir viens no sintētiskajiem DNS ievades vektoru veidiem, kam piemīt vairākas priekšrocības salīdzinot ar citām metodēm, un šīs nanodaļiņas pēdējo gadu laikā pastiprināti pētītas gēnu ievadei *in vitro* un *in vivo*. Šajā pētījumā tika pārbaudīta četru dažādu dendrimēru – ceturtās paaudzes poli(amidoamīna) PAMAM G4, ceturtās paaudzes katjonu fosfora CPD G4, ceturtās paaudzes katjonu fosfora AE2 G4 un otrās paaudzes katjonu oglekļa-silīcija CBD-CS G2 – spēja saistīties ar plazmīdas DNS (pDNS), lai veidotu dendrimēru-pDNS kompleksus. Papildus tika veikti *in vitro* transfekcijas efektivitātes un citotoksicitātes pētījumi. Ar bioluminescences metodi tika pētīta arī divu dendrimēru – CPD G4 un CBD-CS G2 – spēja nogādāt DNS šūnās *in vivo* peļu modelī.

No biofizikālās raksturošanas pētījumiem var secināt, ka visi četri dendrimēri spēj saistīt pDNS un veidot stabilus kompleksus. Dendrimērs CBD-CS G2 bija visefektīvākais šūnu transfekcijai, un būtiska citotoksicitāte netika novērota. *In vivo* pētījumos tika noskaidrots, ka CBS-CS G2 dendrimērs nespēj efektīvi nogādāt DNS šūnās. Pretēji tam, tika novērots, ka veidojot plazmīdas DNS kompleksus ar pieaugošas koncentrācijas CPD G4 denrimēru, bioluminescence pieaug paaugstinoties koncentrācijai. Vislielākā fotonu plūsma tika novērota pelēs, kam tika injicēti kompleksi ar 50 µg CPD G4 dendrimēru. No šiem rezultātiem var secināt, ka CPD G4 dendrimērs no visiem četriem pētītajiem dendrimēriem, ir vispiemērotākais *in vivo* DNS ievadei šūnās. Transfekcijas efektivitātes uzlabošanai nepieciešams optimizēt dendrimēru-DNS kompleksus.

Atslēgas vārdi: gēnu ievade šūnās, transfekcija, elektroporācija, dendrimēri, bioluminescence, nanodaļiņas.

Introduction

Gene therapy applies to diseases that are caused by defect of specific gene. It is based on disease treatment by transfer of necessary genetic material into cells of a patient instead of using conventional drugs (Mulligan, 1993). It is required for gene therapy to identify a specific gene and effectively transfer it to certain cells and tissue. Temporary expression of inserted gene is sufficient for some applications like cancer therapy, however for most genetic diseases long-term gene expression is necessary (Verma and Somia, 1997).

After systemic administration of naked DNA or RNA, these molecules in biological fluids are rapidly degraded. Furthermore, delivery of naked DNA or RNA is highly inefficient because of the large size and negative charge of these molecules that cause electrostatical repulsion from negatively charged cell membranes (Mintzer and Simanek, 2008). Additionally, it is difficult to target the delivery to specific tissue and penetrate cell membrane (Cullis and Hope, 2017), thus a delivery method is needed. The major obstacle for gene therapy implementation in clinical setting is safety and efficiency of delivery vectors (Whitehead et al., 2009).

For genetic material delivery recombinant viruses are widely used. This type of delivery is efficient *in vivo*, however viral particles are not entirely safe to use - they may cause immune response (Bessis et al., 2004), insertional mutagenesis and oncogenesis (Naldini, 2015). A variety of synthetic delivery vehicles has been developed, however they have been reported to have low gene-transfer efficiency and they can be toxic, furthermore these vectors in some cases are not stable *in vivo* (Pack et al., 2005).

Dendrimers are nanosized synthetic polymers formed by layers of monomeric branched molecules diverging on all sides from a central molecule (Tomalia et al., 1990). Dendrimers interact with the negatively charged backbone of DNA, form stable complexes and can be used for gene delivery *in vitro* and *in vivo* (Eichman, 2000). Compared to other non-viral DNA delivery vectors, dendrimers exhibit several advantages, thus these nanoparticles are promising gene delivery vehicles.

The aim of the study: To examine which of four dendrimers – PAMAM G4, AE2 G4, CPD G4 or CBD-CS G2, is the most efficient for *in vitro* and *in vivo* transfection with plasmid DNA.

Tasks:

1. To perform biophysical characterization of dendrimers and their binding to plasmid DNA on example of plasmid encoding luciferase reporter pVax-Luc.
2. To evaluate transfection efficiency and assess cytotoxicity of dendrimers and their complexes with plasmid DNA *in vitro.*
3. To evaluate the efficacy of transfection with plasmid DNA/dendrimer complexes *in vivo* in mice.

This study was conducted in University of Lodz, Department of General Biophysics, Lodz, Poland and A. Kirchenstein Institute of Microbiology and Virology, Riga, Latvia from May 2017 to May 2018 and Karolinska Institutet, Department of Microbiology, Tumor and Cell biology, Stockholm, Sweden from June 2017 to July 2017. The supervisors were dr. habil. Maksim Ionov and PhD Mārtiņš Kālis, for *in vivo* part – Dr. Maria Isaguliants.

Results of this study were presented in international conference "VACCINES & VACCINATION" in Moscow, September 27 – October 1, 2017 as oral presentation - "Dendrimers in *in vitro* and *in vivo* DNA transfection".

This work was financially supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No. 692293 (VACTRAIN), and Project Initiation grant of the Swedish Institute "INNVOIMMUNE" nr. 19806_2016.

1. LITERATURE REVIEW

1.1 Gene therapy

The idea of using genes as treatment for human diseases evolved during late 1960s and early 1970s (Friedmann, 1992). Since then there has been increase in knowledge about gene functions and continuous advancement of DNA delivery techniques into mammalian cells (Couzin-Frankel, 2009 in Giacca and Zacchigna, 2012). By delivering exogenous DNA or RNA into living organisms, disease prevention can be achieved through replacing a mutated gene with a normal copy, introducing protein-coding cDNAs to change cell behavior (Giacca, 2010 in Giacca and Zacchigna, 2012). Another approach is to apply small nucleic acids (DNAs or RNAs) which possess regulatory functions (Giacca and Zacchigna, 2012).

Genetic material for treatment can also be used in DNA vaccines. The principle of DNA vaccination is delivery of plasmids containing genes that encode immunogenic antigens. The aim of DNA vaccines is immune response induction (Silveira et al., 2017). Conventional vaccines against many diseases, such as HIV, malaria, tuberculosis, and others, are ineffective or unavailable, thus DNA vaccines are appealing for treatment. Advantages of DNA vaccines include their ability to mimic live attenuated vaccines, inducing both humoral and cellular immune responses, while reducing safety concerns associated with live vaccines. DNA vaccines are relatively cost-effective, easy to produce and store (Gurunathan et al., 2000), furthermore it is possible to encode several antigens in one vaccine (Silveira et al., 2017).

The main complication with DNA vaccines is that they produce in human trials only modest immune responses. The immunogenicity is thought to be low because of low antigen/body mass ratio in humans when compared to preclinical models, which are less massive. Antigen expression can be promoted by improvement of transfection efficiency and optimization of plasmid vectors. While these methods have yielded significant improvements in preclinical models, the immunogenicity in humans is still low (Zahm et al., 2017). For example, electroporation of plasmid DNA has been reported to improve immunogenicity up to 1000-fold in mice. Translation of this method to human trials has resulted in modest 2-3-fold enhancement over injection of naked DNA (Saade and Petrovsky, 2012). Taking this into consideration, there is still need for effective and safe DNA delivery agent.

1.2 DNA delivery methods

The effectiveness of gene transfer to cell nucleus largely depends on techniques and tools used for gene delivery (Giacca and Zacchigna, 2012). Delivery vector must overcome extracellular barriers, which include binding to DNA and condensing it and maintaining a stable complex in a solution. Delivery vector and DNA complexes also must overcome *in vivo* barriers – stability and survival of complexes in the blood stream and penetrating the surrounding tissue (Pack et al., 2005).

The ideal vector must not evoke an immune response and it must lead to continuous or regulated expression of its genetic cargo. The delivery should be specific to certain types of cells, especially if the target cells are dispersed throughout the body or belong to a heterogeneous population. Furthermore the ability of efficiently transducing both – dividing and non-dividing cells is highly desirable. The integration of the DNA into the chromosome should be site-specific or remain in episomal position, eliminating random integration. The vector should be capable to transport any genetic material disregarding its size, it should be easy to produce inexpensively at high titers (Somia and Verma, 2000).

Delivery vehicles are divided in three large groups and gene transfer can be achieved by biological, physical, or chemical means (Giacca and Zacchigna, 2012).

1.2.1 Viral DNA delivery methods

As a biological tool for DNA introduction, viral vectors are widely used. The therapeutic gene is encapsulated in genetically modified viral particles instead of the viral genome. Viral particles at the same time retain those parts from the viral genome, which are necessary for cell infection. Transduction with these viral vectors result in therapeutic gene introduction and expression in target cells (Kay et al., 2001). Most commonly for genetic material delivery gammaretroviruses, lentiviruses, adenoviruses, adeno-associated viruses, and herpesviruses are used (Giacca and Zacchigna, 2012). Viral vector delivery systems are appealing because of high transfection efficiency *in vivo,* however there are few drawbacks – viral vectors may cause immune response of the host (Bessis et al., 2004), there is limited size of genetic material that can be encapsulated in viral capsid, and they are difficult to engineer, and production of high-titer stocks is expensive (Templeton, 2002).

1.2.2 Non-viral DNA delivery methods

In both - physical and chemical methods - for gene transfer plasmid DNA (pDNA) is used. Ease of preparation and biochemical simplicity as well as stability and low immunogenicity make plasmids appealing as vectors for necessary gene delivery (Herweijer and Wolff, 2003; Wang et al., 2004). However, cellular uptake of pDNA *in vivo* is not effective (Nishikawa and Huang, 2001; Wang et al., 2004), and pDNA has been reported to degrade rapidly *in vivo* with a half-life of 10 minutes after intravenous injection in mice (Kawabata et al., 1995) - physical and chemical approaches are needed to improve it.

Upon physical method application, cell membrane is weakened to promote gene uptake into the cell. Physical methods include needle injections, gene gun, electroporation, and others (Ibraheem et al., 2014).

Genetic material can be introduced with needle injections. pDNA can be administered intramuscularly, however the rate of genetic material integration for stable expression is very low if happens at all (Wang et al., 2004). Another approach is microneedle method, which is suitable for delivery through skin and is simple to use (McCaffrey et al., 2015). It allows delivery of certain dose, control of spatial distribution (Kim et al., 2013 in Dul et al., 2017) and is minimally invasive (Kim et al., 2012). Despite the advantages of microneedle use for gene delivery, transfection efficiency is low and inconvenient for multiple overlapping cell layers (Mellott et al., 2012).

“Gene-gun” is a needle-free method which allows direct genetic material delivery into cell cytoplasm or nucleus by metal microparticles coated with pDNA as a projectile coming out of a pressurized ballistic device. Metal microcarriers are able to reach epidermis crossing stratum corneum (Udvardi et al., 1999 in Mellott et al., 2013). Target tissue can be damaged if cells are bombarded with high amount of microparticles, besides these particles can trigger inflammatory reaction. There is also a limitation for target tissue depth (Sohn et al., 2001) and amount of pDNA that can be precipitated on metal carrier. Moreover, it is challenging to distribute particles equally (Mellott et al., 2013).

In electroporation, pDNA delivery is followed by application of a series of electrical pulses (Gothelf et al., 2003 in Wells, 2004), which is believed to induce hydrophilic pore formation in cell membrane allowing pDNA to enter the cell (Wells, 2004). Pores in cell membrane after electrical pulse application remain for tens of seconds to few minutes, thus the process is reversible (Rems and Miklavčič, 2016). A major advantage to electroporation is high

transfection efficacy – compared to naked plasmid delivery, electroporation increases gene expression by 100- to 1000-fold (Wells, 2004). Electroporation can be applied to any type of cells at any stage of life cycle (Chávez et al., 2009 in Bolhassani et al., 2014), yet efficiency is determined by electrical properties of the cells. The threshold in heterogeneous tissue is variable for different cells - smaller cells require higher voltage pulses, furthermore cells with less conductive contents, such as adipocytes, are less susceptible (Hui, 2008 in Bolhassani et al., 2014). However, electrical field application sometimes can induce permanent membrane permeabilization, cell homeostasis imbalance leading to cell death (Rubinsky, 2007 in Bolhassani et al., 2014). This method also causes significant additional damage compared to other physical methods. Skin edema is frequent outcome when electrical field is applied through the skin because of major potential drop development across the surface instead of the targeted subcutaneous tissues (Hui, 2008 in Bolhassani et al., 2014). Electroporation has shown high transfection efficiency in mouse model, however, translation of this method to human is not effective (Saade and Petrovsky, 2012).

Chemical methods are based on complex formation between positively charged polymers, which can bind negatively charged phosphate groups of DNA. For complexation various chemicals are used: 2-(dieathylamino)ether (DEAE)-dextran, calcium phosphate, artificial lipids, dendrimers and others (Luo and Saltzman, 2000).

(DEAE)-dextran and calcium phosphate form complexes with DNA which are internalized into cells by endocytosis. Despite simplicity and effectiveness, cytotoxicity and difficulties with *in vivo* application are important obstacles for using these methods (Luo and Saltzman, 2000).

Major improvement for chemical gene delivery was synthesis of cationic lipids DOTMA in 1987 by Felgner et al. These lipids are able to spontaneously create liposomes, form liposome-DNA complexes via ionic interactions and fuse with plasma membrane of cells. Transfection efficacy was shown to be 6- to 11-fold higher than using (DEAE)-dextran. Since then various lipid-based systems have been created. Currently lipid-based systems are the leading method for genetic material delivery (Cullis and Hope, 2017). ‘Lipofection’ has been routinely used for both *in vitro* and *in vivo* gene delivery (Pack et al., 2005). Advantages of liposomes are their biodegradability and biocompatibility, because of natural occurring substances in liposome composition. Furthermore, liposomes can protect genetic material from enzymatic degradation, promote uptake in endosomes, deliver large pieces of DNA and are easy to handle and prepare

(Liu et al., 1995 in Jin et al., 2014). The major drawback for this method is that cationic lipids are toxic to cells (Li et al., 1998; Pack et al., 2005) and not effective enough for gene therapy (Li et al., 1998; Zhi et al., 2010 in Jin et al., 2014). It is difficult to reproduce fabricating liposomes and DNA-liposome complexes (Pack et al., 2005).

1.3 Dendrimers

Another approach for genetic material delivery into cells is using nanoparticles - dendrimers. Dendrimers are artificial polymers with regular, three-dimensional architecture. These nanoparticles are synthesized by adding layers of identical branched molecules to core molecule. Addition of each molecule layer leads to higher generation macromolecule formation (Tomalia et al., 1990; Bosman et al., 1999). Identical units, that remain when the core molecule of dendrimer is removed, are called dendrons (fig. 1.) (Lee et al., 2005).

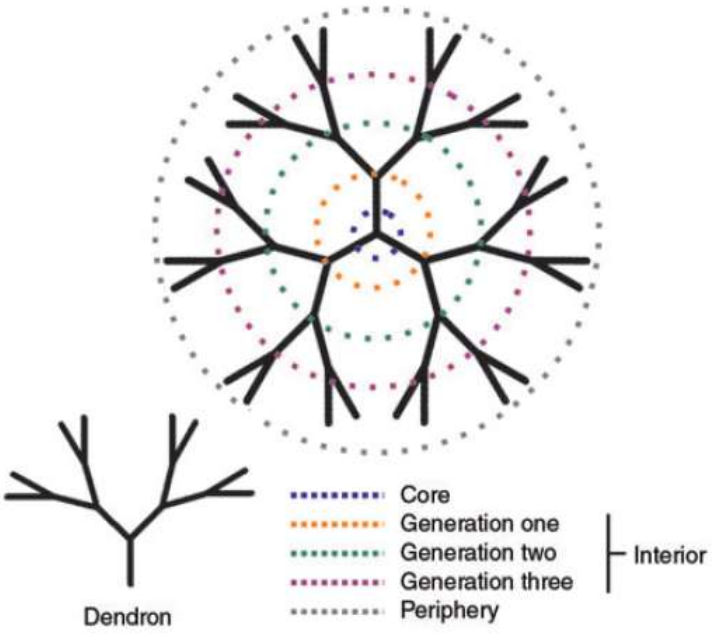


Figure 1. General structure of a dendrimer. Solid black lines represent dendrimer and dendron, coloured lines – dendrimer core (blue), generations (orange, green and violet) and terminal groups (grey) (Lee et al., 2005).

Dendrimers are able to interact with the DNA creating dendrimer-DNA complexes. First, positively charged dendrimers interact with negatively charged phosphate groups of the DNA. The overall charge of dendrimer-DNA complex is positive allowing electrostatical attachment to

negatively charged cell membrane (Eichman et al., 2000). After attachment to cell membrane, dendrimer-DNA complex is internalized by endocytosis (fig. 2) (Pack et al., 2005).

Endosomes then fuse with lysosomes which contain various enzymes that degrade DNA (Pack et al., 2005), however dendrimers can protect genetic material from degradation (Abdelhady et al., 2003; Fröhlich, 2012; Ionov et al., 2015; Ihnatsyeu-Kachan et al., 2017), sterically blocking access of nucleolytic enzymes. Naked pDNA is degraded within minutes, whereas pDNA complexed to polymers is stable for hours (Abdelhady et al., 2003).

Dendrimer-DNA complexes from endolysosomes in cell escape by "proton-sponge" mechanism (Haenser and Szoka, 1993 in Pack et al., 2005; Fröhlich, 2012). Dendrimers buffer H^+ ions and cause influx of Cl^- counter ions in the endolysosome. Increased amount of Cl^- ions in the vesicle eventually cause osmotic swelling and rupture of the membrane and dendrimer-DNA complexes are released into cytosol (Pack et al., 2005).

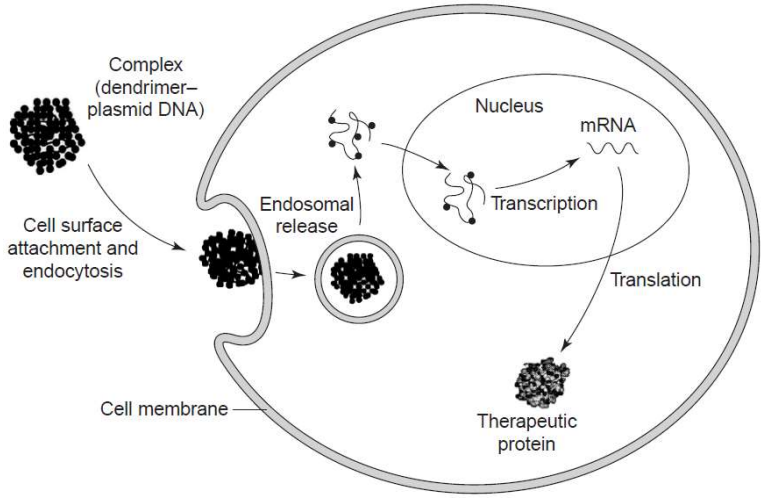


Figure 2. Dendrimer-mediated genetic material delivery into cells. First, positively charged dendrimers interact with negatively charged DNA and form a positively charged complex, which then interacts with negatively charged cell membrane and is internalized into cell by endocytosis. Complex escapes from the endosome by "proton-sponge" mechanism and is translocated to nucleus for transcription and translation (Eichman et al., 2000).

After endosomal escape, genetic material is translocated to nucleus for transcription and translation (Eichman et al., 2000). During this process, at some point vector must release the DNA (Pack et al., 2005). Therefore the polymer must balance sufficient binding strength to initially protect the DNA from degradation with the ability to release the genetic material. Release of genetic material is thought to occur via competitive binding of genomic DNA and

proteins in cell cytoplasm (Schaffer et al., 2000) or enzymatic cleavage of dendrimer (Fröhlich, 2012).

Mechanism of dendrimer-DNA complex transport through cytoplasm relies on movement along microtubules by complex nonspecific interaction with anionic microtubules or motor proteins (Suh et al., 2003). Complexes also could be distributed near the nucleus by the mixing that occurs during cell mitosis. However, mechanisms of complex movement within the cells are not fully understood and need to be characterized better (Pack et al., 2005). It has been reported that cellular localization of positively charged nanoparticles is also size dependent – particles 200 nm and smaller were shown to localize throughout the cell cytoplasm and over longer period of time accumulated in the perinuclear region of the cells, however particles with 500 nm size localized mostly in the periphery of the cells irrespective of the incubation time (Rejman et al., 2004).

Apart from electrostatical interaction with the DNA and the ability to protect genetic material from degradation in cells, dendrimers exhibit several more advantages – they are monodisperse, stable, of low viscosity (Wang et al., 2010 in Ionov et al., 2015) and do not cause significant cytotoxicity in concentrations <1 μM (Shcharbin et al., 2011; Albertazzi et al., 2013; Lazniewska et al., 2013; Dzmitruk et al., 2015). Cytotoxicity of dendrimers depends on the core molecule, but more importantly – dendrimer surface (Duncan and Izzo, 2005). General tendency of dendrimer cytotoxicity shows that toxicity increases along with dendrimer concentration and generation (Duncan and Izzo, 2005; Mukherjee et al., 2010; Dzmitruk et al., 2015).

Dendrimers generally do not have the ability to target specific cells, however various molecules and ligands can be attached to the terminal groups of these nanocarriers, allowing increased cell uptake as well as targeting cells of interest (Pack et al., 2005; Kesharwani et al., 2015; Ahmadzada et al., 2018).

Although dendrimers no not have the ability to actively target cells, it has been shown that nanoparticles can passively accumulate in solid tumor tissue. The so-called passive targeting is based on a phenomenon called enhanced permeability and retention (EPR) effect (Kesharwani et al., 2015). Tumor cells multiply at exponential rates rapidly creating a network of blood vessels. As a result blood vessels are abnormal and highly disorganized, and lymphatic flow from solid tumor tissue is defective (Dreher et al., 2006). In addition, tumor cells secrete increased amount of vascular permeability mediators that promote blood vessel dilation (Maeda et al., 2001). All of these abnormalities lead to extensive leakage of blood plasma components,

macromolecules and nanoparticles into the tumor interstitium (Iyer et al., 2006), and selective accumulation in solid tumor tissue. Thus, as a result of EPR effect, it is possible to attain high local concentration of nanoparticles, including dendrimers, in the tumor tissues with negligible accumulation in other tissues (Kesharwani et al., 2015).

First reports of dendrimer synthesis were published in 1978 by Vögtle et al., who described a procedure of continuous molecule combination to create a branched structure. In 1985 Tomalia et al. synthesized and fully characterized first class of monodisperse dendrimers with certain topology called starburst polymers or poly (amidoamine) (PAMAM) dendrimers. PAMAM were the first dendrimers that were commercialized (Esfand and Tomalia, 2001).

Dendrimers applied for DNA delivery in this study, namely poly(amidoamine) dendrimer of generation four PAMAM G4, cationic phosphorous dendrimer of generation four CPD G4, piperidine terminated phosphorous dendrimer of generation four AE2 G4 and cationic carbosilane dendrimer of generation two CBD-CS G2, are described below in sections 1.3.1 - 1.3.4, with representative structures illustrated in supplementary Figures 1 A, B, and 2 A, B.

1.3.1 Poly(amidoamine) dendrimers PAMAM

PAMAM dendrimers have an ethylenediamine core ($N_c = 4$) with branch cell multiplicity ($N_b = 2$) (Esfand and Tomalia, 2001). Dendrimer is built repetitively from the core adding methylacrylate and the resulting ester is amidated with ethylenediamine (Worner and Mulhaupt, 1993 in Dufes et al., 2005). PAMAM has positively charged terminal amine ($-NH_2$) groups (Tomalia et al., 1990).

PAMAM dendrimers are able to efficiently introduce genetic material in various types of cells *in vitro* (Kukowska-Latallo et al., 1996; Bielinska et al., 1996). Transfection efficiency can be enhanced fracturing dendrimer structure thus creating more flexible branches (Tang et al., 1996), adding positively charged amino acids, for example, L-arginine (Okuda et al., 2004; Choi et al., 2004; Kim et al., 2006) or polysaccharide α-cyclodextrin (Qin et al., 2016), or polyethylene glycol (Luo et al., 2002) and other compounds to terminal surface groups or within the structure of dendrimer.

In contrast with effective *in vitro* transfection, efficiency *in vivo* has been shown to be low with fractured PAMAM dendrimers (Rudolph et al., 2000). However arginine-terminated PAMAM G4 dendrimer has been reported to silence genes effectively in complexes with siRNA in *in vivo* prostate cancer murine models, which indicates successful gene delivery. Furthermore

arginine-decorated PAMAM G4 dendrimers were shown to deliver genes more effectively than PAMAM G4 dendrimer without arginine terminal groups (Liu et al., 2014).

It is observed that lower generation (G0-G3) dendrimers exhibit low transfection efficiency and minimal cytotoxicity, but transfection efficiency as well as cytotoxicity is higher with higher generation dendrimers (G4-G8) (Liu et al., 2012 in Jin et al., 2014). Cytotoxic action of PAMAM dendrimers is correlated with the number of positively charged terminal amino groups – higher numbers of cationic surface charges cause higher cytotoxicity (Naha et al., 2010). PAMAM dendrimers damage plasma membrane of cells – G5 expands already existing damage, G7 causes hole formation, while lower generation G3 dendrimer do not induce defects (Leroueil et al., 2008). PAMAM dendrimers are also associated with elevated ROS levels, lysosomal activity, DNA damage and apoptosis in a generation dependent manner (G4 < G5 < G6) (Mukherjee et al., 2010).

There are several methods for minimizing cytotoxicity – crosslinking PAMAM G2 dendrimers with disulfides has been shown to deliver genes more effectively than PAMAM G5 dendrimer with lower cytotoxic effects (Liu et al., 2012). It is also possible to reduce cell damage by modifying PAMAM terminal amine groups with hydroxyl groups to shield cationic charges that are associated with elevated cytotoxicity (Lee et al., 2003 in Duncan and Izzo, 2005).

1.3.2 Cationic phosphorous dendrimers CPD

Cationic phosphorous dendrimers CPD are characterized by amino thiophosphates in their backbone, phosphorous atoms at each branching point and amines as terminal groups (Solassol et al., 2004). CPD dendrimers have successfully been used for *in vitro* cell transfection (Loup et al., 1999; Maszewska et al., 2003; Shcharbin et al., 2011) and transfection efficiency has been reported to increase along with increasing generation of dendrimer (Loup et al., 1999; Shcharbin et al., 2011). Furthermore, phosphorous containing dendrimers have been reported to have enhanced transfection efficiency in the presence of serum proteins (Loup et al., 1999). Genetic material delivery *in vitro* has been reported to be higher than with PAMAM dendrimer, because of net positive charge on the CPD terminal amine groups, while in case of PAMAM there is no protonation of all amine groups which might reduce the interaction (Shcharbin et al., 2011).

CPD toxicity has been tested on HUVEC cell line and two cancer HEK-293 and HeLa cell lines and the cytotoxicity was lower than lipofectin (Maszewska et al., 2003). Results form similar study showed that CPD G4 dendrimer does not cause significant cytotoxicity to HEK-293

cells, however hMSC cells were more sensitive and cell viability decreased by 50%. (Shcharbin et al., 2011).

1.3.3 Cationic phosphorous dendrimers AE2

AE2 dendrimers have the same structure as CPD dendrimers, except for piperidine terminal groups. The amount of surface groups is the same as for CPD dendrimer, however, piperidine is more voluminous cation than amines on CPD surface. Piperidine terminal groups allow AE2 dendrimer to efficiently bind genetic material in lower molar concentration than CPD, thus reducing the amount of dendrimers for transfection. AE2 dendrimers have been shown to form stable complexes with genetic material and introduce fluorescein-labelled siRNA into cells. Transfection of HeLa cells *in vitro* was observed to be more effective than Lipofectamine 2000. These phosphorous dendrimers also were able to protect genetic material from RNase A. Genetic material release from dendrimer-siRNA complexes was shown to be only partial. Additionally, these dendrimers were non-toxic to HeLa cells in concentrations <1 μM, however cytotoxicity increased along with increasing concentration of dendrimers. AE2 G3 dendrimer was shown to be more toxic than AE2 G4 dendrimer due to more flexible branches with better accessibility of internal groups to cellular content (Ihnatsyeu-Kachan et al., 2017).

1.3.4 Carbosilane dendrimers CBD-CS

Cationic carbosilane dendrimers CBD-CS are characterized by carbon-silicon bonds and cationic terminal amine groups (Chonco et al., 2007; Bermejo et al., 2007; Wrobel et al., 2012). Carbon-silicon bonds are slowly hydrolized in water, resulting in gradual liberation of exterior branches and cargo genetic material inside the cell. It has been estimated that release happens between 4 and 24 hours (Bermejo et al., 2007). Higher generation CBD-CS dendrimers with more terminal groups release genetic material better than lower generation dendrimers with less terminal groups (Weber et al., 2008). Generation two carbosilane dendrimer has been reported to have low cytotoxicity *in vitro* in PBMCs (Ortega et al., 2006) Data from confocal microscopy suggest that carbosilane dendrimer complexes with fluoresceine-labeled oligodeoxynucleotides are internalized in cells (Bermejo et al., 2007). Furthermore, these dendrimers have been shown to protect genetic material from degradation by serum proteins (Chonco et al., 2007) and RNase (Weber et al., 2008; Ionov et al., 2015).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Plasmids

1. pVax1 plasmid #V260–20, Invitrogen, USA
2. Luciferase encoding pVax-Luc plasmid (kindly provided by Roos A. K., Karolinska Institute), constructed by inserting the cDNA of firefly luciferase from pGL2-basic vector (#E1641, Promega, USA) into plasmid vector pVax1 (#V260–20, Invitrogen, USA). Plasmid contains a human cytomegalovirus immediate/early promoter and a polyadenylation signal from the bovine growth hormone gene (Roos et al., 2009).

2.1.2 Dendrimers

1. Poly(amidoamine) PAMAM G4 dendrimer (#412449), Sigma-Aldrich, USA (supplement 1, A)
2. Cationic phosphorous CPD G4 dendrimer, synthesized in Laboratoire de Chimie de Coordination du CNRS, Toulouse, France (supplement 1, B)
3. Cationic phosphorous dendrimer AE2 G4, synthesized in Laboratoire de Chimie de Coordination du CNRS, Toulouse, France (supplement 2, A).
4. Cationic carbosilane dendrimer CBD-CS G2, synthesized in Departamento de Quimica Inorganica, Universidad de Alcala, Spain (supplement 2, B).

2.1.3 Cell lines

1. N2a mouse neuroblastoma cell line
2. mHippoE-18 embryonic mouse hippocampal cell line

2.1.4 Experimental animals

Eight weeks old BALB/cAnNCrl female mice from the breeding facility of the Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institute, Stockholm

2.1.5 Reagents

1. Uranyl-acetate solution, Polysciences, USA
2. 10 mmol/L Na-phosphate buffer (pH 7.4), Sigma-Aldrich, USA
3. Agarose, Sigma-Aldrich, USA
4. 50x TAE buffer (consists of 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) solution), Thermo Fisher Scientific, USA
5. GelRed nucleic acid gel stain, Biotium, USA
6. DNA Gel loading dye (6X), Thermo Fisher Scientific, USA
7. Ethidium bromide, manufacturer Sigma-Aldrich, USA
8. Heparin, manufacturer Sigma-Aldrich, USA
9. 70% ethanol
10. Phosphate buffered saline PBS (pH 7,4), Sigma-Aldrich, USA
11. DMEM medium for N2a and mHippoE-18 cultivation (containing 10% heat inactivated fetal bovine serum and 1% penicillin and streptomycin), Gibco, USA
12. Trypsin, Invitrogen, USA
13. Trypan blue solution 0.4%, Invitrogen, USA
14. Lipofectamine 2000, Invitrogen, USA
15. ONE-Glo™ Luciferase Assay, Promega, USA
16. MTT solution, Sigma-Aldrich, USA
17. DMSO, Sigma-Aldrich, USA
18. Isofluorane, VETMEDIC, Sweden
19. D-luciferin #122796, PerkinElmer, USA

2.1.6 Equipment

1. 200-mesh copper grids with carbon surface, Ted Pella, USA
2. Transmission electron microscope JEM-1010, JEOL, Japan
3. J-815CD spectrometer, JASCO, Japan
4. Quartz cuvettes Hellma® (200 – 300 nm, 0.5 cm path length), Sigma-Aldrich, USA
5. Electrophoresis chamber, BIO-RAD, USA
6. Malvern Instruments Zetasizer Nano-ZS, Malvern, UK
7. Disposable capillary plastic cells DTS1061, Malvern, UK
8. MF-Millipore membrane filter 0.22 μm, Merck Millipore, USA

9. Disposable capillary plastic cells DTS0012, Malvern, UK
10. Black 96-well plates, Thermo Fisher Scientific, USA
11. LS-50B fluorescence/luminescence spectrophotometer, PerkinElmer, USA
12. 75 cm^2 cell culture flask, SPL life sciences, Korea
13. CappController pipette controller, 0.1-100ml, Capp, Denmark
14. Serological pipettes (5, 10, 25 ml), Sarstedt, USA
15. Centrifugation tubes (15, 50 ml), Sarstedt, USA
16. Microcentrifugation tubes (1,5 ml), Sarstedt, USA
17. CO_2 cell incubator New Brunswick Galaxy 48 S, Eppendorf, Germany
18. Capp multi pipettes, 12-channel, 5-50 µl, and 12-channel 20-200 µl, Capp, Denmark
19. Automated cell counter "Countess", Invitrogen, USA
20. Cell counting chamber slides "Countess", Invitrogen, USA
21. Synergy™ H4 Hybrid Multi-Mode Microplate Reader, BioTek Instruments, USA
22. U100 insulin/50 I.U. syringes, Omnican B Braun, Germany
23. Electroporator CUY21 EDIT II, BEX Co., Ltd., Japan
24. BD Plastipak 1 ml syringes, Becton Dickinson, Spain
25. In vivo imaging system IVIS 2000, PerkinElmer, USA

2.2. Methods

2.2.1. Ethidium bromide intercalation assay

For dendrimer and pVax-Luc interaction assay, ethidium bromide (EB) was used (Chen et al., 2000). pVax-Luc plasmid was mixed with 250 µM EB and dendrimers (total volume of 2 ml) in increasing w/w ratios in 1 ml quartz cuvettes in 10 mmol/L Na-phosphate buffer. Formed complexes at final weight ratio were also treated with heparin (0.082 mg/ml) for 5 minutes. Fluorescence of samples with rising pVax-Luc/dendrimer molar ratios was measured with excitation wavelength of 505 nm and emission spectrum of 530 to 680 nm (emission maximum at 590 nm) at 25°C using LS-50B Perkin Elmer fluorescence spectrophotometer. The excitation and emission slit widths were set at 5 nm and 10 nm respectively. The results were calculated as F/F_0 ratio, F – fluorescence intensity of pVax-Luc/dendrimer complexes, F_0 – fluorescence intensity of uncomplexed plasmid.

2.2.2. Elecrtophoretic mobility shift assay

Agarose gel electrophoresis was used to study formation of complexes between dendrimers and pVax-Luc. Dendrimers were complexed with plasmid in 10 mmol/L Na-phosphate buffer in increasing weight ratios. Complexes were analyzed by gel electrophoresis on 1% agarose gel for 40 min at 25 mA with GelRed for plasmid probe staining. After electrophoresis, the gel was visualized in UV light and a digital photography of the stained gel was taken.

2.2.3. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was done to evaluate plasmid structure change in complexes with dendrimers. Plasmid pVax-Luc was mixed with dendrimers in increasing weight ratios in 10 mmol/L Na-phosphate buffer pH 7.4 and measurements were done using J-815 CD spectrometer with quartz cuvettes, 5 mm path length. CD spectra were obtained within the 200 – 320 nm range, 1 nm bandwidth, 2 s response time, scan speed 50 nm/min, 1 nm step resolution. The number of scans varied between three and five for each sample. The mean residue ellipticity (cm^2dmol^{-1}) was calculated using Jasco software.

2.2.4. Zeta potential

For particle surface charge (zeta potential) determination, phase analysis light scattering (PALS) measurements were done. Plasmid pVax-Luc was mixed with dendrimers in 10 mmol/L Na-phosphate buffer in increasing w/w ratios and before sample analysis each sample filtered twice through 0.22 μm filter membrane. Measurements were done using capillary plastic cells DTS1061 at room temperature using Zetasizer Nano-ZS. For each sample five measurements in three repeats were done and averaged for each sample. Zeta potential values were calculated with the Helmholtz-Smoluchowski equation (Smoluchowski, 1921 in Delgado et al., 2005) in Malvern software.

2.2.5. Zeta size

Particle size of dendrimer/pVax-Luc complexes was measured with dynamic light scattering (DLS) method. Wavelength for measurements was set at 633 nm. Plasmid pVax-Luc was mixed with dendrimers in increasing w/w ratios in 10 mmol/L Na-phosphate buffer. Measurements were performed using DTS0012 plastic cells at room temperature with Zetasizer

Nano-ZS. For each sample five measurements in three repeats were done and averaged for each sample. For data analysis Malvern software was used.

2.2.6. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to observe complex formation between dendrimers and pVax-Luc plasmid. Dendrimers were dissolved in deionized water to make100 μM PAMAM G4 and 1 mM AE2 G4, CPD G4 and CBD-CS solutions and 5 μl of these solutions were mixed with 5 μl of pVax-Luc plasmid (500 mg/ml) and incubated for 20 minutes in room temperature. Each sample was placed on 200-mesh copper grid and stained with uranyl acetate saturated solution for another 20 minutes, then washed in deionized water and dried. TEM images were created with JEM-1010 electron microscope at 80 kV and 1.2 second exposure time with 40 000× magnification. For greater contrast, colors of images were inverted.

2.2.7. *In vitro* cell transfection

Two cell lines, N2a and mHippoE-18 were used for transfection. Cells were routinely cultured as attached monolayer in DMEM medium at 37°C, 5% CO_2 atmosphere.

For cell transfection, cells were seeded to a density of 10 000 cells/well in black 96-well plates and grown for 24 hours prior plasmid introduction. Dendrimer and pVax-Luc solutions in PBS were prepared in following w/w ratios: pVax-Luc/PAMAM G4 1 : 35; pVax-Luc/CPD G4 1 : 2.5; pVax-Luc/CBD-CS 1 : 1.13; pVax-Luc/AE2G4 1 : 4.3. pVax-Luc mixture with lipofectamine was prepared according to protocol in cell media without serum. pVax-Luc (100 ng) without dendrimers or lipofectamine was mixed in PBS. All solutions were incubated for 30 minutes before adding to cells. Transfection was done for 24 hours.

2.2.8. Luciferase reporter assay

To determine the efficacy of cell transfection, luciferase reporter assay was performed. After transfection of N2a and mHippoE-18, cell medium was removed and 100μl of reagent ONE-Glo™ Luciferase Assay was added in each well. Subsequently, cells were incubated for 5 minutes in room temperature for complete cell lysis. Luminescence was measured with LS-50B fluorescence/luminescence spectrophotometer.

2.2.9. Cytotoxicity assay

For dendrimer cytotoxicity determination, MTT assay was performed. N2a and mHippo-E18 cells were seeded to a density of 10^4 cells/well in a 96-well plate (100 µl per well) and grown for 24 hours, then treated with all four dendrimers and their complexes with pVax-Luc Lipofectamine with and without plasmid DNA was used as comparison for cell cytotoxicity. pVax-Luc/dendrimer complexes were prepared in the same w/w ratios as for transfection and dendrimers without pDNA were added in the same molar concentration as used for complexation.

After 24 h growth, 26 µl of stock MTT solution (2 mg/ml in PBS) was added to each well and incubated for additional four hours in 37°C. Then cell media was discarded and 150 µl of DMSO was added in each well to dissolve formazan crystals formed by proliferating cells. Absorbance was measured at 570 nm using a micro-plate reader and recorded as percentage relative to untreated cells.

2.2.10. *In vivo* immunization

All experimental mice were kept in the animal facility of MTC, Karolinska Institute under 12h light/12 h dark cycle with unlimited access to food and water. All pain inflicting procedures were done under isofluorane inhalation anesthesia. Experiments were confirmed by the Ethical Committee for the Animal Research of the Northern Stockholm.

All mice, divided into four groups, were immunized with pVax-Luc, empty pVax1 vector or pVax-Luc/dendrimer complexes according to schemes in table 1 and table 2. For *in vivo* gene delivery two dendrimers were chosen – CBD-CS, which showed the highest transfection efficiency *in vitro* and CPD which has been recommended earlier for in vitro transfection of plasmid DNA (Loup et al., 1999; Ionov et al., 2011). Two separate immunizations were done. For secondary immunization mice that did not show any signal from primary immunization were injected again. Before immunization lower back of each mouse was shaved. Immunization was performed with intramuscular injections in the lower back, on both sides at the base of the tail – pVax-Luc, pVax1 and pVax-Luc/dendrimer (w/w ratio for pVax-Luc/CPD 1 : 2.5 and for pVax-Luc CBD-CS 1 : 1.13) complexes were delivered in a volume of 30 µL PBS solution. Injections in group 1 (table 1) and 3.2 (table 2) were followed by electroporation (EP) using three needle fork-plate electrodes. Electrical pulses were generated with the following pattern: one electroporation pulse of 400 V 0,1 ms long and eight 10 ms long driving pulses of 100 V with 20

ms interval between them. Driving current limit was 900 mA. Before each electrical pulse mouse skin resistance was checked to be around 2.3 kΩ.

Table 1. Primary immunization scheme

Group	Treatment	Dose (IM injections)	Number of animals
1	pVax-Luc + EP	2 x 20 μg	5
2	pVax1 + EP	2 x 20 μg	5
3	CBD-CS/pVax-Luc	2 x 20 μg	3
4	CPD G4/pVax-Luc	mouse 1: 2 x 10 μg mouse 2: 2 x 20 μg mouse 3: 2 x 40 μg	3

Table 2. Secondary immunization scheme

Group	Treatment	Dose (IM injections)	Number of animals
3.2	CBD-CS/pVax-Luc, left site EP, right – no EP.	mouse 1: 2 x 5 μg mouse 2: 2 x 10 μg mouse 3: 2 x 20 μg	3
4.2	CPD G4/pVax-Luc	2 x 50 μg	3

2.2.11. *In vivo* bioluminescence imaging

To monitor delivery and expression of luciferase reporter (pVax-Luc) in mice, real-time *in vivo* bioluminescence imaging was performed using a CCD camera (IVIS 2000). Bioluminescence was recorded for 20 days – measurements were performed on day 1, 3, 6, 8, 9, 10, 13, 15, 17 and 20 after immunization. Before each measurement, mice were injected intraperitoneally with 80-100 mg/kg dose of D-luciferin dissolved in PBS. Ten minutes post-injection (time for luciferin to distribute evenly in mouse body), mice were placed in a chamber with anesthetizing gas consisting of 4,5% isofluorane mixture with air. After anesthesia mice were moved to *in vivo* imaging system IVIS 2000 for luminescence measurements. Imaging was performed with automatic exposure time, depending on the intensity of the bioluminescence source. The luminescence was quantified using Living Image software 4.1 (PerkinElmer) as total flux of photons per second (p/s). To determine background bioluminescence level, signal from mice skin was measured in places where pVax-Luc was not injected.

2.2.12. Analysis of data

Calculations and analysis of data was performed using Microsoft Office 365 ProPlus: Excel and GraphPad Prism 7, if not stated otherwise.

For cytotoxicity data analysis uncorrected Fisher's LSD test was used. For *in vitro* transfection efficiency and for *in vivo* protein expression data analysis, Kruskal-Wallis test and two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli was performed. All analysis were performed with 95% confidence interval and p value <0,05 was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Biophysical characteristics

3.1.1. Ethidium bromide intercalation assay

To analyze the complex formation between pVax-Luc and dendrimers the EB fluorescence technique was used. EB is an intercalating fluorescent dye that binds DNA (LePecq and Paoletti, 1967; Boger et al., 2001). Addition of other DNA binding molecules results in EB fluorescence quenching due to its displacement. Loss of fluorescence is directly connected to the other molecule extent of DNA binding (Jenkins 1979; Boger et al., 2001).

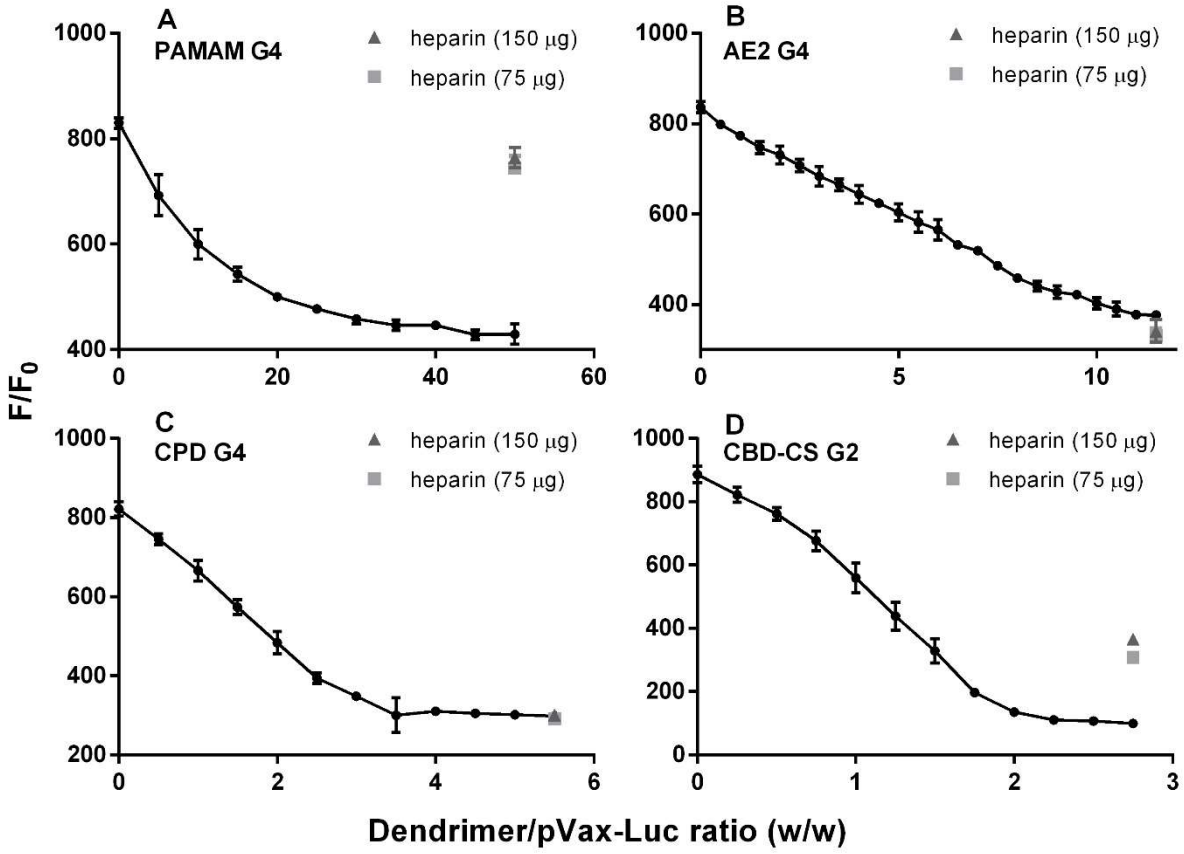


Figure 3. Dependence of the fluorescence intensity of EB-pVax-Luc complex upon increasing PAMAM G4 (A), AE2 G4 (B), CPD G4 (C) and CBD-CS (D) concentration. Plasmid DNA release from complexes upon addition of 75 μg and 150 μg heparin at highest dendrimer concentration.

Addition of dendrimers to EB-pVax-Luc resulted in EB fluorescence quenching following the increase of dendrimer/pVax-Luc w/w ratio (fig. 3), indicating dendrimer binding to the

plasmid, which is consistent with previous findings (Chen et al., 2000; Shakhbazau et al., 2010; Ionov et al., 2015).

pVax-Luc/PAMAM G4 complexes were saturated and the fluorescence reached a plateau at around 1 : 35 w/w ratio, pVax-Luc/CPD G4 at 1 : 3.5, pVax-Luc/CBD-CS at 1 : 2.25. Fluorescence for complexes with AE2 G4 dendrimer was gradually decreasing and did not reach the plateau (fig. 3).

The release of pDNA from dendrimers was studied adding heparin (Xu and Szoka, 1996) to pVax-Luc/dendrimer complexes. Upon heparin addition, fluorescence recovered around 88% for PAMAM, and around 38% for CBD-CS from initial EB-pVax-Luc fluorescence level without dendrimers, however recovery did not happen for CPD and AE2 dendrimers (fig. 3).

These results indicate that PAMAM dendrimer is most efficient in releasing pDNA, but the interaction between pDNA and CPD and AE2 dendrimers stronger. The strong interaction between CPD dendrimer and pDNA could be due to strong electrostatical interaction, since CPD dendrimer has more positive surface charges than PAMAM of the same generation and CBD-CS with lower generation (Shcharbin et al., 2011; Ionov et al., 2015). A similar study was performed where AE2 dendrimer was complexed to siRNA and release of the genetic material upon addition of heparin was observed to be only partial (Ihnatsyeu-Kachan et al., 2017). AE2 dendrimer has the same structure as CPD and it has the same amount of cationic terminal groups. The cationic terminal groups of AE2 dendrimer are more voluminous than of CPD dendrimers (Ihnatsyeu-Kachan et al., 2017), therefore the interaction between pDNA and AE2 could be as strong as with CPD dendrimer resulting in inefficient release of pDNA from complexes.

3.1.2. Electrophoretic mobility shift assay

Complexes were formed at different pVax-Luc/dendrimer weight ratios – plasmid was mixed with increasing concentrations of dendrimers. When pDNA/dendrimer complexes are saturated, they remain in the gel well and fluorescent bands of pDNA are not observed. Results show that complexes with PAMAM dendrimer were fully saturated by pVax-Luc/dendrimer w/w ratio > 1 : 34.8, AE2 > 1 : 5.1, CPD > 1 : 2.9 and CBD-CS complexes > 1 : 1.13 (fig. 4). These results are consistent with EB fluorescence assay data regarding w/w ratio at which saturated complexes are formed with PAMAM, CPD and CBD-CS dendrimers.

CBD-CS dendrimer exhibits the highest binding affinity to pDNA, forming saturated complexes at the lowest w/w ratio, however PAMAM dendrimer has the lowest binding

affinity to pDNA. On the contrary, when dendrimers were complexed to siRNA, the highest affinity was achieved with PAMAM and CPD dendrimers, but CBD-CS dendrimers bound siRNA the least (Ionov et al., 2015). Dendrimer binding affinity to siRNA has been compared also between CPD and AE2 dendrimers. The results indicated that AE2 dendrimers bind siRNA better than CPD, because of piperidine terminal group modification (Ihnatsyeu-Kachan et al., 2017).

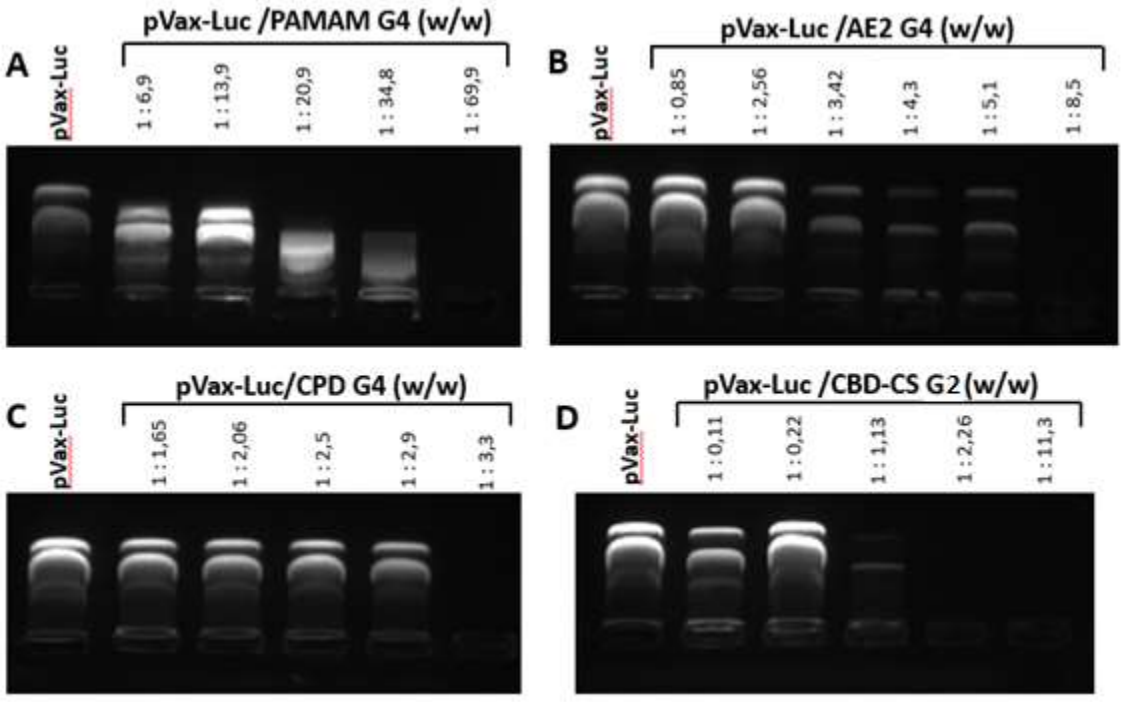


Figure 4. Gel electrophoresis shift analysis of pVax-Luc/dendrimer complexes: pVax-Luc/PAMAM G4 (A), pVax-Luc/AE2 G4 (B), pVax-Luc/CPD G4 (C) and pVax-Luc/CBD-CS (D) in increasing w/w (pVax-Luc : dendrimer) ratios.

3.1.3. Circular dichroism spectroscopy

The CD spectra for pVax-Luc plasmid displayed peaks at 221 nm, 247 nm and 275 nm. The general shape of pVax-Luc plasmid is not significantly changed at the presence of dendrimers in increasing weight ratios, as the CD spectra of pVax-Luc/dendrimer complexes are consistent with the spectrum of naked plasmid (fig. 5). Obtained results indicate that PAMAM, AE2, CPD and CBD-CS dendrimers have only very weak influence on pVax-Luc secondary structure. The weak influence of dendrimers on secondary structure of genetic material has been also observed between PAMAM, CPD and CBD-CS dendrimers and siRNA (Ionov et al., 2015),

however AE2 dendrimer has been reported to have stronger influence on siRNA secondary structure in concentration-dependent manner (Ihnatsyeu-Kachan et al., 2017).

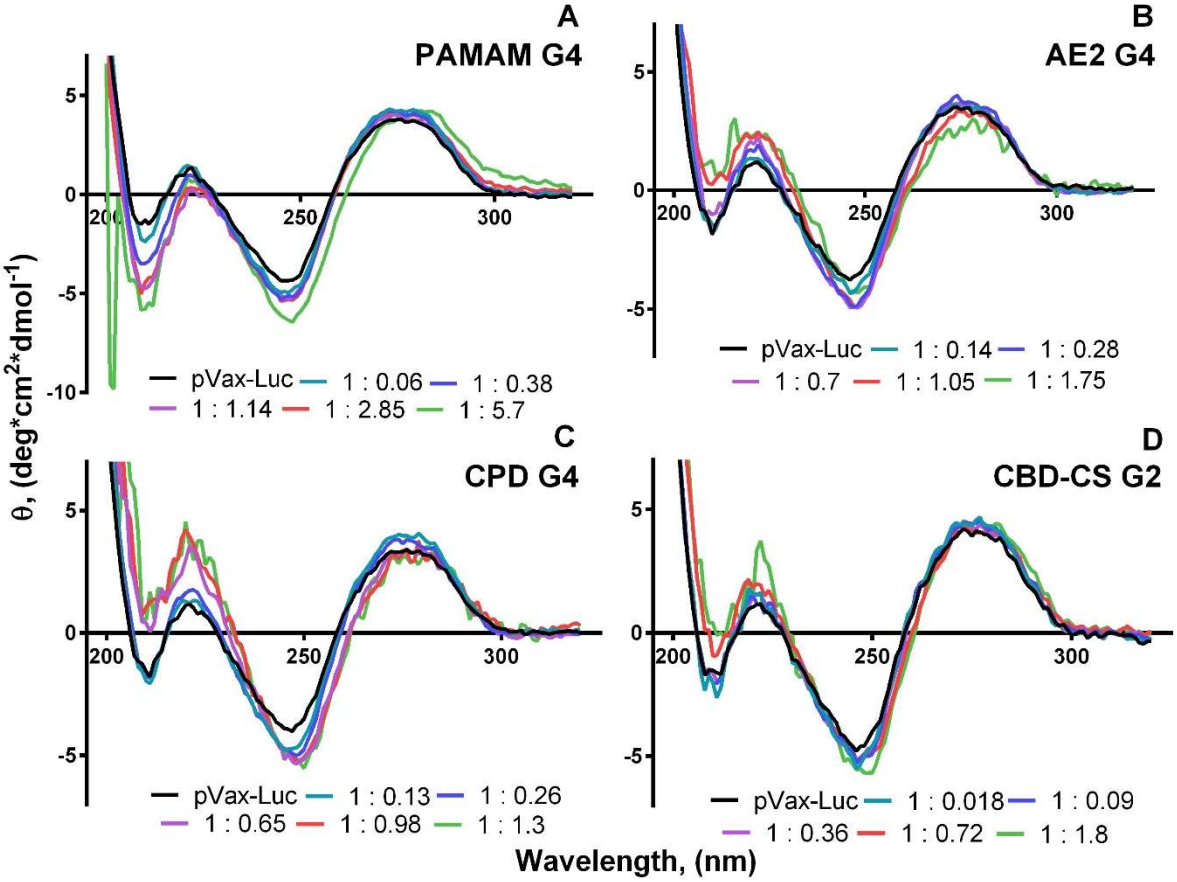


Figure 5. CD spectra of pVax-Luc/PAMAM G4 (A), pVax-Luc/AE2 G4 (B), pVax-Luc/CPD G4 (C) and pVax-Luc/CBD-CS (D) complexes in increasing w/w (pVax-Luc : dendrimer) ratios (colored lines). Black line in all graphs indicate the secondary structure of uncomplexed pVax-Luc plasmid.

3.1.4. Zeta potential and size

Increasing dendrimer/pVax-Luc w/w ratio resulted in zeta potential shift from negative to positive values for all four dendrimers confirming pVax-Luc/dendrimer complex formation. Addition of dendrimers to pVax-Luc suspension changed electrokinetic potential from -22.4 mV to +1.5 mV with PAMAM, from -37.0 mV to +14.7 mV with AE2, from -41.5 mV to +14.9 mV with CPD and from -22.6 mV to +10.8 mV with CBD-CS dendrimer. Zeta potential stabilized at a pVax-Luc/dendrimer w/w ratio >1 : 30 with PAMAM, >1 : 30 with AE2 G4, > 1: 4 with CPD and > 1 : 1.5 with CBD-CS (fig. 6).

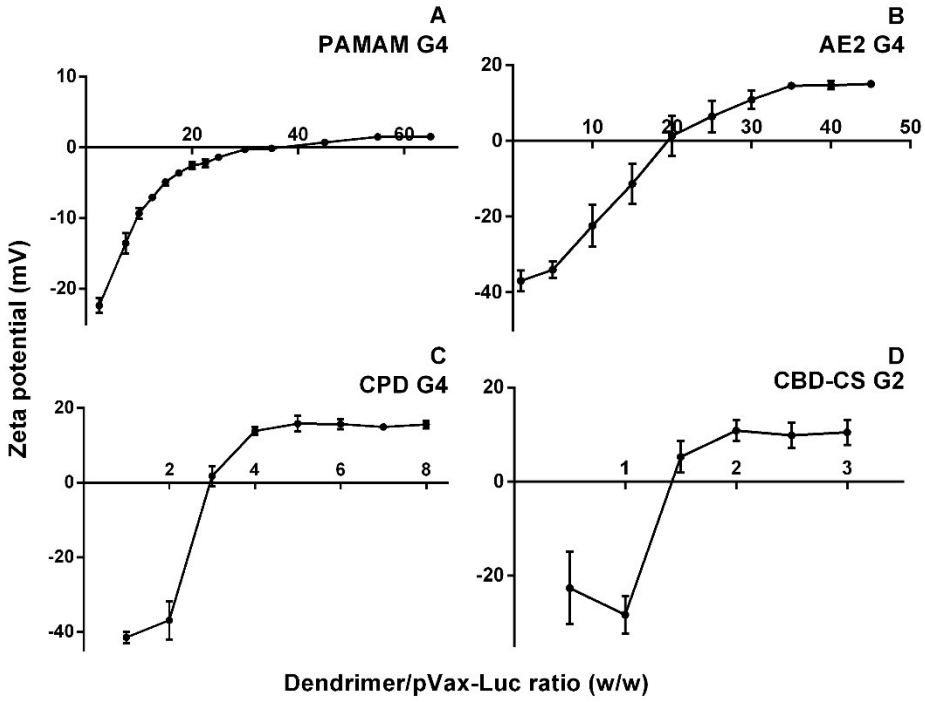


Figure 6. Zeta potential of pVax-Luc plasmid complexed to PAMAM G4 (A), AE2 G4 (B), CPD G4 (C) and CBD-CD (D) dendrimers in increasing w/w (pVax-Luc : dendrimer) ratio.

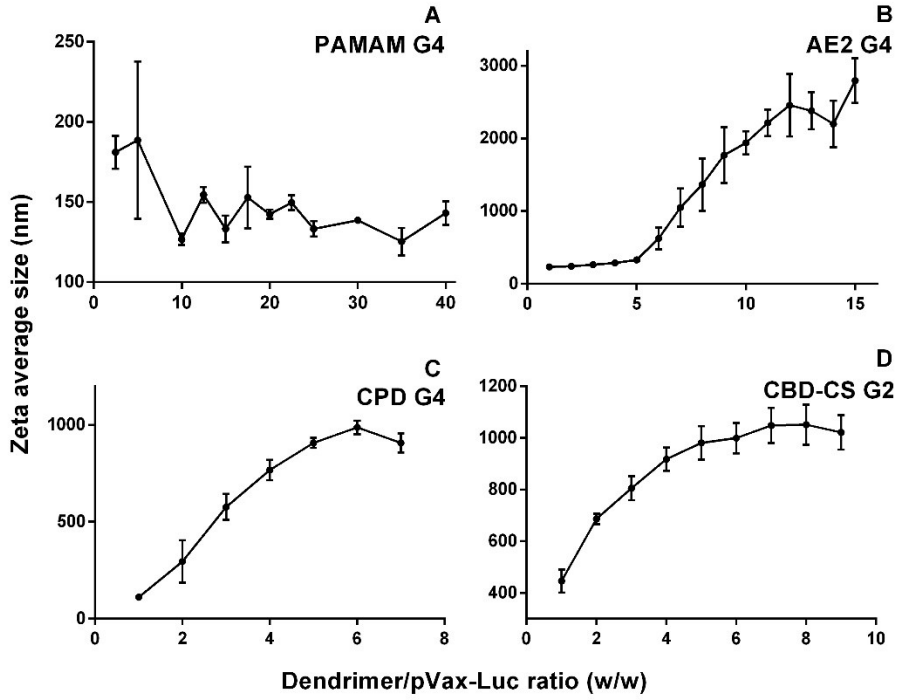


Figure 7. Zeta size (diameter) of pVax-Luc plasmid complexed to PAMAM G4 (A), AE2 G4 (B), CPD G4 (C) and CBD-CD (D) dendrimers in increasing w/w (pVax-Luc : dendrimer) ratio.

The interaction between dendrimers and pVax-Luc resulted in increase of the particle size from 232.9 ± 10 nm to 2458.4 ± 429.6 nm for AE2, from 111.6 ± 3.2 nm to 987.4 ± 34.7 for CPD and from 445.8 ± 45 nm to 1051.1 ± 77.4 nm for CBD-CS dendrimer. PAMAM dendrimer did not cause significant enlargement of particles and complexes remained within a range of 125 – 155 nm. The size of complexes increased up to a pVax-Luc/dendrimer w/w ratio of 1 : 12 for AE2, 1 : 6 for CPD and 1 : 7 for CBD-CS (fig. 7). Zeta size measurements show that all dendrimers except PAMAM form large complexes upon increasing dendrimer concentration.

3.1.5. Transmission electron microscopy

Complex formation between pVax-Luc plasmid and dendrimes was studied by TEM. Naked plasmid DNA was observed as homogenous mass of particles (fig. 8, A). Upon addition of dendrimers, reticular structure formation was observed (fig. 8, B, C, D, E), which confirms pDNA and dendrimer binding.

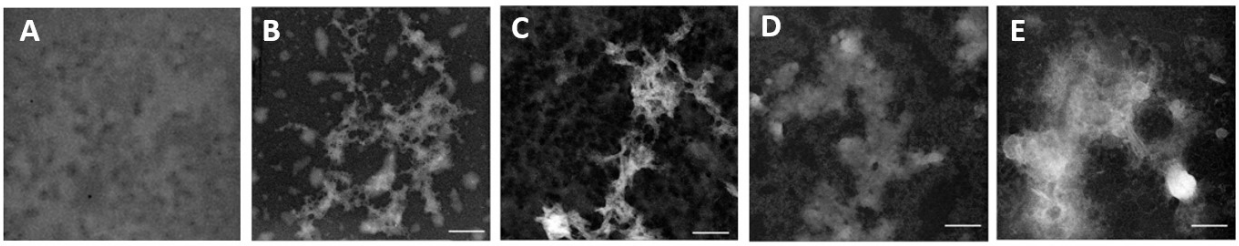


Figure 8. Electron micrographs of pVax-Luc (A) and PAMAM G4 (B), AE2 G4 (C), CPD G4 (D) and CBD-CS G2 (E) mixtures with pVax-Luc. Scale bar – 100 nm.

3.2. DNA delivery efficiency

3.2.1. In vitro cell transfection

Based on electrophoresis mobility assay, pVax-Luc/dendrimer w/w ratio for transfection was chosen to make saturated complexes. For pVax-Luc/PAMAM w/w ratio was 1 : 35, pVax-Luc/AE2 1 : 4.3, pVax-Luc/CPD 1 : 2.5 and pVax-Luc/CBD-CS 1 : 1.13 (fig. 4).

Transfection efficiency in both cell lines was similar and the highest luciferase expression was observed with CBD-CS dendrimer. Transfection with CBD-CS in both cell lines was significantly higher compared to control cells where only pDNA was added ($p<0.05$) (fig. 9, D and fig. 10, D). It was also observed, that transfection in mHippoE-18 cell line is significantly higher with CPD dendrimer ($p<0.05$) compared to control cells (fig. 9, C).

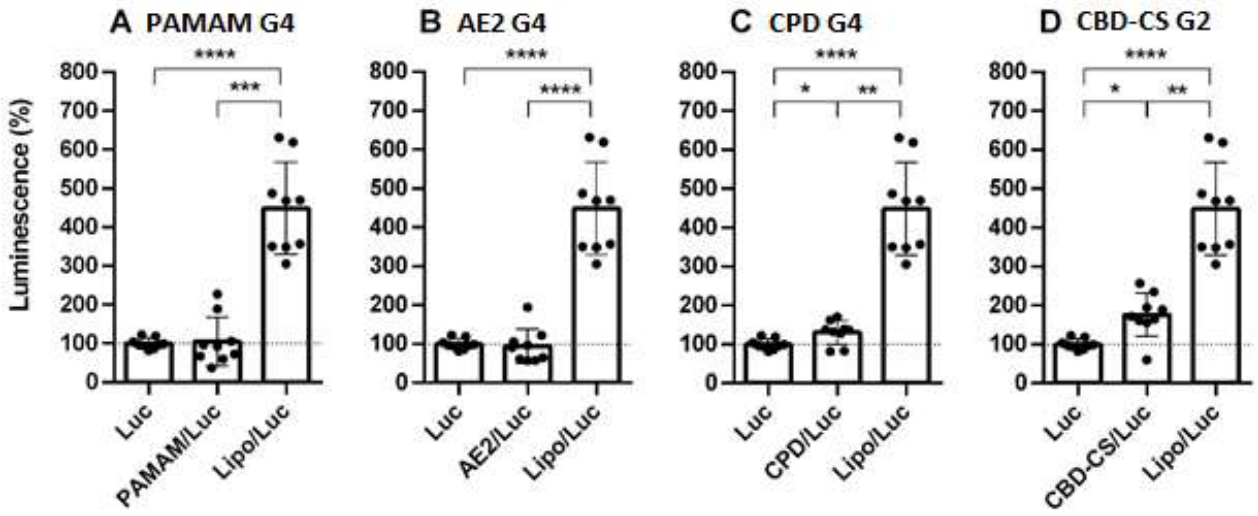


Figure 9. Transfection efficiency of pVax-Luc in mHippoE-18 cell line using dendrimers (A – PAMAM, B – AE2, C – CPD and D – CBD-CS) and lipofectamine. Luc – plasmid pVax-Luc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

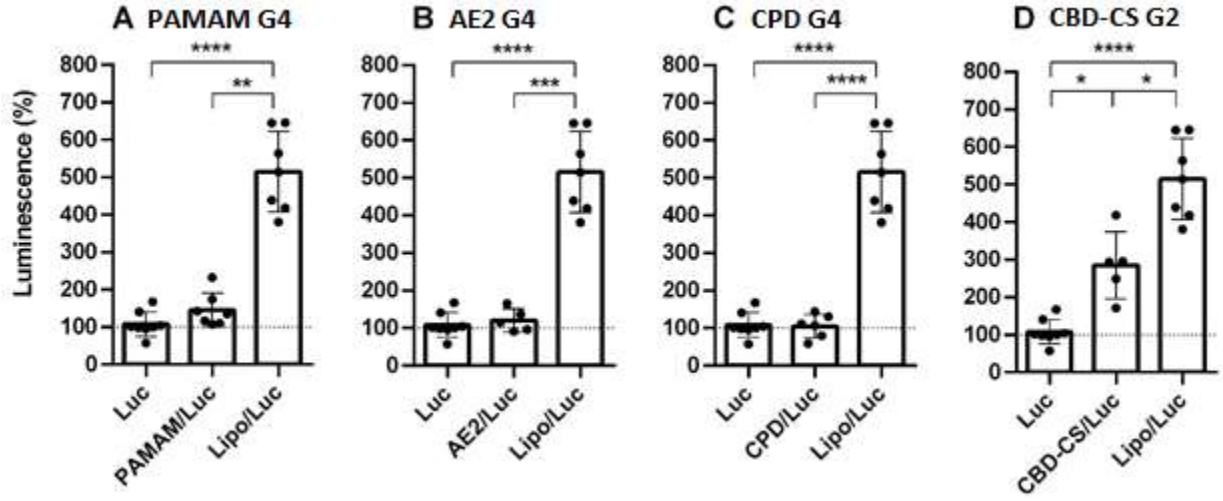


Figure 10. Transfection efficiency of pVax-Luc in N2a cell line using dendrimers (A – PAMAM, B – AE2, C – CPD and D – CBD-CS) and lipofectamine. Luc – plasmid pVax-Luc. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Efficiency of nanoparticle internalization has been shown to be size dependent (Rejman et al., 2004; Fröhlich, 2012). Smaller nanoparticles <200 nm are internalized in cells faster and more efficiently than 200 - 500 nm particles. Nanoparticles <200 nm are internalized in non-phagocytic cells via clathrin-mediated pathway and 500 nm particles are internalized via clathrin-independant pathway (Rejman et al., 2004). Additionally it has been shown that particles up to 3µm size can be internalized in HeLa cells via combination of energy-dependent phagocytosis and clathrin-dependent mechanism (Gratton et al., 2008). In given w/w ratios for transfection size

of pVax-Luc/PAMAM complex is ~125 nm, pVax-Luc/AE2 ~290 nm, pVax-Luc/CPD ~440 nm and pVax-Luc/CBD-CS ~450 nm (fig. 7). Since smaller nanoparticles are shown to internalize more efficiently, the highest transfection efficiency should be obeserved with PAMAM and AE2 dendrimers, however the highest efficiency was observed with CPD and CBD-CS dendrimers (fig. 9 and fig. 10), which formed larger particles. It has been reported that, although the net internalization of larger complexes in cells might be less compared to smaller particles, actual gene release from complexes could be higher due to prolonged time of the complexes in cytoplasm, avoiding rapid lysosomal degradation (Rejman et al., 2004).

Another factor that influences transfection efficiency with dendrimers is zeta potential or complex surface charge (Gratton et al., 2008; Fröhlich, 2012). Positively charged complexes interact with cell membranes and are internalized more effectively than negatively charged complexes (Gratton et al., 2008). In given w/w ratios zeta potential of pVax-Luc/PAMAM complex is ~ +0.2 mV, pVax-Luc/AE2 ~ -35 mV, pVax-Lux/CPD ~ +1.4 mV and pVax-Luc/CBD-CS ~ +4.2 mV (fig. 6). CBD-CS has the highest positive surface charge from all four dendrimers, which explains the highest transfection efficiency observed with this dendrimer.

Transfection efficiency with lipofectamine was significantly higher compared to control cells ($p<0.0001$). Although pVax-Luc pDNA in mHippoE-18 and N2a cells using dendrimers was introduced, transfection efficiency for all dendrimers was still lower than with lipofectamine ($p<0.05$ – $p<0.0001$) (fig. 9 and fig. 10). Transfecion efficiency has been shown to be dependent on the cell line. Lipofectamine is a more effective plasmid carrier than dendrimers also in mesenchymal stem cells (Shakhbazau et al., 2010).

3.2.2. Dendrimer cytotoxicity

For cytotoxicity assay, pDNA/dendrimer complexes were added in the same w/w ratio as for transfection efficiency evaluation. For pVax-Luc/PAMAM w/w was 1 : 35, pVax-Luc/AE2 1 : 4.3, pVax-Luc/CPD 1 : 2.5 and pVax-Luc/CBD-CS 1 : 1.13.

Since cytotoxicity of dendrimers also depend on the cell line (Maszewska et al., 2003; Shcharbin et al., 2011; Fröhlich, 2012), results from MTT cytotoxicity tests suggest that none of the four dendrimers or their complexes with pDNA are significantly toxic to mHippoE-18 and N2a cell lines. Lipofectamine exhibits slightly lower cell viability than dendrimers in both cell lines ($p<0,05$) – it is reduced by ~10%. (fig. 11 and fig. 12). Toxicity of lipofectamine and other cationic lipids has been also previously reported (Pack et al., 2005).

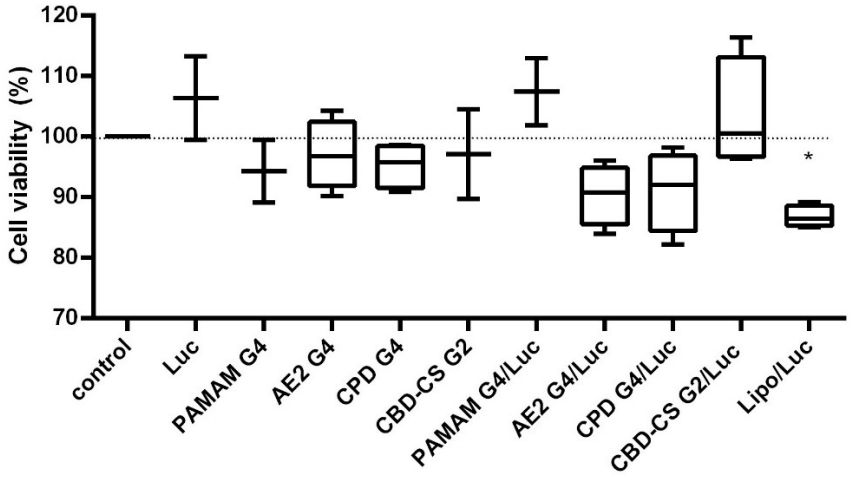


Figure 11. Cytotoxicity of denrimers, dendrimer/pVax-Luc complexes and lipofectamine in mHippoE-18 cell line. Luc – plasmid pVax-Luc. Cell viability was significantly decreased comparing control cells to lipofectamine treatment, *p<0.05.

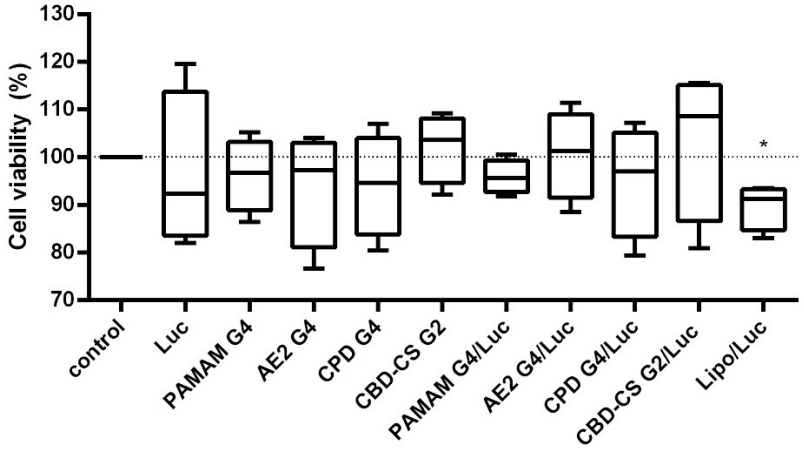


Figure 12. Cytotoxicity of denrimers, dendrimer/pVax-Luc complexes and lipofectamine in N2a cell line. Luc – plasmid pVax-Luc. Cell viability was significantly decreased comparing control cells to lipofectamine treatment, *p<0.05.

For cell cytotoxicity test dendrimers were used in the same concentrations that had been used for cell transfection: PAMAM was 2,6 μM, AE2 – 309,6 nM, CPD – 180 nM and CBD-CS 86,4 nM. PAMAM G4 dendrimer has been reported to be non-toxic in <1 μM concentration (Albertazzi et al., 2013; Dzmitruk et al., 2015) and cell viability is not significantly decreased up to 10 μM PAMAM G4 concentration in HeLa cells (Dzmitruk et al., 2015), however cytotoxicity increases if the concentration is higher (Mukherjee et al., 2010). Similar cytotoxicity has been observed with AE2 dendrimer, where cell viability decreased by 70% with 1 μM AE2 G4 dendrimer, but molar concentration above 1 μM was more cytotoxic (Ihnatsyeu-Kachan et al., 2017). CPD G4 dendrimer has been reported to dramatically decrease murine neural cell viability

in vitro at concentrations >1 μM (Lazniewska et al., 2013) and decrease HeLa cell viability by 60% at 1 μM concentration up to 95% decrease if the molar concentration is higher (Dzmitruk et al., 2015). Similar cell viability decrease was observed with CBD-CS dendrimer, where concentrations <1 μM were not cytotoxic to HeLa cells, however upon increasing dendrimer concentration, cell viability decreased notably (Dzmitruk et al., 2015). Generation two CBD-CS dendrimer has been reported to be non-cytotoxic up to 15 μg/ml in human T cell lymphoblastic Lymphoma SupT1 cell line (Weber et al., 2008).

Data in literature and our results suggest that PAMAM dendrimer is not cytotoxic in 2.6 μM and AE2, CPD, CBD-CS dendrimers in <1 μM concentration, thus can be used for gene delivery.

3.2.3. *In vivo* transfection

After electroporation of 20 μg of pVax-Luc, high bioluminescence signal was observed (fig. 1, A), and it was gradually decreasing during the 20 day imaging period (fig. 13; fig. 14). The pattern of photon flux was similar for both - the left and right injection sites in all mice (data not shown). On day 3 after electroporation, photon flux was decreased already by 64%, on day 6 by 84 % and on day 9 already by 92% from initial signal that was observed on day one after electroporation (fig. 13, B). However on the last day of imaging the signal was 4,8 * 10^5 photons per second, which is significantly higher ($p<0.001$) than the background signal 2,5 * 10^4 p/s (fig. 13, A) and indicates stable, continuous expression of luciferase protein.

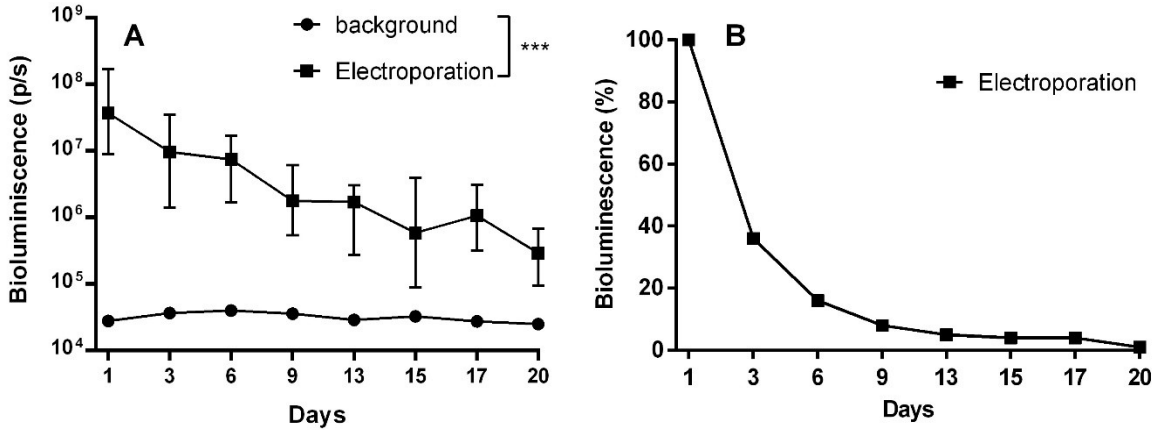


Figure 13. Bioluminescent imaging of mice electroporated with 20μg of pVax-Luc per injection site: (A) photon flux changes during 20 days of imaging, (B) photon flux given as percent change relative to day 1 post electroporation. Significant difference between background and electroporation was observed on days 1 – 20, ***$p<0.001$.

A similar study where luciferase encoding vector was injected intramuscularly (50µg) and electroporated showed that significant signal decrease was only at 42 days with the level of expression being 22% of 3-day levels (Payette et al., 2001). Another study showed that after intradermal injection of pVax-Luc plasmid with subsequent electroporation, high signal could be even detected on day 62 after immunization with pDNA (Roos et al., 2009). Long term protein expression could be observed due to relatively non-immunogenic nature of luciferase protein (Hakamata et al., 2006).

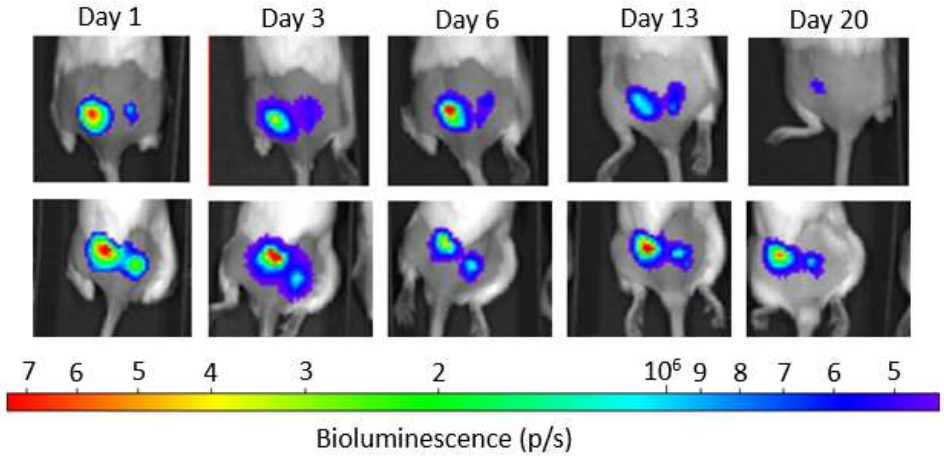


Figure 14. Bioluminescent imaging of mice electroporated with 20µg of pVax-Luc per injection site.

It was also observed that all electroporated mice did not exhibit the same level of bioluminescence (fig. 14). *In vivo* electroporation efficiency depends on skin resistance (Petkov et al., 2013), which was checked on each site before electroporation, and was not the same for all mice (data not shown).

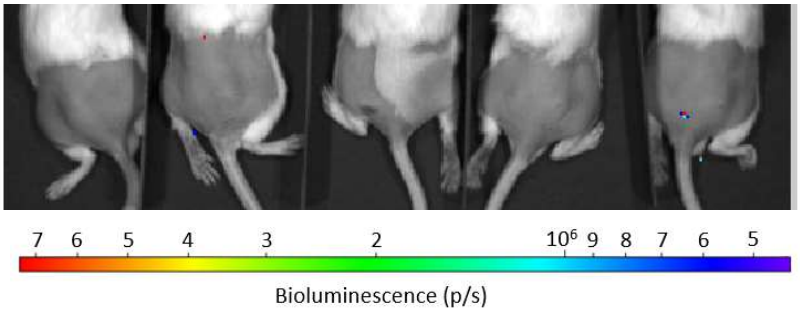


Figure 15. Bioluminescent imaging of mice electroporated with 20µg of empty pVax1 vector per injection site.

Bioluminescence was not observed in mice, which were electroporated with 20 µg of empty pVax1 vector (fig. 15). Photon flux was very low - from 2,3 * 10^4 to 4,5 * 10^4 photons per

second for all mice on both injection sites on day 1 and 3 after electroporation (data not shown). Photon flux in this group was similar to background photon flux in mice electroporated with pVax-Luc (fig. 13, A).

Mice were treated with pDNA/dendrimer complexes in w/w ratio 1 : 1.13 for pVax-Luc/CBD-CS and 1 : 2.5 for pVax-Luc/CPD. Further in result description, only amount of dendrimers is mentioned.

For mice, which were treated with pVax-Luc complexed to 20 µg CBD-CS per injection site, bioluminiscence also not observed (fig. 16, A). On days 1, 3 and 6 photon flux was in the same level as background – from 2,0 * 10^4 to 4,2 * 10^4 photons per second (data not shown).

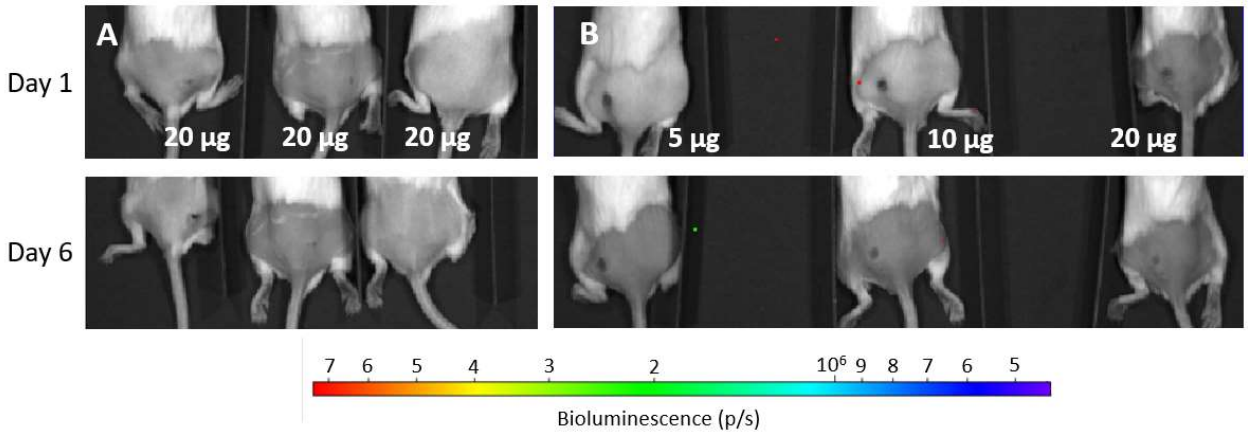


Figure 16. Bioluminescent imaging of mice treated with pVax-Luc complexed to (A) 20 µg CBD-CS and (B) 5 µg, 10 µg and 20 µg CBD-CS, left injection site electroporated.

To try to increase signal from transfection with pVax-Luc/CBD-CS complexes, the experiment was repeated injecting pVax-Luc complexed to 5, 10 and 20 µg CBD-CS. One of injection sites (left) was subsequently electroporated. On days 1, 3 and 6 bioluminescence was not observed (fig. 16, B) and electroporation did not increase photon flux. At the injection site without electroporation, the signal ranged from 2.5 * 10^4 p/s to 3.3 * 10^4 p/s, for the electroporated site – from 2.7 * 10^4 p/s to 4.6 * 10^4 p/s (data not shown). Therefore transfection was not efficient with CBD-CS dendrimer, and the observed photon flux was similar to control group where empty pVax1 was injected.

In mice which were injected with pVax-Luc complexed to CPD dendrimer in increasing concentration, bioluminescence was observed, however the photon flux was very low (fig. 17, A). The highest photon flux was observed in mice treated with 40 µg CPD complex (fig. 17, A) -

the signal was 1,4 * 10^6 p/s on day one. The photon flux had reduced already by 88,7% on day 3 and by 95,8% on day 6. In other mice signal was at the same level as background photon flux (data not shown).

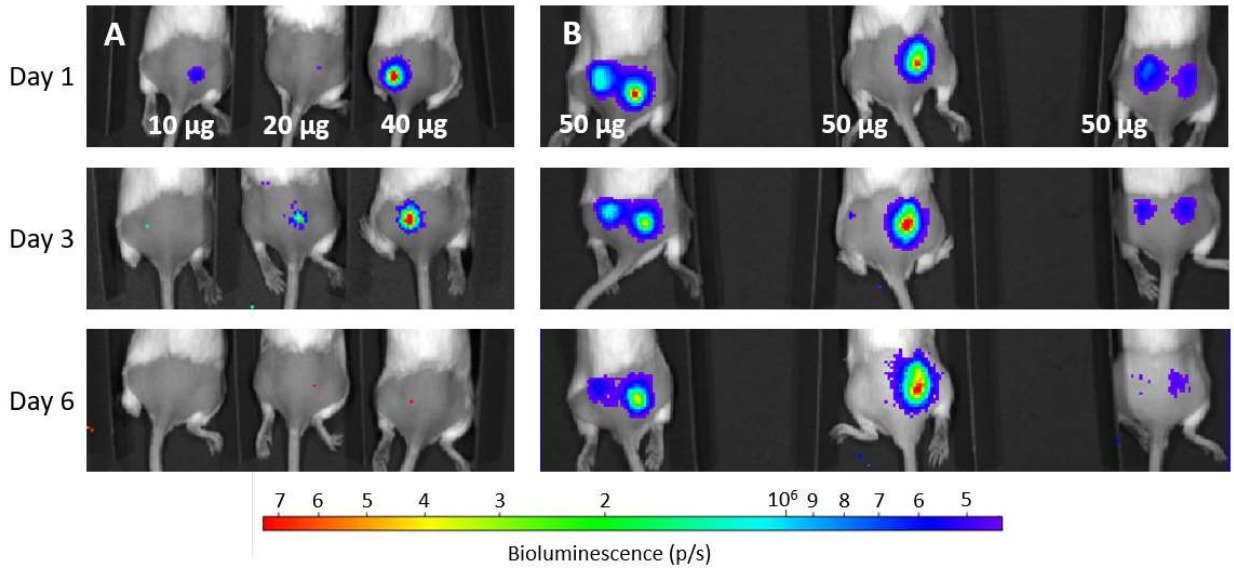


Figure 17. Bioluminescent imaging of mice treated with pVax-Luc complexed to (A) 10 µg, 20 µg and 40 µg and (B) 50 µg of CPD G4 dendrimer.

To try to enhance bioluminescence, a 50 µg dose of CPD dendrimers complexed to pVax-Luc was injected. After injection of this dose, bioluminescence signal was increased (fig. 17, B). Although bioluminescence signal was higher than with 40 µg dendrimer complexes, the photon flux still remained low – the average photon flux on day one was 2 * 10^6 p/s and it was already reduced by 72% on day 3 (average photon flux 5.8 * 10^5 p/s) and by 90% on day 6 (average photon flux 2.0 * 10^5 p/s) (fig. 18).

Compared to electroporation, transfection efficacy was significantly reduced with both - 40 µg CPD ($p<0.001$) and 50 µg ($p<0.05$) (fig. 18). Transfection efficiency with 50 µg of CPD dendrimer was 99% lower than electroporation already on first day after immunization. Transfection efficiency with dendrimers *in vivo* might be decreased due to serum proteins that neutralize positive zeta potential charge (Mirska et al., 2005) suggesting that higher zeta potential of complexes is required for more efficient gene delivery.

Furthermore, transfection efficiency *in vivo* might be affected by aggregation of dendrimer complexes. It is shown, that mixing dendrimers with DNA in increasing ratios, lead to aggregation and even precipitation in different solvents. Complex aggregation leads to polydispersity of size, thus affecting transfection efficiency (Rudolph et al., 2000), as larger

complexes are internalized into cells less effectively than smaller ones (Rejman et al., 2004; Fröhlich, 2012). Complex aggregation was observed also in this study. Increased dendrimer concentration for *in vivo* injections led to heterogeneous mixture formation and aggregation of pDNA-dendrimer complexes – solution became opaque. Aggregation was not observed in pDNA-dendrimer mixtures with low dendrimer concentrations used for *in vitro* cell transfection - the solution remained clear.

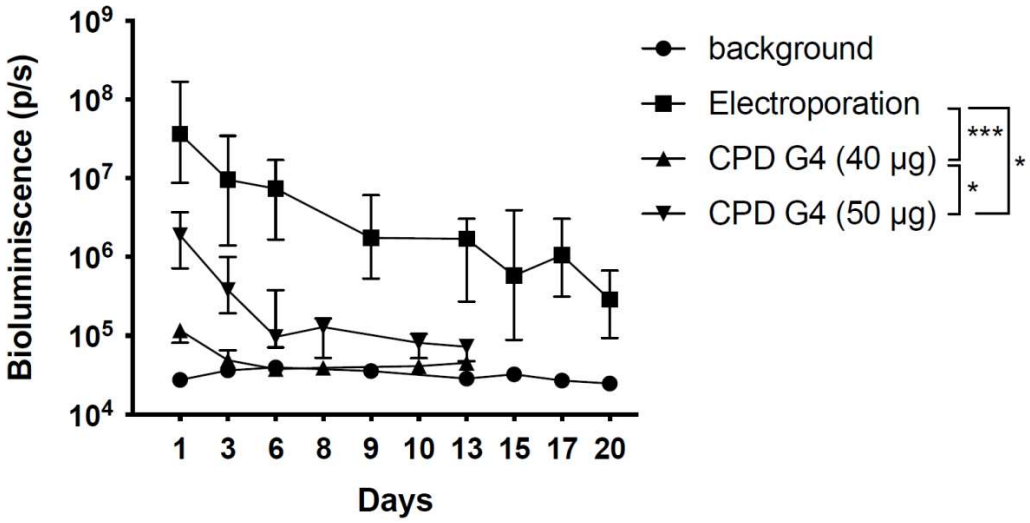


Figure 18. Bioluminescent imaging of electroporated mice and mice treated with pVax-Luc complexed to 40 µg and 50 µg of CPD G4 dendrimer. Significant difference between electroporated mice, mice injected with 40 ug CPD complexes and with 50 ug CPD G4 complexes was observed at days 1 -13, *p<0.05, ***p<0.001.

There is a theory that describes interactions between particles in colloidal suspensions – DLVO theory. It is based on assumption that there are two forces which determine particle dispersion in liquid medium – London - van der Waals force and the electrical double layer force due to the electromagnetic effect of the molecules. London - van der Waals force for two identical particles is always attractive and double layer force is always repulsive. Van der Waals force alone determines the interaction in "simple systems", such as vacuum or non-polar media (oils) (Liang et al., 2007). In this study for *in vivo* injections dendrimers with pDNA were mixed in PBS, because the osmolality and ion concentration matches those of animal and human body, and it is not toxic to cells (Delong and Zhou, 2015). As dendrimer-pDNA complexes are charged positively and dispersed in PBS, which is an ionic solution, the main force that determines the interaction could be electrical double layer force causing repulsion of particles. Electrical double layer consists of two layers – (1) stern layer of counter ions that are opposite to particle surface charge and (2) diffuse layer, which consists of free ions with a higher concentration of the

counter ions. When two particles with the same charge approach each other, their electrical double layers start to overlap, resulting in repulsive force (Liang et al., 2007). However, negative ions from PBS could bind the positively charged outer layer around dendrimer-pDNA complexes and minimize it, thus changing the charge and allowing particle aggregation.

Dendrimer surface can be modified, and it is possible to attach poly ethylene glycol (PEG) to terminal groups, this process is called PEGylation (Gajbhiye et al., 2007). PEG is a large molecule that increases steric hindrance between particles therefore preventing particle collision physically (Yang et al., 2003 in Gajbhiye et al., 2007).

To prevent particle aggregation, dendrimers and pDNA could also be mixed in different solvent. Sugar solutions, such as mannitol or sorbitol solutions do not contain negatively charged ions, yet they are suitable for injections and are used in medicine.

Results from this study indicate that phosphorous CPD G4 dendrimer could be a suitable candidate for *in vivo* gene delivery, however transfection efficiency is much lower than with electroporation. Increase of transfection efficacy with CPD G4 dendrimer requires further optimization of pDNA-dendrimer formulations.

Conclusions

1. Dendrimers PAMAM G4, AE2 G4, CPD G4 and CBD-CS G2 are able to bind plasmid DNA and form stable complexes.
2. Of all four dendrimers, carbosilane CBD-CS G2 dendrimer binds plasmid DNA most efficiently forming complexes with highest positive surface charge and demonstrated the highest transfection efficacy *in vitro*, however the rate of transfection is 54 – 70% lower than with lipofectamine.
3. None of studied dendrimers is significantly cytotoxic to mHippoE-18 and N2a cell lines.
4. The highest gene expression *in vivo* was achieved with 20 ug plasmid DNA complexed to 50 μg phosphorous CPD G4 dendrimer (pVax-Luc /CPD G4 w/w ratio 1 : 2.5), however transfection efficacy was significantly reduced compared to electroporation already on day one after plasmid DNA introduction.

Conferences

Berzina A.*, Michlewska S., Petkov S.P., Podschwadt P., Borikun T., Zadvornyi T., Storchai D., Gordeychuk I., Jansons J., Isaguliants M., Ionov M. Dendrimers in *in vitro* and *in vivo* transfection. International conference “VACCINES & VACCINATION” - Moscow, Russia, September 27-October 1, 2017., abstract book.

* presenting author

Acknowledgements

I would like to express sincere thanks to my supervisors – PhD Mārtiņš Kālis, Dr.hab. Maksim Ionov and Dr. Maria Isaguliants for their expertise, assistance, guidance and patience throughout the process of writing this thesis. I would also like to thank my reviewer Dr. med. Linda Gailīte. Sincere thanks also to colleagues – Sylwia Michlewska for help with *in vitro* experiments, Stefan Petkov, Philip Podschwadt and Ilya Gordeichuk for help with *in vivo* experiments and Šimons Svirskis for help with statistical analysis.

This work was financially supported by the European Union's Horizon 2020 research and innovation programme under grant agreement No. 692293 (VACTRAIN) and Project Initiation grant of the Swedish Institute "INNVOIMMUNE" nr. 19806_2016.

References

Abdelhady, H. G., Allen, S., Davies, M. C., Roberts, C. J., Tendler, S. J., Williams, P. M. 2003. Direct real-time molecular scale visualisation of the degradation of condensed DNA complexes exposed to DNase I. - Nucleic Acids Research, 31(14): 4001-4005.

Ahmadzada, T., Reid, G., McKenzie, D. R. 2018. Fundamentals of siRNA and miRNA therapeutics and a review of targeted nanoparticle delivery systems in breast cancer. - Biophysical reviews, 10(1): 69 – 86.

Albertazzi, L., Gherardini, L., Brondi, M., Sulis Sato, S., Bifone, A., Pizzorusso, T., Ratto G. M., Bardi, G. 2012. In vivo distribution and toxicity of PAMAM dendrimers in the central nervous system depend on their surface chemistry. - Molecular pharmaceutics, 10(1): 249-260.

Bermejo, J. F., Ortega, P., Chonco, L., Eritja, R., Samaniego, R., Müllner, M., de Jesus, E., de la Mata, F. J., Flores, J. C., Gomez, R., Muñoz-Fernandez, A. 2007. Water-Soluble Carbosilane Dendrimers: Synthesis Biocompatibility and Complexation with Oligonucleotides; Evaluation for Medical Applications. - Chemistry-a European Journal, 13(2): 483-495.

Bessis, N., GarciaCozar, F. J., Boissier, M. C. 2004. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. - Gene therapy, 11(S1): S10.

Bielinska, A., Kukowska-Latallo, J. F., Johnson, J., Tomalia, D. A., & Baker Jr, J. R. 1996. Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst PAMAM dendrimers. - Nucleic acids research, 24(11): 2176-2182.

Boger, D. L., Fink, B. E., Brunette, S. R., Tse, W. C., Hedrick, M. P. 2001. A simple, high-resolution method for establishing DNA binding affinity and sequence selectivity. - Journal of the American Chemical Society, 123(25): 5878-5891.

Bolhassani, A., Khavari, A., Orafa, Z. 2014. Electroporation–Advantages and Drawbacks for Delivery of Drug, Gene and Vaccine - Application of Nanotechnology in Drug Delivery. London: InTech, 552 p.

Bosman, A. W., Janssen, H. M., and Meijer, E. W. 1999. About dendrimers: structure, physical properties, and applications. - Chemical reviews, 99(7): 1665-1688.

Buhleier, E. W., Wehner, W., and Vögtle, F. Cascade and Nonskid Chain-like Synthesis of Molecular Cavity Topologies 1978. - Synthesis, 55(2): 155-158

Chen, W., Turro, N. J., & Tomalia, D. A. 2000. Using ethidium bromide to probe the interactions between DNA and dendrimers. - Langmuir, 16(1): 15-19.

Choi, J. S., Nam, K., Park, J. Y., Kim, J. B., Lee, J. K., & Park, J. S. 2004. Enhanced transfection efficiency of PAMAM dendrimer by surface modification with L-arginine. - Journal of controlled release, 99(3): 445-456.

Chonco, L., Bermejo-Martín, J. F., Ortega, P., Shcharbin, D., Pedziwiatr, E., Klajnert, B., de la Mata, F. J., Eritja, R., Gomez, R., Bryszewska, M., Munoz-Fernandez, M. A. 2007. Water-soluble carbosilane dendrimers protect phosphorothioate oligonucleotides from binding to serum proteins. - Organic & biomolecular chemistry, 5(12): 1886-1893.

Cullis, P. R., & Hope, M. J. 2017. Lipid nanoparticle systems for enabling gene therapies. - Molecular Therapy, 25(7): 1467-1475.

Delgado, Á. V., González-Caballero, F., Hunter, R. J., Koopal, L. K., Lyklema, J. 2007. Measurement and interpretation of electrokinetic phenomena. - Journal of colloid and interface science, 309(2): 194-224.

Delong, R. K., Zhou, Q. 2015. Introductory experiments on biomolecules and their interactions. London: Academic Press, 126 pp.

Dreher, M. R., Liu, W., Michelich, C. R., Dewhirst, M. W., Yuan, F., Chilkoti, A. 2006. Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers. - Journal of the National Cancer Institute, 98(5): 335-344.

Dufes, C., Uchegbu, I. F., & Schätzlein, A. G. 2005. Dendrimers in gene delivery. - Advanced drug delivery reviews, 57(15): 2177-2202.

Dul M., Stefanidou M., Porta P., Serve J., O'Mahony C., Malissen B., Henri S., Levin Y., Kochba E., Wong F. S., Dayan C., Coulman S. A., Birchall J. C. 2017. Hydrodynamic gene delivery in human skin using a hollow microneedle device. – Journal of Controlled release 265: 120 – 131.

Duncan, R., Izzo, L. 2005. Dendrimer biocompatibility and toxicity. - Advanced drug delivery reviews, 57(15): 2215-2237.

Dzmitruk, V., Szulc, A., Shcharbin, D., Janaszewska, A., Shcharbina, N., Lazniewska, J., Novopashina D., Buyanova M., Ionov M., Klajnert-Maculewicz B., Gómez-Ramirez, R., Mignani S., Majoral J. P., Munoz-Fernandez A., Bryszewska M. 2015. Anticancer siRNA cocktails as a novel tool to treat cancer cells. Part (B). Efficiency of pharmacological action. - International journal of pharmaceutics, 485(1-2): 288-294.

Eichman, J. D., Bielinska, A. U., Kukowska-Latallo, J. F., Baker Jr, J. R. 2000. The use of PAMAM dendrimers in the efficient transfer of genetic material into cells. - Pharmaceutical science & technology today, 3(7): 232-245.

Esfand, R., Tomalia, D. A. 2001. Poly (amidoamine)(PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. - Drug discovery today, 6(8): 427-436.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop J. P., Danielsen, M. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. - Proceedings of the National Academy of Sciences, 84(21): 7413-7417.

Friedmann, T. 1992. A brief history of gene therapy. - Nature genetics, 2(2): 93.

Fröhlich, E. 2012. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. - International journal of nanomedicine, 7: 5577 - 5591.

Gajbhiye, V., Vijayaraj Kumar, P., Kumar Tekade, R., Jain, N. K. 2007. Pharmaceutical and biomedical potential of PEGylated dendrimers. - Current pharmaceutical design, 13(4): 415-429.

Giacca, M., Zacchigna, S. 2012. Virus-mediated gene delivery for human gene therapy. - Journal of controlled release, 161(2): 377-388.

Gratton, S. E., Ropp, P. A., Pohlhaus, P. D., Luft, J. C., Madden, V. J., Napier, M. E., DeSimone, J. M. 2008. The effect of particle design on cellular internalization pathways. - Proceedings of the National Academy of Sciences, 105(33): 11613-11618.

Gurunathan, S., Klinman, D. M., Seder, R. A. 2000. DNA vaccines: immunology, application, and optimization. - Annual review of immunology, 18(1): 927-974.

Hakamata, Y., Murakami, T., Kobayashi, E. 2006. "Firefly rats" as an organ/cellular source for long-term in vivo bioluminescent imaging. - Transplantation, 81(8): 1179-1184.

Ibraheem, D., Elaissari, A., Fessi, H. 2014. Gene therapy and DNA delivery systems. - International journal of pharmaceutics, 459(1-2): 70-83.

Ihnatsyeu-Kachan, A., Dzmitruk, V., Apartsin, E., Krasheninina, O., Ionov, M., Loznikova, S., Venyaminova A., Milowska K., Shcharbin D., Mignani S. Muñoz-Fernández, M. A., Majoral J. P., Bryszewska M. 2017. Multi-target inhibition of cancer cell growth by siRNA cocktails and 5-fluorouracil using effective piperidine-terminated phosphorus dendrimers - Colloids and Interfaces, 1(1): 6.

Ionov, M., Gardikis, K., Wróbel, D., Hatziantoniou, S., Mourelatou, H., Majoral, J. P., Klajnert, B., Bryszewska, M., Demetzos, C. 2011. Interaction of cationic phosphorus dendrimers (CPD) with charged and neutral lipid membranes. - Colloids and Surfaces B: Biointerfaces, 82(1): 8-12.

Ionov M., Lazniewska J., Dzmitruk V., Halets I., Loznikova S., Novopashina D., Apartsin E., Krasheninina O., Venyaminova A., Milowska K., Nowacka O., Gomez-Ramirez R., Javier de la Mata F., Majoral J. P., Shcharbin D., Bryszewska M. 2015. Anticancer siRNA cocktails as a novel tool to treat cancer cells. Part (A). Mechanisms of interaction. – International Journal of Pharmaceutics 485: 261 – 269.

Iyer, A. K., Khaled, G., Fang, J., Maeda, H. 2006. Exploiting the enhanced permeability and retention effect for tumor targeting.- Drug discovery today, 11(17-18): 812-818.

Jin, L., Zeng, X., Liu, M., Deng, Y., He, N. 2014. Current progress in gene delivery technology based on chemical methods and nano-carriers. - Theranostics, 4(3): 240 – 255.

Kawabata, K., Takakura, Y., and Hashida, M. 1995. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. - Pharmaceutical research, 12(6): 825-830.

Kay, M. A., Glorioso, J. C., Naldini, L. 2001. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. - Nature medicine, 7(1): 33.

Kesharwani, P., Banerjee, S., Gupta, U., Amin, M. C. I. M., Padhye, S., Sarkar, F. H., Iyer, A. K. 2015. PAMAM dendrimers as promising nanocarriers for RNAi therapeutics. - Materials Today, 18(10): 565-572.

Kim, J. B., Choi, J. S., Nam, K., Lee, M., Park, J. S., & Lee, J. K. 2006. Enhanced transfection of primary cortical cultures using arginine-grafted PAMAM dendrimer, PAMAM-Arg. - Journal of controlled release, 114(1): 110-117.

Kim, Y. C., Park, J. H., Prausnitz, M. R. 2012. Microneedles for drug and vaccine delivery. - Advanced drug delivery reviews, 64(14): 1547-1568.

Kukowska-Latallo, J. F., Bielinska, A. U., Johnson, J., Spindler, R., Tomalia, D. A., Baker, J. R. 1996. Efficient transfer of genetic material into mammalian cells using Starburst

polyamidoamine dendrimers. - Proceedings of the National Academy of Sciences, 93(10): 4897-4902.

Lazniewska, J., Milowska, K., Zablocka, M., Mignani, S., Caminade, A. M., Majoral, J. P., Bryszewska M. Gabryelak, T. 2013. Mechanism of cationic phosphorus dendrimer toxicity against murine neural cell lines. - Molecular pharmaceutics, 10(9): 3484-3496.

Lee, C. C., MacKay, J. A., Fréchet, J. M., and Szoka, F. C. 2005. Designing dendrimers for biological applications. - Nature biotechnology, 23(12): 1517.

Leroueil, P. R., Berry, S. A., Duthie, K., Han, G., Rotello, V. M., McNerny, D. Q., Baker J. R., Orr B. G., Banaszak Holl, M. M. 2008. Wide varieties of cationic nanoparticles induce defects in supported lipid bilayers. - Nano letters, 8(2): 420-424.

Li, S. M. A. S. L., Rizzo, M. A., Bhattacharya, S., & Huang, L. 1998. Characterization of cationic lipid-protamine–DNA (LPD) complexes for intravenous gene delivery. - Gene therapy, 5(7): 930 – 937.

Liang, Y., Hilal, N., Langston, P., Starov, V. 2007. Interaction forces between colloidal particles in liquid: Theory and experiment. - Advances in colloid and interface science, 134: 151-166.

Liu, C., Liu, X., Rocchi, P., Qu, F., Iovanna, J. L., Peng, L. 2014. Arginine-terminated generation 4 PAMAM dendrimer as an effective nanovector for functional siRNA delivery in vitro and in vivo. - Bioconjugate chemistry, 25(3): 521-532.

Liu, H., Wang, H., Yang, W., Cheng, Y. 2012. Disulfide cross-linked low generation dendrimers with high gene transfection efficacy, low cytotoxicity, and low cost. - Journal of the American Chemical Society, 134(42): 17680-17687.

Loup, C., Zanta, M. A., Caminade, A. M., Majoral, J. P., & Meunier, B. 1999. Preparation of water-soluble cationic phosphorus-containing dendrimers as DNA transfecting agents. - Chemistry–A European Journal, 5(12): 3644-3650.

Luo, D., & Saltzman, W. M. 2000. Synthetic DNA delivery systems. - Nature biotechnology, 18(1): 33 – 37.

Luo, D., Haverstick, K., Belcheva, N., Han, E., Saltzman, W. M. 2002. Poly (ethylene glycol)-conjugated PAMAM dendrimer for biocompatible, high-efficiency DNA delivery. - Macromolecules, 35(9): 3456-3462.

Maeda, H., Sawa, T., Konno, T. 2001. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. - Journal of controlled release, 74(1-3): 47-61.

Maszewska, M., Leclaire, J., Cieslak, M., Nawrot, B., Okruszek, A., Caminade, A. M., & Majoral, J. P. 2003. Water-soluble polycationic dendrimers with a phosphoramidothioate backbone: preliminary studies of cytotoxicity and oligonucleotide/plasmid delivery in human cell culture. – Oligonucleotides: 13(4), 193-205.

McCaffrey, J., Donnelly, R. F., McCarthy, H. O. 2015. Microneedles: an innovative platform for gene delivery. - Drug delivery and translational research, 5(4): 424-437.

Mellott, A. J., Forrest, M. L., Detamore, M. S. 2013. Physical non-viral gene delivery methods for tissue engineering. - Annals of biomedical engineering, 41(3): 446-468.

Mintzer, M. A., Simanek, E. E. 2008. Nonviral vectors for gene delivery. - Chemical reviews, 109(2): 259-302.

Mirska, D., Schirmer, K., Funari, S. S., Langner, A., Dobner, B., Brezesinski, G. 2005. Biophysical and biochemical properties of a binary lipid mixture for DNA transfection. - Colloids and Surfaces B: Biointerfaces, 40(1): 51-59.

Mukherjee, S. P., Lyng, F. M., Garcia, A., Davoren, M., Byrne, H. J. 2010. Mechanistic studies of in vitro cytotoxicity of poly (amidoamine) dendrimers in mammalian cells. - Toxicology and applied pharmacology, 248(3): 259-268.

Mulligan, R. C. 1993. The basic science of gene therapy. - Science, 260(5110): 926-932.

Naha, P. C., Davoren, M., Lyng, F. M., Byrne, H. J. 2010. Reactive oxygen species (ROS) induced cytokine production and cytotoxicity of PAMAM dendrimers in J774A. 1 cells. - Toxicology and applied pharmacology, 246(1-2): 91-99.

Naldini, L. 2015. Gene therapy returns to centre stage. – Nature: 526(7573), 351.

Okuda, T., Sugiyama, A., Niidome, T., & Aoyagi, H. 2004. Characters of dendritic poly (L-lysine) analogues with the terminal lysines replaced with arginines and histidines as gene carriers in vitro. - Biomaterials, 25(3): 537-544.

Ortega, P., Bermejo, J. F., Chonco, L., de Jesus, E., de la Mata, F. J., Fernández, G., Flores, J. C., Gomes, R., Serramia, M., J., Muñoz-Fernandez, M. A. 2006. Novel water-soluble carbosilane dendrimers: synthesis and biocompatibility. - European journal of inorganic chemistry, 2006(7): 1388-1396.

Pack, D. W., Hoffman, A. S., Pun, S., & Stayton, P. S. 2005. Design and development of polymers for gene delivery. - Nature Reviews Drug discovery, 4(7): 581 – 593.

Payette, P. J., Weeratna, R. D., McCluskie, M. J., & Davis, H. L. 2001. Immune-mediated destruction of transfected myocytes following DNA vaccination occurs via multiple mechanisms. - Gene therapy, 8(18): 1395.

Petkov, S. P., Heuts, F., Krotova, O. A., Kilpelainen, A., Engström, G., Starodubova, E. S., Isaguliants, M. G. 2013. Evaluation of immunogen delivery by DNA immunization using non-invasive bioluminescence imaging. - Human vaccines & immunotherapeutics, 9(10): 2228-2236.

Qin, L., Cao, D., Huang, H., Ji, G., Feng, M., Chen, J., & Pan, S. 2016. Improvement of Cellular Uptake and Transfection Ability of pDNA Using α-Cyclodextrin-Polyamidoamine Conjugates as Gene Delivery System. - Journal of biomedical nanotechnology, 12(2): 261-273.

Rejman, J., Oberle, V., Zuhorn, I. S., & Hoekstra, D. 2004. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. - Biochemical journal, 377(Pt 1): 159 – 169.

Rems, L., Miklavčič, D. 2016. Tutorial: electroporation of cells in complex materials and tissue. - Journal of Applied Physics, 119(20): 201101.

Roos, A. K., Eriksson, F., Walters, D. C., Pisa, P., & King, A. D. 2009. Optimization of skin electroporation in mice to increase tolerability of DNA vaccine delivery to patients. - Molecular Therapy, 17(9): 1637-1642.

Rudolph, C., Lausier, J., Naundorf, S., Müller, R. H., Rosenecker, J. 2000. In vivo gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. - The journal of gene medicine, 2(4): 269-278.

Saade, F. Petrovsky, N. 2012. Technologies for enhanced efficacy of DNA vaccines. - Expert review of vaccines, 11(2): 189-209.

Schaffer, D. V., Fidelman, N. A., Dan, N., Lauffenburger, D. A. 2000. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. - Biotechnology and bioengineering, 67(5): 598-606.

Shakhbazau, A., Isayenka, I., Kartel, N., Goncharova, N., Seviaryn, I., Kosmacheva, S., Potapnev M., Shcharbin D., Bryszewska, M. 2010. Transfection efficiencies of PAMAM dendrimers correlate inversely with their hydrophobicity. International journal of pharmaceutics - 383(1-2): 228-235.

Shcharbin, D., Dzmitruk, V., Shakhbazau, A., Goncharova, N., Seviaryn, I., Kosmacheva, S., Potapnev M., Pedziwiatr-Werbicka E., Bryszewska M., Talabaev M., Chernov, A., Kulchitsky V., Caminade A. M., Majoral J. P. 2011. Fourth generation phosphorus-containing dendrimers: prospective drug and gene delivery carrier. - Pharmaceutics, 3(3): 458-473.

Silveira, M. M., Oliveira, T. L., Schuch, R. A., McBride, A. J. A., Dellagostin, O. A., Hartwig, D. D. 2017. DNA vaccines against leptospirosis: A literature review. - Vaccine, 35(42): 5559-5567.

Sohn R. L., Murray M. T., Schwarz K., Nyitray J., Purray P., Franko A. P., Palmer K. C., Diebel L. N., Dulchavsky S. A. 2001. In-vivo particle mediated delivery of mRNA to mammalian tissues: ballistic and biologic effects. - Wound Repair and Regeneration, 9(4): 287-296.

Solassol, J., Crozet, C., Perrier, V., Leclaire, J., Beranger, F., Caminade, A. M., Meunier B., Dormont D., Majoral J. P., Lehmann, S. 2004. Cationic phosphorus-containing dendrimers reduce prion replication both in cell culture and in mice infected with scrapie. - Journal of general virology, 85(6): 1791-1799.

Somia, N., Verma, I. M. 2000. Gene therapy: trials and tribulations. - Nature Reviews Genetics, 1(2): 91.

Suh, J., Wirtz, D., J. 2003. Efficient active transport of gene nanocarriers to the cell nucleus. - Proceedings of the National Academy of Sciences, 100(7): 3878-3882.

Tang, M. X., Redemann, C. T., & Szoka, F. C. 1996. In vitro gene delivery by degraded polyamidoamine dendrimers. - Bioconjugate chemistry, 7(6): 703-714.

Templeton, N. S. 2002. Cationic liposome-mediated gene delivery in vivo. - Bioscience reports, 22(2): 283-295.

Tomalia D. A., Baker H., Dewald J., Hall M., Kallos G., Martin S., Roeck J., Ryder J. and Smith P. 1985. A New Class of Polymers: Starburst-Dendritic Macromolecules. - Polymer Journal 17(1): 117 – 132.

Tomalia, D. A., Naylor, A. M., Goddard, W. A. 1990. Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology, and flexibility from atoms to macroscopic matter. - Angewandte Chemie International Edition, 29(2): 138-175.

Verma, I. M., Somia, N. 1997. Gene therapy-promises, problems and prospects. - Nature, 389(6648): 239 – 242.

Wang Z., Troilo P. J., Wang X., Griffiths II T. G., Pacchione S. J., Barnum A. B., Harper L. B., Pauley C. J., Niu Z., Denisova L., Follmer T. T., Rizzuto G., Ciliberto G., Fattori E., Monica N. L., Manam S., Ledwith B. J. 2004. Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. - Gene Therapy 11(8): 711 – 721.

Weber, N., Ortega, P., Clemente, M. I., Shcharbin, D., Bryszewska, M., de la Mata, F. J., Gómez, R., Muñoz-Fernández, M. A. 2008. Characterization of carbosilane dendrimers as effective carriers of siRNA to HIV-infected lymphocytes. - Journal of Controlled Release, 132(1): 55-64.

Wells, D. J. 2004. Gene therapy progress and prospects: electroporation and other physical methods. - Gene therapy, 11(18): 1363.

Whitehead, K. A., Langer, R., & Anderson, D. G. 2009. Knocking down barriers: advances in siRNA delivery. - Nature reviews Drug discovery, 8(2): 129 – 138.

Wrobel, D., Kłys, A., Ionov, M., Vitovic, P., Waczulikowa, I., Hianik, T., Gomez-Ramirez, R., de la Mata, F. J., Klajnert, B., Bryszewska, M. 2012. Cationic carbosilane dendrimers–lipid membrane interactions. - Chemistry and physics of lipids, 165(4): 401-407.

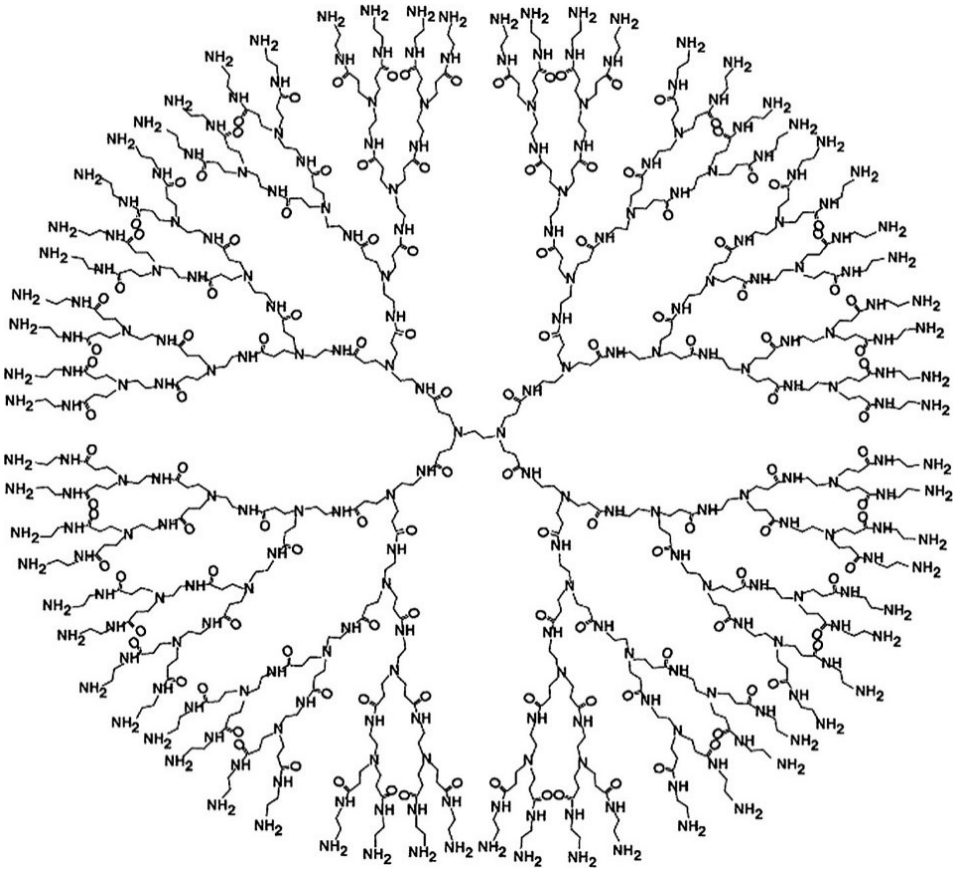
Xu, Y., & Szoka, F. C. 1996. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. - Biochemistry, 35(18): 5616-5623.

Zahm, C. D., Colluru, V. T., McNeel, D. G. 2017. DNA vaccines for prostate cancer. - Pharmacology & therapeutics, 174: 27-42.

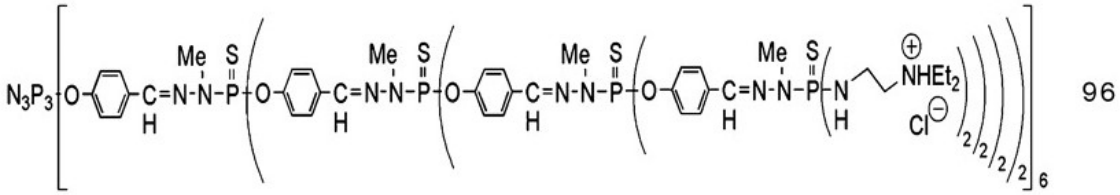
Supplements

Supplement 1.

Molecular strucrure of PAMAM G4 (Kesharwani et al., 2015), 64 surface cationic end groups, molecular weight: 14214.17 g/mol; $NH_2(CH_2)_6NH_2$. (A)

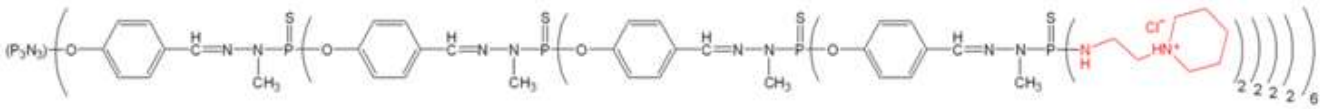


Molecular structure of CPD G4 dendrimer (Solassol et al., 2004), 96 surface cationic end groups, molecular weight: 33 702 g/mol; $C_{1296}H_{2256}N_{375}Cl_{96}O_{90}P_{93}S_{90}$ (B)

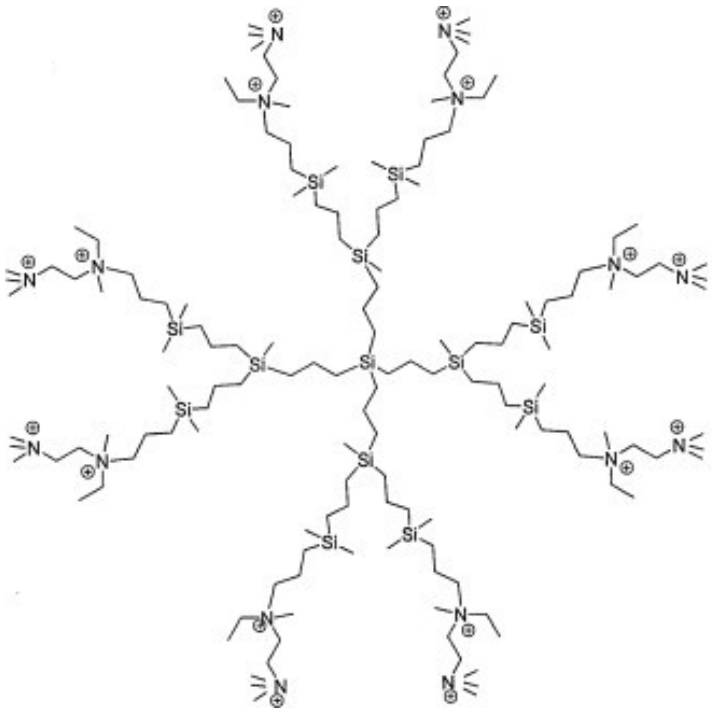


Supplement 2.

Molecular structure of AE2 G4 dendimer (Ihnatsyeu-Kachan et al., 2017), 96 surface cationic end groups, molecular weight: 34 856 g/mol (A)



Molecular structure of CBD-CS G2 dendimer (Wrobel etl al., 2012), 16 cationic end groups, molecular weight: 4603.56 g/mol; $C_{128}H_{316}I_{16}N_{16}O_8Si_{13}$ (B)



Maģistra darbs „ Dendrimēri kā luciferāzi saturošas plazmīdas transfekcijas vektori, in vitro un in vivo pētījumi” izstrādāts LU Bioloģijas fakultātē.

Ar savu parakstu apliecinu, ka pētījums veikts patstāvīgi, izmantoti tikai tajā norādītie informācijas avoti un iesniegtā darba elektroniskā kopija atbilst izdrukai.

Autors: Anita Bērziņa 01.06.2018

Rekomendēju darbu aizstāvēšanai
Vadītājs: PhD Mārtiņš Kālis 01.06.2018.

Recenzents: Dr. Med. Linda Gailīte

Darbs iesniegts Bioloģijas fakultātē:
Metodiķis:

Darbs aizstāvēts maģistra gala pārbaudījuma komisijas sēdē
07.06.2018. prot. Nr. ___, vērtējums _____

Komisijas sekretārs/e: